MICROBIAL EXOPOLYSACCHARIDES: FROM GENES TO APPLICATIONS

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MICROBIAL EXOPOLYSACCHARIDES: FROM GENES TO APPLICATIONS

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Schematic representation of the different research in the field of microbial exopolysaccharides. The whole process "From genes to applications" is depicted, including genome and structural analysis, process optimization as well as development of different applications. Artwork and copyright by sonnensprosse.de

Microbial polysaccharides represent an attractive alternative to those from plants or macro algae. They can be produced from renewable sources including lignocellulosic waste streams. Their production does not depend on geographical constraints and/or seasonal limitations. Additionally the manipulation of biosynthetic pathways to enhance productivity or to influence the chemical polysaccharide composition is comparatively easy in bacteria. Microbial exopolysaccharides

represents a valuable resource of biogenic and biodegradable polymers, suitable to replace petro based polymers in various technical applications. Furthermore, biocompatible exopolysaccharides are very attractive in medical applications, such as drug delivery systems, use as vaccines or nanoparticles. This research topic will depict the status quo, as well as the future needs in the field of EPS and biofilm research. Starting from the unexplored diversity of microbial polysaccharide producers to production processes and possibilities for modifications, to enhance the already high number of functionalities based on the chemical structures. An overview of the recent and future applications will be given, and the necessity in unravelling the biosynthesis of microbial exopolysaccharide producers is depicted, highlighting the future trend of tailor made polymers. Constraints in structure analysis of these highly complex biogenic polymers are described and different approaches to solve the restrictions in imaging and NMR analysis will be given. Therefore; this research topic comprises the whole process from genes to applications.

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EDITORIAL
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Editorial: Microbial Exopolysaccharides: From Genes to Applications

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The Editorial on the Research Topic

Microbial Exopolysaccharides: From Genes to Applications

The Research topic "Microbial exopolysaccharides from genes to applications" covers 12 articles dealing with the highly diverse class of microbial exopolysaccharides (EPSs). Many bacteria, archaea, yeast, and filamentous fungi are able to produce EPSs under different conditions. These biopolymers significantly differ in monomer composition, substituent decoration, degree and type of branching as well as molecular weight. Therefore, both chemical diversity and functionality of biopolymers is enormous. Their natural roles range from adhesives, to storage compounds, protective hulls, as well as pathogenicity factors. The complete field of putative natural applications is not fully understood up to now. A similar situation is observed for the different biosynthetic pathways. Only minimal information is available for EPS biosynthesis in fungi and similarly, little is known for cyanobacterial and archaeal polysaccharide synthesis routes. In addition to these challenges from the biological point of view, the current methods for polysaccharide analysis are still limited due to many different constraints, which in conjunction make exopolysaccharides a challenging topic of study. One of these limitations is the low achievable EPS concentration often caused by their high viscosity, which, for example, lowers the efficiency of NMR analysis.

Despite, all the remaining challenges and obstacles concerning the study of molecular processes underlying formation of EPS and their chemical characterization, many aspects of this highly diverse class of biopolymers are already known sustaining them as biomolecules of industrial interest.

In the series of articles presented in this book, the authors provide an overview of the different fields involved in microbial EPS production, characterization, and applications. Particular emphasis is directed toward the molecular mechanisms of EPS biosynthesis and modification as well as their regulation. Additionally, the production of fungal EPSs is also explored to show the potential use of these currently less understood microbial biopolymers. Furthermore, the problems and future perspectives related to polysaccharide characterization are summarized and described. Finally, the various aspects of microbial polysaccharide application covering a wide range of uses are also discussed. In summary this research topic deals with the following aspects.

Effective microbial EPS production is based on the identification of novel and efficient production strains. Therefore, Rühmann et al. describe and compare the different available methods

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to identify EPS producers, including the characterization of monomer composition of these polysaccharides. They finally disclose the most promising and currently available novel techniques, as the main input for boosting the exploration and discovery of new EPS-producers in the short term.

The review of Schmid et al. gives a comprehensive overview of the different biosynthetic pathways known for bacterial EPS-producers and compares in detail the different kinds of EPS. It summarizes the regulatory mechanisms of bacterial EPS production and describes present and future engineering strategies toward tailor-made EPS variants.

The minireview of Becker goes much deeper into this topic, with a specific focus on xanthan and succinoglycan biosynthesis. It includes challenges and perspectives in combinatorial assembly of the biosynthetic pathways in order to obtain tailored variants.

The enhanced bacterial persistence due to enzymatic EPS modifications is described in the review of Whitfield et al. They describe the effect of different enzymatic activities, which are involved in the modification of different EPSs such as alginate, Pel polysaccharide as well as a nitrogen-containing EPS, and link them to their biological function with respect to enhanced survival of the producing microorganisms, either in pure cultures or in biofilms.

The contribution of Ertesvåg especially focuses on alginate modifying enzymes, and discusses how the different modifications influence on material properties of the respective alginate variants. This review represents a comprehensive overview of enzymatic tools suitable for tailoring alginate polysaccharides.

In the original research article of Jachlewski et al. different techniques are presented for the targeted isolation of various EPS and further polymeric compounds, such as DNA and proteins, from biofilms of extremophilic archaea. The authors present a combined approach of proteome and EPS analyses, which provides further insight into the composition and functionality of extremophile biofilms and alludes to the potential of biofilms in future applications.

The special class of microbial EPSs produced by lactic acid bacteria is described in detail by Torino et al. The authors give a comprehensive outlook of either capsular or exopolysaccharides produced by lactobacilli and summarize their traditional and novel applications in food and beverage manufacturing. To complete the landscape, the authors provide a wide description of relevant EPS characteristics and the enzymes involved in their biosynthesis.

The section of rare-sugar-containing EPSs, such as fucose or rhamnose, is described by Roca et al. These polymers are uncommon or at least, rarely identified up to now, and open new frontiers for special applications. In this brief review, EPSs containing rare sugars as well as the respective producing strains are presented, along with the cultivation conditions influencing their monomer pattern. Additionally, the authors focus on their downstream processing and discuss the applications of these special polymers in various fields such as e.g., cosmetics, foodstuff, pharmaceuticals, and biomedical applications.

An overview of current and future biomedical applications of microbial EPSs is given by Moscovici. This article explores the various EPS applications starting from the first tested medical applications, such as the use of dextran as plasma expander, up to the latest innovations in the field, like micro- and nanoparticle-based EPS formulations.

The complete scleroglucan production process, one of the few fungal representative EPSs commercially available, is revisited by Castillo et al. In this comprehensive review, they describe the complete fermentative production process as well as some downstream processing clues which have influence on the final EPS properties. The utilization of non-conventional complex media as efficient carbon-sources for sustainable production of EPS is also outlined and discussed.

Putative future solutions for resolving one of the main obstacles of EPS characterization, i.e., the efficient determination of the EPS structure by NMR analysis, are outlined in the article by Larsen and Engelsen. They present a simple but very efficient combination of NMR spectroscopy and molecular modeling as an efficient EPS analytical tool. This approach might be efficiently used to determine the chemical and three-dimensional EPS structures in fast and reliable way in the near future.

Further, insights into the microbial EPS structure characterization by using novel imaging technologies at various length scales are summarized by Lilledahl et al. The authors present the use of different high-resolution microscopic techniques along with the combination of different approaches in order to support the elucidation of structural features of isolated and secreted EPSs in the cell environment.

In conclusion, these contributions summarize important aspects related to microbial biopolymer research. The technical limitations for analyzing/characterizing microbial EPSs, the urgent need to advance our understanding on EPS biosynthetic pathways, as well as the relevance of producing tailor-made EPSs, are widely explored and discussed employing various representative examples. On the other hand, a range of current and future applications of microbial EPSs is presented, which either already, or in the near future, will contribute to a biobased industry.

In summary, a wide readership with interest in bio-polysaccharides and their promising future is expected to find in this research topic a clear overview assessing the currents gaps in our understanding of EPS while already taking advantage of the current knowledge in the field of microbial polysaccharide research, thus identifying still unmet needs informing future R&D programmes.

AUTHOR CONTRIBUTIONS

JS initiated the research topic and invited editors JF, BR, and VS. The editorial was written jointly by the editors of the topic.

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This topic was organized to update the current status of research in the field of microbial polysaccharides. We would like to

take this opportunity to specially thank all the researchers who contributed their valuable work to this topic, not only enriching this book but also, encouraging the scientific community to become more involved in this challenging investigation field. As well, the corresponding financial supports for EPS worldwide research, all the other "hidden" people who silently support our day-to-day commitment with EPS proposals and, the reviewers who contributed their respected criticism for improving this work are gratefully acknowledged.

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MINI REVIEW
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Methods to identify the unexplored diversity of microbial exopolysaccharides

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Microbial exopolysaccharides (EPS) are a structurally very diverse class of molecules. A number of them have found their application in rather diverging fields that extend from medicine, food, and cosmetics on the one side to construction, drilling, and chemical industry on the other side. The analysis of microbial strains for their competence in polysaccharide production has therefore been a major issue in the past, especially in the search for new polysaccharide variants among natural strain isolates. Concerning the fact that nearly all microbes carry the genetic equipment for the production of polysaccharides under specific conditions, the naturally provided EPS portfolio seems to be still massively underexplored. Therefore, there is a need for high throughput screening techniques capable of identifying novel variants of bacterial EPS with properties superior to the already described ones, or even totally new ones. A great variety of different techniques has been used in screening approaches for identifying microorganisms that are producing EPS in substantial amounts. Mucoid growth is often the method of choice for visual identification of EPS producing strains. Depending on the thickening characteristics of the polysaccharide, observation of viscosity in culture broth can also be an option to evaluate EPS production. Precipitation with different alcohols represents a common detection, isolation, and purification method for many EPS. A more quantitative approach is found in the total carbohydrate content analysis, normally determined, e.g., by phenol-sulfuric-acid-method. In addition, recently a new and reliable method for the detailed analysis of the monomeric composition and the presence of rare sugars and sugar substitutions has become available, which could give a first hint of the polymer structure of unknown EPS. This minireview will compare available methods and novel techniques and discuss their benefits and disadvantages.

Keywords: polysaccharide, screening, high throughput, carbohydrate fingerprint, colorimetric assays

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Introduction

The global production of bacterial polymers is increasing rapidly, caused by the growing demand for biobased polymers. The natural variety of different exopolysaccharides (EPS) with specific properties has a huge potential for industrial utilization. Based on the Bacterial Carbohydrate Structure Data Base (Toukach et al., 2007) ca. Four hundred different EPS variants with different chemical structures have been published, of which some can be linked to specific strains or genera. Additionally many reports can be found, which describe microbes to be capable of producing EPS, without giving structural information. This impressively demonstrates the

high diversity of naturally available EPS and the capacity for new variants to be of technical and commercial interest. Especially the growing demand of sustainable products further increases the need for the replacement of petro-based polymers such as polyacrylates or polyvinyl alcohol. An example of one of those new products is the biobased lubricant Berufluid®. Furthermore, in fields such as medicine (Colegrove, 1983; Costerton et al., 1999), cosmetics (Thibodeau, 2005; Prajapati et al., 2013), water treatment (Srinivasan, 2013), agriculture (Colegrove, 1983), enhanced oil recovery (Rau and Brandt, 1994), and construction chemistry (Schmidt et al., 2013) new and innovative EPS variants are used. In order to trap the full potential of EPS fast and reliable screening methods are important to identify novel EPS with innovative properties to enhance the field of applications. Here we describe the most common and publicly available screening approaches that have been used including the different methods for identification of EPS on which they are based and discuss their advantages and disadvantages (**Table 1**) as well as their compatibility for high-throughput (HT).

Screening Approaches for Solid Media

Detection of EPS Producing Phenotypes

Exopolysaccharides producers can be identified by their phenotypes on solid as well as liquid media. This technique is the most prevalent method to date and has been successfully used within the past several years to identify bacteria that are used for EPS production today. Generally, the terms "ropy," "mucoid," and "slimy" are used for this visual characterization. "Ropy" in liquid cultures is characterized via high resistance to flow through serological pipettes as well as via formation of viscous strands during "free fall" from the pipette tip (Vedamuthu and Neville, 1986). Furthermore, "ropy" colonies form long filaments when extended with an inoculation loop (Dierksen et al., 1997). The "mucoid" colonies have a glistening and slimy appearance on agar plates and do not form a filament during this process. One successful example for a screening via "mucoid" and "slimy" morphology was performed by Ortega-Morales et al. (2007) for a prescreening. Biomass of positive, "mucoid" and "slimy" strains was then scraped from the agar plate surface and diluted, before cells were removed and EPS was precipitated. The problem of this method is that it leads to false negative strains, which are excluded. After dissolving the precipitate the total carbohydrate content was determined via phenol-sulfuric-acid-method. The advantage of this screening method is that it can be easily performed without the need of any special equipment. The weakness of the method is that strain selection via colony morphology occurs by human interpretation and can hardly be standardized. Novel interesting polymers might not be detected due to a missing obvious slime formation. Ruas-Madiedo and De Los Reyes-Gavilán (2005) also pointed out that the nomenclature used to describe the different EPS producing phenotypes of lactic acid bacteria (LAB) can be confusing. The terms "ropy," "mucoid" and "slimy" have been used indistinctly in literature, without any consequence and therefore lack of a clear definition.

A good way to evaluate mucoid and slimy colonies is the comparison of colony morphology between induced and non-induced EPS production. This can be efficiently used for strains showing extracellular sucrase activity, which is known to be inducible and strongly substrate dependent. Therefore, sucrose and raffinose as supplemented to the agar plates induce glucan- and fructansucrase activity of the EPS producers. Malik et al. (2009) screened 63 LAB-strains with this method and identified 29 isolates, from which 18 were randomly selected and proven via PCR to carry sucrase genes by use of degenerated primers. Tallgren et al. (1999) screened 600 strains for mucoid and slimy phenotypes and identified 170 interesting strains. Of these they only selected 10% (randomly chosen 17 strains) for further characterization of the monomeric composition, since no fast and reliable HT-screening methods for the determination of the monomeric composition of the produced polymers was available.

Agar-Plates with Dyes

Some dyes are known to interact with polysaccharides with different specificities. This phenomenon can be used to identify different EPS-producers by a fast and easy agar plate based screening approach. Aniline Blue fluorochrome (Sinofluor) for example shows an intense fluorescence when bound to β-(1-3)-glucans. Additionally, the relative fluorescence with different types of other polysaccharides is well studied (Evans et al., 1984). Ma and Yin (2011) screened for EPS producing bacteria from different environments on LB-agar-plates supplemented with aniline blue. They identified 89 EPS producing strains and selected eight of them for further physiological, biochemical, and genetic analysis. The same technique, but with a different dye, was successfully utilized for the identification of EPS-defective mutants of *Rhizobium meliloti* (Leigh et al., 1985). Calcofluor White binds to succinoglycan as well as pure β-(1-3)- and β-(1-4)-glucans and exhibits a blue-green fluorescence when irradiated by long-wave UV light. Thereby, fluorescence negative colonies can easily be identified on agar-plates. This makes this method additionally suitable for a fast screening and characterization of a large number of mutagenized strains. Furthermore, there exist several dyes for various applications. Congo Red, for example, is known to interact with β-(1-3)- and β-(1-4)-glucans (Wood and Fulcher, 1978) and was successfully used to identify biofilm formation by different *Staphylococci* strains (Darwish and Asfour, 2013).

The use of dyes can be very useful when working with one or two defined polymers or if the screening targets a specific class of polysaccharide, e.g., β-(1-3)-glucan. However, screening for novel polymers containing different sugars, uronic acids as well as deoxy-and amino-sugars cannot be performed by use of these dyes, since interactions of the dyes to new EPS are unpredictable rendering this technique useless to identify novel EPS variants.

Screening Approaches for Liquid Media

Precipitation

When screening approaches are carried out in liquid media instead of solid media different techniques for EPS identification

TABLE 1 | Overview and description of different screening approaches including benefits and disadvantages.

Method/Reference	*Example of Screening Approach*/Description	Pros and Cons
Detection of exopolysaccharides (EPS) producing phenotypes		
Colony morphology Ortega-Morales et al. (2007)	*Screening of 34 strains for biofilm formation:* – replication of isolates – selection of mucoid (slimy) colonies (indication for their ability to produce exopolymeric substances) – biomass scraped from the agar surface – dissolving and centrifugation – precipitation with two volumes cold ethanol – dissolving and detection via phenol-sulfuric acid method	+ simple experimental setup and low cost + only small amounts needed – preselection via visual observation (mucoid colonies) may result in many false negative – only EPS which precipitate detected – only determination of total carbohydrate content
Colony morphology Tallgren et al. (1999)	*Screening of 600 strains for EPS production from sugar beets* – first screening round: 170 out of 600 strains detected via slimy colony morphology – 17 randomly chosen isolates – centrifugation of liquid culture – precipitation with two volumes isopropanol – collection of precipitate by centrifugation and freeze dried – hydrolysis: 1N H_2SO_4 for 60 min at 120°C – neutralization with 5 M sodium hydroxide – monosaccharide composition: via high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)	+ preselection: simple experimental setup and low cost + detailed monosaccharide analysis of selected strains – preselection via visual observation (mucoid colonies) may result in many false negatives – randomly chosen isolates for further monosaccharide analysis – only 10% of positive strains were analyzed – not manageable in high throughput
Agar-plate with dyes		
Aniline Blue Ma and Yin (2011)	*Screening for EPS producers in different environments* – interacts with β-(1-3)-glucans – visual observation of colony color and morphology	+ manageable in high throughput and low cost – fluorochrome Sinofluor is only an impurity in Aniline Blue
Calcofluor White Leigh et al. (1985)	*Screening of defective mutants in succinoglycan production:* – visual observation under UV-light – Calcofluor-dark mutants are defective in EPS production.	+ manageable in high throughput and low cost + binding of Calcofluor White to succinoglycan and (1-4)-β and (1-3)-β-glucans
Congo Red Darwish and Asfour (2013)	*Biofilm formation ability in Staphylococci* – interacts with β-(1-3)- and β-(1-4)-glucans – visual observation of colony color and morphology	+ manageable in high throughput and low cost
Precipitation		
Precipitation Van Geel-Schutten et al. (1998)	*Screening of 182 LAB strains for EPS production:* – centrifugation of liquid culture – precipitation with two volumes cold ethanol, stored overnight at 4°C – collection of precipitate by centrifugation – dissolving in original volume, second precipitation – drying at 55°C and measuring the dry weight – dissolving in original volume – detection via phenol-sulfuric-acid-method and HPAEC-PAD	+ simple experimental setup and low cost + detailed monosaccharide analysis of selected strains – difficult redissolving – limited to EPS that can be precipitated – time consuming
Viscosity		
Culture viscosity Visual observation Garai-Ibabe et al. (2010)	*Screening of 147 LAB strains for β-glucans*: – EPS positive strains showed a ropy liquid culture and deposit formed a long string – identification of the gene (*gtf*) encoding for β-glucan-synthase – β-glucan agglutination test with *Streptococcus pneumoniae* type 37-specific antisera – centrifugation of liquid culture – precipitation with two volumes cold aceton and washing – dissolving and detection via phenol-sulfuric acid method – EPS characterization via NMR studies	+ preselection: simple experimental setup and low cost + specific identification of the β-glucan-synthase gene + specific β-glucan immunoprecipitation via antisera + NMR studies of selected EPS – preselection via visual observation may result in many false negative (viscosity) – molecular characterization is time consuming – only EPS which precipitate were detected – only determination of total carbohydrate content
Microhaematocrit capillaries Ricciardi et al. (1997)	– inversing tubes and measuring the time taken by the liquid to reach by gravity the opposite extremity of the tube – precipitation with three volumes cold ethanol – dissolving and detection via phenol-sulfuric acid method	+ simple experimental setup and low cost + only small volume needed – manual handling – only viscous EPS are detected
Carbohydrate screening		
Uronic acid determination with m-hydroxydiphenyl Mojica et al. (2007)	*Screening for biofilm formation:* – dissolving of washed and dried biofilms – intense vortexing for 2.5 min, 2 min resting – adding reagent solution 1, vortex 45 s	+ manageable in high throughput + fast determination of UA + no interference with neutral sugars

(Continued)

TABLE 1 | Continued

Method/Reference	*Example of Screening Approach*/Description	Pros and Cons
	– heating 100°C for 5 min – adding reagent solution 2, vortex – absorbance read at 520 nm after 4 min	– only detection of UA – no discrimination of UA's – different color development for ManUA, GalUA, GlcUA
Modular exopolysaccharide screening platform Rühmann et al. (2015b)	*HT-Screening of 96-strains per day* – 96-well cultivation and cell removal – visual observation of viscosity and precipitation assay – 96-well gel-filtration and hydrolysis – PMP-derivatization and UHPLC-UV-ESI-MS analysis	+ combination of different detection systems + detailed carbohydrate fingerprint + high throughput method – only aldoses can be detected as ketoses cannot be derivatized with PMP

are required. Most EPS are highly soluble in aqueous solutions, whereas the solubility can be drastically decreased by using water miscible solvents by extracting water molecules from the hydration shell. Accordingly, for various EPS (xanthan gum, gellan gum, welan gum, diutan gum, succinoglycan, colanic acid) precipitation with alcohols or acetone is a common purification and isolation method (Phillips and Williams, 2000). In the same way it can also be applied for identification of EPS in screening approaches. The efficiency of precipitation of polymers depends on their chemical structure, molecular weight, and the final concentration of polymer and alcohol used for precipitation (Smidsroed and Haug, 1967; Swennen et al., 2005). Most importantly, it has to be taken into account that other biopolymers like DNA, RNA, proteins, and polyglutamat are also precipitating in the same manner (Schmid et al., 2013; Kreyenschulte et al., 2014). The appearance of the precipitate can help to distinguish the different polymers. Polysaccharides usually precipitate as fibers, when alcohols such as ethanol or 2-propanol are used as precipitant. However, this has to be considered with care as some EPS, like, e.g., hyaluronic acid precipitate more in the form of flakes.

Van Geel-Schutten et al. (1998) screened 182 LAB strains in de Man, Rogosa, and Sharpe (MRS) medium supplemented with different sugars. After 3 days of incubation at 37°C, the cells were removed from the cultures via centrifugation and EPS were precipitated with cold ethanol. This prescreening identified 60 strains that showed pellet formation. These were dried at 55°C, dissolved in water and the EPS-content was determined by measuring the total carbohydrate content with the phenol-sulfuric-acid-method (Dubois et al., 1956) and a detailed monosaccharide analysis for 17 selected strains was performed. However, one drawback of this method is that not all polysaccharides precipitate under the same conditions with alcohols or acetone (Sutherland, 1990; Azeredo and Oliveira, 1996). This has to be taken into account as limiting factor in the search for novel EPS structures, when only the precipitate is used for further analysis. On the other hand, the precipitation process is fast, easy to handle and at the same time purifies the EPS concerning remaining sugars and salts of the cultivation media (Kumar et al., 2007). This enables a better quantification of the total sugar content of the EPS and the polymer concentration can be increased by using less volume for dissolving the precipitate. This can lead to a higher sensitivity in the subsequent analysis. When the precipitate is dried after the supernatant is removed, the dry weight of the polymer can be determined to compare the productivity of different strains. However, in combination with HT-screening approaches, determination of the polymer dry weight is not applicable due to the use of multi-well format. Generally, an additional disadvantage of drying the precipitated polymer is an altered dissolving property of dried EPS, leading to inhomogeneous suspensions and therefore, to non-reliable results in subsequent analytics. Xu et al. (2010) screened 60 LAB strains in a similar way, but without drying the precipitate and without performing a detailed monosaccharide analysis. In this screening all LAB's demonstrated their ability to produce EPS in different amounts. Only the strain showing the highest productivity was selected to be further optimized and characterized.

Taken together, precipitation is a fast and easy way to detect, isolate and purify polysaccharides. However, it is unsuitable for HT. It is difficult to handle precipitation in small volumes and even more though it is challenging to ensure the correct dissolving of the different precipitates (with or without drying) in 96-well format.

Viscosity

Visually observable properties such as viscosity of the liquid culture have also been used for the evaluation of EPS production. A rapid screening for β-glucans based on this technique was performed by Garai-Ibabe et al. (2010). All strains that showed "ropy" liquid culture and had a long string formed by the cell deposit were selected. This criterion was used for a preselection to identify strains from which genes encoding β-glucansynthases could be amplified. However, not all viscous cultures show "ropy" behavior and sometimes there is just no correlation between phenotypic characterization on the one side and viscous behavior in liquid cultures on the other side Another reliable method is the determination of texture properties for example of LAB via a sensory and rheological screening (Folkenberg et al., 2006). These techniques for LAB and all other bacterial strains are very time consuming, though. Up to now there are no HT-screening applications in this field. Ricciardi et al. (1997) developed a rapid and convenient screening method for viscosity of LAB cultures based on the measurement of efflux time in microhaematocrit capillaries. For the simple experimental setup only small volumes of the samples are needed. However, the manual handling of this approach makes it more difficult to be manageable in HT.

Specific Carbohydrate Screening

One possibility to search for specific carbohydrates is the targeted screening for uronic acids in biofilms (Mojica et al., 2007). This method is based on the method of Blumenkrantz and Asboe-Hansen (1973). The combination of hydrolyzing the sample and the specific color reaction caused by the uronic acids present in the polymer leads to a method capable for HT-applications. Mannuronic-, glucuronic-, and galacturonic-acids show different calibration curves and therefore, can be quantified reliably when only one known uronic acid is present in the biofilm. Discrimination of various uronic acids cannot be performed. However, this method presents a rapid screening for uronic acids in biopolymers without interference of neutral sugars.

To deal with all these issues, Rühmann et al. (2015b) recently developed a EPS screening platform, that combines visual observation of viscosity, and precipitation with a detailed monosaccharide analysis via UHPLC-UV-ESI-MS. All steps are handled in 96-well format, starting from the strain cultivation up to the carbohydrate fingerprint. Cells are removed via centrifugation and filtration steps and polymers are purified by removal of remaining low molecular compounds such as glucose and salts by gel-filtration. An aliquot of the gel-filtrate is then hydrolyzed, derivatized, and analyzed via an optimized HT-PMP method (HT-1-phenyl-3-methyl-5-pyrazolone; Rühmann et al., 2014). This method allows the simultaneous analysis of hexoses, pentoses, deoxy, and amino-sugars, uronic acids as well as different sugar modifications in one single run. Double detection via UV- and ESI-MS-detector makes that quantification very reliable. Additionally, the method is also very fast. The carbohydrate fingerprint of 96 different strains can be analyzed within only 1 day. Other detection modules like precipitation and visual observation of viscosity are handled in parallel and therefore, there is not only one screening method causing a preselection. This allows to trap the full potential of various EPS producers. Additionally, the carbohydrate fingerprint enables a targeted screening. The throughput can be even increased by the factor of eight when coupling the detailed carbohydrate fingerprint analysis with a modular automated prescreening system (Rühmann et al., 2015a).

96-Well Colorimetric Carbohydrate Assays

Usually, methods for the determination of the total carbohydrate content are combined with other screening methods as shown in **Table 1**. Mostly they are placed at the end of the screening approach and for that reason will not be applied to all strains. However, to grab the full potential of all the different polysaccharides it would be more suitable to place the determination of total carbohydrate content as first detection system for EPS in order not to produce false negatives. **Table 2** gives an overview of some 96-well micro-titer-plate (MTP) based methods for the rapid analysis of carbohydrate content in parallel. The phenol-sulfuric-acid-method still represents the common procedure for the fast determination of total carbohydrate content of bacterial and plant polysaccharides (Ortega-Morales et al., 2007; Xu et al., 2010). This method was first described by Dubois et al. (1956) and later adapted to a 96-well application (Masuko et al., 2005). Masuko et al. (2005) optimized it toward a really rapid method (<15 min) with only one heating step and without any mixing step. They reached a linear calibration range from 4.5 to 676 mg/L with a coefficient of determination (R^2) of 0.988. The major disadvantages of this method are the need of a carcinogenic phenol reagent and the incubation in a water bath, what necessitates manual drying of the plate. In addition the method does not provide real quantitative values as the molar extinction coefficient of different sugars varies causing unequal responses of different heteropolysaccharides.

Albalasmeh et al. (2013) developed a non-carcinogenic assay by removing the phenolic component. They also reduced the reaction time to a minimum and eliminated the heating step. By this setup they reduced the error in measurement and developed a more reliable method. This simple approach would be easily transferred to an automated liquid handling station to perform an HT-screening. However, a MTP version of the method was not reported up to now and has to be validated first. Additionally, the lack of phenol eliminates the ability to produce a color reaction and therefore, the UV absorbance at 315 nm strongly interferes with protein and/or flavonoid impurities.

Another non-carcinogenic assay was developed by Laurentin and Edwards (2003). This group used anthrone as reaction chemical, which mainly interacts with hexoses, and shows only slight reactivity with pentoses, or uronic acids. However, the limit of quantification (LOQ) is 10 times lower than for the phenol-sulfuric-acid-method. Furthermore, special equipment as well as many additional and time consuming handling steps are necessary to perform this assay.

Cesaretti et al. (2003) reported an MTP based assay with carbazole to detect uronic acids in glycosaminoglycans. The uronic acids were stabilized with tetraborate and hydrolyzed via sulfuric-acid in the first incubation step. The color reaction caused by carbazole takes place during a second incubation step. The method showed the lowest value for the coefficient of determination (R^2) with 0.975 and at the same time the widest linear calibration range (20–2,000 mg/L) of all methods as summarized in **Table 2**. Another assay for the determination of glycosaminoglycans was reported by Van Den Hoogen et al. (1998). The hydrolysis in the first step of this method is similar to the first step of the carbazole assay. Afterward, the brownish background color reaction caused by neutral sugars was measured at 540 nm. In the second incubation step the color reaction with *m*-hydroxydiphenyl and uronic acids takes place at room temperature and once again the absorbance was measured at 540 nm. The background subtraction enables a robust method where an excess of neutral sugars (up to 20-fold) does not interfere with the determination of the uronic acids. Both described assays are capable for targeted screening approaches for uronic acid containing polymers.

However, when screening for various kinds of polymers only total carbohydrate content methods are appropriate. They are able to detect all kinds of sugars and determine their total content, but are not able to distinguish between

TABLE 2 | Overview and description of different micro-titer based colorimetric assays including benefits and disadvantages.

Colorimetric assay/ Reference	Description	Calibration range/R^2	Pros and Cons
Phenol-sulfuric-acid Masuko et al. (2005)	50 μL sample + 150 μL concentrated sulfuric acid + 30 μL 5% phenol (w/v) Incubation: 5 min at 90°C in a water bath, cooling down Measurement: at 490 nm	4.5–676 mg/L 0.988 R^2	+ Very fast (<15 min) + No mixing step + One heating step – Carcinogenic – Manual drying of the plates after incubation in the water bath
Sulfuric-acid w/o phenol Albalasmeh et al. (2013)	1 mL sample + 3 mL of concentrated sulfuric acid rapidly mixed in a test tube Shaking: vortex for 30 s Measurement: at 315 nm	10–70 mg/L 0.992 R^2	+ Non-carcinogenic phenol + Reduced measurement time and error + No extra heating step – Not tested for high throughput in micro titer plates – Small linear calibration range – Interference by protein and/or flavonoid impurities
Anthrone-sulfuric-acid Laurentin and Edwards (2003)	40 μL sample, cover plate with cling film, vortex gently Incubation: 15 min at 4°C (preparing reagent) 100 μL reagent (2 g/L anthrone in concentrated sulfuric acid), seal with plate sealer, vortex gently Incubation: 3 min at 92°C; 5 min at RT; 15 min at 45°C Measurement: at 630 nm	50–400 mg/L 0.982 R^2	+ Non-carcinogenic + Selective to hexoses – Requirements needed (e.g., cling film, acetat film) – Two heating steps – 11 main steps for preparing the assay – Time consuming preparation (∼2.5 h)
Carbazole Cesaretti et al. (2003)	50 μL sample, 200 μL (25 mM sodium tetraborate in sulfuric acid) Incubation: 10 min at 100°C in an oven, cooling down 15 min Carefully adding 50 μL (0,125% carbazole in ethanol) Incubation: 10 min at 100°C in an oven; cooling down Measurement: at 550 nm	20–2,000 mg/L 0.975 R^2	+ Detection of glycosaminoglycans (e.g., heparin, chondroitin and hyaluronic acid) – Two heating steps – Long preparation time (∼1 h) – Low interferences with hexoses
***m*-Hydroxydiphenyl** Van Den Hoogen et al. (1998)	40 μL sample + 200 μL (120 mM sodium tetraborate in sulfuric acid) Incubation: 1 h at 80°C Measurement: at 540 nm (background) 40 μL reagent (100 μL (100 mg/mL m-hydroxydiphenyl in dimethyl sulfoxide) mixed with 4.9 mL 80% (v/v) sulfuric acid just before use) Incubation: 15 min at room temperature Measurement: at 540 nm	12.5–200 mg/L	+ Detection of glycosaminoglycans + Background subtraction + No interference of 20-fold excess of neutral sugar to uronic acids + Lower hydrolysis temperature – Long preparation time (∼1.5 h) – Small linear calibration range
Phenol-sulfuric-acid coupled with glucose-assay Rühmann et al. (2015a)	20 μL gel-filtrate + 180 μL phenol-sulfuric-acid (30 μL 5% (w/v) phenol in ddH_2O + 150 μL concentrated sulfuric-acid, mixed before on ice) Shaking: 5 min at 900 rpm Incubation: 35 min at 80°C in an oven; cooling down Measurement: at 480 nm	50–5,000 mg/L 0,999 R^2	+ Background subtraction of remaining glucose after cultivation via glucose assay – One mixing step – Medium preparation time (∼1 h)

different monomeric, oligomeric, or polymeric carbohydrates within the samples. Taking this into account, the choice of a suitable cultivation medium is essential to obtain reliable results. Complex media containing oligomeric or polymeric carbohydrate compounds (like, e.g., yeast extract) might lead to falsely increased values. Therefore, these complex media compounds should be avoided. Remaining carbohydrates from the cultivation process can also negatively interfere with the measurement of the EPS content, since often high amounts of sugars are used as C-source for cultivation of EPS producing strains. Therefore, a precipitation step is usually necessary to separate the monomeric and oligomeric material from the polymeric precipitate. After precipitation the dissolved polymer can be applied to further measurements. However, both steps, precipitation and dissolving, are hardly suitable for HT-screening approaches. A different possibility would be to eliminate oligomeric substances in the media, to determine the remaining monomeric carbohydrates and to subtract this value from the total carbohydrate content. Ruijssenaars et al. (2000) investigated the biodegradability of extracellular polysaccharides and therefore, calculated the EPS content. This was done by measuring the total carbohydrate content via phenol-sulfuric-acid-method and subtraction of the reducing sugar concentration determined with dinitrosalicylic-acid (DNS) method (Miller, 1959). Another group adapted the DNS method toward a 96-well format (Negrulescu et al., 2012). Generally, the determination of reducing sugars is of special interest for the saccharification analysis of biomass and massive research is performed in this field. Whitehead et al. (2012) reported a completely automated analysis of reducing sugars via 3-methyl-2-benzothiazolinone hydrazone (MBTH). Other methods use enzymatic glucose oxidation, coupled with a color reaction to determine the remaining glucose after cultivation (Rühmann et al., 2015a). In combination with the total carbohydrate content this method

enables the selective detection of EPS without precipitation and dissolving. This method is designed for automated handling as well as for manual execution in 96-well format.

Conclusion

Different techniques for EPS screening approaches are available. Identification of EPS via colony morphology, dye based plate-assays, precipitation, and visual evaluation of viscosity are techniques, which do not require special equipment. They all have their own benefits and limitations, which have to be taken into account. During the past few years colorimetric assays have been more and more transferred to 96-well format to determine different sugars in various applications and to increase the throughput. Further development in the carbohydrate analysis via UHPLC and MS technology enables a fast and reliable determination of the carbohydrate fingerprint, which gives a more detailed overview of the monomeric composition. Additionally, automatization platforms are available and even further increase the throughput for screening of various strains. However, until now there are no HT-screening approaches available for the determination of the molecular weight distribution and/or rheological properties of microbial polymers. Development of such techniques might be hard to realize, but would drastically enhance our knowledge on microbial EPS. Other techniques, such as NMR, already give further information about the sequence of the repeating unit including linkages in-between these heteropolysaccharides. As soon as they are available in HT this would revolutionize the targeted screening of EPS.

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REVIEW
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Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies

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Bacteria produce a wide range of exopolysaccharides which are synthesized via different biosynthesis pathways. The genes responsible for synthesis are often clustered within the genome of the respective production organism. A better understanding of the fundamental processes involved in exopolysaccharide biosynthesis and the regulation of these processes is critical toward genetic, metabolic and protein-engineering approaches to produce tailor-made polymers. These designer polymers will exhibit superior material properties targeting medical and industrial applications. Exploiting the natural design space for production of a variety of biopolymer will open up a range of new applications. Here, we summarize the key aspects of microbial exopolysaccharide biosynthesis and highlight the latest engineering approaches toward the production of tailor-made variants with the potential to be used as valuable renewable and high-performance products for medical and industrial applications.

Keywords: bacterial exopolysaccharides, tailor-made exopolysaccharides, polysaccharide engineering, biosynthesis, gene clusters

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Introduction

Polysaccharides produced by microbes can be generally classified by their biological functions into intracellular storage polysaccharides (glycogen), capsular polysaccharides which are closely linked to the cell surface (e.g., K30 O-Antigen) and extracellular bacterial polysaccharides (for example, xanthan, sphingan, alginate, cellulose, etc.) that are important for biofilm formation and pathogenicity. This article will focus on the latter, also termed EPS, which are secreted to the surrounding environment, and therefore can be efficiently harvested from cell-free culture supernatant in a continuous and cost-effective manufacturing process. At present four general mechanisms are known for the production of these carbohydrate polymers in bacteria: (i) the so called Wzx/Wzy-dependent pathway; (ii) the ATP-binding cassette (ABC) transporter-dependent pathway; (iii) the synthase-dependent pathway and (iv) the extracellular synthesis by use of a single sucrase protein. The precursor molecules, which are necessary for the stepwise elongation of the polymer strands, are realized by various enzymatic transformations inside the cell, and follow in principle the same concept of producing activated sugars/sugar acids in the first three cases of different biosynthesis pathways. For the extracellular production, the polymer strand is elongated by direct addition of monosaccharides obtained by cleavage of di- or trisaccharides.

In the Wzx/Wzy dependent pathway individual repeating units, which are linked to an undecaprenol diphosphate anchor (C55) at the inner membrane, are assembled by several glycosyltransferases (GT's) and translocated across the cytoplasmic membrane by a Wzx protein

(so called flippase). In a next step their polymerization occurs at the periplasmic space by the Wzy protein before they will be exported to the cell surface (Cuthbertson et al., 2009; Morona et al., 2009; Islam and Lam, 2014). Transport of the polymerized repeat units from the periplasm to the cell surface has been shown to be dependent upon additional protein(s) assigned to the polysaccharide co-polymerase (PCP) and the outer membrane polysaccharide export (OPX; formerly OMA) families (Cuthbertson et al., 2009; Morona et al., 2009). All polysaccharides assembled by the Wzx/Wzy pathway have a highly diverse sugar pattern (up to four or five types of sugar within their chemical structure are common) and are therefore classified as heteropolymers (e.g., xanthan). All strains using this pathway carry the genes for the flippase (Wzx) and the polymerase (Wzy) within their extracellular polysaccharide operons.

The second pathway of bacterial exopolysaccharide biosynthesis is the ABC transporter dependent pathway which is mainly present in capsular polysaccharide (CPS) biosynthesis (Whitney and Howell, 2013). These polysaccharides do not really represent EPS, since they are still linked to the cell surface. Like the Wzx/Wzy dependent EPS, the CPS synthesized via the ABC-transporter dependent pathway are assembled by the action of GT's at the cytoplasmic face of the inner membrane, resulting in homopolymers when only a single GT-containing operon is involved, or in heteropolymers when multiple GT's are used for the assembly process (Whitney and Howell, 2013). The export across the inner membrane and translocation to the cell surface, however, is different as it is realized by a tripartite efflux pump like complex. The complex is composed of ABC-transporters spanning the inner membrane, and periplasmatic proteins of the PCP and OPX family (Cuthbertson et al., 2009; Morona et al., 2009). These proteins are closely related to OPX and PCP proteins involved in secretion process of the Wzx/Wzy pathway (**Figure 1**). CPSs produced via this pathway all carry a conserved glycolipid at the reducing terminus composed of phosphatidylglycerol and a poly-2-keto-3-deoxyoctulosonic acid (Kdo) linker. This represents one of the main differences of the Wzx/Wzy and the ABC dependent pathways. Just recently novel insights into the early steps in CPS biosynthesis were provided by new discoveries of the structure of this conserved lipid terminus (Willis and Whitfield, 2013; Willis et al., 2013).

The third pathway is the synthase dependent pathway, which secretes complete polymer strands across the membranes and the cell wall, and is independent of a flippase for translocating repeat units. The polymerization as well as the translocation process is performed by a single synthase protein, which in some cases (alginate, cellulose) is a subunit of an envelope-spanning multiprotein complex (Rehm, 2010). Synthase dependent pathways are often utilized for the assembly of homopolymers requiring only one type of sugar precursor. This is observed in curdlan biosynthesis for example, here only β-(1-3)-linked glucose is found in the polymer. Another example of a strict homopolymer is bacterial cellulose, consisting only of β-(1-4)-linked glucose units. In the case of alginates, the preliminary polymer is synthesized as polymannuronic acid, which is processed by different epimerases and further modifying enzymes to glucuronic/mannuronic acid block-polymers, which can differ in the ratio and sequence of G/M building blocks (Rehm and Valla, 1997). The biosynthesis of hyaluronic acid (HA) is catalyzed by a single enzyme (hyaluron synthase), which performs both steps, polymerization and secretion. Assembly of the polymeric disaccharide is realized by use of the two different precursors; glucuronic acid and GlcNAc (Chong et al., 2005). Therefore, HA synthesis differs from the other synthase dependent pathways, but at the same time shows a high degree of similarities at protein level.

Most of the enzymatic steps for exopolysaccharide precursor biosynthesis appear inside the cell while polymerization/secretion is localized in the cell envelope. But there also exist some examples of extracellular synthesized polysaccharides, such as, e.g., dextran or levan. The biosynthesis of these occurs via GT's, which are secreted and covalently linked to the cell surface (**Table 1**).

In alignment with the various EPS biosynthesis pathways, the chemical structure and material properties of the final polymers are quite variable (**Figure 2**). The genes involved in the different biosynthesis pathways encode various types of GT's, polymerizing and branching enzymes, as well as enzymes responsible for addition of substituents or modifications of sugar moieties. Not all steps in the various pathways are currently understood, and sometimes the differences between the pathways become less defined. The genes encoding these enzymes can be found in most of the EPS producing microbes clustered within the genome or on large plasmids (Finan et al., 1986; Rehm, 2010). This condensed appearance of several GT's and polymerizing as well as secreting enzymes (one to more than 23) facilitates the identification of EPS operons, even if only partially sequenced draft genomes are available (**Figure 3**). Even though many gene clusters responsible for EPS biosynthesis have been known for several years, the function and mode of action of most of the genes and proteins is not completely clarified. An overview of the most relevant, commercial available EPS, including the biosynthesis pathway they are produced by is given in **Table 1**.

Since the ABC transporter dependent pathway is mainly involved in the biosynthesis of (CPSs, only a schematic outline of its synthesis will be presented here, and the interested reader is referred to several outstanding reviews in this field (Whitfield, 2006; Cuthbertson et al., 2010; Willis and Whitfield, 2013).

General Strategies for Engineering of Bacterial Polysaccharides

Bacterial polysaccharides have diverse unique properties for food applications and are used as viscosifiers, stabilizers, emulsifiers, or gelling agents. Due to these valuable properties several studies were performed to genetically engineer the producing organisms in order to generate novel polysaccharide variants and to improve production. Putative targets for engineering are the molecular weight, composition and sequence of co-monomers as well as addition of substituents. Bacterial polysaccharides by their diversity inherently exhibit a tremendous design space toward the production of new valuable materials.

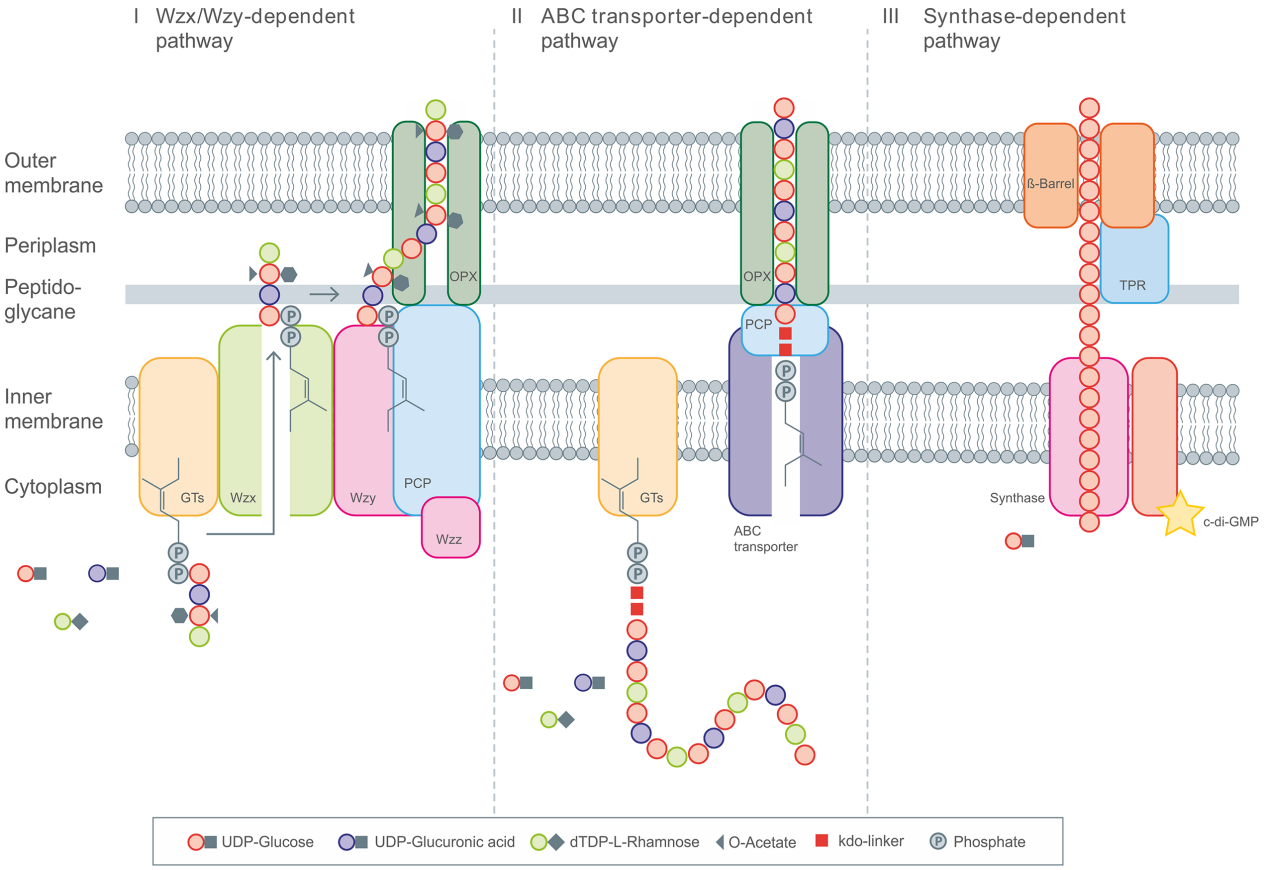

FIGURE 1 | General overview of the three different intracellular EPS biosynthesis pathways. (I) Display the Wzx/Wzy dependent pathway with the repeating unit assembled by various Glycosyltransferases (GT's) at the C55-lipid linker and the following translocation toward the periplasm by Wzx flippase. Polymerization occurs via Wzy polymerase and the polysaccharide co-polymerase protein (PCP). The PCP and OPX proteins realize the transport across the membranes. (II) The ABC transporter dependent pathway assembles the polysaccharide chain anchored on the poly-kdo-linker, which is then transported across the membranes and cell wall under involvement of PCP and OPX proteins. (III) The synthase dependent pathway realizes the polymerization and transport by the synthase complex, spanning the complete cell envelope, including the tetratricopeptide repeat (TPR) proteins.

Within the last years intensive research focused on providing insight into the mechanisms underlying bacterial exopolysaccharide biosynthesis pathways. High through-put genome sequencing, functional genomics, protein structure analysis and new bioinformatics tools aid toward identifying new EPS biosynthesis pathways and to understand the principles of EPS formation.

Depending on the purpose, engineering strategies can be subdivided into different categories. One goal of EPS production engineering is an increased volumetric productivity to cost-effectively produce the various EPS. These studies were mostly aiming at increasing the pool of sugar nucleotides (i.e., EPS precursors) to enhance the carbon flux toward the final polymer. In particular genes of precursor biosynthesis were overexpressed. This strategy was demonstrated to be successful for some EPS producers, but failed in some cases (Thorne et al., 2000; Videira et al., 2000; Huang et al., 2013; Schatschneider et al., 2013; Wu et al., 2014). Additionally, in some cases the overexpression of genes involved in the EPS assembly (e.g., GTs, Wzx, Wzy) resulted in enhanced yields and precursor conversion rates while in other cases it had a negative effect presumably due to distorting the multidomain protein complex involved in polymerization and secretion (van Kranenburg et al., 1999a). These approaches included overexpression of single genes as well as whole gene clusters (Pollock et al., 1996; Harding et al., 2011; Jones, 2012). Additionally the targeted engineering of regulatory proteins could increase productivity by increasing transcription of the operons, which encode the EPS biosynthesis proteins. Furthermore, the disruption of pathways competing for precursors used for EPS formation did also increase the productivity (Pena et al., 2002; Galindo et al., 2007). Single gene knock-outs were also described to enhance yield as well as to alter the chemical structure of the EPS (Nunez et al., 2009; Gaytan et al., 2012). Unfortunately, the titer of bacterial polysaccharides is limited in the production because the highly viscous polysaccharides have a massive negative influence on mass transfer (Seviour et al., 2011). However, the strategy to enhance productivity based on genetic engineering might be interesting for EPS with reduced viscosifying properties, for example due to lower molecular

TABLE 1 | Overview of the most relevant bacterial exopolysaccharides concerning monomer composition, substituent decoration, applications, and biosynthesis pathway routes.

EPS	Components	Substituents	Applications	Biosynthesis pathway	Reference
Alginate	GulA, ManA	Ace	Food, feed, medicine, research	Synthase dependent	Rehm and Valla (1997), Rehm (2010)
Cellulose	Glc		Food, medicine, acoustics	Synthase dependent	Ross et al. (1987), Rehm (2010)
Colanic acid	Glc, Fuc, GlcA, Gal	Ace, Pyr	N.a.	Wzx/Wzy dependent	Goebel (1963), Stevenson et al. (1996), Rehm (2010)
Curdlan	Glc		Food, cosmetics, medicine, construction chemistry	Synthase dependent	Stasinopoulos et al. (1999), Rehm (2010)
Dextran	Glc		Medicine, chromatography	Extracellular, dextransucrase	Dols et al. (1998), Rehm (2010)
Diutan	Glc, Rha, GlcA,	Ace	Construction chemistry,	Wzx/Wzy dependent	Plank (2005), Pollock (2005)
Gellan	Glc, Rha, GlcA	Ace, Gly	Construction chemistry, food, feed	Wzx/Wzy dependent	Pollock (1993), Plank (2005)
Hyaluronic acid	GlcA, GlcNAc		Medicine, cosmetics	Synthase dependent	Thonard et al. (1964), Dougherty and van de Rijn (1994), Rehm (2010)
Levan	Fru, Glc		Food (prebiotic), feed, medicines, cosmetics, industry, glue	Extracellular, Levansucrase	Ceska (1971), Combie (2003), Srikanth et al. (2015)
Succinoglycan	Glc, Gal	Ace, Pyr, Suc	Oil industry, cosmetics	Wzx/Wzy dependent	Glucksmann et al. (1993b), Becker et al. (1995a)
Welan	Glc, Rha, GlcA, Man	Ace	Construction chemistry,	Wzx/Wzy dependent	Plank (2005), Pollock (2005)
Xanthan	Glc, Man, GluA	Ace, Pyr	Food, feed, technical applications, oil drilling	Wzx/Wzy dependent	Ielpi et al. (1981), Vorholter et al. (2008), Rehm (2010)

Colanic acid represents an EPS with no commercial application, but is of high interest due to pathogenicity studies. Glc, glucose; Rha, rhamnose; Fuc, fucose; Fru, fructose; Gal, galactose; Man, mannose; GlcA, glucuronic acid; ManA, mannuronic acid; GulA, guluronic acid; GalA, galacturonic acid; GlcNAc, N-acetyl-glucosamine; Pyr, pyruvate; Ace, acetate; Gly, glycerate; Suc, succinate, N.a., not announced.

weight. The optimization of manufacturing process parameters might be more promising than engineering EPS biosynthesis for many established industrial EPS producers. The highest titers of highly viscous EPS such as xanthan are around 30–50 g/L (Sieber et al., 2006; Hublik, 2012) and represent the current maximum amount, which is manageable by existing bioprocess units.

Another strategy of engineering EPS biosynthesis is aiming at tailor-made variants with desirable material properties for medical and industrial applications. Here the aim is to alter the molecular structure and therefore the behavior and material characteristics of the final polymer. For example these modifications can be based on deleting substituents or monomeric sugars from the side chain. On the other hand new or more substituents might be attached to change the ratio of decoration. Most efforts were done in engineering the degree of acetylation and pyruvylation of various polymers, in order to control their rheological behavior (Hassler and Doherty, 1990; Donati and Paoletti, 2009). Additionally an altered degree of pyruvylation results in varied charge density of the polysaccharide (Skjåk-Bræk et al., 1989). Targeted modification of the molecular weight via overexpression or mutation of genes involved in the polymerization/degradation process (e.g., synthase, Wzy, PCP/lyases, glucosidases) represents another possibility to adjust rheology of the final product and was reported for some EPS (Rehm, 2010; Diaz-Barrera et al., 2012; Galván et al., 2013).

The early engineering approaches of xanthan biosynthesis as performed by Hassler and Doherty (1990) gave interesting insights in general structure-function relationships. The strongest influence on rheology was observed by altering the substituent decoration. The degree of acetylation and pyruvylation has opposite effects on viscosity. A high degree of pyruvylation resulted in higher viscosity, whereas more acetyl groups decreased viscosity of the resulting EPS. This finding is a general rule for polysaccharides and can be used in tailoring the viscosity of EPS. The degree of acetylation can be adjusted by *in vivo* as well as *in vitro* approaches or even process parameters applied during the production process (Skjåk-Bræk et al., 1989; Pena et al., 2006; Donati and Paoletti, 2009; Diaz-Barrera et al., 2010; Gaytan et al., 2012; Rehm, 2015). Further engineering approaches with respect to the production of xanthan variants included the targeted engineering of the length of the side chain. These approaches are of great interest, since they might be transferred to other EPS variants. A truncated tetramer xanthan version, obtained by deletion of the terminal mannose via inactivation of the GT GumI results in a much lower viscosity. The further removal of the glucuronic acid from the side chain by inactivation of GumK (a GT) resulted in enhanced viscosity compared to the wild type when measured as a function of concentration (Hassler and Doherty, 1990). The influence of acetylation on viscosity of the truncated versions showed an irregular result. The acetylated polytetramer version showed decreased viscosity as observed for the wild type xanthan. The acetylation of the polytrimer (only one mannose as side chain) resulted in similar viscosity as the non-acetylated version. Pyruvylation of the outer mannose also blocks acetylation of this sugar, therefore enhancing the viscosity. In the wild type xanthan gum acetylation of the inner or the outer mannose showed similar viscosities, which indicated that the extend of acetylation affected viscosity,

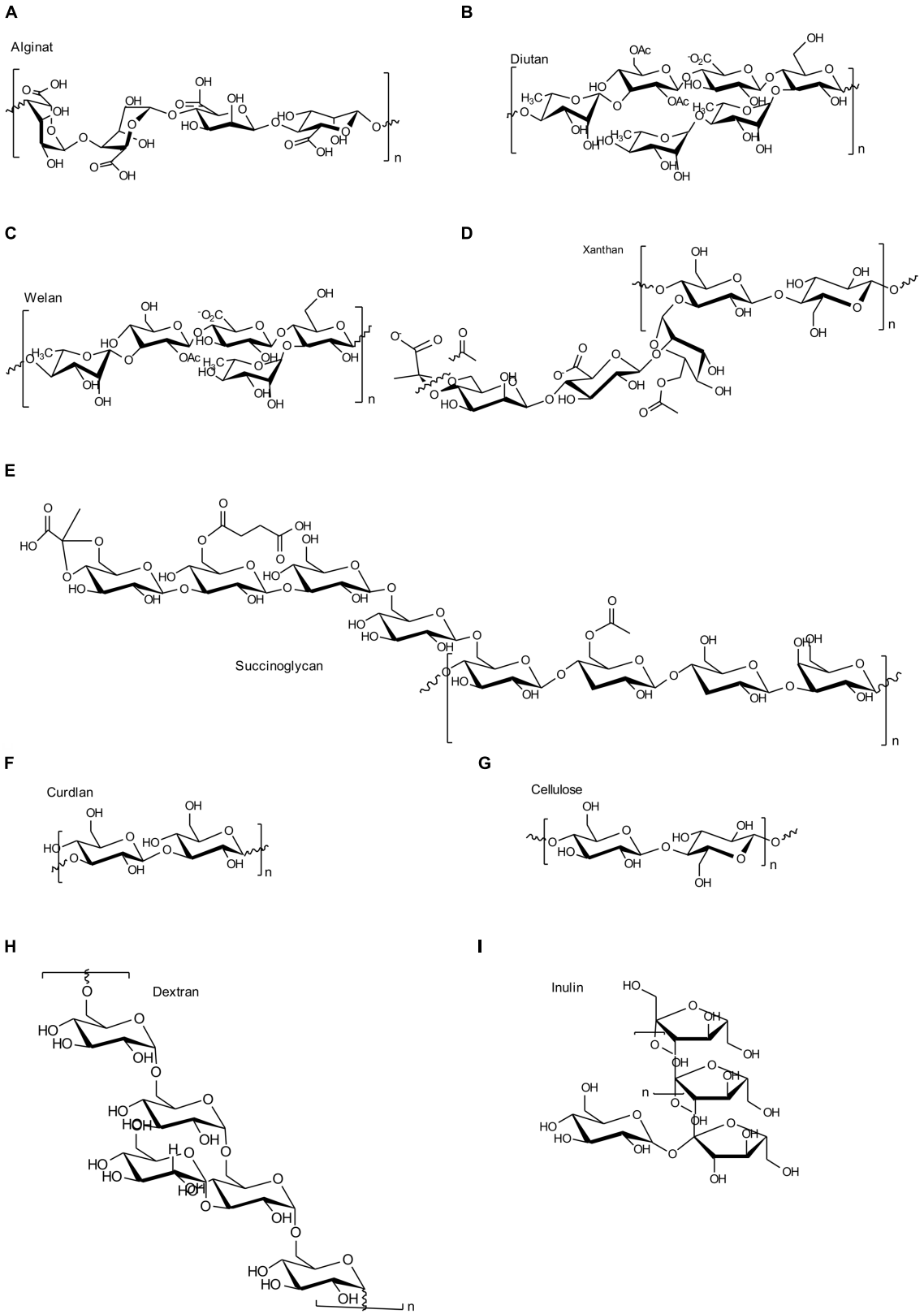

FIGURE 2 | Chemical structures of the most important EPS as described in this manuscript. (A) Alginate; **(B)** Diutan; **(C)** Welan; **(D)** Xanthan; **(E)** Succinoglycan; **(F)** Curdlan; **(G)** Cellulose; **(H)** Dextran; **(I)** Inulin like fructan.

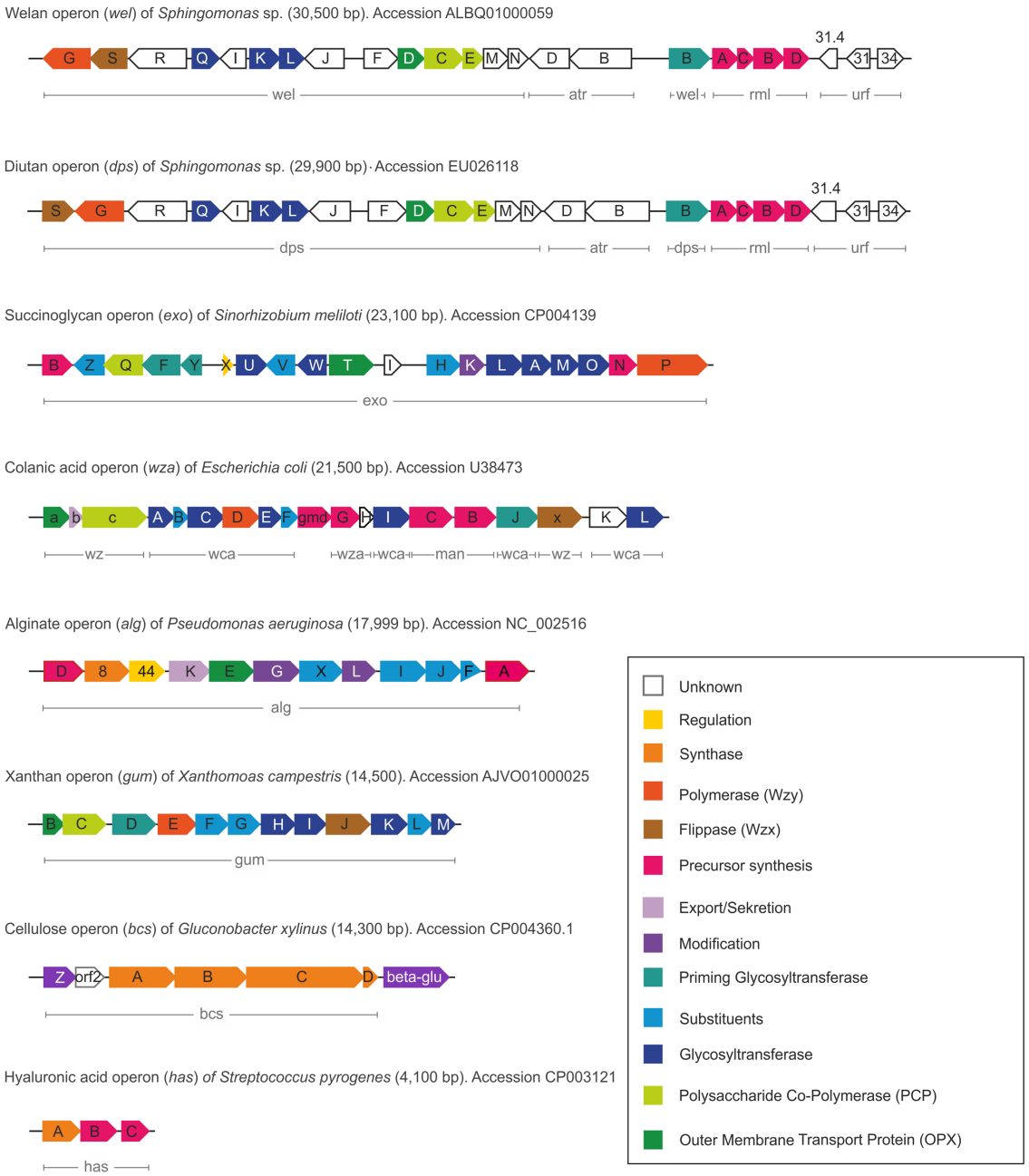

FIGURE 3 | Comparison of the different gene clusters including functions of the various encoded proteins.

but the position within the polymer is less critical. Whether the change in viscosity results from the substituents itself, or from conformational changes of the polymers remains elusive up to now. Several studies describe the occurrence of conformational changes by side chain pyruvylation and acetylation (Morris et al., 1977; Lecourtier et al., 1986; Muller et al., 1986; Liu et al., 1987). Just recently, the molecular weight of xanthan was synthetically adjusted by controlling the expression level of the Wzy polymerase GumE (Galván et al., 2013). For alginate a similar effect was observed by an overexpression of alginate polymerase *alg8/alg44* in *Azotobacter vinelandii* resulting in a high molecular weight alginate variant (Diaz-Barrera et al., 2012).

Relatively little information is available on EPS with varying monomer composition. The transfer of complete gene clusters toward alternative host strains was reported to result in altered compositions of the repeat units (Pollock et al., 1997; van Kranenburg et al., 1999a). This effect might result from the different enzymatic equipment of the host strains for nucleotide precursor synthesis (Stingele et al., 1999). These heterologous expression strategies were mostly combined with lowered

production levels of the foreign polymers (Stingele et al., 1999). Complementation experiments of single GTs and further proteins involved in the biosynthesis pathway were performed (van Kranenburg et al., 1999a,b). These results showed a relatively broad specificity of the Wzx and Wzy proteins in regard of the chemical structure of the repeating units in several strains, indicating a high potential for modifying these and still using the same secretion and polymerization machinery.

Exopolysaccharides Produced Via the Wzx/Wzy-Dependent Pathway

Xanthan – A Highly Diverse Heteropolysaccharide with Long Side Chain

Xanthan gum as produced by *Xanthomonas campestris* consists of a cellulose like backbone [β-(1-4)-linked glucose] and a side chain made of two mannose units and one glucuronic acid (Jansson et al., 1975; **Figure 2D**). Xanthan is produced by the two precursor's glucose- and fructose 6-phosphate, the key intermediates of the central carbohydrate metabolism. At the moment five different genomes of *X. campestris* pv. *campestris* are available (Schatschneider et al., 2013). Comparative genomics of three of these identified a common core genome of about 3,800 genes, with a diverse amount (∼500) of unique genes, but simultaneously highly conserved xanthan operon and *xanAB* genes (precursor synthesis; Vorholter et al., 2008). Just recently the draft genome of *X. campestris* NRRL B-1459 (ATCC 13951) was published which might further enhance the insights in conserved xanthan biosynthesis pathway (Wibberg et al., 2015). *X. campestris* is capable of utilizing a vast amount of carbohydrates (Vorholter et al., 2008) and several transcriptomic and genome wide analytical approaches were performed for *X. campestris* strains (Chung et al., 2007; Serrania et al., 2008; Vorholter et al., 2008; Zhou et al., 2011; Li et al., 2014). Just recently there was published a large scale *in silico* based metabolomics network, verified by experimental data (Schatschneider et al., 2013). This model gave further insight into stimulated growth and xanthan production in complete accordance with the experimental data for xanthan as well as biomass production. This verified model represents the first one focusing on microbial polysaccharide biosynthesis and might dramatically enhance the knowledge for generalized enhanced product titers.

The biosynthesis pathway as encoded by the *gum* cluster comprises 13 genes involved in assembly of the repeat unit, polymerization, translocation as well as decoration with substituents. The bifunctional genes providing the nucleotide precursors (*xanAB*) are not located within the gum-cluster. In detail, the assembly of the pentasaccharide repeating unit starts with the transfers of the first glucose unit toward the phosphorylated lipid linker (C55) anchored to the inner membrane via the priming GT GumD. In a next step, the cytosolic GT GumM attaches the second glucose unit by a β-(1-4)-bond to the first glucose. Catalyzed by GumH the first mannose unit is linked by an α-(1-3)-glyosidic bond, followed by the cytosolic glycosyltransferase activity of GumK which adds a β-(1-2)-linked glucuronic acid. Finally the repeating unit is completed by action of GumI, attaching the terminal mannose via a β-(1-4)-bond. In general most of the GTs involved in biosynthesis of EPS following the Wzx/Wzy–pathway appear to be monofunctional and the same applies specifically also for the xanthan biosynthesis (Breton et al., 2006). The genes encoding GumF, GumG, and GumL are known to be involved in acetylation and pyruvylation of the repeating units of xanthan. The GumL protein is known to incorporate pyruvyl residues to the external β-mannose, while the acetyl residues are incorporated into the internal α-mannose by GumF, and into the external β-mannose by GumG (Becker et al., 1998). Whether the decoration with substituents occurs before the spatial reorientation toward the periplasm or within the periplasm is not elucidated up to now. In some cases there was observed a decreased activity of the GT's when acetylated precursors were used as in the case of GumK (Barreras et al., 2004). These findings might indicate that at least a final repeat unit is necessary for decoration, but the last proof of spatial action of GumFGL still remains speculative.

The translocation process of the complete repeating unit is realized by the flippase GumJ (Wzx-protein) at which the repeat unit is still linked to the C55 anchor, which might play an important role in the targeted transport of the repeating unit (Islam and Lam, 2014).

The general topology of Wzx proteins shows several transmembrane helices (TMHs), 10 in the case of GumJ (Vorholter et al., 2008). Data of tertiary structure, which will give further insights into the mechanism and functionality of these highly complex membrane proteins, is still missing and only a low amount of homology models is available (He et al., 2010; Islam and Lam, 2014). Polymerization of the translocated repeating units occurs via the action of GumE, a membrane protein, showing 12 TMHs and a large periplasmatic loop as described for other Wzy-proteins (Whitfield, 2006; Vorholter et al., 2008; Islam et al., 2010; Marczak et al., 2013). The exact mechanism as well as substrate specificity of GumE and most other Wzy-proteins remain elusive up to now.

A putative adaption mechanism toward the length, as well as acceptance of repeating units with modified side chains was observed for some Wzy-proteins, characterizing them to be well suited for acceptance of tailored repeating units as obtained by genetic engineering (Nyman et al., 1979; Reeves et al., 2013; Islam and Lam, 2014).

The PCP proteins as present in the Wzx/Wzy pathway are assumed to be responsible for chain length control of the final polymer (Cuthbertson et al., 2009) and much more information is available compared to Wzx and Wzy proteins, even on structural level (Islam and Lam, 2014; Schmid and Sieber, 2015). GumC as inner membrane protein belongs to the PCP-2a sub-family. These proteins are distinguished by their common topology, which consist of a large periplasmatic domain, flanked by two transmembrane fragments. The PCP-2a sub-family normally shows an additional C-terminal cytoplasmic kinase domain, which is not the case for GumC topology (Cuthbertson et al., 2009; Galván et al., 2013). This domain is normally autophosphorylated at several tyrosine residues and

seems to be essential for assembly of high molecular weight EPS, rendering GumC to be somehow different (Cuthbertson et al., 2009; Bianco et al., 2014). For further reinforcement of this finding, no kinase partner has been identified in the *X. campestris* genome (Cuthbertson et al., 2009). The general characteristics of the different gum genes as identified in *X. campestris* are given in **Table 2**.

Just recently the crystal structure of the soluble form of GumB was published, revealing its structure to be a tetramer of ∼100 kDa (Jacobs et al., 2012; Bianco et al., 2014). GumB represents the corresponding OPX proteins as necessary for the final stage of polymer secretion (Vorholter et al., 2008). GumB is an OPX protein containing the polysaccharide export sequence (PES) motif which is characteristic for OPX proteins and can be categorized to the OPX-C family as defined by Cuthbertson et al. (2009). Interestingly there are no transmembrane regions identified by *in silico* prediction, but GumB is located in membrane fractions as identified by subcellular location experiments (Galván et al., 2013). The OPX and PCP protein (GumB and GumC) comprise a molecular scaffold that spans the cell envelope (Cuthbertson et al., 2009). Early engineering approaches already revealed the absolutely necessity of *gumB* and *gumC* in xanthan biosynthesis. No xanthan production was observed when *gumB* or *gumC* were inactivated, but assemblage of the repeat unit was still realized (Katzen et al., 1998). Co-overexpression of *gumB* and *gumC* results in higher molecular weight xanthan as well as higher viscosity, therefore indicating direct interaction of both proteins (Galván et al., 2013). Even if more and more information of the interplay of GumB and GumC is available, there would still be the need for further experiments to elucidate the interaction and functionality of this trans- periplasmic/membrane spanning complex.

Sphingan – A Family of Similar but Different Heteropolysaccharides

Different heteropolysaccharides with closely related chemical structures, but strongly differing material properties belongs to the family of sphingans as produced by several *Sphingomonas* and *Pseudomonas* strains, (Pollock, 2005). The backbone of most sphingans is composed of Rha-Glc-GlcA-Glc (Pollock, 2005) with small variation in the sugar composition of the backbone (Rha or Man) as well as the side chains, when existent (Jansson et al., 1983, 1986; O'Neill et al., 1983; Jansson and Widmalm, 1994). EPS included in the sphingan family are gellan, welan, diutan, rhamsan, S-7 and S-88 (Pollock, 2005). The differences in the chemical structures are encoded in differently composed gene operons, as just recently reviewed (Schmid et al., 2014b). The genes involved in the synthesis of the rhamnose precursor (*rmlABCD*) are placed on the highly conserved gene operon; the genes necessary for the other nucleotide sugar precursors are randomly distributed within the genome (Harding et al., 2004; Wang et al., 2012a). The assembly of the repeat unit of the different sphingans follows a strict procedure, encoded in the corresponding sphingan operons. In the case of the three sphingans, gellan, welan and diutan, the genes involved in the biosynthesis are named according to the corresponding polymer, *gel* for gellan, *wel* for welan and *dsp* for diutan, respectively. To facilitate the general description of the biosynthesis of this highly similar EPS the introduction of gene nomenclature *spn*, for genes involved in sphingan biosynthesis was suggested and will be used here (Schmid et al., 2014b). The assembly of the identical backbone of the repeat unit for gellan, welan and diutan occurs by the transfer of glucose toward the C55-anchor, via the priming glycosyltransferase SpnB. In a next step, the glucuronic acid is linked toward the

TABLE 2 | General characteristics of the several *gum* genes as present in the *xanthan gum*-cluster from *Xanthomonas campestris*.

Gene	Length (aa)	Localization	Protein family	Mechanism	Additional information	Reference
GumM	263	Cytosol	CAZY 26	Transferase, inverting	PFAM WecB-GT family	Becker et al. (1998), Vorholter et al. (2008)
GumH	380	Cytosol	CAZY 4	Transferase, retaining	PFAM GT family 1	Becker et al. (1998), Vorholter et al. (2008)
GumK	400	Membrane	CAZY 70	Transferase, inverting	Membrane associated	Barreras et al. (2004), Vorholter et al. (2008)
GumI	349	Membrane	CAZY unclassified	Transferase, putative retaining	GT-B, Monotopic	Becker et al. (1998), Vorholter et al. (2008)
GumJ	492	Membrane	Wzx	Flippase	PFAM PS-biosynthesis protein family (10 TMHs)	Becker et al. (1998), Vorholter et al. (2008)
GumE	428	Membrane	Wzy	Polymerase	10–12 TMHs predicted	Becker et al. (1998), Vorholter et al. (2008)
GumC	479	Membrane	PCP2a	Export	Oligomeric, no glycosylation	Vorholter et al. (2008), Galván et al. (2013)
GumB	232	Membrane	OPX-C	Export	Tetramer, PES domain	Jacobs et al. (2012), Galván et al. (2013)
GumD	484	Membrane	Undecaprenyl-Glc-GT	Transferase	Priming GT	Ielpi et al. (1981, 1993)
GumL	264	Membrane?	Pyruvyltransferase	Transferase		Harding et al. (1987), Becker et al. (1998), Vorholter et al. (2008)
GumF	364	Membrane	Acetyltransferase	Transferase	PFAM family 3, 9 TMHs	Vorholter et al. (2008)
GumG	351	Membrane	Acetyltransferase	Transferase	PFAM family 3, 9 TMHs	Vorholter et al. (2008)

Length in amino acids is given for the proteins as identified in Xanthomonas campestris JX.

priming glucose by a β-(1-4)-bond catalyzed by SpnK. As a third glycosyltransferase involved in the assembly process, SpnL transfers the second glucose to the nascent repeat unit and finally SpnQ transfers the rhamnose unit by linking it via an α-(1-3)-bond (**Figure 4**).

The next steps are different in gellan, welan and diutan. Gellan represents the unbranched version of sphingans, which only shows substituent decorations of glycerol and acetyl at the second of the two glucose units of its backbone.

Welan is described to carry only one acetyl group as substituent, and a side branch of α-(1-3)-linked rhamnose or mannose in the ratio of 2:1 as present at the first glucose of the repeat unit (Stankowski and Zeller, 1992; Jansson and Widmalm, 1994). Diutan has a dimeric L-rhamnose side chain attached to the first glucose residue of the growing repeating unit (Chowdhury et al., 1987) and two acetyl groups are attached per repeating unit to the C2 and C6 positions of the second glucose in the repeating unit (Diltz and Zeller, 2001; **Figures 2C,D**). The genes involved in incorporation of the side chains for welan and diutan have not been exactly functionally assigned at present. But the genes *urf31.4*, *urf31*, and *urf34*, which are labeled as "unknown reading frames" are assumed to be involved (Coleman et al., 2008). These findings are in accordance with the different amount of *urf* genes present in the *spn* operons encoding the differently branched sphingan variants (Harding et al., 2003, 2004; Coleman et al., 2008; Schmid et al., 2014b). Up to now only one acetyltransferase, outside the *spn* operon was verified to be involved in acetylation of gellan (Harding et al., 2003).

Whether the addition of the side chain sugars and non-sugar substituents occurs as final step of repeat unit assembly or at the nascent repeat unit remains speculative up to now, but as observed for xanthan, it can be assumed that already decorated repeat unit intermediates might reduce the activity of the GT's involved in assembly of the repeat unit (Barreras et al., 2004). The next steps of sphingan biosynthesis follow the same order for all variants and include activity of the Wzx-protein flippase (SpnS), the Wzy-polymerase (SpnG) as well as the PCPs, which are thought to be SpnC and SpnE and seem to be involved in chain length

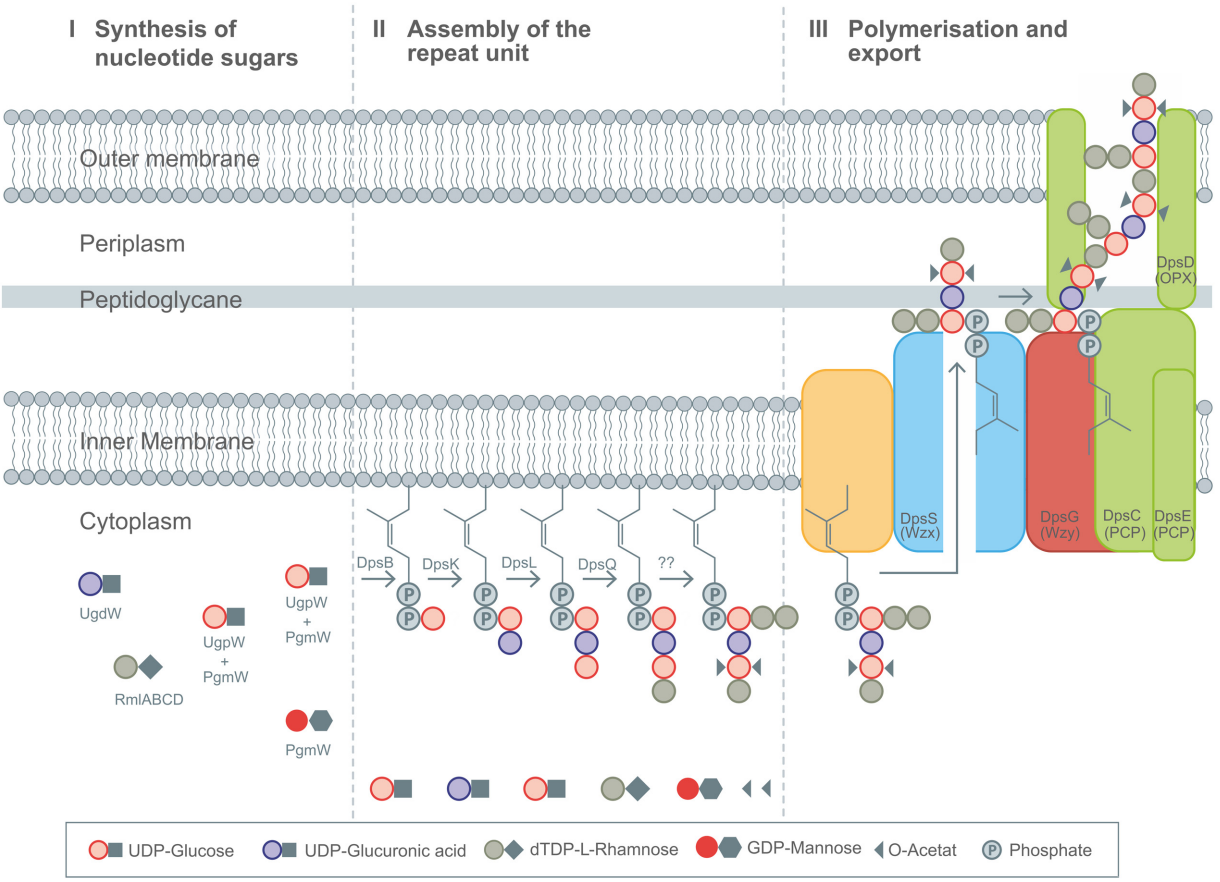

FIGURE 4 | Detailed description of the Wzx/Wzy-pathway as present in diutan biosynthesis by *Sphingomonas sp.* ATCC 53159. (I) Synthesis of nucleotide sugars in the cytoplasm. (II) Stepwise assembly of the repeating unit at the membrane bound C55 linker including decoration with substituents by action DpsBKL and DpsQ. The proteins involved in branching and transfer of acetyl groups remain elusive up to now. (III) Polymerization and translocation process across the membranes as guided by Wzx (DpsS), Wzy (DpsG), PCP (DpsC and DpsE), and OPX (DpsD) proteins. Figure modified according to Fialho et al. (2008), Schmid et al., (2014a,b), and Schmid and Sieber (2015).

regulation, having the typical kinase domains in their sequences (Moreira et al., 2004). The complete mechanism of their function is still speculative. Secretion of the finally polymerized sphingans occurs via OPX protein SpnD and the two PCP proteins which comprise a molecular scaffold spanning the cell envelope.

Succinoglycan – A Heteropolysaccharide with Large Repeating Unit

Succinoglycan (SG) is an acidic EPS produced by several *Rhizobium*, *Agrobacterium*, *Alcaligenes,* and *Pseudomonas* strains (Harada and Yoshimura, 1964; Zevenhuizen, 1997). The model organism for SG production is *Rhizobium meliloti* RM 1201. SG is a branched heteropolysaccharide consisting of an oligosaccharide repeat unit with several substituent decorations, such as succinate, pyruvate and acetate. The monomers included in the repeat unit are β-linked glucose and galactose in the ratio of 7:1 (**Figure 2E**). Pyruvate is present in stoichiometric ratio, whilst succinate and acetate decoration depends on strain origin and culture conditions (Stredansky, 2005). The pyruvate residues are linked at the positions O4 and O6 of the terminal glucose residue of the side chain. SG plays an essential role in plant symbiosis (Leigh and Walker, 1994) and next to its biological importance shows industrially attractive properties (Sutherland, 1990; Stredansky and Conti, 1999). Biosynthesis of SG uses a set of 19 genes, which are labeled as *exo* genes, and two additionally *exs* genes which are also involved in SG biosynthesis (Glucksmann et al., 1993b; Becker et al., 2002). *ExoC*, *exoB* and *exoN* are involved in the biosynthesis of the precursors UDP-galactose and UDP-glucose encoding the corresponding phosphoglucomutase, UDP-glucose-4-epimerase and UDP-pyrophosphorylase, respectively (Uttaro et al., 1990; Buendia et al., 1991; Stredansky, 2005). ExoY represents the priming GT, which initiates the synthesis of the repeating unit by transferring the galactosyl residue to the lipid carrier having a high similarity on nucleotide level to the priming GT GumD in xanthan biosynthesis. Interestingly ExoY needs the gene product of *exoF* to successfully transfer the galactose toward the lipid linker (**Figure 3**). The genes *exoA*, *exoL*, *exoM*, *exoO*, *exoU,* and *exoW* code for the GTs, subsequently elongating the octasaccharide repeating unit by addition of a glucose unit (Glucksmann et al., 1993a; Reuber and Walker, 1993). ExoP (PCP-2a), ExoQ (Wzy), and ExoT (OPX) represent the necessary translocation end polymerization machinery, including chain length control of SG (Becker et al., 1995b, 2002; Niemeyer and Becker, 2001). The ExoP protein has the typical periplasmic domain flanked by two transmembrane regions and an additional cytoplasmic domain. It shows autophosphorylating protein tyrosine kinase activity and is involved in molecular weight distribution of SG (Niemeyer and Becker, 2001) The transmembrane proteins ExoZ, and ExoH decorate the repeating unit with acetyl and succinyl substituents. ExoV protein is a ketalase transferring a pyruvyl group to the terminal glucose (Leigh et al., 1987; Glucksmann et al., 1993b). Interestingly, SG producing strains encode genomic information for extracellular endoglycanases (ExoK and ExsH), to reduce the high molecular weight of the product (Becker et al., 1993; York and Walker, 1997; Jones et al., 2007).

The majority of the 21 genes involved in SG biosynthesis are clustered on a megaplasmid (Glucksmann et al., 1993b; Becker et al., 1995a) and only *exoC* is located on the chromosome (Glucksmann et al., 1993a). The *exs* genes can be found adjusted to the *exo* genes upstream of the *exoB* gene. Two of the *exs* (*exsA*, *exsB*) genes were identified to be involved in SG biosynthesis, *R. melioti* strains carrying mutated *exsA* (high similarity to ABC-transporter) variants showed an altered ratio of high molecular SG to low molecular SG, indicating involvement of *exsA*, without further knowledge of the detailed function. *ExsB* gene product was shown to have a negative influence on SG biosynthesis, resulting in lowered product titers (Becker et al., 1995a). The phenomenon of plasmid based EPS operons is widespread, especially in the field of *Lactobacilli* (Kranenburg et al., 1997). Another gene cluster encoding the second EPS of *Rhizobium* (galactoglucan) is localized on the same megaplasmid, but more than 200 kb away (Charles and Finan, 1991; Becker et al., 1997). This EPS also consists of galactose and glucose, but in the ration of 1:1 (Chandrasekaran et al., 1994).

This phenomenon of the genetic equipment for the production of more than one EPS is also very widespread amongst microbes (Christensen et al., 1985; Sutherland, 2001; Wozniak et al., 2003b; Laue et al., 2008) and complicates the defined analysis of sugar moieties making up the polymer (Rühmann et al., 2015).

Colanic Acid

Colanic acid (CA) also is known as the M antigen and is described to be an EPS (Goebel, 1963). CA is mainly found in *Enterobacteria* and is made from a repeat unit of glucose, fucose, galactose, and glucuronic acid. Decorations of acetyl and pyruvate are present as substituents in non-stoichiometric amount (Grant et al., 1969; Garegg et al., 1971). CA biosynthesis has been linked to a cluster of 19 genes mainly named following the general nomenclature for polysaccharide biosynthesis genes as suggested by Reeves et al. (1996). The genes for synthesis of the fucose nucleoside sugar precursors are placed within the CA gene cluster (Stevenson et al., 1996; Stout, 1996). The genes *manB* and *manC* are directly involved in the biosynthesis mechanism of GDP-mannose, which is converted via a three-step pathway toward GDP-fucose (Stevenson et al., 1996; Andrianopoulos et al., 1998). These three steps are catalyzed by GDP-mannose dehydratase (GMD) followed by an epimerase and reductase reaction as catalyzed by the bifunctional *wcaG* gene, which encodes the fucose-synthase (Andrianopoulos et al., 1998; Albermann et al., 2000). The genes for the synthesis of the other nucleotide precursors can be found dispersed in the genome. The stepwise assembly of the repeat unit is initiated via the action of WcaJ, which will transfer the first glucose unit toward the C55 lipid carrier (Johnson and Wilson, 1977; Patel et al., 2012). The next sugar monomers will be transferred by the action of WcaA, WcaC, WcaE, WcaI, and WcaL. The order of synthesizing steps is not completely clarified and mainly is based on sequence similarities and not on biochemical experiments (Stevenson et al., 1996). Due to its

location within the fucose synthesizing genes, WcaI might be involved in transfer of fucose units (Stevenson et al., 1996). The structural similarity as found for WcaL suggest involvement in transfer of galactose or glucuronic acid (Stevenson et al., 1996).

For WcaB and WcaF a high similarity with the family of acetyltransferases is observed, but no precise role of WcaB or WcaF in acetylation process or explanation for presence of two acetyltrasnferases is given up to now (**Figure 3**). The Wzx protein was identified within the CA gene cluster by its typical transmembrane segments and the large periplasmic loop. WcaD is predicted to span the inner membrane with nine transmembrane segments, and to polymerize the repeat units of CA (Stevenson et al., 1996), therefore representing the Wzy polymerase. The OPX protein involved in the secretion process in concert with the PCP proteins is encoded by *wza* and can be categorized as OPX group A protein, which can functionally replace its homolog in K30 biosynthesis pathway (Reid and Whitfield, 2005; Cuthbertson et al., 2009). Wzc forms the typical contiguous molecular scaffold that spans the cell envelope together with Wza and belongs to the PCP-2a family. The Wzb protein represents the protein tyrosine phosphatase, which controls the phosphorylation state of Wzc, the corresponding tyrosine kinase. The detailed regulatory interactions between Wzb and Wzc were recently characterized for the first time (Temel et al., 2013). Several characterization and mutation experiments were performed for the K30 analog of Wza and Wzc, giving further insights into mechanism and structure (Whitfield, 2006; Cuthbertson et al., 2009; Willis and Whitfield, 2013; Willis et al., 2013).

Exopolysaccharides Produced Via the Various Synthase-Dependent Pathways

Homopolysaccharides

Curdlan, A Bacterial β-(1-3)-Glucan

Curdlan is a water insoluble β-(1-3)-glucan (glucose homopolymer) without any substituents, produced by, e.g., *Agrobacterium* (**Figure 2F**). Four genes are involved in curdlan biosynthesis (*crdA*, *crdS*, *scrdR,* and *crdB*). The curdlan synthase (CrdS), is the key enzyme of curdlan biosynthesis, showing a high similarity to cellulose synthases (Stasinopoulos et al., 1999). The lack of experimental characterization of curdlan synthase makes it difficult to determine its mechanism of biosynthesis and secretion (Whitney and Howell, 2013). Many genomic as well as transcriptomic and proteomic information is available for curdlan producing *Agrobacterium* strains (McIntosh et al., 2005; Zheng et al., 2007; Jin et al., 2008; Wu et al., 2008; Ruffing and Chen, 2010, 2012; Ruffing et al., 2011; Jin and Lee, 2014). Results as obtained by that approaches displayed metabolic structures and pathway distributions indicating that energy efficiency, rather than substrate availability is the major constraint for improving curdlan yield (Zheng et al., 2007). Curdlan displays an EPS of high industrial interest and product titers of around 70 g/L were reported (Wu et al., 2008). The biosynthesis via the CrdS is believed to occur by the repetitive addition of glucosyl residues from the sugar nucleotide donor UDP-glucose to form polymeric β-(1-3)-glucan chains (Hrmova et al., 2010). The CrdS is confined within cell membranes and belongs to GT2 family of glycosyltransferases, predicted to adopt a GT-A fold and using an inverting reaction mechanism that is mediated through a single displacement reaction via a glycosyl-enzyme intermediate (Coutinho et al., 2003). First insights into the putative structure and mechanism were obtained and might enhance the understanding of curdlan synthases in the future (Hrmova et al., 2010). Additionally a cell-free protein synthesis was recently realized for the curdlan synthase in nanodiscs and was followed by X-ray scattering to obtain further structural information (Periasamy et al., 2013).

Cellulose, A Bacterial β-(1-4)-Glucan

Cellulose is a major component of several bacterial biofilms and has been increasingly considered as biomaterial for medical applications. The first description of cellulose synthesizing (*celS*) genes from *Acetobacter xylinum* (*acsABCD*) was given in Wong et al. (1990). These encoded proteins showed very low sequence identity to the corresponding plant homologues (<30%) of the cellulose synthases. The bacterial cytoplasmic membrane cellulose synthase (Bcs) proteins also belong to the GT2 family and are composed of three subunits (BcsA, BcsB, and BcsC; Ross et al., 1991). In some bacterial species BcsA and BcsB can be found fused as a single polypeptide (Umeda et al., 1999; Kawano et al., 2002). Cellulose biosynthesis occurs by polymerization of UDP-glucose nucleotide sugar precursors (Umeda et al., 1999). *BcsA* encodes the catalytic subunit of cellulose synthase and binds UDP-glucose to guarantee supply of monomers for polymerization. BcsB represents the regulatory subunit of the synthase complex, whereas the functional role of BcsC and BcsD are not functionally assigned yet. BcsC is proposed to function as pore formation protein to enable cellulose secretion, whereas BcsD seems to be involved in control of crystallization process of cellulose nanofibrils (Lee et al., 2014). Additionally, the genes *bcsZ* encoding for an endo-β-(1-4)-glucanase and the *orf2* encoding for the "cellulose completing protein" are placed up-stream of the cellulose synthase operon (Kawano et al., 2002). Both genes are essential for cellulose biosynthesis (Kawano et al., 2002). The function of *orf2* has not been determined up to now, but is essential in cellulose synthesis (Standal et al., 1994). Located downstream from the cellulose synthase operon is a gene encoding a β-glycosidase, which hydrolyzes glucose units consisting of more than three monomers and has an essential role in cellulose production (Kawano et al., 2002). The cellulose synthesizing operon as recently identified in *Gluconacetobacter xylinus* E25 (Kubiak et al., 2014) is given in **Figure 3**. The typical GT-A family catalytic fold of cellulose synthase has been verified by crystallization experiments (Cantarel et al., 2009) and is composed of two juxtaposed β/α/β-folds which form a β-sheet surrounded by α-helices (Lairson et al., 2008). Morgan et al. (2013) the crystal structure and the reaction mechanisms of the cellulose synthase CelS (A and B subunits) from

Rhodobacter sphaeroides were solved. It was shown that the large central domain of BcsA contains a single UDP-glucose binding site, and that cellulose synthesis occurs within the cytosolic domain of BcsA. BcsB is located in the periplasm and might assist in guidance of the cellulose chain during the secretion process (Slabaugh et al., 2014). Several functional motifs as well as the function of different conserved amino acids were elucidated (Sethaphong et al., 2013). The GT-A fold comprised the motifs DDG and DCD, which coordinate the UDP as well as the essential divalent cation. An internal helix, which is very close to these conserved motifs, interacts with the cellulose acceptor substrate. These findings supported the hypothesis of cellulose elongation at the non-reducing end, by a single active side. The characteristic cellulose structure (**Figure 2G**) can be explained for the first time by the steric environment presented by the preceding glucose and the β-(1-4)-linkage, thus reversing the direction of terminal glucose rotation with every additional glucose monomer (Slabaugh et al., 2014).

Heteropolysaccharides

Alginate, A Gel-Forming Exopolysaccharide Produced Via an Envelope-Embedded Multiprotein Complex

Alginates are EPS made of variable amounts of (1-4)-linked β-D-mannuronic acid and its C5-epimer α-L-guluronic acid (**Figure 2A**). These comonomers are arranged in blocks of continuous mannuronic acid residues (M-blocks), guluronic acid residues (G-blocks), or as alternating residues (MG-blocks; Rehm and Valla, 1997; Gutsche et al., 2006). Alginates are synthesized by brown seaweeds and by bacteria belonging to the genus *Pseudomonas* and *Azotobacter* (Rehm, 2009).

The arrangement and sequence of comonomer residues and in particular the formation of G-blocks were similar in algal and *A. vinelandii* alginates. However, alginates derived from *pseudomonads* do not contain G-blocks (Skjak-Braek et al., 1986). Based on these structural differences the various alginates adopt different material properties which align with their biological role such as, e.g., the formation of a tough cyste wall (a dormant stage) in *A. vinelandii* or the viscous biofilm matrix material in regard to *pseudomonads*.

The intracellular biosynthesis steps of the activated precursor GDP-mannuronic acid are well known (Hay et al., 2013, 2014). In contrast the molecular mechanisms underlying polymerisation and modification are less understood. Recent studies on secretion of alginate illuminated the underlying concepts based on the involvement of the secretion pore AlgE in the outer membrane of *Pseudomonas aeruginosa* (Rehm et al., 1994; Hay et al., 2010a; Whitney et al., 2011; Rehman and Rehm, 2013). Polymerisation and secretion are linked via an envelope-spanning multiprotein complex composed of at least six subunits (Alg8, Alg44, AlgG, AlgX, AlgK, AlgE) as was shown by protein–protein interaction and mutual stability studies (Gutsche et al., 2006; Hay et al., 2012, 2013; Rehman et al., 2013). A schematic overview of alginate biosynthesis pathway is given in **Figure 5**.

GDP-mannuronic acid biosynthesis starts from the central metabolite acetyl-CoA which is converted to oxaloacetate via the citric acid cycle. Oxaloacetate enters gluconeogenesis leading to fructose-6-phosphate which is then converted by alginate-specific enzymes into the activated alginate precursor GDP-mannuronic acid (Rehm and Valla, 1997). GDP-mannuronic acid is polymerized to alginate presumably by membrane-anchored Alg8, a GT, which is a subunit of the multiprotein complex (Remminghorst and Rehm, 2006b; Remminghorst et al., 2009). Only recently experimental evidence for the multiprotein complex spanning the cytoplasmic membrane (Alg8, Alg44), the periplasm (AlgX, AlgK, AlgG, AlgL) and the outer membrane (AlgE) was obtained (Rehman and Rehm, 2013). A scaffold of periplasmic proteins has been proposed to guide the nascent alginate chain through the periplasm for secretion by the alginate-specific channel protein AlgE in the outer membrane (Gutsche et al., 2006; Rehman and Rehm, 2013; Rehman et al., 2013). AlgE is linked to the periplasmic scaffold via an interaction with the lipoprotein AlgK which was found to interact with AlgX which links the periplasmic scaffold to the polymerase subunits via an interaction with Alg44. Alg44 has been described as co-polymerase which also binds c-di-GMP required for activation of alginate polymerisation (Remminghorst and Rehm, 2006a; Merighi et al., 2007). Recently, the membrane-anchored sensor protein MucR was found to be required to specifically activate alginate formation by generating a localized c-di-GMP pool likely in proximity to Alg44. However, regulation of alginate biosynthesis is highly complex and recruits a network and cascades of integrated regulatory processes as has been recently reviewed in Hay et al. (2014). It involves two-component signal transduction systems FimS/AlgR and KinB/AlgB, transcriptional regulation through various DNA-binding proteins, σ/anti-σ factors, posttranscriptional regulation through the Gac/Rsm sRNA system, posttranscriptional regulation through a natural antisense transcript (MucD-AS) that promotes alginate production by blocking the translation of *mucD* mRNA in addition to the previously mentioned c-di-GMP mediated activation. Directly following the model for xanthan biosynthesis there was published a genome-scale model for mapping the global effects of MucA on transcriptional level in *P. fluorescens*, giving great insights into correlation of alginate biosynthesis and biomass production (Borgos et al., 2013).

Bacterial alginate production could be increased by overexpressing biosynthesis genes and by inactivation of negative regulators (Hay et al., 2010b). Controlling expression of alginate modifying enzymes (lyases, epimerases, acetyltransferases) as well as their use *in vitro* enables the production of alginates with tailored molecular weight, M/G arrangement and acetylation degree, i.e., alginates exhibiting a range of desirable material properties can be obtained (Rehm, 2010). Future research will target to engineer stable and improved bacterial production strains toward biotechnological production of a wide range of defined alginates suitable for high value medical applications (Rehm, 2005). The unique material properties of alginates has been already harnessed for variety of industrial applications

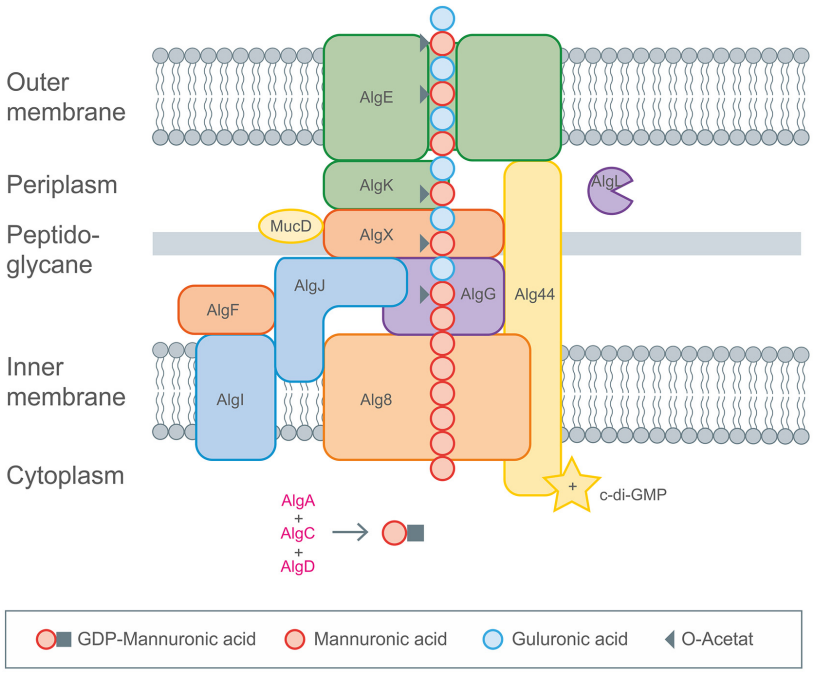

FIGURE 5 | Detailed description of alginate biosynthesis as performed by *Pseudomonas aeruginosa*. The activated mannuronic acid precursors obtained by cytoplasmic enzyme activities are polymerized by the membrane anchored GT Alg8. The transport across the membranes occurs by the inner membrane spanning protein complexes (Alg8, Alg44), the periplasm spanning (AlgX, AlgK, AlgG, AlgL) and the outer membrane (AlgE) proteins. Decoration with acetyl groups and epimerization toward glucuronic acid occurs via AlgIJX and AlgG, respectively. Figure modified according to Hay et al. (2013).

such as, e.g., stabilizing, thickening, and gelling agent in food production, or to immobilize cells in pharmaceutical and biotechnology industries (Paul et al., 1986). Commercial alginates are currently exclusively produced from brown seaweeds.

Hyaluronic Acid, A Heteropolysaccharide Following the Synthase Dependent Pathway

Hyaluronic acid or hyaluronan is a linear, extremely hydrophilic polymer of alternating β-D-glucuronic acid and β-D-*N*-acetyl-glucosamine residues linked via β-(1-4) and β-(1-3)-glycosidic bonds (Atkins and Sheehan, 1971; Toole, 2004; Weigel and DeAngelis, 2007). HA can be produced by vertebrates and prokaryotes and finds a wide range of applications especially in medicine and cosmetics due to its biocompatibility, high water retention capacity and excellent viscous behavior. The HA operon is composed of three genes, but for assembly of the polymer only the cytosolic membrane embedded HA synthase (HasA) is the key player (DeAngelis and Weigel, 1994; Chong et al., 2005; Liu et al., 2011). The genes *hasC* and *hasB* encode for UDP-glucose pyrophosphorylase, catalyzing the synthesis of UDP-glucose from UTP and glucose-1-phosphate and the UDP-glucose dehydrogenase activity, catalyzing the oxidation toward UDP-glucuronic acid, respectively.

UDP-N-acetyl-glucosamine results from phosphorylation reactions encoded by genes of the global cell metabolism (Chong et al., 2005). The bacterial HasA proteins (Class I) are GTs integrated in the membrane polymerizing the precursor molecules by adding new moieties to the reducing end of the polysaccharide chain (Weigel and DeAngelis, 2007; Badel et al., 2011). As cellulose and curdlan synthases they contain a core of four TMHs connected to at least one intracellular loop, which contains the consensus sequence of processive GT's (Saxena et al., 1995; Heldermon et al., 2001). Different hypothesis for the HA synthase reaction mechanism exist, such as combined glycosyltransferase and translocase activity (Tlapak-Simmons et al., 1999; Weigel and DeAngelis, 2007; Thomas and Brown, 2010) or inclusion of an HA secreting ABC transporter (Ouskova et al., 2004; Schulz et al., 2007). Just recently it was shown by *in vitro* analysis based on proteoliposomes that the synthesis and translocation process spatially aligned (Hubbard et al., 2012). In combination with the latest results as obtained for cellulose and curdlan synthases, the mechanism of HA synthesis might soon be solved.

The first commercial bacterial HA was produced via *Streptococcus zooepidemicus,* but due the production of streptolysin (exotoxin), causing β-hemolysis, as observed during cultivation of streptococci strains, recombinant HA production was of high priority even in the early stage of commercialization (Thonard et al., 1964; Widner et al., 2005; Chien and Lee, 2007; Liu et al., 2011). The high-price segments in which HA can be applied motivated metabolic engineering approaches to enhance and tailor HA synthesis (Chong et al., 2005), identifying a balanced availability of both precursor molecules to influence yield and molecular weight (Chen et al., 2009; Jia et al., 2013).

Extracellularly Synthesized Polysaccharides

Dextran and Derivatives – Sucrase Based Glucan-Homopolymers

The most common sucrase activity based polymer is dextran, which mainly consists of α-(1-6) linked glucose (**Figure 2H**). Polymerization is realized outside the cell by the dextransucrase the key enzyme for dextran synthesis. Dextransucrases (or more general glucansucrases) belong to the enzyme class of transglucosidases that are part of the glucosyltransferases which are classified as glycoside hydrolase family 70 (GH70; Cantarel et al., 2009). Glucansucrases (GS) catalyze the transfer of glucose from sucrose onto a growing chain of α-glycosidic linked oligo- and polysaccharides. Depending on the glucansucrase the resulting linkages can be formed to each of the free hydroxyl groups of the sugar moiety. Besides the purely α-(1-6) linked dextran there are dextrans containing a small amount of α-(1-3) or even α-(1-2) linkages, mutan with mostly α-(1-3) linkages, alternan with strictly alternating α-(1-3) and α-(1-6) linkages and reuteran consisting mainly of α-(1-4) linkages that are interspersed with α-(1-6) linkages (Dols et al., 1998; Leemhuis et al., 2013). The polymers formed from glucansucrases are branched to different degrees with possible branching points at all hydroxyl groups.

Dextransucrases are secreted and anchored to the cell wall. They have average molecular weights in the range of 110–160 kDa and are built of multiple domains (Vujičić-Žagar et al., 2010). Recently three dimensional structures of truncated GH70 proteins have become available (Vujičić-Žagar et al., 2010; Ito et al., 2011; Brison et al., 2012) and the structural models revealed surprisingly different structures than expected from sequence alignments. The catalytic core of the GSs consists of three domains, with two extra domains attached to the core domains (Leemhuis et al., 2013). Only the C-domain consists of one contiguous polypeptide and is involved in glucan production and/or glucan binding (Lis et al., 1995; Kingston et al., 2002; Kralj et al., 2004).

There has been some debate over the years on the enzymatic mechanism, especially concerning chain initiation and chain elongation. Some evidence was provided that elongation occurs at the reducing end, which led to the proposal of two nucleophilic sites being involved, where the growing chain remains covalently bound to the enzyme and is transferred from site one to the glucose moiety bound at site two and *vice versa* (Robyt et al., 2008). This mechanism would explain the high processivity of the enzyme as polymer length is inversely proportional to the number of enzymes (Robyt et al., 2008). Other studies and the recently solved crystal structures of glucansucrases, however, propose a much simpler mechanism (Leemhuis et al., 2013). Here, during elongation sucrose is hydrolyzed resembling the action of a retaining glycosyltransferase where a covalent β-glycosyl-enzyme intermediate is formed via a carboxylic acid residue of the enzyme. Within this step the high energy of the glycosidic bond of sucrose is retained. Hydrolysis of this intermediate with concomitant release of glucose is possible but not favored by the enzyme. Instead a transfer occurs of the glucose onto any suitable hydroxyl group. Accordingly elongation occurs at the non-reducing end. In this reaction model the acceptor hydroxyl group is determined by what molecule is bound to the active site and how this is held in position, which might be mediated by a carbohydrate binding module in the multidomain protein (Janecek et al., 2011). This model would also explain the branching to be a more statistic phenomenon caused by movement of the chain or by intermediate release and rebinding. The mechanism of initiation has also been debated and different monomers and oligomers have been found or proposed as primers, depending on the experimental setup. Since the presence of bound oligomers seems to promote the transfer reaction the initiation should occur on various molecules with sufficiently high concentration to bind (Seibel et al., 2006).

Levan and Inulin

Sucrases can also act as fructose transferases producing polyfructan. Two types of linkages have been described here, α-(2-6) and α-(2-1). Levan, which is produced by levansucrases, mainly consist of the former with occasional α-(2-1) branches. Inulin type polyfructan is obtained from inulinosucrase and shows the opposite α-(2-1) chain with α-(2-6) branches (**Figure 2I**). Levansucrases are widely distributed among Gram-positive bacteria and several plant pathogens carry more than one enzyme (Khandekar et al., 2014); inulinosucrases are only present in lactic acid bacteria (Srikanth et al., 2015). Mechanism of reaction is similar to glucansucrases. When fed with maltose as sole priming substrate fructansucrases can also produce maltosylfructosides (Diez-Municio et al., 2013).

The synthesis of dextran, levan and derivatives is directly induced in the presence of sucrose for some *Leuconostoc mesenteroides* strains (Kim and Robyt, 1994) and some strains express the genes constitutively (Schwab et al., 2007).

Biosynthesis Regulation

Regulation of the various EPS biosynthesis pathways is complex and occurs at different levels. Some general regulation strategies such as extensive transcriptional regulation involving two-component signal transduction pathways, quorum sensing, alternative RNA polymerase σ-factors and anti-σ-factors, as well as integration host factor (IHF)-dependent and cyclic di-GMP dependent regulatory mechanisms are available and will be summarized here.

Nitrogen limitation was shown to induce EPS biosynthesis for many different EPS producing strains by use of the different components of the nitrogen signaling cascade (Ruffing and Chen, 2012). Additionally, and in general the presence of numerous c-di-GMP synthesizing diguanylate cyclase proteins which contain the conserved GGDEF motif as catalytic active side (Ausmees et al., 2001; Simm et al., 2004) play an important role in regulation of EPS-biosynthesis (Hengge, 2009; Ruffing and Chen, 2012; Liang, 2015). Transcriptomic analysis of **curdlan** biosynthesis displayed up-regulation of GGDEF protein

encoding genes under nitrogen limited conditions and lowered EPS production (57%) by knocking out c-di-GMP synthases (Ruffing and Chen, 2012). Several EPS such as cellulose, alginate, and xanthan are regulated by c-di-GMP, which can serve as an allosteric regulator of cellulose and alginate synthases (Hay et al., 2014; Liang, 2015). *Agrobacterium* sp. ATCC 31749 for example encodes 31 proteins containing GGDEF domains (Ruffing and Chen, 2012). The regulation of EPS biosynthesis strongly differs amongst the bacterial species as indicated by the highly varying amount of genes encoding for c-di-GMP synthesizing and degrading proteins (EAL or HD-GYP motifs). This amount ranges from zero in *Staphylococcus aureus* to more than 80 in *Kineococcus radiotolerans*, as recently reviewed (Liang, 2015). *Xanthomonas* genomes encode around 40 of these proteins for example.

The nitrogen regulatory mechanisms are centrally controlled by conserved sets of nitrogen sensor proteins called PII and the signal is transmitted by a two component signal transduction mechanism involving NtrB and NtrC. In the cascade NtrC is phosphorylated by NtrB under nitrogen deplete conditions. Nitrogen regulatory operon (*ntrBC*) also includes *nifR* whose function is unknown. Knock-outs results in 30% reduction of curdlan production, whereas the knock-out experiments of σ-factor *rpoN* result in a 30% increased curdlan biosynthesis (Ruffing and Chen, 2012). Similar to alginate, the regulation circuits of curdlan are expected to be multifaceted. Curdlan biosynthesis is dependent on pH-value and the acidocalcisome as well as polyphosphate. Knock-out experiments of a putative acidocalcisome gene (*rrpP*, membrane bound proton translocating pyrophosphatase) resulted in a 70% decrease in curdlan production. Energy storage via polyphosphate influences curdlan biosynthesis as well as maintains the intracellular pH (Ruffing and Chen, 2012). Just recently it was shown that the helix-turn-helix transcriptional regulatory protein (*crdR*) is essential for curdlan production by operating as positive transcriptional regulator of the curdlan operon in ATCC31749. The potential binding region of *crdR* is located upstream from the *crdA* start codon (Yu et al., 2015).

Several EPS synthases/copolymerases contain so called PilZ domains, which bind c-di-GMP, as shown for example for Alg44 in alginate biosynthesis (Merighi et al., 2007; Hay et al., 2009) and for AcsA/BcsA in **cellulose** synthesis, within BcsA's C-terminus right next to the GT-domain as well as several more structural hints for binding possibilities (Weinhouse et al., 1997; Amikam and Galperin, 2006; Ryjenkov et al., 2006; Fujiwara et al., 2013; Morgan et al., 2013). This stimulating effect is assumed to be based on an induced conformational change of BcsA that allows UDP-glucose to access the catalytic site more effective (Morgan et al., 2014).

Alginate biosynthesis is controlled by one of the best examined regulatory networks due to the clinical significance of *P. aeruginosa*. The regulatory network of alginate biosynthesis is highly complex and occurs on different levels (Hay et al., 2014). The master regulator of alginate biosynthesis is the alternative σ-factor AlgU, classified as an extra cytoplasmic function σ - factor, which confers resistance to several environmental stress factors affecting the cell envelope (Hay et al., 2014). AlgU promotes the expression of several genes involved in alginate biosynthesis (*algD*, *algC*), regulation (*algR*, *algB*, *algZ*) as well as its own operon (Yu et al., 1997; Firoved et al., 2002; Wozniak et al., 2003a; Muhammadi and Ahmed, 2007). The anti σ-factor MucA is embedded in the cytoplasmic membrane and directly binds to AlgU and exert its function in concert with MucB, MucC, and MucD (Boucher et al., 1996; Wood and Ohman, 2006; Cezairliyan and Sauer, 2009). The complete process of regulation of alginate biosynthesis based on envelope stress following the complex regulated intermembrane proteolysis (RIP) cascade was recently reviewed in detail (Hay et al., 2014). Next to AlgU, transcriptional regulation occurs by the activity of the sigma factors AlgQ, which blocks the general housekeeping σ-factor RpoD (σ 70), thus allowing AlgU to mediate transcription of the alginate biosynthesis operon (Pineda et al., 2004; Yin et al., 2013). In nitrogen rich conditions the alternative σ-factor RpoN (σ 54) binds to *algD* promotor and therefore suppresses alginate production (Boucher et al., 2000). The alginate biosynthesis operon expression is further regulated by several DNA-binding proteins occupying the promoter region (Kato et al., 1990; Baynham et al., 2006). Other DNA-binding proteins, such as AlgQ and AlgP are described to be positive regulators of alginate biosynthesis (Deretic et al., 1992; Delic-Attree et al., 1997; Ledgham et al., 2003; Yin et al., 2013). Another DNA-binding protein (Vfr) is more indirectly involved in regulation of alginate biosynthesis with a more speculative mechanism (Hay et al., 2014). Additionally two-component signal transduction systems are involved in alginate biosynthesis regulation as observed by the autophosphorylation mechanism of the sensor kinase proteins KinB and FimS, which activate the corresponding response regulators AlgB and AlgR respectively. The detailed mechanism remains elusive up to now, but AlgB and AlgR are known to activate expression of alginate biosynthesis genes by binding to *algD* promotor (Hay et al., 2014). Generally, the stimulation of two-component systems is induced by environmental signals, which are not clearly identified for AlgB and AlgR (Wozniak and Ohman, 1991; Leech et al., 2008). Posttranscriptional regulation is observed by non-coding small RNAs whose transcription is activated by a two-component system (GacS-GacA), known to be involved in regulation of several independent pathways including such as, e.g., oxidative stress and quorum sensing (Timmermans and Van Melderen, 2010; Ghaz-Jahanian et al., 2013). The posttranslational regulation of alginate biosynthesis occurs by the already described c-di-GMP binding to the PilZ domain of Alg44 and the assumed conformational change, which might bring the nucleotide sugar closer to the active site of the corresponding glycosyltransferase active site (Alg8) as recently shown for the BscA protein in cellulose synthesis (Morgan et al., 2014). A corresponding cytoplasmic GGDEF protein was recently identified, which might provide the necessary pool of c-di-GMP, and at the same time might be involved in c-di-GMP degradation by having an additional EAL motif (Galperin et al., 2001; Hay et al., 2009; Li et al., 2013).

For ***Xanthomonas*** strains several genome wide expression profiles were recorded under different conditions, revealing single ORFs of the gum operon to be transcribed as polycistronic

mRNA, from *gumB* to *gumN*, while glucose seems to induce xanthan biosynthesis (Yoon and Cho, 2007; Serrania et al., 2008; Liu et al., 2013). in this plant pathogenic bacterium the virulence and exopolysaccharide production are closely connected and controlled by cell–cell signaling mediated by diffusible signal factors (DSFs) and an uniquely evolved two-component regulatory system (RpfC/RpFG; Barber et al., 1997). The DSF signaling is mediated by c-di-GMP and the two-component system RpfC/RpfG senses and transduces the DSF signal. RpfG contains a HD-GYP domain indicating regulation via c-di-GMP. Additionally, the global transcriptional regulator Clp mediates the DSF signaling through a hierarchical regulatory network, directly acting on xanthan production (He et al., 2007). Knowledge with respect to the extensive metabolic network might be used toward a better understanding of the regulation of xanthan biosynthesis (Schatschneider et al., 2013).

Factors inducing **CA** biosynthesis were identified to mainly rely on stress conditions for cell envelope structure (osmotic stress) and seem to be mainly controlled by the Rcs (regulation of capsule synthesis) proteins, as reviewed by Majdalani and Gottesman (2005). The complex signal transduction system comprises several proteins, whereas RcsA, RcsB, and RcsD play the major role in CA biosynthesis (Majdalani and Gottesman, 2005). RcsC is a sensor protein which directly interacts with the response regulator RcsB, the signal for RcsC response is still unknown (Arricau et al., 1998; Chen et al., 2001; Majdalani and Gottesman, 2005). A helix-turn-helix DNA binding motif of the RcsB protein is phosphorylated by RcsC when stimulated by environmental cues (Takeda et al., 2001; Clarke et al., 2002). The phosphorylated version of RcsB interacts with RcsA and forms a heterodimer by utilization of an additional helix-turn-helix DNA motif as present in RcsA (Stout et al., 1991). Both positive regulators RcsA and RcsB act as a heterodimer to activate transcription at the single promoter upstream of the CA operon (Stout, 1996; Rahn and Whitfield, 2003).

One of the main differences of CA regulation is the absence of CA production in wild-type strains grown at 37°C, whereas cultivation at lowered temperatures seems to induce CA biosynthesis (Whitfield, 2006). This temperature sensitivity was examined by RT-PCR experiments, showing that expression of the CA operon was only slightly altered by growth at 19, or 37°C. Additionally the expression profile of the Rcs machinery was only marginally altered. Navasa et al. (2011), were providing experimental evidence for the underlying mechanism.

Regulation of **succinoglycan** is occurring mainly at transcriptional and posttranscriptional level (Keller et al., 1995; Becker et al., 2002; Janczarek, 2011) and employs by several proteins involved in different regulatory mechanisms. The *exoS* encoded membrane sensor together with the gene product of *chvI* (response regulator) constitute a two-component regulatory system which controls the expression of the *exo* genes. ExoR as a negative regulator directly acting on transcription and translation levels of most of the *exo* genes with exception of *exoB* (Reuber and Walker, 1993). Another posttranscriptional regulatory effect such as lowered EPS yields was observed by overexpression of *exoX* and increased EPS yields by overexpression of *exoY*, respectively without influencing any of the genes involved in succinoglycan biosynthesis (Reed et al., 1991; Leigh and Walker, 1994). The *mucR* encoded regulatory protein that plays a key role in control of biosynthesis EPS of *Rhizobium* (Keller et al., 1995). MucR causes overexpression of *exoK*, *exoY,* and *exoF* genes, resulting in high production levels. Expression can be induced by ammonia, phosphate and sulfate present in the cultivation media. Increased succinoglycan production was recently obtained by overexpression of the priming GT *exoY* (Jones, 2012).

Negative regulation of the single promotor controlling **HA** synthesis was shown to involve the two-component regulatory system CovR/S (Dougherty and van de Rijn, 1994; Heath et al., 1999). This system consist of the CovS sensor kinase and the response regulator CovR, which binds to the AT-rich elements of the *has* promotor (Bernish and van de Rijn, 1999; Miller et al., 2001; Federle and Scott, 2002). Just recently novel elements including two additional promotors, one initiating transcription of a small RNA, and an additional intrinsic transcriptional terminator were identified (Falaleeva et al., 2014). Negative regulation occurs via CovR.

Production of the extracellular **sucrases** can be induced by sucrose, which directly induces reversible phosphorylation of the sacY anti-terminator as observed in many *Bacillus* species (Idelson and Amster-Choder, 1998; Biedendieck et al., 2007). Additionally high salt concentrations induce sucrose expression by activating the DegSU-two component system (Biedendieck et al., 2007). For other genera the involvement of the negative regulators RcsA and RcsB is described to reduce the amount of levansucrase (Bereswill and Geider, 1997). Differences in Gram-negative and Gram-positive bacteria were observed, as well as dependency of expression in the stationary phase (Abhishek et al., 2009). For some levansucrases a temperature dependent expression is described which occurs at around 18°C.

Bioengineering Strategies Toward Tailor-Made Exopolysaccharides

In addition to the already existing techniques for realization of tailor-made variants of microbial polysaccharides (exchange/overexpression of single genes or complete operons), approaches implementing domain shuffling of GTs revealed to be of high potential to enhance the portfolio of EPS variants (Hancock et al., 2006). The increasing insights into fundamental mechanisms and functions of genes and proteins involved in the different EPS producing biosynthesis pathways in combination with the growing portfolio of techniques unraveling metabolic networks have the potential to be applied for the production of tailor-made polysaccharides. Additionally, identification of novel EPS encoding gene clusters by next generation sequencing approaches will enhance our understanding of EPS synthesis pathway variation and modification. Combining bioinformatics, with high throughput data obtained by systems biology approaches as well as structural information of proteins and EPS will enable the implementation of synthetic biology approaches for tailoring microbial EPS. Synthetic biology approaches such as utilization of regulatory bio bricks might enable the targeted

induction of EPS biosynthesis, aiming to uncouple it from natural regulatory networks. In the case of heteropolysaccharides, the complete biosynthesis machinery might be used to design tailor-made polysaccharides. Growing insights into mechanism and structures of the various GT's as involved in assembly of the repeat units will enable the targeted shuffling of GT's and therefore might enable the design of artificial repeat units in the future. The insights in Wzx and Wzy topology and mechanism might open up the opportunity for incorporation of desired sugars or sugar derivatives resulting in modified EPS structures with hitherto unknown material properties (Marolda et al., 2006; Hong et al., 2012; Wang et al., 2012b; Hong and Reeves, 2014; Islam and Lam, 2014; Rehm, 2015). In combination with the enhanced understanding of EPS biosynthesis regulation as obtained by systems and synthetic biology approaches, a novel era of bacterial EPS might be reached. Completely synthetic gene clusters could be obtained by application of domain swapping approaches, exchange of elements between different gene clusters as well as introduction of additional elements.

Alternatively protein engineering can be applied to EPS modifying enzymes for *in vivo* as well as *in vitro* modification of EPS. The latter strategy was successfully applied to alginate and succinoglycan, both having in common the secretion of enzymes for EPS modification. Utilization of these secreted enzymes might allow a tight control of the material properties. Epimerases of *A. vinelandii* have been employed to modify alginate exhibiting a range of material properties (Martinsen et al., 1989; Morch et al., 2007; Reese et al., 2015). Such variants can be of particular interest for specific applications because of their specific material properties. Future engineering strategies of alginate variants will include the design of block-copolymers to obtain tailored properties. This might include other monomers in addition to guluronic acid and mannuronic acid in order to broaden the range of applications. For homopolymers such as, e.g., curdlan and cellulose, targeted modification of the synthases involved in the biosynthesis process might enable the modification of the molecular weight as well as selectivity of glyosidic bonds within the backbone structure. As seen by the latest results from insights into the cellulose synthase machinery, the strict order of β-(1-4)-glyosidic linkages might be modified by altering the substrate binding pocket of the synthase, which is responsible for the orientation of the UDP-glucose. These structural insights might also give further information for related GT2 proteins, such as involved in the synthesis of hyaluronan, alginate, and chitin (Weigel and DeAngelis, 2007; Hubbard et al., 2012; Hay et al., 2014).

Only recently an innovative bi-enzymatic process was reported for the production of short chain fructooligosaccharides and oligolevans from sucrose. This system was based on an immobilized levansucrase and an endo-inulase, resulting in a highly efficient synthesis system with a yield of more than 65% and a productivity of 96 g/L/h (Tian et al., 2014). The utilization and combination of several carbohydrate modifying enzymes create the potential for industrial production of different low molecular weight oligo- or polysaccharides with applications as food additives (prebiotics) or in medicine and for industry.

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Challenges and perspectives in combinatorial assembly of novel exopolysaccharide biosynthesis pathways

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Because of their rheological properties various microbial polysaccharides are applied as thickeners and viscosifiers both in food and non-food industries. A broad variety of microorganisms secrete structurally diverse exopolysaccharides (EPS) that contribute to their surface attachment, protection against abiotic or biotic stress factors, and nutrient gathering. Theoretically, a massive number of EPS structures are possible through variations in monosaccharide sequences, condensation linkages and non-sugar decorations. Given the already-high diversity of EPS structures, taken together with the principal of combinatorial biosynthetic pathways, microbial polysaccharides are an attractive class of macromolecules with which to generate novel structures via synthetic biology approaches. However, previous manipulations primarily focused on increasing polysaccharide yield, with structural modifications restricted to removal of side chains or non-sugar decorations. This article outlines the biosynthetic pathways of the bacterial heteroexopolysaccharides xanthan and succinoglycan, which are used as thickening and stabilizing agents in food and non-food industries. Challenges and perspectives of combining synthetic biology approaches with directed evolution to overcome obstacles in assembly of novel EPS biosynthesis pathways are discussed.

Keywords: polysaccharide, synthetic biology, glycosyltransferase, synthase-dependent pathway, ABC transporter-dependent pathway, Wzx/Wzy-dependent pathway

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Introduction

A broad variety of polysaccharides are naturally produced by bacteria, fungi, algae, and plants. Bacteria are able to synthesize surface polysaccharides including lipopolysaccharides (LPS) constituting the outer leaflet of the outer membrane of Gram-negative bacteria, capsular polysaccharides (CPS) bound to the cell surface, and secreted exopolysaccharides (EPS). As the interface between the bacterial cell and the environment, surface polysaccharides play important roles in protection against abiotic or biotic stress factors, nutrient gathering, surface attachment, motility, and interactions with host immune systems (Rehm, 2009; Ullrich, 2009; Donot et al., 2012). Variations in monosaccharide composition, condensation linkages, non-sugar decorations, and molecular weight give rise to an enormous diversity of structures that contributes to their diverse biological functions. This diversity also accounts for an attractive spectrum of physical and rheological properties of microbial EPS opening up commercial applications in industrial, food and medical sectors as thickening, emulsifying, chelating, or stabilizing agents (Freitas et al., 2011). Many microbial polysaccharides have properties similar to traditionally applied gums originating

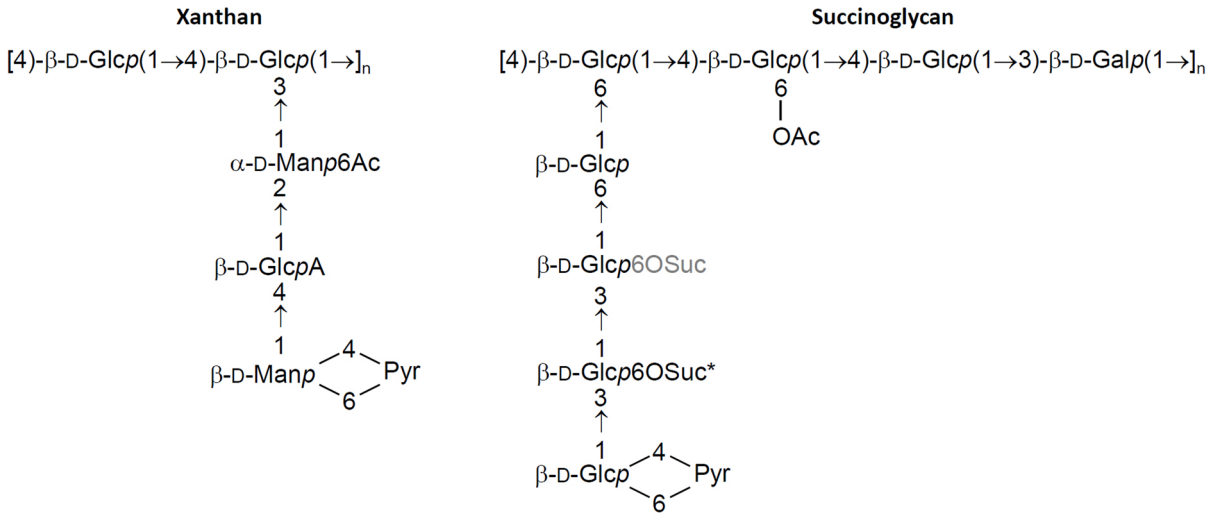

FIGURE 1 | Repeat unit structure of xanthan and succinoglycan. The polysaccharides consist of a variable number of these repeats. While carbohydrate structure is uniform along the polysaccharide chain, decorations with non-sugar groups are variable. Ac, acetyl group; Gal, galactosyl group; Glc, glucosyl group; GlcA, glucuronyl group; Man, mannosyl group; Pyr, pyruvyl group; Suc, succinyl group, variable and fixed positions of succinylation are denoted in grey and by an asterisk, respectively.

from plants or algae. Prominent examples of commercially applied microbial EPS are xanthan gum, gellan, and alginate. While xanthan is primarily used in cosmetics, food and oil industry (Becker et al., 1998), gellan and alginate are also applied in pharmacy and medicine, e.g., in wound healing, tissue engineering and drug delivery (Lee and Mooney, 2012; Osmalek et al., 2014). Furthermore, an increasing number of algal and microbial polysaccharides with novel properties are being discovered (Paniagua-Michel Jde et al., 2014).

Exopolysaccharides are either homo- or heteropolymers which are frequently decorated by non-carbohydrate substituents, such as acetyl, pyruvyl, or succinyl groups, which confer anionic properties to the polysaccharide. Heteropolymeric EPS are typically composed of identical repeat units that may only vary by the presence of decorating groups. Assembly of the repeat units to the polymer can result in branched structures.

Exopolysaccharides biosynthesis is a multistep process comprising the

(i) synthesis of nucleotide sugar precursors
(ii) synthesis of oligosaccharide repeat units or direct synthesis of the polysaccharide by successive or progressive activity of glycosyltransferases
(iii) assembly of the polysaccharide from the repeat units
(iv) export of the product

Nucleotide diphosphates (NDPs) or nucleotide monophosphates (NMPs) are the common precursors for the carbohydrate components of polysaccharide biosynthesis pathways. They serve as activated donors for the glycosyltransferase-catalyzed transfer of the sugar to a lipid carrier or a carbohydrate. Polysaccharides are assembled and exported by one of three known distinctive types of mechanisms: the synthase- (Whitney and Howell, 2013), ATP-binding cassette (ABC) transporter- (Greenfield and Whitfield, 2012; Willis and Whitfield, 2013), and Wzx/Wzy-dependent (Islam and Lam, 2014, and summarized by Schmid et al., 2015) pathways.

The broad range of structural diversity of secreted branched heteropolysaccharides makes their biosynthetic pathways ideal candidates for design of novel structures by synthetic biology approaches. Such polysaccharides are typically built from repeat units that are assembled by the Wzx/Wzy-dependent pathway. Through more detailed elucidation of this biosynthetic pathway, novel tailored EPS may eventually be generated via combinatorial strategies using an engineered modular apparatus. However, to date the most successful engineering approaches addressed improvements in the yield or production process, alterations in the degree of polymerization, removal of side chains or non-sugar substituents, or heterologous expression of EPS biosynthesis gene clusters (Rehm, 2009; Ullrich, 2009). This review outlines the well-studied biosynthetic pathways of the acidic heteroexopolysaccharides xanthan and succinoglycan applied in cosmetics, food and oil industry (Becker et al., 1998; Fink, 2003a,b; De et al., 2015; **Figure 1**). It discusses obstacles, perspectives, and the needs for research of molecular mechanisms operating at different steps of biosynthesis to promote synthetic biology approaches toward assembly of pathways producing novel EPS structures.

Biosynthesis of Xanthan and Succinoglycan by the Wzx/Wzy-dependent pathway

Xanthan produced by *Xanthomonas campestris* is composed of pentasaccharide repeat units, forming a cellulose backbone

with trisaccharide side-chains of [β-D-Man*p*-(1→4)-β-D-Glc*p*A-(1→2)-β-D-Man*p*-(1→)] attached to alternate glucose residues in the backbone by α-1,3 linkages (Jansson et al., 1975; Melton et al., 1976; **Figure 1**). The terminal mannose residues can be modified by a pyruvic acid group attached by a ketal linkage and acetyl groups often decorate as 6-O substituents the internal mannose residues. Some external mannoses carry a second 6-*O*-acetyl substituent (Ielpi et al., 1981, 1983; Stankowski et al., 1993). Succinoglycan produced by *Sinorhizobium meliloti* is made of octasaccharide repeat units containing one galactose and seven glucose residues joined by β-1→3, β-1→4, and β-1→6 linkages (**Figure 1**). The terminal glucose residue is substituted by a pyruvyl group while acetyl and succinyl groups decorate inner glucose residues (Aman et al., 1981; Reinhold et al., 1994).

Typically, genes directing synthesis, polymerization and export of a specific polysaccharide are clustered in the bacterial genome. In contrast, genes involved in the synthesis of common nucleotide sugar precursors required for the production of more than one oligo- or polysaccharide are frequently uncoupled from the specific biosynthesis gene clusters (Harding et al., 1993). However, many clusters contain additional copies of these genes or genes for the synthesis of nucleotide sugar precursors specific to the polysaccharide. In *X. campestris* and *S. meliloti*, the 16 kb *gum* and the 24 kb *exo* gene cluster, respectively, encode glycosyltransferases, enzymes catalyzing the addition of non-sugar decorations, and proteins involved in the terminal steps of xanthan and succinoglycan biosynthesis (Becker and Pühler, 1998; Becker et al., 1998; **Figure 2**). While in the succinoglycan biosynthesis gene cluster, *exoB* and *exoN* encode a UDP glucose 4-epimerase and a UDP-glucose pyrophosphorylase, respectively, the xanthan biosynthesis gene region does not encode enzymes involved in synthesis of nucleotide sugar precursors.

Both EPS are synthesized by the Wzx/Wzy-dependent pathway named after the key components involved in flipping the lipid carrier with the repeat unit from the cytoplasmic to the periplasmic face of the inner membrane (Wzx) and assembly of the repeat units to the polymer (Wzy). Repeat units are synthesized on a C55-undecaprenol phosphate (und-P) lipid carrier located in the inner leaflet of the cytoplasmic membrane by the sequential activity of glycosyltransferases as has been revealed by the accumulation of lipid-carrier bound oligosaccharide intermediates in glycosyltransferase mutants (Ielpi et al., 1993; Reuber and Walker, 1993). Initiation of the repeat unit synthesis is catalyzed by a member of the polyisoprenylphosphate hexose-1-phosphate transferases (**Figure 2**): in the succinoglycan biosynthesis pathway, ExoY transfers galactosyl 1-phosphate from UDP-galactose to und-P whereas synthesis of the xanthan repeat unit is started by GumD-catalyzed transfer of glucosyl 1-phosphate from UDP-glucose to the lipid carrier. Serial activities of the glycosyltransferases ExoA, ExoL, ExoM, ExoO, and ExoU complete the synthesis of the succinoglycan repeat unit that is acetylated and succinylated to varying degrees by ExoZ and ExoH, respectively. While these modifications are not required for assembly of the polysaccharide, pyruvylation of the terminal glucose residue by ExoV seems to be essential for this process (Becker et al., 1993; Reuber and Walker, 1993). The xanthan repeat unit is completed by the glycosyltransferases GumM, GumH, GumK, and GumI. Non-sugar decorations are added to the mannose residues of the repeat unit at varying degrees by the pyruvyltransferase GumL and the acetyltransferases GumF and GumG (Ielpi et al., 1993; Stankowski et al., 1993; Becker et al., 1998). Accumulation of intermediates carrying non-sugar substituents suggests that modification occurs at the level of repeat unit synthesis in the cytoplasm, before assembly to the polysaccharide (Ielpi et al., 1993; Reuber and Walker, 1993).

Und-PP-linked repeat units are then transported by the Wzx flippase to the periplasmic face of the inner membrane where they are polymerized to the polysaccharide by Wzy. In succinoglycan and xanthan biosynthesis, ExoT/ExoQ and GumJ/GumE represent the Wzx/Wzy proteins, respectively (González et al., 1998; Becker and Vorhölter, 2008). Wzx and Wzy protein sequences are poorly conserved in different bacteria which may reflect strict substrate specificities (Islam and Lam, 2013, 2014; Hong and Reeves, 2014). As revealed by topology mapping experiments, Wzx proteins typically contain 12 transmembrane helices (Mazur et al., 2005; Cunneen and Reeves, 2008; Islam et al., 2010; Marolda et al., 2010). They belong to the polysaccharide transporter (PST) family that is part of the multidrug/oligosaccharidyl-lipo/polysaccharide (MOP) exporter superfamily. Within this superfamily, the PST family is the most closely related to the multidrug and toxin extrusion (MATE) family of efflux proteins (Hvorup et al., 2003). Although a X-ray crystal structure of a Wzx protein so far has not been established, a structure for the *Pseudomonas aeruginosa* Wzx protein was reported based on homology modeling using the MATE family protein NorM from *Vibrio cholerae* (He et al., 2010) as a suitable template, followed by genetic, bioinformatic, and biochemical structure validation (Islam et al., 2012). The structure model as well as data from site-directed mutagenesis suggest a cationic lumen which has a role in substrate binding during translocation and a cationic exit portal at the periplasmic face of the protein. MATE efflux proteins use proton- or sodium-coupled antiport (Kuroda and Tsuchiya, 2009). Since protons were reported to affect gating of and be taken up by the *P. aeruginosa* Wzx protein, the flippase mechanism likely utilizes proton-mediated antiport (Islam et al., 2013a). Cross-species and cross-strain complementations of *wzx* genes indicate that Wzx flippases demonstrate remarkable substrate specificity for their complete native repeat units (Hong et al., 2012; Wang et al., 2012; Hong and Reeves, 2014). Certain non-native repeat units can still be flipped, albeit very inefficiently, a limitation that can be partially compensated for through overexpression of the particular flippase; however, such non-native complementations have thus far only been shown to be successful when the flippase and the non-native repeat both originate from systems in which the und-PP-linked sugar is the same (Marolda et al., 2006; Hong and Reeves, 2014).

Wzy-dependent polymerization of und-PP-linked repeat units takes place in the periplasm. Wzy catalyzes transfer of the growing chain to the new und-PP-linked repeat unit resulting in growth of the polysaccharide chain at the reducing end (Robbins et al., 1967; Woodward et al., 2010). In this respect, Wzy has glycosyltransferase activity (Islam and Lam, 2014). The tertiary structure of Wzy proteins is unknown. Membrane topology

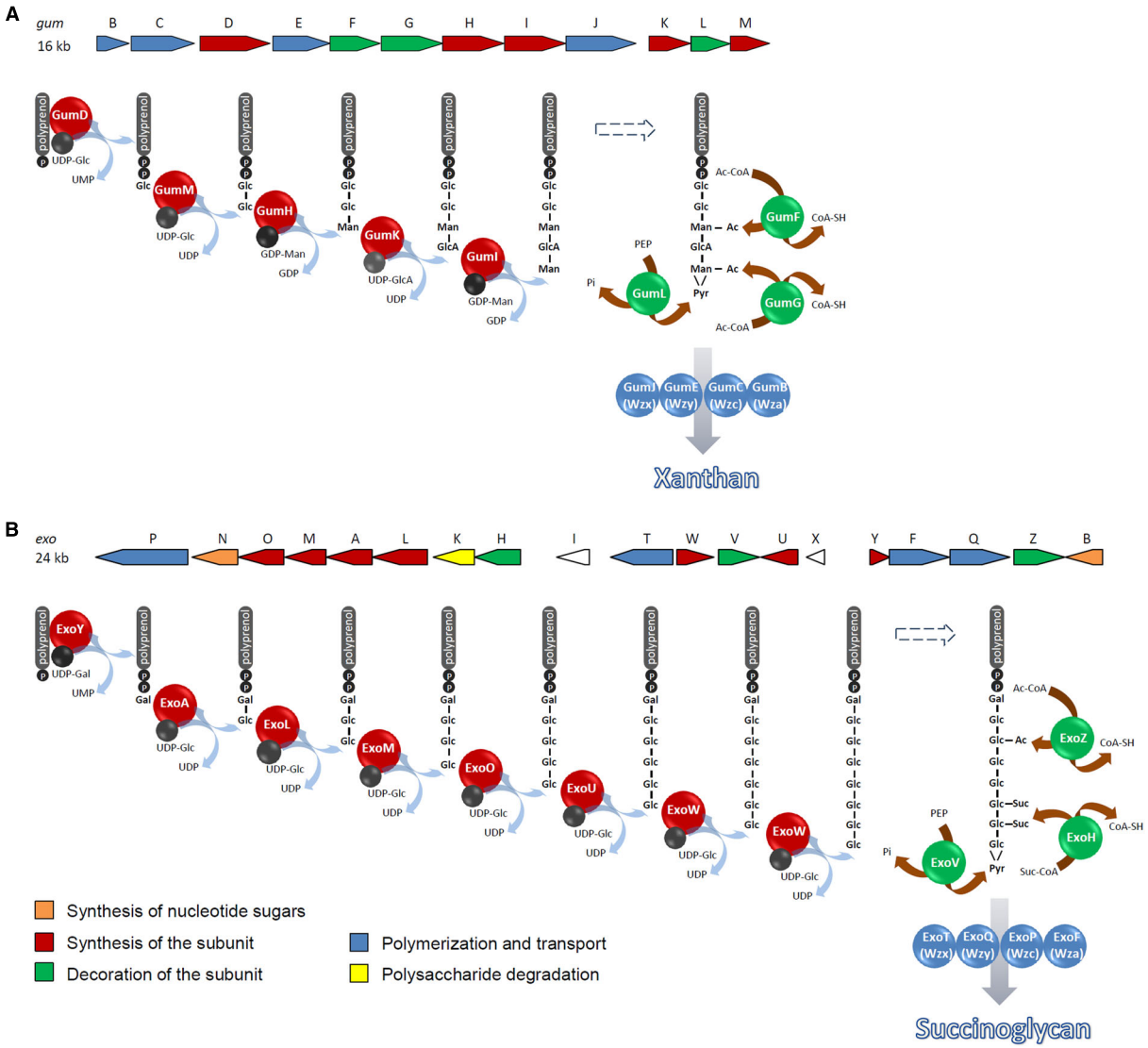

FIGURE 2 | Biosynthetic pathways of xanthan in *X. campestris* (A) and succinoglycan in *S. meliloti* (B). Repeat units are synthesized on a C55-undecaprenol phosphate lipid carrier at the inner face of the cytoplasmic membrane. Polymerization occurs by transfer of the lipid carrier-bound growing chain to a monomeric lipid carrier-bound repeat unit in the periplasm at the outer face of the cytoplasmic membrane. Modifications may occur during synthesis of the repeat unit before completion of the oligosaccharide. E.g., acetylated intermediates were detected in succinoglycan biosynthesis (Reuber and Walker, 1993). Ac, acetyl group; Ac-CoA, Acetyl-CoA; Gal, galactosyl group; Glc, glucosyl group; GlcA, glucuronyl group; Man, mannosyl group; PEP, phosphoenolpyruvate; Suc, succinyl group; Suc-CoA, succinyl-CoA.

mapping indicates 12–14 transmembrane helices and two large periplasmic loops, the largest in the C-terminal and a smaller one in the N-terminal half (Daniels et al., 1998; Mazur et al., 2003; Islam et al., 2010). It is hypothesized that one of these loops binds the incoming und-PP-linked repeat unit while the other is binding the growing polysaccharide chain linked to und-PP (Islam et al., 2011, 2013b). A catch-and-release mechanism was proposed with one binding site responsible for recruiting the new subunit and the other binding site retaining the growing chain with a lower affinity that allows release and rebinding of the polysaccharide chain after each elongation step (Islam et al., 2011). Knowledge of the substrate specificity of Wzy proteins is very limited (Islam and Lam, 2014). Few examples have been reported for tolerance of Wzy proteins involved in O-antigen synthesis for differences in side-branch sugars and sensitivity for the repeat length of the main chain (Nurminen et al., 1971; Nyman et al., 1979; Reeves et al., 2013). In the biosynthetic pathway of succinoglycan and xanthan, ExoT/ExoQ and GumJ/GumE are the homologs of Wzx/Wzy (González et al., 1998; Becker and Vorhölter, 2008).

Transport through the periplasm and across the outer membrane is mediated by proteins of the PCP (polysaccharide copolymerase) and OPX (outer membrane polysaccharide export) families named Wzz/Wzc and Wza, respectively. After the termination of polymerization for O-antigen chains (Daniels

et al., 2002), the polysaccharide is transferred from the und-PP lipid carrier to the lipid A–core oligosaccharide acceptor by WaaL (Abeyrathne and Lam, 2007; Han et al., 2011; Ruan et al., 2012) to complete a molecule of LPS. Wzz proteins affect the length distribution of the O-antigen chain (Woodward et al., 2010). Although the sequence shows low conservation among different bacteria these integral inner membrane proteins of the polysaccharide copolymerase 1 (PCP-1) family have a characteristic topology with a large periplasmic domain flanked by an N-terminal and a C-terminal transmembrane helix (Morona et al., 1995; Whitfield et al., 1997). Oligomers of 5, 8, and 9 protomers were found analyzing X-ray crystal structures of different Wzz periplasmic domains (Tocilj et al., 2008; Kalynych et al., 2012). However, cryo-electron microscopy studies of full-length Wzz proteins indicated an invariant bell-shaped oligomer recently resolved as an octamer (Larue et al., 2009; Kalynych et al., 2015). Lack of Wzz results in unregulated O-antigen chains instead of the modal length of the O-antigen chain characteristic to a specific LPS. Several models for Wzz protein function in chain-length regulation have been put forward (for a review, see Islam and Lam, 2014). The most recent, termed the "Chain-Feedback–Ruler" mechanism reconciles the majority of published data. It suggests that the growing chain is bound to the outer surface of the Wzz bell as soon as the oligosaccharide has reached the appropriate length for this binding. Interaction of Wzy, Wzz and the growing chain keeps the polymer in position for addition of further subunits. As the chain becomes longer it adopts higher-order structures that weaken the interaction with Wzz, which is thought to occur when the chain length exceeds the tip of the Wzz bell acting as a ruler. Through mechanical feedback this structural change is transmitted to the active site of Wzy resulting in release of the growing chain and ligation to the lipid A-core oligosaccharide.

Wzc proteins belong to the polysaccharide copolymerase 2a (PCP-2a) family engaged in assembly of high molecular weight CPS and EPS (Morona et al., 2000). These integral membrane proteins resemble Wzz proteins in their N-terminal half. This part represents a large periplasmic domain anchored in the inner membrane by two flanking transmembrane helices. The C-terminal part of Wzc proteins constitutes a cytosolic tyrosine autokinase domain transphosphorylating at multiple tyrosine residues (Cuthbertson et al., 2009). Wzc is a component of the trans-envelope polysaccharide translocation complex. Continued polymerization of repeat units requires Wzc and mutational studies imply that phosphorylation plays an important role in this process (Niemeyer and Becker, 2001; Paiment et al., 2002). In addition to Wzx, Wzy, and Wzc, capsule biosynthesis gene clusters frequently encode a tyrosine phosphatase (Wzb) which is likely involved in switching between phosphorylated and unphosphorylated states of Wzc (Wugeditsch et al., 2001; Cuthbertson et al., 2009). This cycling is thought to be important for polymerization of repeat units. Although, deletion of *wzc* has a drastic effect on assembly of repeat units to the polymer, its exact role is still enigmatic. Models for the functional mechanism of Wzc propose a role as copolymerase that interacts with the polymerase Wzy or the initial glycosyltransferase to directly regulate polymerization or the supply of repeat units to the polymerization and export machinery (Cuthbertson et al., 2009). Another model proposes that Wzc oligomers serve as scaffold for organization of polysaccharide polymerization and export complexes (Cuthbertson et al., 2009). PCP-2a proteins required for polymerization of CPS and EPS differ from members of the PCP-1 family involved in O-antigen synthesis mainly by the presence of the kinase domain. Therefore, it was hypothesized that the kinase activity plays an important role particularly in biosynthesis of high molecular weight polysaccharides. In succinoglycan and xanthan biosynthesis ExoP and GumC are the Wzc homologs (Niemeyer and Becker, 2001; Becker and Vorhölter, 2008). In *Sphingomonas elodea* the periplasmic domain and the kinase domain of Wzc are encoded by two separate polypeptides, GelC and GelE (Moreira et al., 2005). Interestingly, GumC lacks the kinase domain and a kinase partner could not be identified in *X. campestris* (Cuthbertson et al., 2009) suggesting that this activity may not be required for synthesis of the high molecular weight EPS xanthan.

Wza is an OPX lipoprotein that was shown to interact with Wzc (Reid and Whitfield, 2005). While cryo-EM analysis suggested that Wzc forms tetramers, Wza forms octamers with a central channel through which the polysaccharide chain is transported across the outer membrane (Dong et al., 2006; Nickerson et al., 2014). Thus, both proteins build a machinery that accomplishes the transport of the polysaccharide from the periplasm to the cell surface (Collins et al., 2007). ExoF and GumB are the homologs of Wza in the biosynthetic pathway of succinoglycan and xanthan (Becker and Vorhölter, 2008; Cuthbertson et al., 2009).

Challenges and Perspectives in Biosynthesis of Tailored EPS

Exploiting the structural space of polysaccharides by combinatorial synthesis of novel EPS biosynthetic pathways is an attractive opportunity for synthetic biology. Major obstacles that need to be overcome on this route are substrate specificities at the levels of repeat unit biosynthesis, polymerization and export of polysaccharides that hinder free combination of biosynthetic components originating from different pathways. Failure to combine these components in one pathway may also arise from requirements for specific interactions of these proteins with other protein components of an EPS biosynthetic complex.

The CAZy (Carbohydrate Active Enzymes) database (Cantarel et al., 2009; Lombard et al., 2014) offers a wealth of information on glycosyltransferases. These enzymes catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules resulting in the formation of glycosidic bonds. During this reaction the stereochemistry of the substrate is either retained or inverted (Lairson et al., 2008). According to their domain structure, nucleotide sugar-dependent glycosyltransferases are classified into two major families. GT-A family enzymes comprise a single Rossman-like domain while GT-B enzymes contain two of these domains (Lairson et al., 2008; Cantarel et al., 2009). However, inverting and retaining enzymes are both present in the GT-A and GT-B families indicating that

these different domain structures do not correlate with the catalytic mechanism (Lairson et al., 2008). Glycosyltransferases are further sub-classified according to catalytic activities and sequence similarities (Lombard et al., 2014). Nonetheless, for many subfamilies relationships between sequence and specificity are poorly understood impeding substrate predictions. Thus, enzymes with different substrate specificities frequently are members of the same family. Another problem arises from glycosyltransferases composed of several modules which may have different catalytic activities. Such proteins can be assigned to more than one family. The CAZy database is an invaluable resource for mining of glycosyltransferases to identify candidate enzymes for combinatorial synthesis of novel polysaccharides. Tens of thousands of potential glycosyltransferase genes have been revealed to be encoded in the numerous genomes and metagenomes that have been sequenced to date. Still, for the vast majority of these enzymes substrate and acceptor specificities are unknown. Future progress in linking sequence information to enzyme specificities, increasingly integrating protein structure information, will significantly accelerate the field. Yet, functionally important interactions of glycosyltransferases with other proteins of the EPS biosynthetic complex represent an additional gap of knowledge to be filled.

Furthermore, specific protein-protein interactions and substrate specificities strongly apply to assembly and export of the polymer. The low conservation of proteins involved in these terminal steps of EPS biosynthesis as well as swapping experiments of these components between different pathways imply substrate specificities related to the whole repeat unit structure. Only few examples of these components being conserved but structurally different polysaccharides being produced have been reported (Islam and Lam, 2014). Such cases may provide fundamental insights into the structural and mechanistic basis of substrate specificities.

Based on the current state of knowledge ensuring the availability of precursors, such as nucleotide sugars, appears to be feasible. In contrast, more structural insights into the interactions of glycosyltransferases with the substrate and other components of the biosynthetic complex are required to chose the most promising candidates for combinatorial and directed evolution strategies toward novel or optimized specificities or for design-based engineering of these enzymes to function in a novel pathway. Even larger is the lack of fundamental knowledge of the terminal steps accomplished by the membrane protein complex that in Gram-negative bacteria spans the cytoplasmic membrane, the periplasm and the outer membrane. Recent progress in technologies that allow structural analysis of large membrane protein complexes, such as cryo-EM combined with data from crystal structure analysis, open new windows into a deeper mechanistic understanding of EPS polymerization and export. This would be crucial for succeeding in knowledge-based combinatorial assembly of novel functional EPS biosynthetic pathways in the future, particularly when components originating from different organisms are to be combined in one pathway. Yet another aspect that should receive consideration in pathway assembly as well as in replacement or addition of individual genes is clustering of biosynthetic genes which may promote membrane protein complex formation. Combinatorial assembly of novel pathways will also largely benefit from statistical design of experiments (DoE) strategies in bioengineering to meet unpredicted impacts of factor interactions and non-linear effects (Weissman and Anderson, 2014).

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Enzymatic modifications of exopolysaccharides enhance bacterial persistence

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Biofilms are surface-attached communities of bacterial cells embedded in a self-produced matrix that are found ubiquitously in nature. The biofilm matrix is composed of various extracellular polymeric substances, which confer advantages to the encapsulated bacteria by protecting them from eradication. The matrix composition varies between species and is dependent on the environmental niche that the bacteria inhabit. Exopolysaccharides (EPS) play a variety of important roles in biofilm formation in numerous bacterial species. The ability of bacteria to thrive in a broad range of environmental settings is reflected in part by the structural diversity of the EPS produced both within individual bacterial strains as well as by different species. This variability is achieved through polymerization of distinct sugar moieties into homo- or hetero-polymers, as well as post-polymerization modification of the polysaccharide. Specific enzymes that are unique to the production of each polymer can transfer or remove non-carbohydrate moieties, or in other cases, epimerize the sugar units. These modifications alter the physicochemical properties of the polymer, which in turn can affect bacterial pathogenicity, virulence, and environmental adaptability. Herein, we review the diversity of modifications that the EPS alginate, the Pel polysaccharide, *Vibrio* polysaccharide, cepacian, glycosaminoglycans, and poly-*N*-acetyl-glucosamine undergo during biosynthesis. These are EPS produced by human pathogenic bacteria for which studies have begun to unravel the effect modifications have on their physicochemical and biological properties. The biological advantages these polymer modifications confer to the bacteria that produce them will be discussed. The expanding list of identified modifications will allow future efforts to focus on linking these modifications to specific biosynthetic genes and biofilm phenotypes.

Keywords: Biofilm, exopolysaccharide, PNAG, PIA, alginate, PEL, VPS, cepacian

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Abbreviations: Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; CPS, capsular polysaccharide; EPS, exopolysaccharide; G-blocks, guluronate blocks; GAGs, glycosaminoglycans; GBS, Group B *Streptococcus*; GlcA, glucuronic acid; GulA, guluronate; GulNAcA, *N*-acetyl guluronate; HS, heparan sulfate; IdoA, iduronic acid; LPS, lipopolysaccharide; M-blocks, mannuronate blocks; ManA, mannuronate; MBOAT, membrane-bound *O*-acetyltransferase; MG-blocks, mixed mannuronate and guluronate blocks; PEL, pel polysaccharide; PIA, polysaccharide intercellular adhesin; PNAG, poly-*N*-acetylglucosamine; polyM, polymannuronate; ROS, reactive oxygen species; VPS, *Vibrio* polysaccharide.

Introduction

Bacteria faced with fluctuating or stressful environmental conditions can undergo a number of physiological changes. One common tactic that bacteria use to adapt to their surroundings is to grow as a multicellular community or biofilm. Biofilm formation begins with attachment of the bacteria to a surface or, in the case of some infectious biofilms, embedding of the bacteria in host-derived tissue or mucous. This is followed by bacterial aggregation, colony development, and the secretion of self-produced polymeric substances, which form a matrix that encapsulates and protects the bacteria (Ohman, 1986; Allesen-Holm et al., 2006). This matrix is composed of nucleic acids, proteins, lipids, and extracellular polysaccharides (EPS; Flemming and Wingender, 2010), with the types and ratio of each component varying between bacterial species and environmental conditions. Once the biofilm has matured into a robust structure it becomes exceedingly difficult to eradicate, and is typically capable of enduring mechanical, biological, and chemical means of elimination. Bacteria form biofilms in nearly all environments studied to date (Bjarnsholt et al., 2013b), and are implicated in the contamination of surfaces as diverse as the International Space Station (Kim et al., 2013), ship hulls (Schultz et al., 2011), and oil storage and transfer infrastructure (Lenhart et al., 2014). Biofilms are also of major concern in medical settings, where they are responsible for the chronic infection of burn wounds, eye and skin lacerations, and pneumonia in CF patients (Lyczak et al., 2000). The contamination of medical devices such as catheters, prosthetic joints, and ventilators (Veerachamy et al., 2014) has also been well documented. In these environments EPS often contribute to the formation, growth, and preservation of biofilm architecture and also serve to protect the bacteria against antibiotics, desiccation, and the host's immune defenses.

Biosynthesis of EPS begins in the cytoplasm with the generation of activated precursor sugars. These precursors are often taken from common cellular sugar pools and are modified for specific use in EPS biosynthesis pathways, prior to polymerization (**Figure 1**). In Gram-negative bacteria, the polymer is transported across the inner membrane to the periplasm during synthesis; whereas in Gram-positive bacteria the polymer is transported directly to the extracellular space. Modifications to the polymer can occur in the cytoplasm (Atkin et al., 2014) or the periplasm (Colvin et al., 2013; Baker et al., 2014; Little et al., 2014b; Wolfram et al., 2014) prior to export across the outer membrane, and in the extracellular space in both Gram-positive and Gram-negative bacteria (Rozeboom et al., 2008; Little et al., 2014a).

The chemical structure of EPS from different bacterial species, or even within the same organism, can vary greatly. Bacterial EPS are usually composed of hexose sugars, but pentose sugars

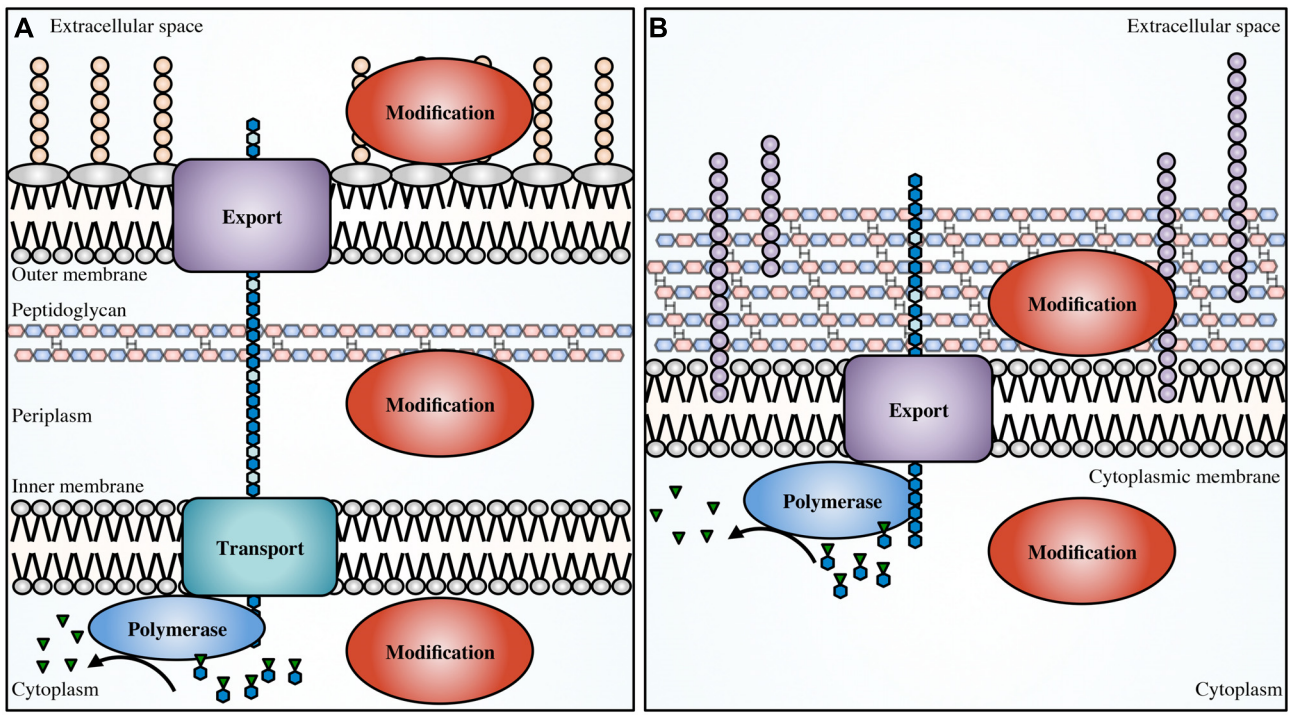

FIGURE 1 | Generalized EPS biosynthetic platforms. *Not to scale.* In Gram-negative bacteria **(A)** modifications to the polymer can occur in the cytoplasm, periplasm and in the extracellular space. In Gram-positive bacteria **(B)** modifications can occur in the cytoplasm and extracellular space. Polymer biosynthetic systems are represented here as follows: activated sugars (blue hexagons with green inverted triangles) are assembled by a polymerase (blue), transported across the inner membrane (teal; polymerization and transport may be coupled and performed by a single protein), and exported (purple) across the outer membrane in Gram-negative bacteria, or exported across the cytoplasmic membrane in Gram-positive bacteria. Modifications can be performed in any of these cellular compartments (red). Also shown, LPS (tan circles), teichoic acids (light purple circles), and EPS (blue hexagons).

have also been identified. *Rhizobium huakuii* EPS contains ribose (Hisamatsu et al., 1997), while some marine bacteria produce EPS with xylose and ribose moieties (Kwon et al., 2002). EPS can be homo- or hetero-polymers, and have branching side chains (Byrd et al., 2009; Cescutti et al., 2010) or be simple linear sugar polymers (Linker and Jones, 1966; Maira-Litràn et al., 2002). They can be as short as dimers and trimers, or thousands of saccharide repeat units long (González et al., 1998), depending on the mechanisms of chain length regulation, and can even be woven together to form fibers (Benziman et al., 1980).

Exopolysaccharides can be modified by the action of transferases and hydrolases which add or remove functional groups such as acetyls, pyruvyls (Marzocca et al., 1991), glyceryls (Kuo et al., 1986), succinyls (Reuber and Walker, 1993), lactyls (Maalej et al., 2014), or a combination of these, leading to variations in polymer surface electrostatics and solubility. Additionally, epimerization can drastically alter the structural conformation of polysaccharides, affecting polymer interactions within the biofilm (Steigedal et al., 2008). Some of these modifications have been studied with respect to their importance in bacterial virulence, pathogenesis, biofilm formation, or symbiosis (**Figure 2**; Ridout et al., 1997), as well as their commercial utility in the food and cosmetic industries.

Despite this wealth of knowledge, there remain a number of unresolved questions regarding the biological implications of EPS modifications. In this review, we explore the modifications that biofilm-forming EPS produced by human pathogenic bacteria undergo and discuss the proteins involved in modification, as well as the role modifications play in bacterial persistence in the environment and host.

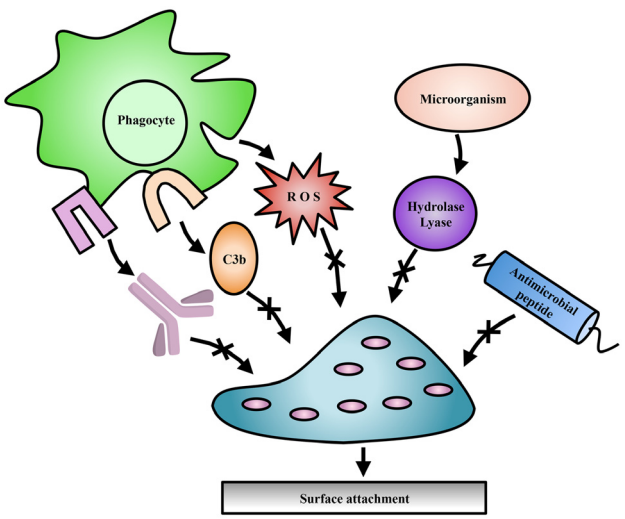

FIGURE 2 | Exopolysaccharide modifications offer protection to bacteria. Modifications to EPS contribute to evasion of host immune mechanisms such as complement deposition (C3b), and specific antibody production (lavender). Modifications have also been shown to protect against ROS produced by immune cells, antimicrobial peptides, and EPS degradation enzymes produced by competing microorganisms.

Alginate

Alginate synthesis has been characterized in several species of brown algae, as well as in the genera *Azotobacter* and *Pseudomonas* (Gorin and Spencer, 1966; Evans and Linker, 1973; Govan et al., 1981; Gacesa, 1988). Bacterial alginate is a high molecular weight, linear polysaccharide composed of β-1,4-linked D-ManA and variable amounts of its C5 epimer L-GulA (Smidsrød and Draget, 1996). In *Pseudomonas aeruginosa* and *Azotobacter vinelandii*, alginate is initially synthesized as polyM in the cytoplasm and is shuttled across the inner membrane to the periplasm, where it is randomly acetylated at the O2 and/or O3 hydroxyl positions (**Table 1**; **Figure 3**; Franklin and Ohman, 1993, 1996, 2002). ManA residues that are not acetylated serve as substrates for epimerization at the C5 position by the enzyme AlgG in the periplasm, leading to the formation of mixed ManA and GulA segments (MG-blocks) as well as non-epimerized sections (M-blocks; Gacesa, 1988; Jain et al., 2003).

Pseudomonas aeruginosa chronic lung infections in CF patients are the leading cause of morbidity and mortality. In these infections the production of alginate is often linked to poorer patient prognosis (Lyczak et al., 2000). In the lung, the clinical isolate *P. aeruginosa* FRD1 displays an alginate-overproducing, or mucoid phenotype due to mutations in negative regulatory elements, providing *P. aeruginosa* with the capability of adhering to respiratory tract epithelial cells and mucin (Marcus and Baker, 1985; Doig et al., 1987; Ramphal et al., 1987). In addition, alginate production has been linked to the hindrance of host cell-mediated phagocytosis and neutralization of ROS (Learn et al., 1987; Mai et al., 1993; Pier et al., 2001). Mucoid conversion is still not well understood, but *in vitro* experimentation has shown that nutrient and aeration levels (Buckmire, 1984; Krieg et al., 1986; Speert et al., 1990) and external stressors (Terry et al., 1991; Damron et al., 2011; Limoli et al., 2014) can induce mucoidy.

Azotobacter vinelandii is a soil-borne bacterium that is often used as a model organism for nitrogen fixation studies (Bulen et al., 1964). Although *A. vinelandii* is not a human pathogen, understanding the process of alginate biosynthesis in this organism has provided valuable insight into the biological significance of alginate production by *P. aeruginosa*. In *A. vinelandii,* alginate plays a unique and essential role where, under conditions of nutrient and environmental stress such as nitrogen starvation, *A. vinelandii* converts from a vegetative cell to a dormant cyst (Socolofsky and Wyss, 1961). Cyst development proceeds through deposition of a protective extracellular material composed primarily of alginate. The cyst layers are rich in proteins, lipids and carbohydrates, with the exine (outer layer) and intine (inner layer) containing carbohydrate material consisting of approximately 40 and 72% polyuronic acids, respectively (Lin and Sadoff, 1969). Cyst formation, much like the biofilm matrix, protects *A. vinelandii* from desiccation, and only when environmental conditions become more favorable will *A. vinelandii* convert back to the vegetative state by degrading the alginate barrier. In contrast to *P. aeruginosa*, *A. vinelandii* contains seven additional extracellular epimerases (AlgE1-7),

TABLE 1 | Exopolysaccharide modifying enzymes of pathogenic bacteria.

Protein name	GenBank Accession number	Organism(s) studied[a]	Function	Cellular localization	PDB code[b]	Additional comments	Reference
AlgG	NP_252235.1 AAP46694.1 YP_002798298.1	*P. aeruginosa* *P. fluorescens* *A. vinelandii*	Mannuronan C5-epimerase; introduces MG-blocks into polyM alginate	Periplasm	**4NK6**	Required for alginate production and epimerization in *P. aeruginosa* and *P. fluorescens*; cannot epimerize acetylated alginate	Chitnis and Ohman (1990), Franklin et al. (1994), Rehm et al. (1996), Morea et al. (2001), Gimmestad et al. (2003), Jain et al. (2003), Wolfram et al. (2014)
AlgE1 AlgE2 AlgE3 AlgE4 AlgE5 AlgE6 AlgE7	YP_002802178 YP_002802177 YP_002802176 YP_002802179 YP_002800496 YP_002802180 YP_002802182	*A. vinelandii*	Mannuronan C5-epimerases; alginate lyase (AlgE2, AlgE7); varying MG- and G-block forming activities	Extracellular	**AlgE4: 2PYG (active domain), 2AGM (regulatory domain)** **AlgE6: 2ML2 and 2ML3 (regulatory domains)**	Ca^{2+}-dependent; modular architecture composed of one or more epimerase active domains and activity enhancing regulatory domains	Ertesvåg et al. (1994, 1995, 1998), Ertesvåg and Valla (1999), Svanem et al. (1999, 2001), Aachmann et al. (2006), Rozeboom et al. (2008), Buchinger et al. (2014)
AlgJ	NP_252239.1	*P. aeruginosa*	Exhibits *in vitro* acetylesterase activity	Periplasm - IM tethered	**4O8V**	Required for alginate acetylation	Franklin and Ohman (1996, 2002), Baker et al. (2014)
AlgF	NP_252240.1 YP_002798293.1	*P. aeruginosa* *A. vinelandii*	Acetylation; specific role unknown	Periplasm	ND	Required for alginate acetylation	Franklin and Ohman (1993), Shinabarger et al. (1993), Vazquez et al. (1999), Franklin and Ohman (2002)
AlgI	NP_252238.1	*P. aeruginosa*	Acetylation; predicted MBOAT	Inner membrane	ND	Required for alginate acetylation	Franklin and Ohman (1996, 2002), Franklin et al. (2004)
AlgX	NP_252236.1	*P. aeruginosa* *A. vinelandii*	Acetylation; exhibits *in vitro* acetylesterase activity; terminal alginate acetylase	Periplasm	**4KNC**	Required for alginate production and acetylation	Monday and Schiller (1996), Robles-Price et al. (2004), Riley et al. (2013), Baker et al. (2014)
PelA	NP_251754.1	*P. aeruginosa*	Deacetylation; *in vitro* deacetylase activity	Periplasm	2VYO (30%; 511-794)	Modeled region of deacetylase, also has N-terminal hydrolase domain	Colvin et al. (2013)
BceO BceS BceU	YP_001116903 YP_001116907 YP_001116910	*B. cepacia* complex	Acetyltransferase	Inner membrane (predicted)	ND	Deletion of *bceS* – reduced acetylation	Ferreira et al. (2010)
VpsC VpsG	NP_230566.1 NP_230570.1	*V. cholerae*	Acetyltransferase (proposed)	ND	1T3D (92%; 2-172) 1T3D (97%; 1-140)	Modeled protein is an acetyltransferase	Fong et al. (2010)
PgaB	NP_415542.1	*E. coli*	Deacetylase; C-terminal carbohydrate binding module facilitates PNAG export	Outer membrane lipoprotein	**4F9J/4F9D**	Low catalytic efficiency allows for partial PNAG deacetylation	Wang et al. (2004), Itoh et al. (2008), Little et al. (2012, 2014b)
HmsF	NP_415542.1	*Y. pestis*	Deacetylase	Outer membrane	4F9D (88%; 43-646)	Modeled on PgaB	Forman et al. (2006)
IcaB	AAC06118.1 AAD52057.1	*S. epidermidis* *S. aureus*	Deacetylase	Extracellular	**4WCJ**	Low catalytic efficiency allows for partial PIA deacetylation	Pokrovskaya et al. (2013), Little et al. (2014a)
IcaC	AAC06119.1 AAD52058.1	*S. epidermidis* *S. aureus*	Succinyltransferase (proposed)	Membrane embedded	ND	Not experimentally determined	Atkin et al. (2014)

[a]*Genus abbreviations as follows: P. aeruginosa, Pseudomonas aeruginosa; P. fluorescens, Pseudomonas fluorescens; A. vinelandii, Azotobacter vinelandii; B. cepacia complex, Bcc; V. cholerae, Vibrio cholerae; E. coli, Escherichia coli; Y. pestis, Yersinia pestis; S. epidermidis, Staphylococcus epidermidis; S. aureus, Staphylococcus aureus.*

[b]*Accession code for structure or structurally related protein, bold letters indicate that the structure has been experimentally determined. Where applicable, protein coverage (%) and residues modeled are listed in brackets. ND: not determined, Phyre[2] (Kelley and Sternberg, 2009) was unable to model the protein.*

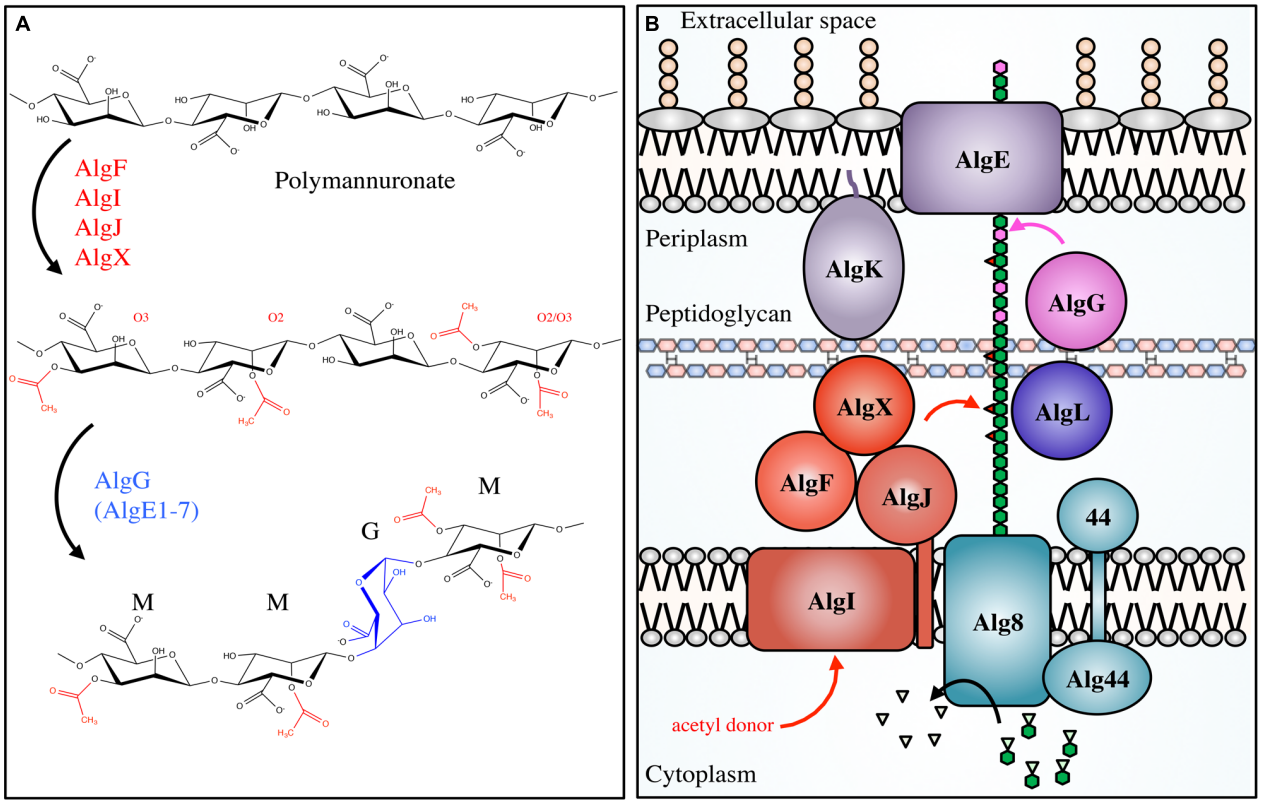

FIGURE 3 | *Pseudomonal* alginate modifications and biosynthetic apparatus. *Not to scale.* **(A)** Alginate is first polymerized as β-sD-(1→4)-polyM (top) before being acetylated at the O2 and/or O3 positions by the concerted actions of AlgIJFX. Acetylated ManA residues (middle) cannot be epimerized to L-GulA by AlgG (bottom). **(B)** The proteins involved in alginate biosynthesis in *Pseudomonas aeruginosa*. PolyM (green hexagon chain) is synthesized in the cytoplasm from the nucleotide-sugar precursor GDP-ManA (green hexagons with inverted triangles). It is polymerized, and transported across the inner membrane via Alg8/44, acetylated (red triangles) by the concerted action of AlgFIJX, and epimerized to GulA (magenta) by AlgG. AlgL possesses lyase activity; AlgK is a lipoprotein required for efficient localization of AlgE, the porin required for alginate export.

capable of generating polyguluronate segments (G-blocks) in addition to MG-blocks (**Table 1**; Fischer and Dorfel, 1955; Haug et al., 1966, 1967). Despite the importance of alginate in cyst formation (Campos et al., 1996), alginate production by *A. vinelandii* has been studied primarily for its potential use as an alternative source of commercial alginate in place of traditional seaweed harvesting approaches.

Given the importance of alginate for the virulence of *P. aeruginosa* in the CF lung, the protective characteristics of *A. vinelandii* cysts, and the use of bacterial alginate as a convenient substitute for commercial eukaryotic alginate sources, there has been a drive to understand the consequences of alginate acetylation and epimerization on these processes.

Alginate Acetylation

Alginate acetylation in *P. aeruginosa* requires the collective actions of the proteins AlgF, AlgI, AlgJ, and AlgX for the addition of acetyl groups to the O2 and/or O3 hydroxyl positions of polyM in the periplasm prior to epimerization and export (**Table 1**; **Figure 3**). Specifically, it is thought that AlgI transfers an acetyl group from an as yet unidentified cytoplasmic donor to AlgJ or AlgX, where it may be passed between them before transfer to the polymer by AlgX (Riley et al., 2013; Baker et al., 2014). AlgF is an important part of the acetylation process but has not yet been assigned a role. Based on the peptidoglycan *O*-acetylation machinery (Moynihan and Clarke, 2011), it seems that the presence of a MBOAT for acetyl-donor transport (AlgI), and an acetyltransferase (AlgX) should be sufficient for polysaccharide *O*-acetylation. As acetylation occurs at both the O2 and O3 positions, one hypothesis is that the proteins AlgJ and AlgF govern specificity. Although they have not yet been shown to directly bind alginate, they may still be able to regulate the position and frequency of acetyl addition under different environmental conditions. Genetic and biochemical experiments targeting the degree of alginate acetylation in combination with structural data may provide insight into these questions.

One of the purposes of EPS production is to serve as a structural component of the biofilm matrix. Alginate lacking *O*-acetyl groups has been analyzed for its ability to form structured biofilms using an acetylation defective *P. aeruginosa* FRD1 mutant. These studies revealed that the mutant was only

TABLE 2 | Biological implications of EPS modifications.

Modification	Proteins involved	Organism studied	Implication of modification	Reference
Alginate				
Acetylation	AlgF, AlgI, AlgJ, AlgX	*Pseudomonas aeruginosa*	Required for surface attachment and formation of structured microcolonies	Nivens et al. (2001), Tielen et al. (2005)
			Increased polymer viscosity	Tielen et al. (2005)
			Decreased neutrophil locomotion and lymphocyte transformation	Mai et al. (1993)
			Reduced activation of complement and opsonic killing by phagocytes	Pier et al. (2001)
			Scavenging of ROS	Learn et al. (1987)
			Reduced susceptibility to enzymatic degradation	Farrell and Tipton (2012)
			Increased gel thickness	Skjåk-Braek et al. (1989)
Epimerization	AlgG, AlgE1-7 (*Azotobacter vinelandii)*	*P. aeruginosa*	Improved gel forming ability (cohesion)	Grant et al. (1973), Donati et al. (2005)
			Upregulation of virulence factors through Ca^{2+} sequestration	Horsman et al. (2012)
		A. vinelandii	Maintain biofilm structure during changing environmental conditions	Ertesvåg et al. (1998)
			Preserve N_2-fixing capability	Sabra et al. (2000)
			Required for formation of functional cyst coat	Steigedal et al. (2008)
PEL				
Deacetylation	PelA	*P. aeruginosa*	Required for biofilm formation (in PSL deficient strains)	Colvin et al. (2013)
Cepacian				
Acetylation	BceOSU	*Bcc*	Reduced susceptibility to enzymatic degradation	Cescutti et al. (2006)
			Scavenging of ROS	Cuzzi et al. (2012)
***Vibrio* polysaccharide (VPS)**				
Acetylation	VpsG	*Vibrio cholerae*	Required for robust biofilm formation and wild-type phenotypes	Fong et al. (2010)
Poly-*N*-acetyl-glucosamine (PNAG)				
Deacetylation	PgaB (*Escherichia coli*), HmsF (*Yersinia pestis*) IcaB (*Staphylococcus epidermidis* and *S. aureus*)	*S. epidermidis*	Required for biofilm formation and surface attachment	Vuong et al. (2004a)
			Resistance to human cationic antimicrobial peptides	
			Resistance to neutrophil phagocytosis	
			Persistence in mouse model of infection	
		S. aureus	Required for biofilm formation and surface attachment	Cerca et al. (2007)
			Resistance to phagocytosis	
			Persistence in mouse model of infection	
		E. coli	Required for export of polymer and biofilm formation	Itoh et al. (2008)
		Y. pestis	Required for biofilm formation	Forman et al. (2006)
Succinylation	IcaC	*S. aureus*	Modulation improves *in vitro* fitness	Brooks and Jefferson (2014)

able to produce small, unstructured microcolonies that sparsely populated the examined surface, suggesting an attachment defect (**Table 2**; Nivens et al., 2001). In contrast, FRD1 formed extensive biofilm structures that exhibited significant structural heterogeneity. In a separate study, an aggregation defect was revealed when the capacity for an acetylation-deficient FRD1 mutant to adhere to a steel surface was tested (Tielen et al., 2005). Additionally, the viscosity of extracellular material from the acetylation-defective mutant was significantly reduced in comparison to FRD1, suggesting that the loss of *O*-acetyl groups led to weakening of inter- and intra-polymer interactions within the biofilm matrix. This is supported by rheological studies of FRD1 biofilms, which suggested that inter-chain alginate interactions occur primarily through physical entanglements (Wloka et al., 2005). These entanglements supported an elastic biofilm architecture, which differed from *O*-acetylation-defective FRD1 mutants which produced weaker biofilms with reduced resistance to tensile forces. Based on these results, it was suggested that *O*-acetyl groups in alginate act as molecular hooks that improve the resistance of the entangled alginate structural network against applied forces (Wloka et al., 2005). While the importance of alginate acetyl groups for cell aggregation and microcolony formation *in vitro* is well established, the influence of *O*-acetyl groups on biofilm formation phenotypes in clinically relevant *P. aeruginosa* infections or related *in vivo* model systems of infection remain uncharacterized.

P. aeruginosa biofilm formation in the CF lung has been shown to provide significant protection from a variety of host immune

factors. For example, decreased locomotion of neutrophils, as well as reduced lymphocyte transformation, have been observed when these cell types are incubated with alginate (Simpson et al., 1988). However, chemical removal of acetyl groups from alginate led to a complete loss of these inhibitory effects on neutrophil and lymphocyte function, suggesting that alginate *O*-acetylation is essential for their suppression (Mai et al., 1993). The activation of complement is also affected by the presence of acetyl groups (Pier et al., 2001). This is not surprising given that interactions between alginate and the complement component C3b likely occurs through unsubstituted hydroxyl groups (Hostetter et al., 1982), suggesting that the addition of acetyl groups to alginate in *P. aeruginosa* may have evolved as a mechanism for complement evasion. Activation of the alternative pathway of complement can lead to phagocytic killing, which is also impaired by the presence of *O*-acetyl groups. Opsonic killing of the FRD1 *O*-acetylation deficient mutant by phagocytes was readily observed, while wild-type FRD1 was resistant to these attacks (Pier et al., 2001). Alginate is also known to scavenge ROS produced by phagocytic cells during infection. Hypochlorite is a common ROS produced by phagocytes, and the presence of alginate in mucoid *P. aeruginosa* provides a significant protective advantage against hypochlorite over non-mucoid cells *in vitro* (Learn et al., 1987). This protective effect was, in part, attributed to the *O*-acetyl groups, as chemically deacetylated alginate exhibited impaired hypochlorite scavenging. Furthermore, addition of hypochlorite to native alginate led to a decrease in viscosity, similar to that seen for the chemically deacetylated alginate, suggesting that hypochlorite may be specifically reacting with *O*-acetyl groups from native alginate (Learn et al., 1987).

When bacteria are contending for control of the same environment, they can release extracellular enzymes to degrade critical structural components of cohabiting organisms to give them a competitive advantage (Korotkov et al., 2012). This is observed in the CF lung, where instances of multi-species biofilms are common (Elias and Banin, 2012). During colonization of the CF lung, alginate acetyl groups may serve as a protective mechanism to prevent unwanted degradation of alginate within the biofilm by bacteria that could secrete an AlgL-like lyase as an offensive tactic. The *P. aeruginosa* alginate lyase AlgL preferentially degrades deacetylated alginate or polyM over mature, acetylated alginate (Farrell and Tipton, 2012). Furthermore, *O*-acetyl groups prevent the epimerization of ManA to GulA by the epimerases AlgE1-7 in *A. vinelandii*, which may allow for control over the degree of epimerization and, in turn, regulation of the cyst coat composition.

Alginate acetylation content ranges from 4 to 57%, depending on the percentage of ManA present (Skjåk-Braek et al., 1986). The degree of *O*-acetylation is often observed to vary not only between different alginate-producing organisms, but also between different strains of the same organism and even within the same strain under differing growth conditions (Marty et al., 1992; Peña et al., 2006). For example, modulation of carbon source during growth for a single alginate-producing *P. syringae* strain led to significant differences in acetyl content, ranging from 9 to 34% of total uronic acids bearing an acetyl group (Day, 1988). In another study, the alginate produced by several different strains of *P. aeruginosa* grown on nutritionally distinct media was examined. This study revealed that between different strains *O*-acetyl content of alginate varied between 2 to 56% (Marty et al., 1992). Furthermore, in both studies alginate acetyl content changed over the course of a single growth experiment by as much as 40%, possibly owing to the availability of acetyl-CoA, the proposed acetyl donor (Lee and Day, 1998). In addition to acetyl-CoA availability, differences in acetyl content could conceivably be a means to optimize attachment, nutrient uptake, or nutrient diffusion within the biofilm in the face of different media compositions and nutrient sources. This notion is supported by findings which suggest that alginate *O*-acetylation can enhance the swelling ability of calcium alginate gels (Skjåk-Braek et al., 1989). Deacetylated alginate exhibited poor swelling ability in comparison to chemically acetylated variants, with increasing degrees of acetylation leading to greater swelling volume. Conversely, increased *O*-acetylation led to a decrease in the affinity of alginate gels for calcium ions (Skjåk-Braek et al., 1989). Thus, alginate acetyl content has specific consequences with respect to calcium ion binding and the thickness of alginate gels, which may influence nutrient diffusion in the biofilm. These findings could potentially be extrapolated to other components of the growth media, and suggests a mechanism by which alginate-producing bacteria could regulate the uptake of essential nutrients.

Alginate Epimerization

Pseudomonas aeruginosa has a single alginate C5-epimerase in the periplasm, AlgG. In *A. vinelandii*, there is an AlgG ortholog that performs the same function, and seven additional extracellular epimerases, AlgE1 through AlgE7 (**Table 1**). Alginate can form strong gels through interactions with GulA residues, mediated by Ca^{2+} ions. This feature was thought to be limited to alginates containing G-blocks, and would therefore exclude the MG-block alginates produced by *P. aeruginosa* (Grant et al., 1973). However, it is now thought that alginates containing exclusively MG-blocks can also form gels in the presence of Ca^{2+} (Donati et al., 2005), suggesting that epimerization by AlgG in *P. aeruginosa* may serve as a mechanism to improve the cohesion of alginate during biofilm formation. It was found that addition of $CaCl_2$ to growth media led to the production of biofilms that were 10- to 20-fold thicker than that produced in the absence of Ca^{2+} (Sarkisova et al., 2005). Ca^{2+}-alginate interactions also regulate virulence factor expression, as chelation of Ca^{2+} by alginate induces expression of the Type 3 secretion system (Horsman et al., 2012). Therefore, it appears that there are mechanisms in place in *P. aeruginosa* for virulence factors to be upregulated by the expression of another, thus allowing for concerted actions that improve fitness (**Table 2**). Despite advances in understanding the interplay between Ca^{2+} and alginate in *P. aeruginosa*, and the extensive studies performed on acetyl-deficient alginate, there are no reports on the effects of epimerization on biofilm formation, pathogenicity, or virulence.

In contrast, the role of epimerization in *A. vinelandii* cyst formation has been well characterized. One hypothesis regarding

the ability of *A. vinelandii* to express multiple epimerases with unique activities is that these enzymes allow the alginate structures to be tailored to different layers of the cyst under diverse environmental conditions. For example, the epimerase AlgE1 has two catalytic domains that introduce primarily MG-blocks and G-blocks, respectively (Ertesvåg et al., 1998). Decreasing the availability of Ca^{2+} in the presence of AlgE1 *in vitro* led to greater incorporation of G-blocks into polyM alginate. This may provide a means *in vivo* to maintain the strength of Ca^{2+}-mediated inter-alginate bonds in the face of decreased environmental Ca^{2+} availability (Ertesvåg et al., 1998). Regulation of alginate structure is also observed during vegetative growth of *A. vinelandii*, where nitrogen fixation is mediated by the expression of highly oxygen-sensitive nitrogenases. In this state, alginate is utilized as a barrier to prevent the diffusion of oxygen into the cell. In the presence of increasing environmental O_2 concentrations, *A. vinelandii* was able to produce alginate with greater G-content, which led to the formation of a thicker, denser alginate layer around the cell and thus limited oxygen penetration (Sabra et al., 2000). The expression of different mannuronan C5-epimerases is also regulated over the course of the *A. vinelandii* life cycle, including during vegetative growth, cyst development, and cyst germination (Høidal et al., 2000). Although the exact biological function for the expression of specific epimerases at unique points in the life cycle of *A. vinelandii* has not been determined, preferential expression of AlgE7 during cyst germination could be linked to the apparent lyase activity of this enzyme, which may be utilized to degrade the cyst coat (Høidal et al., 2000).

Unlike AlgG in *P. aeruginosa*, the importance of the AlgE1-7 epimerases in the formation of the cyst coat and tolerance to desiccation has been explored. Inactivation of the AlgE1-7 epimerases, either through chromosomal deletion in *A. vinelandii* (Steigedal et al., 2008) or by inactivating the Type 1 secretion system responsible for their export (Gimmestad et al., 2006) led to the production of low G-content alginate, suggesting that the periplasmic *A. vinelandii* AlgG is active but not very efficient. In both cases, these mutants were unable to form a cyst coat and could not survive desiccation. In contrast, deletion of individual *algE* genes, with the exception of *algE3*, did not have an appreciable effect on the G-content of alginate. Deletion of *algE3* showed a significant reduction in G-content (Steigedal et al., 2008). However, since each of the individual *algE* deletion mutants was able to form a functional cyst and survive desiccation, it appears that no single epimerase is absolutely essential for cyst formation or germination. This suggests that the presence of multiple extracellular epimerases may increase redundancy of epimerase activity to ensure formation of a functional cyst coat (Steigedal et al., 2008). It remains to be determined whether cyst formation under unique stressful conditions may require specific epimerases, and little work has been done to date to examine the role of mannuronan C5-epimerases during the vegetative stage of *A. vinelandii* growth.

While a great deal is understood about the regulation of alginate biosynthesis and its modification at the genetic and protein level (Hay et al., 2014), the implications of alginate acetylation and epimerization in terms of biofilm formation, pathogenicity and virulence, environmental adaptability and survivability remain largely uncharacterized.

The Pel Polysaccharide

In addition to alginate, *P. aeruginosa* is capable of synthesizing two other polymers that have been implicated in biofilm formation, the Pel and Psl polysaccharides (PEL and PSL; Franklin et al., 2011). Unlike alginate, PEL and PSL are primarily associated with the establishment of non-mucoid biofilms. PSL is a neutral, branched polysaccharide with a five-sugar repeat unit composed of D-mannose, D-glucose, and L-rhamnose and is not thought to undergo any modifications after polymerization (Byrd et al., 2009). The exact structure of PEL is currently unknown, but it is predicted to be glucose rich (Friedman and Kolter, 2004; Ma et al., 2012). Colvin and colleagues have demonstrated that the PEL biosynthesis protein PelA has deacetylase activity *in vitro* and when residues predicted to be required for deacetylation were mutated, this activity was lost. Introduction of these PelA deacetylation mutations in *P. aeruginosa* PA14, which uses PEL as the primary EPS, led to a biofilm deficient phenotype and a lack of material recognizable by PEL-reactive antisera (**Table 1**; Colvin et al., 2013). Given the localization of PelA to the periplasm (Colvin et al., 2013), deacetylation of PEL following polymerization may be necessary for biofilm formation, and suggests that an acetylated sugar is likely a feature of the PEL. Our current understanding of PEL biosynthesis is limited, and it remains to be determined whether PelA acts directly on PEL, the degree of PEL deacetylation by PelA, and what effect this modification has on virulence.

Cepacian

The Bcc is a group of at least 17 different bacterial species, including beneficial environmental isolates, as well as rhizosphere parasites, and plant and animal pathogens (Mahenthiralingam et al., 2002; Vanlaere et al., 2009). The Bcc have become increasingly important as opportunistic pathogens of immunocompromised individuals and those with CF (Mahenthiralingam et al., 2005). In CF patients, Bcc infections occasionally develop into a form of necrotising pneumonia, known as cepacia syndrome, which often leads to patient death (Govan and Deretic, 1996). The majority of both clinical and environmental Bcc isolates produce the EPS cepacian (Ferreira et al., 2010); a known virulence factor that contributes significantly to bacterial pathogenicity. Cepacian is composed of glucose, GlcA, mannose, rhamnose, and galactose in a 1:1:1:1:3 ratio and is decorated with acetyl groups (**Figure 4**; Cérantola et al., 1999; Cescutti et al., 2000; Linker et al., 2001). The acetyl groups can be found at 12 different locations on the polymer repeating unit with an average of three acetyl groups present per repeat unit (Cescutti et al., 2011). The genes responsible for cepacian acetylation were discovered by Ferreira and colleagues, and include the putative acetyltransferases *bceO*, *bceS*, and *bceU* (Ferreira et al., 2010). Mutations in *bceS* produced cepacian with

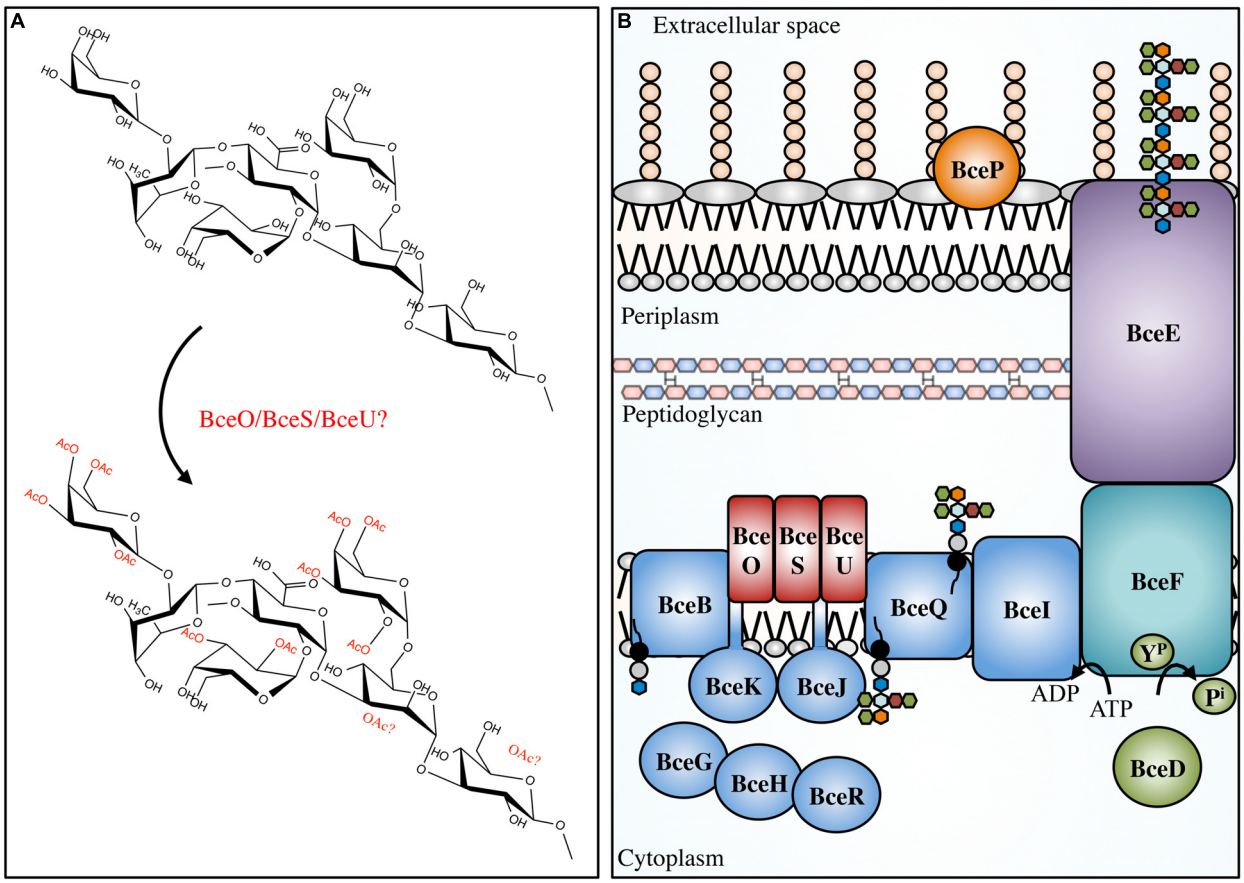

FIGURE 4 | Cepacian modifications and biosynthetic apparatus. *Not to scale.* **(A)** Cepacian may be *O*-acetylated at various locations by BceOSU, leading to a number of unique combinations with an average of three acetyls per repeat unit. **(B)** Cepacian repeat units are synthesized in the cytoplasm on an isoprenoid lipid carrier (black circle/gray circle), initiated by BceB and continued by glycosyltransferases BceGHJKR. BceGHR are cytoplasmic, while BceJK are integral membrane proteins. BceOSU are predicted to be membrane embedded acetyltransferases that decorate the repeat unit with acetyl groups. BceQ translocates the repeat units across the inner membrane, followed by addition of repeat units to the growing polymer at the periplasmic side, which is dependent on BceI. Polymerization and export requires BceF, a tyrosine kinase. BceD is a protein tyrosine phosphatase which dephosphorylates BceF. BceE is the channel for polymer export across the OM. BceP is putatively involved in polysaccharide degradation, though its role and localization is unkonwn. ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate; Y^P, phosphorylated tyrosine residue; P^i, inorganic phosphate. Green hexagon, galactose; orange hexagon, GlcA; light blue hexagon, mannose; maroon hexagon, rhamnose; blue hexagon, glucose.

approximately 20% fewer acetyl groups, implicating this protein in partial cepacian acetylation (Ferreira et al., 2010). The roles of *bceO* and *bceU* in cepacian acetylation have not been evaluated, nor has a triple mutant been generated to discern the fitness of an acetyl-deficient cepacian producer.

An enzyme with lyase activity that specifically degrades cepacian has been isolated from culture supernatants of *Bacillus* sp. This enzyme has significantly higher activity against the chemically deacetylated polymer than native cepacian, implicating acetylation in protective mechanisms (**Table 2**; Cescutti et al., 2006). As with alginate acetylation, cepacian acetylation may have evolved as a defensive mechanism to prevent polymer cleavage in the presence of competitive organisms. Furthermore, like alginate, cepacian acetyl groups have been shown to provide protection against ROS, specifically hypochlorite (Cuzzi et al., 2012). Acetyl groups were the first structural features to undergo damage following hypochlorite treatment, which led to a loss of polymer-polymer interactions and cepacian unfolding, increasing the susceptibility of the cepacian backbone to hypochlorite degradation (Cuzzi et al., 2012). Consequently, cepacian acetylation improves polymer robustness to hypochlorite-mediated damage and increases the amount of polymer reactive groups that could neutralize hypochlorite prior to reaching the cellular surface. Further study of cepacian producing Bcc pathogens is necessary to determine, whether like alginate, there is a role for cepacian acetyl modification in immune evasion.

Vibrio Polysaccharide (VPS)

Vibrio cholerae is a human pathogen that causes the diarrhoeal disease cholera (Kaper et al., 1995; Faruque et al., 1998). This bacterium is a natural inhabitant of aquatic ecosystems, where

it forms biofilms on a variety of surfaces, including plankton, plants, crustaceans, insects, and sediment (Huq et al., 1983, 1995; Halpern et al., 2004; Broza et al., 2005). In areas where cholera is endemic, *V. cholerae* has been shown to form suspended biofilm-like aggregates in surface waters, however, when particles >20 μm in diameter are removed from water sources, the incidence of cholera can be reduced (Huq et al., 1996; Colwell et al., 2003). Furthermore, it has been shown that the average infectivity of the aggregate form of *V. cholerae* is significantly higher than that of planktonic cells (Faruque et al., 2006), and biofilm formation within aquatic ecosystems significantly improves *V. cholerae* fitness and persistence (Matz et al., 2005). A major component of the biofilm produced by *V. cholerae* is an EPS called VPS. This polymer is thought to be produced during infection and contributes to bacterial colonization and survival (Yildiz and Schoolnik, 1999; Fong et al., 2010). The chemical structure of VPS revealed a backbone containing the unusual constituent GulNAcAGly: the amide formed from 2-acetamido-2-deoxy-L-guluronic acid and glycine (**Figure 6**; Yildiz et al., 2014). Of the genes involved in VPS biosynthesis, originally identified using a transposon mutagenesis screen (Yildiz and Schoolnik, 1999), two putative acetyltransferases, *vpsG* and *vpsC*, were identified (Fong et al., 2010). Deletion of *vpsG* results in reduced biofilm formation and altered biofilm-related phenotypes, as well as weak reactivity with VPS antisera, suggesting that it may modify the polymer, perhaps through acetylation (**Table 1**). In contrast, deletions of *vpsC* do not affect biofilm formation or VPS production, suggesting that *vpsC* is inactive, not expressed, or is performing some other function in VPS biosynthesis besides polymer modification (Fong et al., 2010). The chemical composition of VPS produced by *vpsG* and *vpsC* mutants was not studied for alterations in acetyl content. The presence of GulNAcA in VPS may be the result of epimerization by the predicted GDP-mannose dehydrogenase VpsB, a conversion similar to the ManA to GulA epimerization catalyzed by AlgG in the biosynthesis of alginate by *P. aeruginosa* (Wolfram et al., 2014; Yildiz et al., 2014). The unusual glycine modification in VPS requires further exploration, as the enzyme responsible for its addition is presently unknown. Given the important role of VPS in *V. cholerae* pathogenesis and environmental persistence, and the recent determination of its precise chemical structure, we anticipate that the proteins involved in VPS modification will soon be identified and characterized.

Glycosaminoglycans (GAGs)

GAGs are a group of polymers that are typically composed of a disaccharide repeat unit containing an amino sugar and a hexuronic acid (Laurent and Fraser, 1992; Esko and Lindahl, 2001; DeAngelis, 2002; Silbert and Sugumaran, 2002). GAGs were initially thought to exist only in the animal kingdom, where they serve essential biological functions, however, there has been an emergence of GAG-like polymers amongst prokaryotes (Vann et al., 1981; Rodriguez et al., 1988; DeAngelis et al., 2002). Prokaryotic GAGs are typically less complex than their eukaryotic counterparts due to an absence of modifications such as sulfation (Raedts et al., 2011). HS, for example, is an essential GAG in animals and is composed of repeating disaccharides of GlcA and GlcNAc (Kjellén and Lindahl, 1991). HS can be modified post-polymerization by a glucuronyl C5-epimerase, which converts GlcA to IdoA, as well as by the addition of sulfate groups to GlcNAc or IdoA moieties. Mouse embryos lacking the GlcA C5-epimerase display a lethal phenotype characterized by skeletal malformations and lung defects (Li et al., 2003), highlighting the importance of HS epimerization in murine development. Interestingly, the K5 antigen of *E. coli* O10:K5:H4 has an identical structure to heparosan, the unsulfated, non-epimerized backbone structure of HS (Vann et al., 1981). K5 heparosan is a form of molecular camouflage, as it imparts low immunogenicity to the bacterium in humans and hence increased pathogenicity (Vann et al., 1981).

Although sulfation has not yet been observed amongst prokaryotic GAGs, IdoA residues have been found to be constituents of bacterial GAGs (**Figure 6**). The identification of the bacteria glucuronyl C5-epimerase has proven elusive (Raedts et al., 2011), however, an enzyme (RED65_08024) from the marine bacterium *Bermanella marisrubi* that shares 37% sequence similarity with the human glucuronyl C5-epimerase has been characterized and shown *in vitro* to convert GlcA to IdoA in de-sulfated mouse HS (Raedts et al., 2013). This glucuronyl C5-epimerase represents the first prokaryotic protein capable of generating IdoA residues, and is the only identified epimerase that can function on bacterial polysaccharides post-polymerization, besides AlgG and AlgE1-7. Unfortunately, the EPS produced by *B. marisrubi* has not been characterized, so its target remains unknown. Nevertheless, the ability of bacteria to more closely replicate the structures of essential human polysaccharides by expression of homologous modification enzymes likely serves as a mechanism to mask their presence from the host immune system (Cress et al., 2014).

Poly-β-1,6-*N*-Acetyl-glucosamine (PNAG)

Poly-β-1,6-*N*-acetyl-glucosamine is a poly-GlcNAc polymer that is produced by a wide range of Gram-positive and Gram-negative bacterial pathogens, including *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, *Yersinia pestis*, *Bordetella* sp., *Acinetobacter baumanii*, *Actinobacillus pleuropneumoniae*, *Burkholderia cepacia* complex (Bcc), and *Aggregatibacter actinomycetemcomitans* (Cramton et al., 1999; Vuong et al., 2004b; Wang et al., 2004; Izano et al., 2007, 2008; Parise et al., 2007; Bobrov et al., 2008; Choi et al., 2009). These organisms are responsible for a wide spectrum of diseases, including but not limited to, hospital acquired infections, toxic shock syndrome, plague, and whooping cough. Depending on the source or organism in question, PNAG may also be referred to as PGA (polyglucosamine, in Gram-negative bacteria), PIA (in Gram-positive bacteria), poly-NAG, hms+ (in *Y. pestis*), or BPS (*Bordetella* polysaccharide, in *Bordetella* sp.). Given the differences in PNAG modifications between Gram-positive and

Gram-negative bacteria, as described below, we will use PGA and PIA to refer to PNAG polymer produced by Gram-negative and Gram-positive organisms, respectively.

Initially *S. epidermidis* was thought to produce several different polymers, but the discovery of the *icaADBC* operon (Heilmann et al., 1996a,b; Gerke et al., 1998) revealed that only a single polymer, PIA, was produced (Tojo et al., 1988; Christensen et al., 1990; Heilmann et al., 1996a; Mack et al., 1996; McKenney et al., 1998). PIA is a partially deacetylated β-1,6-GlcNAc polymer. In *S. epidermidis* and *S. aureus* 15–20% of the *N*-acetyls are removed by the extracellular, cell-surface associated polysaccharide deacetylase IcaB (**Table 1**; **Figure 5**; Vuong et al., 2004a; Cerca et al., 2007). In addition to deacetylation, approximately 6 and 10% of GlcNAc residues in *S epidermidis* and *S. aureus*, respectively, are *O*-succinylated (Joyce et al., 2003; Sadovskaya et al., 2005). This modification is thought to be performed by the membrane localized protein, IcaC (Atkin et al., 2014). Interestingly, a mechanism of phase variation, where bacteria modulate virulence phenotypes at the genome level in a rapid on/off fashion, was noted in *S. aureus* wherein slipped-strand mispairing led to inactivation of *icaC* (Brooks and Jefferson, 2014). This phenotype confers a fitness advantage that was not seen when the *ica* operon was deleted, which may be a response to modulate PIA *O*-succinylation and thus decrease the overall anionic charge of the polymer.

The production of PGA has been extensively characterized in *E. coli*, where the *pgaABCD* operon encodes the proteins necessary for its biosynthesis (Wang et al., 2004). In *E. coli*,

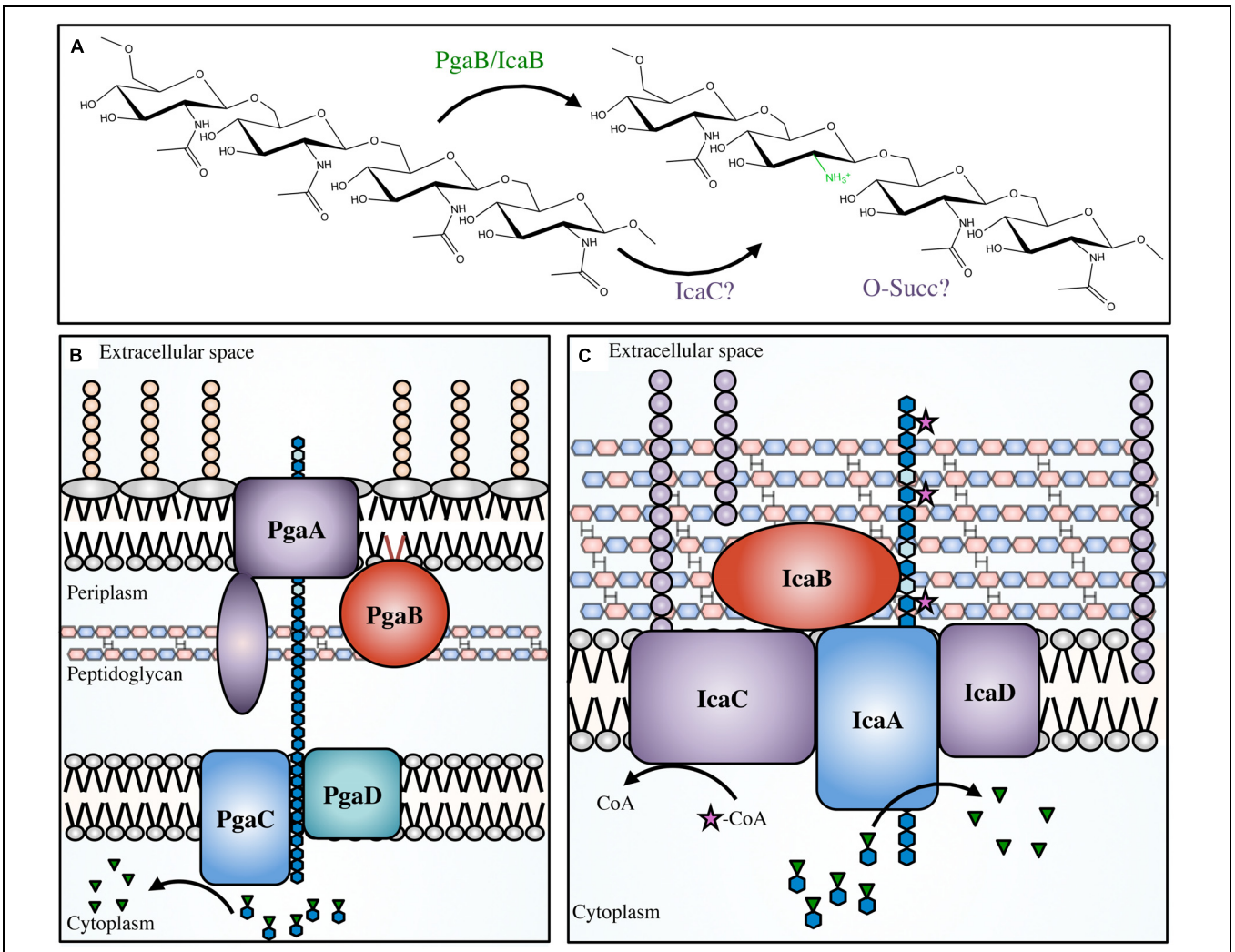

FIGURE 5 | The PNAG and PIA modifications and biosynthetic apparatus. *Not to scale.* **(A)** PNAG polymers are partially deacetylated by PgaB in *Escherichia coli*, or IcaB in *Staphylococcal* species. It has been proposed that IcaC *O*-succinylates the polymer in certain *Staphylococcal* species, however, the location of the succinyl groups and the order of deacetylation/succinylation has not yet been determined. The proteins involved in PNAG biosynthesis in *E. coli* **(B)**, and PIA synthesis in *Staphylococcal* species **(C)**. PNAG (blue hexagon chain) is synthesized in the cytoplasm from the nucleotide-sugar precursor UDP-GlcNAc (blue hexagons with green inverted triangles). The polymer is transported across the inner membrane *via* PgaCD, deacetylated (light blue hexagons) by PgaB in the periplasm, and then exported through the PgaA porin. PIA is transported across the cytoplasmic membrane by IcaAD, then partially deacetylated by IcaB in the extracellular space. PIA has been proposed to be *O*-succinylated by IcaC (magenta star).

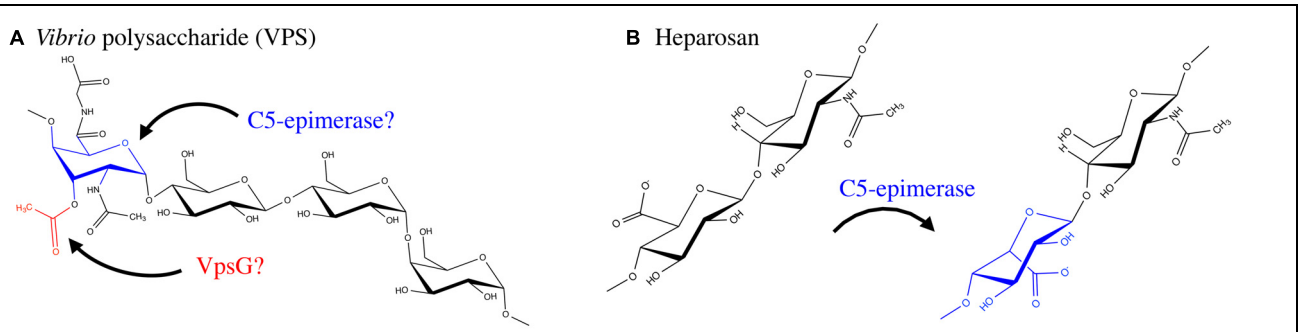

FIGURE 6 | Exopolysaccharide modifications of VPS and Heparosan. **(A)** VPS is produced by *Vibrio cholerae* O1 El Tor and contains an *O*-acetyl group, likely added by VpsG. In addition the polymer has a glycine modification and an *N*-acetyl group; the enzymes responsible for these modifications have not been determined. VPS contains a GulA residue which is epimerized by an unknown C5-epimerase. **(B)** Heparosan, a GAG composed of a disaccharide repeating unit of GlcA and GlcNAc, produced by select bacteria such as the urinary tract pathogen *E. coli* O10:K5:H4. A C5-epimerase introduces IdoA residues. The proteins involved in, and mechanism of, biosynthesis of the above EPS have not been fully resolved.

approximately 3–5% of *N*-acetyls are removed by the lipoprotein PgaB (Wang et al., 2004; Itoh et al., 2008; Little et al., 2012). The N-terminal domain of PgaB is homologous to IcaB in Gram-positive bacteria but the protein is located on the inner leaflet of the outer membrane. There is no IcaC ortholog in the *pgaABCD* operon, which is consistent with an observed lack of *O*-succinyl groups in PGA.

Partial deacetylation of PNAG by both IcaB and PgaB is important for a variety of biofilm-associated phenotypes in *S. epidermidis*, *S. aureus*, and *E. coli*. Deletion of *icaB* in *S. epidermidis* led to the production of fully acetylated PIA, suggesting that IcaB is not necessary for polymer production (Vuong et al., 2004a). However, the fully acetylated polymer was not retained at the cellular surface and was shed into the culture media, which led to deficiencies in biofilm formation and surface attachment (**Table 2**). The lack of deacetylation led to a loss of cationic charge in the polymer, which may be essential for interactions with the anionic cell surface of *S. epidermidis* (Vuong et al., 2004a). Furthermore, *icaB*-deficient mutants of *S. epidermidis* were more susceptible to human cationic antimicrobial peptides and phagocytosis by neutrophils, and were unable to persist in a mouse model of device-related infections (Vuong et al., 2004a). Deletion of *icaB* in *S. aureus* produced similar phenotypes (Cerca et al., 2007). Intriguingly, the production of wall teichoic acids, the predominant anionic component of the Gram-positive bacterial envelope, was dispensable for adherence of PIA to the cell surface of *S. aureus* (Vergara-Irigaray et al., 2008), suggesting that other less prevalent anionic species mediate this interaction.

In contrast to IcaB, inactivation of PgaB in *E. coli* prevented polymer export, suggesting that partial deacetylation is necessary for export through the predicted outer membrane porin PgaA (Itoh et al., 2008). This is in line with findings that suggest conformational changes in the C-terminal domain of PgaB, upon binding of deacetylated PNAG, assist in targeting PNAG for export (Little et al., 2014b). Deacetylation has also been studied in *Y. pestis*, where a PNAG-like polymer is thought to mediate biofilm formation. Biofilm formation in *Y. pestis* is crucial for its zoonotic transmission (Jarrett et al., 2004). In the flea, the proventriculus, a feeding tube covered in spines that connects the midgut to the esophagus, provides a platform for the adhesion of *Y. pestis* aggregates. Subsequent colonization impedes blood passage and leads to transposition of *Y. pestis* from flea to mammal when a flea attempts excessive feeding due to a partial or completely blocked proventriculus (Jarrett et al., 2004). The *hmsHFRS* operon in *Y. pestis* is orthologous to the *pgaABCD* operon, where HmsF is the outer membrane localized deacetylase with structural similarity to PgaB (Forman et al., 2006). Mutation or deletion of *hmsF* led to a deficiency in biofilm formation. This suggests HmsF in *Y. pestis* may be analogous to PgaB in *E. coli* in terms of de-*N*-acetylation activity and importance for polysaccharide export and biofilm formation.

While PNAG production has been studied primarily in *S. aureus*, *S. epidermidis*, and *E. coli*, there are a multitude of additional pathogenic bacteria, fungi, and protozoans that may produce this polymer (Cywes-Bentley et al., 2013). PNAG could represent the first example of an EPS that is broadly utilized by pathogenic organisms as a mechanism to improve fitness in the environment or during infection.

Insights from Modification of Other Microbial Polysaccharides

The implications of EPS modifications in pathogenic bacteria have been studied to some extent, particularly in alginate and PNAG producing bacteria (**Figure 2**; **Table 2**). However, the breadth of our knowledge in this field remains limited. Despite this, comparable modifications found on LPS and CPSs have been studied extensively in an effort to identify vaccine targets, and can be used for comparison purposes to generate new hypotheses regarding EPS modifications (Cody et al., 2003; Pinto and Berti, 2014). In particular, the study of polysaccharide acetyl modifications has clarified their role in mediating a variety of survival mechanisms.

Many of the protective benefits of EPS acetyl modifications described above have been noted for other bacterial pathogens.

For instance, in *Haemophilus influenzae*, an opportunistic pathogen of the upper respiratory tract, acetylation of LPS by the acetyltransferase OafA leads to increased resistance to complement-mediated killing by human serum (Fox et al., 2005). Similarly, in *S. aureus*, the acetyltransferase Cap5H, which is responsible for the *O*-acetylation of type 5 CPS, confers protection against opsonophagocytic killing and improves propagation in a murine model of infection (Bhasin et al., 1998). Type 5 CPS producers also exhibit increased survival rates in murine models of bacteremia and renal abscess formation and resistance to killing in whole mouse blood and opsonophagocytic assays, in comparison to producers of the structurally similar type 8 CPS which have reduced levels of *N*-acetylation (Watts et al., 2005). Beyond the prokaryotic domain, acetyl modifications are also incorporated into the CPS of the pathogenic fungus *Cryptococcus neoformans* to evade complement activation (Fujihara et al., 1997), decrease the efficiency of capsule clearance by the host (Kozel et al., 2003), and inhibit neutrophil migration (Ellerbroek et al., 2004) during cryptococcosis. An excellent example of the benefits of polysaccharide acetyl modifications comes from a survey of clinical isolates of *Streptococcus pneumoniae* and *E. coli* K1, which found that the bacteria expressing acetyl-decorated polymers were more virulent and invasive than those that expressed polymers lacking the modification (Frasa et al., 1993; Melin et al., 2010). Therefore, the immunomodulatory characteristics of acetyl modifications are utilized by a wide range of pathogenic organisms and likely represent a general mechanism for survival and proliferation within the host.

The above notion is firmly supported by studies of serotype variation within the context of CPS biosynthesis. In *Streptococcus pneumoniae*, a causative agent of meningitis, bacteremia, and pneumonia, there are more than 90 different capsule serotypes with unique carbohydrate structures and biosynthetic loci. This has evolved, in part, as a mechanism to overcome serotype-specific host mechanisms of adaptive immunity that can efficiently clear infections. In some serotypes, such as 9V/9A, 11A/11E, and 15B/15C, the CPS structures differ only in the degree of *O*-acetylation (Jansson et al., 1987; Rutherford et al., 1991; Zartler et al., 2009). Mechanisms within *S. pneumoniae* have been revealed that allow for serotype switching during infection as a means to actively evade the host immune response. In the case of serotypes 9V and 11A, inactivating mutations in the acetyltransferase-encoding gene *wceJ* led to expression of non-acetylated 9A and 11E capsule (Calix and Nahm, 2010; Calix et al., 2011). Moreover, certain *wceJ* mutations only partially inhibit acetyltransferase activity, which have led to intermediate 9V/9A and 11A/11E phenotypes (Calix et al., 2011, 2014). In the case of 15B/15C serotype switching, the process is reversible due to slipped-strand mispairing of the acetyltransferase-encoding gene *wciZ* (van Selm et al., 2003). Regardless of the mechanism, this mid-infection serotype variation provides significant protective advantages to *S. pneumoniae* in terms of antibody evasion. For example, antibodies generated against *O*-acetylated serotype 15B were unreactive with non-acetylated 15C polymer (Rajam et al., 2007), and serotype 9V specific antibodies exhibited reduced specificity for 9A polymer (Calix et al., 2012). Furthermore, 10–20% of individuals receiving a *S. pneumoniae* vaccine targeted against the 9V polysaccharide did not generate antibodies targeting serotype 9A (McNeely et al., 1998). In addition, the ability of acetyl groups to mask protective epitopes of bacterial polysaccharides has been noted for the Vi antigen of *Salmonella typhi* (Szu et al., 1991), *Salmonella typhimurium O*-antigen (Kim and Slauch, 1999) and *Neisseria meningitidis* serogroup A, C, and Y CPS (Michon et al., 2000; Berry et al., 2002; Fusco et al., 2007). Therefore, through modulation of acetyl groups on the polymer, a wide variety of pathogenic bacteria are able to evade host-mediated mechanisms of adaptive immunity.

The above examples illustrate scenarios in which acetyl modification is an all-or-nothing response to adaptive immunity, however, in the case of GBS, acetyl levels on its sialic acid CPS can be fine-tuned by the actions of the acetyltransferase NeuD and the acetylesterase NeuA (Lewis et al., 2006, 2007). Different degrees of *O*-acetylation in GBS CPS have been linked to different stages of invasion and infection. For instance, it is thought that during the asymptomatic stages of initial colonization and persistence in the human gastrointestinal and vaginal tracts, GBS produces an extensively acetylated form of CPS to protect against degradation by sialidases introduced by competing microbes in these environments (Weiman et al., 2009). However, highly acetylated CPS renders GBS more susceptible to killing by neutrophils and reduces virulence during stages of opportunistic infections (Weiman et al., 2010). Therefore, during infection it is thought that GBS produces a sparsely acetylated form of CPS that improves resistance to neutrophil-mediated killing through reduced neutrophil activation and production of pro-inflammatory cytokines, and enhances survival in the murine bladder (Kline et al., 2011). Interestingly, this variant of CPS is also able to promote the persistence of uropathogenic *E. coli* in co-culture urinary tract infection models (Kline et al., 2012). Therefore, in certain pathogens, specific degrees of polysaccharide acetylation allow for adaptation during different stages of colonization and infection.

The above examples of acetyl modulation in LPS and CPS not only reinforce the importance of EPS acetylation for pathogenicity and persistence, but also provide additional perspectives in considering the variability of this modification observed in alginate and cepacian. For instance, cepacian has on average three acetyl groups per repeat unit, each located on one of 12 potential positions (Cescutti et al., 2011). Therefore, each cepacian repeat unit can have one or more acetyl groups at any of 12 positions, generating an overwhelming number of unique acetyl decoration patterns. Given the importance of acetyl groups in forming or masking antibody epitopes, this level of diversity would make the generation of protective antibodies or the development of an effective vaccine extraordinarily difficult. Furthermore, production of such a heterogeneous polymer likely requires an arsenal of regulatory factors and/or acetyltransferases, very few of which have been discovered in the context of cepacian biosynthesis (Ferreira et al., 2011). Similar to *S. pneumoniae* and *N. meningitidis* CPS production, members of the Bcc may modulate the presentation of cepacian acetyl groups through an as yet unknown mechanism as a means to evade host adaptive immune mechanisms.

The degree of acetylation and epimerization of alginate has long been known to differ depending on the organism and strain, as well as the composition of the growth medium (Day, 1988; Marty et al., 1992; Peña et al., 2006). This reflects, in part, a need to adapt to the specific conditions imposed by different nutritional media, and may mirror other features of the environment from which the organism was isolated. In line with this concept, additional promoters within the alginate operon have been identified upstream of *algG* and *algIJF* in *P. aeruginosa*, suggesting that there may be modes of regulating the levels of these modifying enzymes independently of the rest of the alginate biosynthesis machinery (Paletta and Ohman, 2012). The upregulation of *O*-acetylation machinery would not only increase alginate acetyl content, but would also decrease the availability of substrate for AlgG and thus decrease epimerization levels. Conversely, upregulation of *algG* would increase the number of G-residues that cannot act as substrates for *O*-acetylation. Therefore, there is the potential for a complicated regulatory interplay between these processes, much like the reciprocal *O*-acetylation/de-*O*-acetylation of GBS CPS that allows for fine-tuning of acetyl levels at different stages of infection.

The degree of PNAG de-*N*-acetylation is not known to vary considerably, and the exact processes involved in VPS acetylation and epimerization, PIA *O*-succinylation, PEL deacetylation and GAG epimerization are poorly understood. However, the ability to perform these types of modifications in a random fashion may increase the difficulty in generating antibodies that recognize specific epitopes on these EPS, either during host adaptive immune responses or in vaccine development (Gening et al., 2010). As such, EPS modifications are capable of imparting beneficial characteristics upon polymers that improve persistence, survival, or evasion of the immune response in their cognate bacteria regardless of their frequency, mechanism of addition to the polymer, or chemical properties.

Reflection and Future Perspectives

Identifying and characterizing biofilm EPS is difficult and there are a number of hurdles that need to be overcome. One of the initial challenges involves culturing biofilm-forming bacteria. Identification of an appropriate medium and growth conditions is required to study EPS production of certain microorganisms in the laboratory (Stewart, 2012). Of those that can be cultured, it is imperative to use similar growth conditions when making experimental comparisons in the literature, as variations can affect the presence or degree of different polysaccharide modifications. With alginate, varying levels of acetylation and epimerization are observed depending on the culture conditions, as well as varying biofilm phenotypes of identical *P. aeruginosa* strains (Pier et al., 2001; Tielen et al., 2005). Additionally, conflicting studies on the levels of pyruvyl and *O*-acetyl modifications to xanthan gum were attributed to different media conditions (Bradshaw et al., 1983). Different media or culturing equipment may also affect experiments such as surface attachment assays. For example, different types of plastics were found to affect PIA-mediated surface attachment in microtitre plates (Maira-Litràn et al., 2004). This suggests that during preliminary analyses, multiple types of media and different materials including plastics and glass should be tested to ensure the validity of observed biofilm phenotypes. Variations in the abundance or type of modifications on a given polymer under different experimental conditions can be difficult to quantify; however, this variation likely reflects the ability of different bacteria to adapt to unique situations. Many EPS-producing bacteria naturally exist in diverse environments and are also able to infect various hosts and survive in specific tissues. Additionally, during the course of infection, the environment within the host will change as immune mechanisms attempt to eradicate the bacteria and the surrounding tissue suffers damage. Variations in the degree of modifications under different growth conditions or stressors may therefore provide valuable insight into bacterial adaptation.

The majority of biofilm studies focusing on EPS modifications have been performed using *in vitro* model systems with mono-species cultures. However, the majority of biofilms from chronic infections differ significantly from those studied in the laboratory (Bjarnsholt et al., 2013a). One significant issue lies in the use of abiotic surfaces, such as cover slides or the plastic surfaces of 96-well plates for the growth and study of biofilms. While some systems can closely approximate the conditions encountered *in vivo*, such as flow cell models for catheter associated infections, biofilms from infections like CF pneumonia or those encountered in epidermal wounds are thought to involve attachment to host cells or host-derived molecules (Bjarnsholt et al., 2009). In these instances it is challenging to extrapolate results obtained *in vitro* to chronic biofilm infections, as modifications that appear important for attachment to abiotic surfaces may be disposable for attachment to host tissues, or vice versa (Leuck et al., 2014). Furthermore, many biofilms encountered in the clinic are not comprised of a single species of bacteria, but rather contain mixtures that can include pathogenic or non-pathogenic bacteria of host or environmental origin, as well as fungi (Wargo and Hogan, 2006; Elias and Banin, 2012). These types of microbial interactions have been studied extensively in the CF lung, where *P. aeruginosa* has been shown to interact with the Bcc opportunistic pathogens *B. cepacia* and *B. cenocepacia* (Chattoraj et al., 2010; Schwab et al., 2014), as well as the pathogenic fungus *Aspergillus fumigatus* (Chotirmall and McElvaney, 2014). In the case of mixed *P. aeruginosa* and *B. cenocepacia* biofilms, the production of alginate by *P. aeruginosa* has been shown to promote *B. cenocepacia* persistence in a mouse model of CF (Chattoraj et al., 2010). This highlights how the production of EPS in mixed species biofilms can have implications that extend beyond the source organism, and suggests that data obtained regarding the presence or absence of EPS modifications in monospecies biofilms *in vitro* may have additional significance in multispecies biofilms.

Following bacterial culturing, difficulties in polysaccharide isolation due to the compositional complexity of the biofilm matrix or polymer insolubility may necessitate extensive optimization of purification protocols. Typically, separation of the polymer from the cell surface may require the use of procedures that lyse the associated bacteria, introducing

additional contaminants (Bales et al., 2013). Therefore, it is preferable if possible to isolate EPS from culture supernatants; however, such polymers may exhibit different properties from their cell-associated counterparts leading to discrepant analysis (Maira-Litràn et al., 2002). In either case, once the polymer has been obtained from the cell surface or supernatant, contaminants such as DNA, RNA and protein must be removed. This can be achieved through enzymatic digestion or chemical precipitation of contaminants, or precipitation of EPS. Contaminating carbohydrates can be removed by chromatographic techniques such as size exclusion or ion exchange (Bales et al., 2013). In some cases, purification of EPS leads to insufficient yields for subsequent compositional or structural analysis, often as a result of polymer insolubility. As a result, harsher conditions may need to be employed to solubilize the polymer, including the use of strong acids or bases. During initial structural studies of PIA, strong alkaline purification conditions led to the incorrect identification of *N*-succinyl groups on the polymer (McKenney et al., 1999; Maira-Litràn et al., 2002), which was actually a degradation product of glucosamine monosaccharides (Sadovskaya et al., 2005). Additionally, phosphates have been reported in the monosaccharide composition of polymers, which are occasionally remnants from purification buffers, or teichoic acids in the case of Gram-positive bacteria (Sadovskaya et al., 2005). Harsher conditions can also partially or completely remove functional groups, such as acetyls, or even degrade the polymer, leading to incorrect calculations of molecular weight. The EPS described in this review are the select few whose structures have been determined, or for which we have biochemical and genetic data supporting the importance of the polysaccharide modifications. With improved culturing, purification and structural determination procedures, additional EPS will be discovered and their modifications characterized. We will then be able to formulate trends between different types of modifications and their effect on biofilm formation, pathogenicity, and virulence. Furthermore, the details regarding the genes and proteins involved in the addition of polysaccharide modifications remain largely unavailable. Characterization of these genes and proteins will likely provide details on how the levels and types of modifications are regulated under different conditions. Such findings will have a significant impact on our understanding of bacterial pathogenicity, and may reveal novel drug targets aimed at inhibiting the biosynthesis of these important virulence factors.

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REVIEW
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Alginate-modifying enzymes: biological roles and biotechnological uses

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Alginate denotes a group of industrially important 1-4-linked biopolymers composed of the C-5-epimers β-D-mannuronic acid (M) and α-L-guluronic acid (G). The polysaccharide is manufactured from brown algae where it constitutes the main structural cell wall polymer. The physical properties of a given alginate molecule, e.g., gel-strength, water-binding capacity, viscosity and biocompatibility, are determined by polymer length, the relative amount and distribution of G residues and the acetyl content, all of which are controlled by alginate modifying enzymes. Alginate has also been isolated from some bacteria belonging to the genera *Pseudomonas* and *Azotobacter*, and bacterially synthesized alginate may be *O*-acetylated at O-2 and/or O-3. Initially, alginate is synthesized as polymannuronic acid, and some M residues are subsequently epimerized to G residues. In bacteria a mannuronan C-5-epimerase (AlgG) and an alginate acetylase (AlgX) are integral parts of the protein complex necessary for alginate polymerization and export. All alginate-producing bacteria use periplasmic alginate lyases to remove alginate molecules aberrantly released to the periplasm. Alginate lyases are also produced by organisms that utilize alginate as carbon source. Most alginate-producing organisms encode more than one mannuronan C-5 epimerase, each introducing its specific pattern of G residues. Acetylation protects against further epimerization and from most alginate lyases. An enzyme from *Pseudomonas syringae* with alginate deacetylase activity has been reported. Functional and structural studies reveal that alginate lyases and epimerases have related enzyme mechanisms and catalytic sites. Alginate lyases are now utilized as tools for alginate characterization. Secreted epimerases have been shown to function well *in vitro*, and have been engineered further in order to obtain enzymes that can provide alginates with new and desired properties for use in medical and pharmaceutical applications.

Keywords: alginate, alginate lyase, mannuronan C-5 epimerases, alginate acetylation, alginate deacetylase

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Introduction

Alginate is defined as a linear polymer consisting of 1-4-linked mannuronic acid (M) and guluronic acid (G). The polysaccharide is currently manufactured from brown algae, and used as a viscosifier, emulsifier and gel forming agent in many different applications (Skjåk-Bræk et al., 2015). Alginate has also been found in some red algae (Usov et al., 1995) and is produced by some bacteria belonging to the genera *Azotobacter* (Gorin and Spencer, 1966) and *Pseudomonas*

(Linker and Jones, 1966). Genes encoding putative alginate biosynthetic gene clusters are present in many other sequenced bacterial genomes.

Initially alginate is synthesized as mannuronan by polymerization of GDP-mannuronic acid (**Figure 1**). This homopolymer is then modified by alginate modifying enzymes. Four groups of such enzymes have been characterized; alginate acetylases, alginate deacetylases, alginate lyases, and mannuronan C-5-epimerases (**Figure 1**). As a result of the action of these alginate-modifying enzymes the term alginate denotes a family of polysaccharides with differing chemical composition and properties. In this review the relationship between the composition of a specific alginate and its physical properties are briefly described as are the uses of alginates by the alginate-producing organisms. The different enzyme groups are then addressed separately, before the review concludes by describing how alginate lyases and mannuronan C-5 epimerases may be used *in vitro*.

Post-Polymerization Modifications Determine the Functional Properties of Alginates

Alginates are utilized industrially because they bind cations, bind water, provide viscosity to a solution, and may form gels. It has long been known that both the ratio and distribution of G-residues and the polymer length influence these properties, and it is conceptually better to consider alginate as a group of chemically related polymers with different properties. A given alginate molecule can formally be described as composed of at least one of the three possible block-types: consecutive M-residues (M-blocks), consecutive G-residues (G-blocks) and strictly alternating M and G-residues (MG-blocks; **Figure 1**). The length of these may vary from two residues and upward. G-blocks and MG-blocks may be cross-linked by divalent cations and form gels. Currently NMR is the preferred method used to characterize alginate composition, since it makes it possible to determine the frequencies of the monomers M and G, the diads MM, MG/GM, and GG, and the eight different triads (Grasdalen et al., 1981; Grasdalen, 1983).

The block types and block lengths influence the flexibility of the polymer and thus the viscosity of the solution as well as the stability, permeability, and syneresis of gels (Skjåk-Bræk et al., 2015). Studies have shown that alginates with a high M-content are immunogenic, while G-blocks does not stimulate an immune response (Espevik et al., 1993), and this has important implications when alginate capsules for implantation of cells in humans are designed. Alginate synthases produce polymannuronic acid, and the G-residues are later introduced by mannuronan C-5 epimerases. Many alginate-producing organisms even encode several C-5 epimerases enabling them to tailor the synthesized alginates to a specific use.

Bacterial alginates may be *O*-acetylated at some of the C-2 and C-3 carbons of the mannuronic acid residues, and acetylation takes place during transport through the periplasm (Baker et al., 2014). Acetyl groups affects the viscosity and flexibility of the molecule and increases its ability to bind water (Skjåk-Bræk et al., 1989b).

Polymer length is another variable important for viscosity and gel strength. Any given alginate preparation contains a mixture of molecules with different lengths, the lengths, and degree of polydispersity might be found by SEC-MALLS (Bakkevig et al., 2005). It is not known what causes the synthesis of a particular alginate molecule to terminate. During manufacturing of alginate from brown algae some depolymerization takes place, both due to physical forces, but also by alginate lyases produced by bacteria living on the seaweeds.

Alginates Have a Protective Role in the Alginate-Producing Organisms

The cell walls of brown algae contain a network of alginate molecules where G-blocks in the molecules are cross linked by Ca^{2+}. Alginate is the most abundant component of the cell wall, which also contains fucoidan, cellulose, polyphenols, and proteins (Michel et al., 2010). Polyphenols have been shown to be linked to the alginate network, thus further strengthening the cell wall (Deniaud-Bouët et al., 2014). It has been suggested that the genes involved in biosynthesis of alginate in brown algae were acquired through a horizontal gene transfer from an

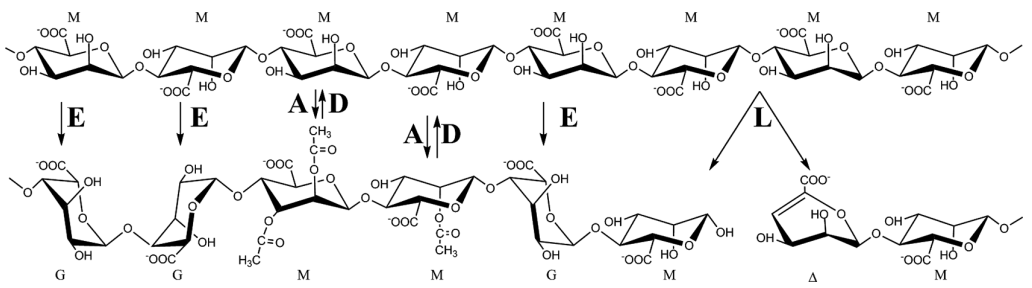

FIGURE 1 | Enzymatic modifications of alginate. Alginate is synthesized as polymannuronan (top molecule). The mannuronic acid residues (M) may then be epimerized to guluronic acid residues (G) by mannuronan C-5 epimerases (E), O2 or O3 acetylated by mannuronan acetylases (A), or degraded by alginate lyases (L) resulting in an unsaturated 4-deoxy-L-erythro-hex-4-enepyranosyluronate residue (Δ). Acetyl groups may be removed by deacetylases (D).

actinobacterium and that this enabled the formation of complex multicellular organisms within the group of Stramenopiles (Michel et al., 2010; Deniaud-Bouët et al., 2014).

Wild type strains belonging to the genus *Azotobacter* seem to produce alginate constitutively, at least under laboratory conditions. Alginate is surrounding the vegetative cells, however, alginate-negative mutants do not display impaired growth or fitness in the laboratory. Most studies addressing nitrogen fixation in *Azotobacter vinelandii* are utilizing mutants that are unable to produce alginate due to a transposon insertion in the sigma factor gene necessary for alginate production (Setubal et al., 2009). When bacteria belonging to the genus *Azotobacter* sense a lack of carbon source, the cell will enter a dormant stage where it is protected by a G-block-containing cyst coat. The cyst can survive desiccation for years, and when conditions again allow for growth the cyst will germinate (**?**). It has later been proven that only alginate-producing cells may produce cysts that are able to germinate after storage (Campos et al., 1996), showing that the ability to produce alginate containing G-blocks is important for the long-time fitness of these bacteria in nature.

Only a few species belonging to the genus *Pseudomonas* are able to produce alginate, and alginate production in these species are tightly regulated and only turned on in response to specific stimuli; typically those causing cell wall stress (Wood and Ohman, 2012). It has been shown that alginate participates in forming the matrix of the *P. aeruginosa* biofilm and in providing resistance toward the host immune system (Pier et al., 2001; Tielen et al., 2005; Harmsen et al., 2010). Acetylated M residues are also protected from cleavage by some alginate lyases (Skjåk-Bræk et al., 1989a) and from epimerization.

Alginate Lyases

In nature alginate lyases are synthesized by organisms that utilize alginate as a carbon source, as well as by the alginate-producing bacteria. So far no alginate lyase produced by brown algae has been identified. Several alginate lyases have been thoroughly studied, and this has provided a detailed understanding of the catalytic mechanism of these enzymes.

Structure-Function Studies of Alginate Lyases

As depicted in **Figure 1** an alginate molecule may contain four different bonds; M–M, M–G, G–M, and G–G, and most lyases display very different reaction rates toward the different bonds. Alginate lyases are divided into G-specific (EC4.2.2.11) and M-specific (EC4.2.2.3) enzymes. However, this classification does not distinguish between enzymes cleaving for instance either M–G or G–M. Moreover, not all alginate lyases accept acetylated substrates (Wong et al., 2000).

Polysaccharide lyases are divided into families based on sequence similarities. By the end of 2014 22 polysaccharide lyase families had been identified in the CAZY database, moreover some characterized enzymes are not yet classified in a family (Lombard et al., 2010). Alginate lyases are found in the polysaccharide lyase families PL5, PL6, PL7, PL14, PL15, PL17, and PL18, and in the group of unclassified polysaccharide lyases. The assignment to this number of families implies that alginate lyases structurally are quite diverse. The polysaccharide lyase families PL5, PL15, and PL17 have been found to have an $(\alpha/\alpha)_n$ toroid fold structure, PL6 has a β-helix fold structure, while PL7, PL14, and PL18 are folded as β-jelly rolls. The structural folds of the polysaccharide lyases have been reviewed recently (Garron and Cygler, 2010, 2014) and will not be further discussed here.

Most studied alginate lyases functions endolytically, i.e., they cleave the alginate molecules internally, and do not produce significant amounts of oligomers at the beginning of the reaction. If the reaction is allowed to proceed, the final products usually are dimers, trimers, tetramers, or pentamers. However, several exolyases which removes single residues from the end of the polymer have been described (Ochiai et al., 2010; Thomas et al., 2012; Park et al., 2014).

Gacesa (1987) first proposed the reaction mechanism for alginate lyases. Firstly the negative charge on the carboxylate anion is shielded by the enzyme. This allows for the proton to be abstracted from C-5. The intermediate enolate ion is proposed to be stabilized by resonance. Finally, electron transfer from carboxyl group results in the formation of a double bond between C-4 and C-5 and cleavage of the *O*-glycosidic bond. Cleavage has been found to be facilitated by an amino acid residue acting as an acid (Garron and Cygler, 2010). The new non-reducing end will contain a 4-deoxy-L-*erythro*-hex-4-enepyranosyluronate (Δ) as depicted in **Figure 1**. This double bond absorbs at 235 nm and is used to quantitate alginate lyase activity (Preiss and Ashwell, 1962a). For most alginate lyases the negative charge is stabilized by glutamine, arginine or asparagine. It is important for the catalytic mechanism that for M-residues the C-5-proton and the departing oxygen on C-4 lie *syn* relative to each other, while they lie *anti* relative to each other for G-residues (**Figure 1**). For the studied alginate lyases it has been found that for M-specific lyases the C-5 proton is abstracted by a tyrosine that also acts as the acid that facilitates the cleavage of the *O*-glycosidic bond. For lyases acting on G-residues the C-5-proton is abstracted by a histidine while a tyrosine again acts as the acid (Garron and Cygler, 2014). Alginate lyases belonging to the PL6 family do not follow this scheme. They use Ca^{2+} as neutralizer, lysine as the proton-abstracting residue, and arginine as the acid (Garron and Cygler, 2014).

Alginate Lyases in Alginate-Producing Bacteria

So far no alginate-producing bacteria have been reported to use alginate as a carbon source. Still, in all known alginate-producing bacteria an alginate lyase is encoded in the gene cluster encoding the other proteins needed for alginate biosynthesis. This periplasmic alginate lyase seems to be involved in clearing the periplasm for mislocated alginate molecules originating from non-functional export complexes. Alginate is a polyanion, which attracts small cations. Free alginate molecules in the periplasm consequently result in an increased osmotic pressure that finally will cause lysis, and alginate-producing cells without this lyase do not survive (Bakkevig et al., 2005; Jain and Ohman, 2005). It has also been found that mutants that do not produce AlgK, AlgX, or AlgG do not produce alginate, but instead unsaturated oligomers, which would be the expected product of AlgL-degraded alginate

(Jain and Ohman, 1998; Gimmestad et al., 2003; Jain et al., 2003; Robles-Price et al., 2004). These data is compatible with a model (**Figure 2**) in which the polymerized alginate is transported through a protein complex composed of the polymerase Alg8, the copolymerase Alg44, the periplasmic proteins AlgG, AlgX, and AlgK, and the outer membrane pore AlgE; and AlgL would only have access to alginate molecules that were aberrantly released to the periplasm because of a non-functional protein complex (Bakkevig et al., 2005).

Pseudomonas aeruginosa also encodes a second alginate lyase, Pa1167 (Yamasaki et al., 2004), but the biological function of this lyase is unknown. *A. vinelandii* encodes two alginate lyases, AlyA1 and AlyA2, which are homologous to Pa1167. AlyA1 was dispensable for growth in the laboratory, while AlyA2 was necessary for normal growth (Gimmestad et al., 2009). *A. vinelandii* further encodes two secreted alginate lyases, AlyA3 and AlgE7. AlyA3 contains an alginate lyase module homologous to AlyA1 and AlyA2 linked to three calcium-binding modules homologous to the R-modules of secreted mannuronan C-5 epimerases (See Mannuronan C-5 Epimerases). It has been shown that R-modules can bind to alginate, although the binding strength is dependent on the specific R-module as well as the structure of the alginate molecule (Buchinger et al., 2014). The cysts of *Azotobacter* sp. will germinate when they are placed in an environment with a suitable carbon source (**?**), and AlyA3 is needed to open the cyst capsule in order to allow the vegetative cell to escape and start growing (Gimmestad et al., 2009). AlgE7 is a bifunctional enzyme belonging to the family of secreted mannuronan C-5- epimerases and displays both mannuronan C-5-epimerase and alginate lyase activity (Svanem et al., 2001). In the absence of AlgE7 it is more difficult to separate alginate from the cells by centrifugation (Gimmestad et al., 2009), indicating that AlgE7 is used to detach cells from alginate. *A. vinelandii* also encodes an exolyase, AlyB, the biological function of which is unknown (Ertesvåg, unpublished data). The genome sequence of *A. chroococcum* was recently released (Robson et al., 2015). In addition to *algL*, the *A. chroococcum* genome contains four other genes homologous to *A. vinelandii* alginate lyase genes: *Achr_16810* is homologous to *A. vinelandii alyA1* and *alyA2*, *Achr_5040* is homologous to *A. vinelandii alyB* and two genes, *Achr_39560* and *Achr_39570*, are homologous to AlgE7.

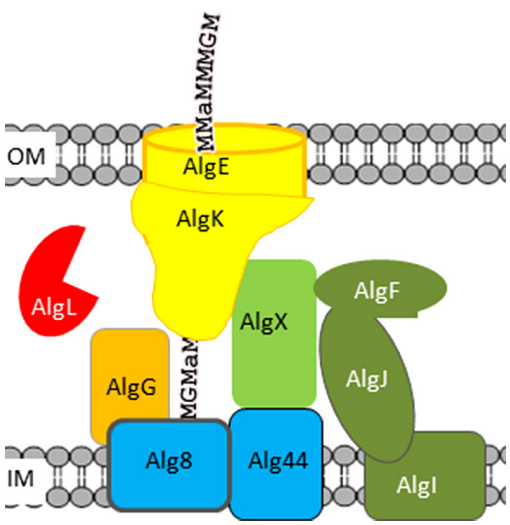

FIGURE 2 | Model of the *Pseudomonas aeruginosa* alginate biosynthetic complex. Proteins involved in polymerization are colored blue, the alginate lyase red, the epimerase orange, and proteins involved in acetylation green. The names of proteins assumed to be part of the alginate polymerization and transport complex are written using black letters. Alginate is depicted as a text string; "a" denotes acetyl-groups.

Alginate Lyases in Alginate-Utilizing Organisms

Alginate lyases are produced by many organisms that utilize alginate as a carbon source; bacteria, animals, and viruses (Wong et al., 2000). These organisms are either marine, utilizing the alginate produced by brown algae, or soil bacteria presumably utilizing alginate produced by *Azotobacter* sp. or alginate-producing *Pseudomonas* sp. Many bacteria have been found to secrete alginate lyases that degrade alginate into smaller oligomers that can be taken up by the cells. The oligosaccharides are further degraded by oligoalginate lyases and exotype alginate lyases into 4-deoxy-*l*-erythro-5-hexoseulose uronic acid, which then is converted to pyruvate and glyceraldehyde-1-phosphate (Preiss and Ashwell, 1962b). As an example the marine bacterium *Zobellia galactanivorans* contains two operons encoding several alginate lyases as well as the dehydrogenase and kinase needed for converting alginate into substrates used in the central carbon metabolism (Thomas et al., 2013).

Secretion of alginate lyases inflicts the risk that other cells might import the alginate oligomers generated by such extracellular enzymes. The soil bacterium *Sphingomonas* sp. A1 has developed an alginate up-take system whereby the polymer is imported into the cell through a dedicated ABC-transporter and then degraded by a series of intracellular alginate lyases with different substrate specificities. This unique system has been reviewed elsewhere (Hashimoto et al., 2010).

Mannuronan C-5 Epimerases

Alginates isolated from brown algae and from bacteria belonging to the genus *Azotobacter* contain consecutive G-residues, while alginates isolated from *Pseudomonas* sp. so far only has been found to contain single G-residues. This can be related to the need brown algae and *Azotobacter* have in forming alginate gel networks that can provide strength to their cell wall and cyst coat, respectively. As long as Pseudomonads only use alginates to form a loosely connected capsule or as a minor component of their biofilms, they may not have the same need for G-blocks in their alginates. Still, some *Pseudomonads* encode a protein that is able to introduce G-blocks *in vitro* (Bjerkan et al., 2004a). Two types of mannuronan C-5-epimerases have been described; the Ca^{2+}-independent AlgG-type, and the Ca^{2+}-dependent AlgE-type.

AlgG-Type Epimerases are Found in All Alginate-Producing Organisms

All alginate-producing bacteria encode a periplasmic mannuronan C-5- epimerase, AlgG, in the alginate biosynthesis gene cluster and the secreted alginate contains some G-residues. Polymannuronic acid forms fairly stiff molecules due to the diequatorial linkages, while introduction of G-residues provides equatorial-axial bonds that increase the flexibility of the chain (Smidsrød et al., 1973), and this might explain why a mannuronan-producing organism has not been identified in nature. In the laboratory mannuronan-producing strains encoding a mutated form of AlgG have been isolated (Chitnis and Ohman, 1990; Gimmestad et al., 2003).

Brown algae encode a large family of mannuronan C-5-epimerases that are related to the bacterial AlgG-epimerases (Nyvall et al., 2003; Michel et al., 2010). No algal epimerase gene have yet been reported expressed in any heterologous system, thus nothing is known about how these enzymes might differ as to which kind of alginate they are producing. It is, however, known that the G-content of alginates synthesized by brown algae differ between species, within different parts of the plant and with the environment of the individual plant. Since genes encoding mannuronan C-5-epimerases have been found to be differentially expressed in *Laminaria digitata* (Tonon et al., 2008), it seems probable that different epimerases are able to introduce distinct G-distribution patterns that confers the crosslinking and ionic binding properties needed in a given tissue and environment.

The Secreted AlgE-Type Epimerases are Used to Produce G-Block-Containing Alginates in Bacteria

In contrast to what seems to be the case in algae the consecutive G-residues found in *A. vinelandii* alginates are not a result of its AlgG epimerase (Steigedal et al., 2008). However, as early as in 1969 a secreted, Ca^{2+}-dependent mannuronan C-5 epimerase from *A. vinelandii* was described (Haug and Larsen, 1969), and this was the first example of epimerases active on a polymer. The enzyme attracted interest because it was able to increase the G-content in alginates *in vitro*. The earliest studies were not able to ascertain whether *A. vinelandii* secreted one or more different epimerases, but this was resolved when the genes encoding seven secreted epimerases (AlgE1-7) were identified, cloned and expressed in *E. coli* (Ertesvåg et al., 1999). These epimerases have been shown to introduce different G-distribution patterns and to be expressed differentially during the life cycle of the cell (Høidal et al., 2000). *A. vinelandii* also encodes another protein, AlgY, which is homologous to the AlgE-epimerases, but does not display any activity on mannuronan. These early studies on the secreted epimerases have been extensively reviewed earlier (Ertesvåg et al., 2009).

The AlgE epimerases consist of two different protein modules where the A-module always is succeeded by from one to four R-modules (**Figure 3**). AlgE1 and AlgE3 each contain two A-modules. The A-modules are sufficient for both epimerization and for determining the epimerization pattern (Ertesvåg and Valla, 1999). The R-modules are needed for full activity (Ertesvåg and Valla, 1999) and has been shown to participate in substrate binding (Buchinger et al., 2014). At their N-terminal ends each R-module contain four to seven copies of a nine amino acid long motif known to be involved in binding of Ca^{2+} (Baumann et al., 1993; **Figure 3**).

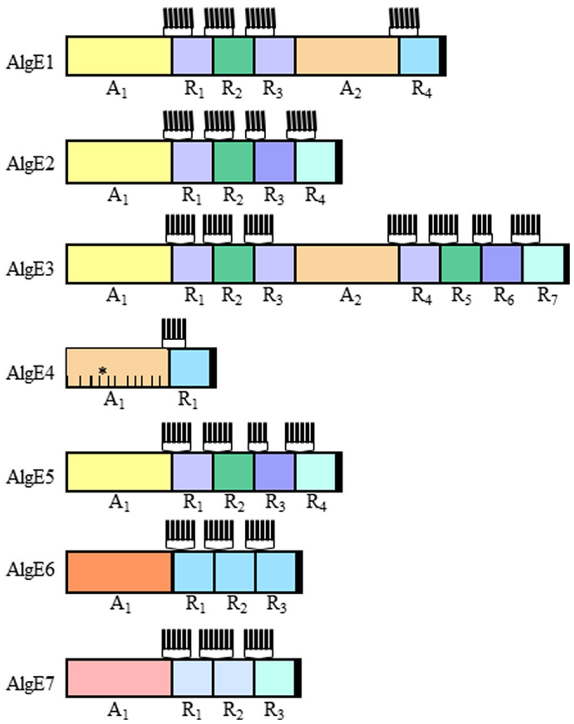

FIGURE 3 | The modular structure of the secreted epimerases from *Azotobacter vinelandii*. The AlgE epimerases from *A. vinelandii* is composed of varying numbers of the two modules designated A and R. The relatedness between individual modules (confer **Figure 4**) is indicated by the similarity of the colors. The Ca^{2+}-binding motifs found at the N-terminal end of each R-module are indicated by vertical lines. The proposed substrate-binding subsites in the A-module of AlgE4 are indicated by lines within the module, the catalytic subsite is marked with an asterisk.

The A-modules of the *A. vinelandii* enzymes differ when it comes to the G-distribution pattern they introduce in the alginate substrate. E1A2, E3A2, and E4A mainly introduce single G residues, while E1A1, E2A, E3A2, E5A, and E6A introduce consecutive G-residues as well as some single G residues. However, AlgE6A introduces longer G-blocks than the other A-modules. As mentioned above, AlgE7 is a bifunctional lyase/epimerase. It is able to introduce G-blocks, but also to cleave the alginate at an introduced G-residue. **Figure 4A** displays the similarity between the different A-modules showing that there is a clear relationship between the primary structure and the product. When it comes to the R-modules this is not the case; they tend to group mostly according to their position relative to the A-module (**Figure 4B**).

Six of the *A. vinelandii* AlgE epimerases are found clustered in the genome at around 5.2 Mb, while AlgE5 is found around 3.4 Mb. The *A. chroococcum* genome encodes only three putative

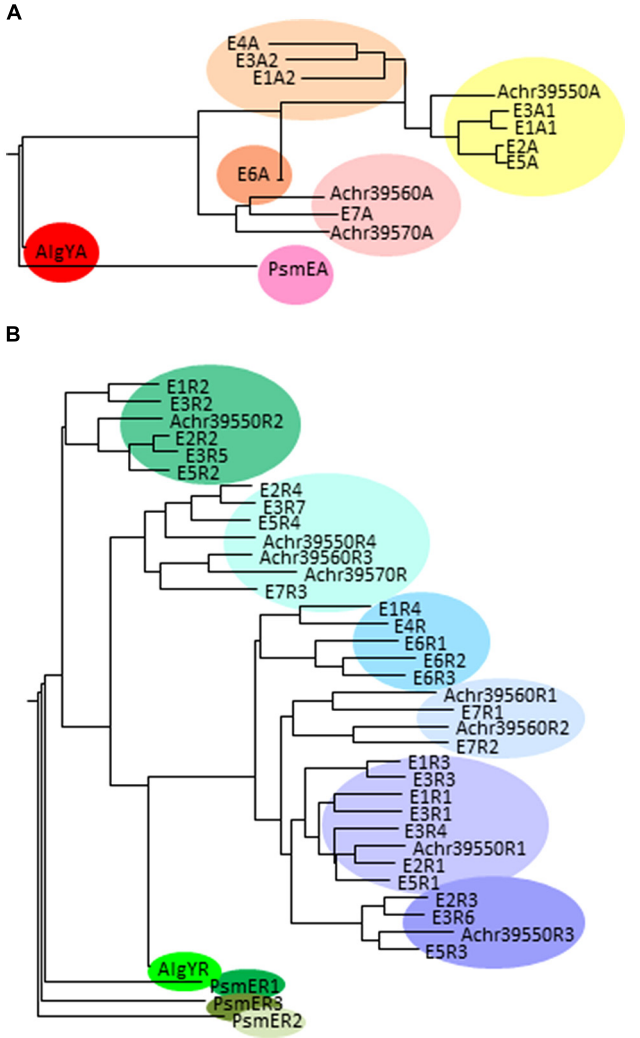

FIGURE 4 | Phylogenetic trees displaying the relationship between the A-modules (A) and R-modules (B) in different secreted mannuronan C-5 epimerases. The *A. vinelandii* epimerases are denoted by E and enzyme number, AlgY is a protein homologous to AlgE-epimerases in *A. vinelandii*, PsmE is the *P. syringae* secreted epimerase, and the putative *A. chroococcum* epimerases are identified by their loci numbers. When one enzyme contains several A or R-modules, these are each numbered according to **Figure 3**.

secreted epimerases. These genes are found together in the same genomic context as the cluster containing six *A. vinelandii AlgE* genes. Both the A-module and the four R-modules of the protein encoded by *Achr_39550* are homologous to the corresponding modules of *A. vinelandii* AlgE2 (**Figure 4**). *Achr_39560* is transcribed in the same direction as *Achr_39550* and encodes a protein most similar to the bifunctional lyase/epimerase AlgE7. The third gene, *Achr_39570,* is transcribed in the opposite direction of *Achr_39560*. These two genes are fairly homologous except that 1058 nucleotides encoding amino acids 336-721 in *Achr_39560* is missing in *Achr_39570*. This truncated protein thus contain only nine of the 11 suggested subsites for substrate binding usually found in the A-module (Tøndervik et al., 2013), and only one R-module. Nothing is as yet known about the activities of these putative epimerases.

AlgG and AlgE Epimerases Display Similar Structure and Catalytic Site Residues as Alginate Lyases

The structure of the E4A-module has been determined using x-ray crystallography (Rozeboom et al., 2008), while the structures of the R-modules of AlgE4 and of AlgE6 have been determined using NMR (Aachmann et al., 2006; Buchinger et al., 2014). These studies showed that both modules form right-handed β-helixes. A SAXS analysis of full length AlgE4 indicates that it forms a rod-shaped molecule (Buchinger et al., 2014). Both the A- and the R-modules participate in substrate binding, and it has been calculated that the A-module bind 11 uronic acid residues (**Figure 3**; Tøndervik et al., 2013), while the R-module can bind five residues (Buchinger et al., 2014). It has further been shown that different R-modules have different binding strength, and that this property influences the degree of processivity displayed by a given epimerase (Buchinger et al., 2014). The structure of *P. syringae* AlgG was recently published (Wolfram et al., 2014) and AlgG was found to form a long right-handed parallel β-helix similar to that of AlgE4. AlgG was calculated to bind at least nine uronic acid residues.

The catalytic mechanisms of lyases and epimerases were early postulated to be similar (Gacesa, 1987). Indeed, the close relationship between epimerases and lyases are emphasized by the bifunctional lyase/epimerase AlgE7 where the same catalytic site are needed for both activities (Svanem et al., 2001). Both epimerases and lyases need to neutralize the negative charge on the carboxyl group and to abstract the C-5-proton. For a lyase the last step then is cleavage of the glycosidic bond, while an epimerase needs an amino acid residue that is able to donate a proton to C-5 from the opposite side of the pyranose ring. The overall structure of both AlgG and the A-module of AlgE4 display structural similarities to some pectate- and pectin lyases of the PL1 family. While the negative charge in the Ca^{2+}-dependent pectate lyases and AlgE4 is likely to be shielded by bound calcium, the calcium-independent pectin lyases, and AlgG use an arginine residue (Vitali et al., 1998; Wolfram et al., 2014).

The catalytic site of the epimerases otherwise seem to be more similar to that of alginate lyases from PL5 and PL7 (Rozeboom et al., 2008). Studies using AlgG mutants indicate that the histidine residue in the conserved DPHD motif found in both AlgG and the AlgE epimerases probably abstract the proton, while Tyr314 in AlgG coordinates a water molecule that might act as the proton donor (Wolfram et al., 2014). This tyrosine residue is also present in the catalytic site of AlgE4 (Rozeboom et al., 2008).

Enzymes Involved in Determining the Degree of Acetylation

Alginate acetylation increases the water-binding capacity of the polymer (Skjåk-Bræk et al., 1989b). The acetyl groups will also increase the viscosity of the alginate and this might aid in keeping

the polymer as a protective film close to the bacterium and hamper the movement of for instance immune cells. Acetylation also renders the mannuronic acid residue inaccessible for many alginate lyases, thus protecting the produced alginate from degradation (Skjåk-Bræk et al., 1989b). So far no studies have been published that compare the acetylation pattern for bacteria cultivated using different laboratory conditions or different strains.

Four Proteins Encoded in the Alginate Biosynthetic Gene Cluster are Necessary for Alginate Acetylation

When the model describing the alginate polymerization and transport complex (**Figure 2**) were proposed a decade ago (See Alginate Lyases in Alginate-Producing Bacteria) it was known that AlgF, AlgI, and AlgJ were necessary for alginate acetylation, but also that none of these are necessary for polymer formation, indicating that they are not a part of the biosynthetic protein complex. The model could not explain how any of these enzymes could get access to the nascent alginate within the protein complex and acetylate it while AlgL could not degrade the polymer when it was surrounded by a functional complex. This enigma was recently resolved when it was shown that AlgX is the mannuronan *O*-acetylase (Riley et al., 2013; Baker et al., 2014).

AlgI is a membrane-bound *O*-acetyl-transferase that transfers an acetyl group from an unknown cytoplasmic acetyl donor (Franklin et al., 2004). The crystal structures of AlgX and AlgJ have been published and both enzymes belong to the SGNH hydrolase superfamily and further contain the same amino acids needed for *O*-acetyl-transferase activity (Riley et al., 2013; Baker et al., 2014). AlgJ is attached to the inner membrane (Franklin and Ohman, 2002), while AlgX is part of the alginate synthase, and transport complex. AlgX further contains a carbohydrate-binding module and has been shown to be able to acetylate mannuronan *in vitro* (Baker et al., 2014). AlgF is necessary for acetylation (Franklin and Ohman, 1993), however, it is not known which particular function AlgF has.

The alginate synthesis and export complex contains more proteins than for instance the cellulose synthesis and export complex encoded by *Gluconoactetobacter xylinus*, probably because alginate is modified during its passage through the periplasm (Whitney and Howell, 2013). *P. fluorescens,* which produces acetylated cellulose, encodes a modified cellulose synthase and export complex containing homologs of all four proteins necessary for alginate acetylation (Spiers et al., 2003).

It has been shown that bacterial alginates with a relatively high degree of acetylation has a lower degree of epimerization (Skjåk-Bræk et al., 1986). Given that the periplasmic epimerase AlgG and the acetylase AlgX are part of a protein complex, they may be considered as immobilized enzymes acting on a passing polymeric substrate. Both enzymes are able to bind several M-residues (Baker et al., 2014; Wolfram et al., 2014), and may exhibit some degree of processivity. It is not known if AlgG and AlgX are competing for binding to the alginate chain or if their potential access to any specific M-residue is separated in time.

Alginate Deacetylation

Deacetylation may take place spontaneously, but the reaction is slow at normal pH. Extracellular epimerases are necessary for producing G-block containing alginate. However, these enzymes will not epimerize acetylated residues, and residues close to acetylated residues are probably also protected from epimerization. The crystal structure of AlgE4 indicates that this is due to steric constraints (Rozeboom et al., 2008). In this way the acetyl groups attached during the passage through the transport complex limit the amount of guluronic acid residues that can be formed later by the secreted epimerases.

So far no species of *Pseudomonas* have been found to produce alginate containing consecutive G-residues. However, *P. syringae* pv *glycinea* were found to a gene encoding a secreted mannuronan C-5-epimerase designated PsmE (Ullrich et al., 2000; Bjerkan et al., 2004a). PsmE was found to introduce consecutive G-residues *in vitro*. Similar to the secreted epimerases from *A. vinelandii,* PsmE contains a catalytic A-module and three Ca^{2+}-binding R- modules. These all form a separate cluster in the phylogenetic trees (**Figure 4**). The enzyme contains two other Ca^{2+}-binding modules, M and RTX and a seventh module called N containing 273 amino acid residues. In contrast to the secreted *A. vinelandii* epimerases, PsmE is able to epimerize acetylated substrates, and it was shown that the N-module is necessary for removing acetyl groups prior to epimerization by the A-module (Bjerkan et al., 2004a). Like AlgX and AlgJ, the N-module is predicted to belong to the SGNH hydrolase superfamily, and it shows some homology to esterases active on acetylated polysaccharides. Homologous genes are found in the genomes of many strains of *P. syringae* and related species (e.g., *P. savastanoi, P. amygdali, P. fluorescens,* and *P. avellanae*). Synthesis of bifunctional deacetylases/mannuronan C-5 epimerases would enable these strains to both decrease the amount of acetyl groups and to increase the G-content of its alginate.

Use of Alginate Modifying Enzymes

For bulk applications such as food and feed, water treatment, or textile printing (Onsøyen, 1996) the alginate price may be as low as 5 USD per kg. However, alginate is also used in pharmaceutical and biotechnological industries, and for well-defined and ultrapure qualities the price may be as high as 100 USD/g. Examples of such applications are alginates for wound healing, encapsulation of pancreatic cells, and tissue engineering (Hunt and Grover, 2010; Skjåk-Bræk et al., 2015). The secreted mannuronan C-5 epimerases have been found to enable the upgrading of alginate *in vitro*, creating alginates with long G-blocks and/or replacing stretches of M-blocks with MG-blocks. The product may thus be tailored as to the need for gel strength, porosity, or biocompatibility (Skjåk-Bræk et al., 2015). It is also possible to tailor mannuronan using a chemoenzymatic approach in which some residues are modified and the material are then epimerized to introduce gel-forming G-blocks (Donati et al., 2005). In a recent paper this procedure was

used to produce RGD-peptide modified alginates that were used to culture myoblasts in 2D and 3D cultures (Sandvig et al., 2015). Alternatively, specific epimerases might be used to obtain a homogenous population of alginate molecules with desired properties, which then are chemically modified (Arlov et al., 2014).

It is now possible to obtain pure mannuronan from bacteria synthesizing mutant AlgG proteins (Gimmestad et al., 2003). This may then be used as a substrate for recombinantly produced AlgE4, resulting in nearly pure MG-alginate (Holtan et al., 2006). G-blocks can be isolated from G-rich alginates from *L. digitata* (Haug et al., 1974). Such pure substrates are valuable when the substrate specificities of alginate lyases are to be determined.

Initial studies aimed at altering the substrate specificities of alginate lyases have been performed. The G-specific alginate lyase AlyA from *Klebsiella pneumonia* is able to degrade both G-M, and G-G bonds. The gene was subjected to random mutagenesis, cloned in a plasmid and transferred to *E. coli*. Protein extracts from 6700 randomly picked clones were prepared and assayed for lyase activity using isolated G-blocks and MG-alginate as substrates. One of the clones still retained sufficient activity on G-G bonds, while the activity on G-M-bonds was very low (Tøndervik et al., 2010). Recently it was also shown that the polysaccharide lyase Smlt1473 is active on several different polysaccharides, and, even more important, it was demonstrated that the activity and specificity of Smlt1473 toward polymannuronan and polyglucuronan could be increased by site-directed mutagenesis (MacDonald and Berger, 2014).

Alginate lyases are currently used to quantify alginate in a solution and may also be used to quantify the G-content (Østgaard, 1992). Still, as mentioned in Section "Post-Polymerization Modifications Determine the Functional Properties of Alginates" NMR is the most commonly used method to characterize alginate. However, NMR only yields knowledge of the statistical distribution of the M and G-residues. A new method utilizing a combination of alginate lyases specific for certain alginate bonds and HPAEC-PAD (Aarstad et al., 2012) enables a measurement of the block-length distribution to be performed, and this method has been used to show that algal alginates contain some very long G-blocks (Aarstad et al., 2013).

The homology and modular structure of the *A. vinelandii* AlgE epimerases has initiated several studies where modules or parts of modules are exchanged between the enzymes. Combining parts of the A-modules of AlgE2 and AlgE4 resulted in epimerases that introduced new G-distribution patterns (Bjerkan et al., 2004b). This approach was taken further when the DNA-fragments encoding the A-modules from AlgE1–AlgE6 was combined using error-prone PCR and cloned in front of the R-module from AlgE4. Nine hundred and sixty mutants from the resulting library were then screened for the ability to make long G-blocks and two mutant enzymes which met this criterion were identified (Tøndervik et al., 2013). This screen depended on the use of alginate lyases, included the GG-specific mutant lyase described above, to characterize alginate (Østgaard, 1992).

These studies illustrate that a random approach combined with an increased knowledge of the structure of the catalytic sites as well as the substrate binding sites of alginate lyases and epimerases will enable the design of new enzymes with more desired properties as to processivity, recognition site, substrate specificities, or catalytic activity. Engineered epimerases may then be used alone or in chemoenzymatic approaches to create biomaterials with new and desired functionalities.

Acknowledgment

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Isolation of extracellular polymeric substances from biofilms of the thermoacidophilic archaeon *Sulfolobus acidocaldarius*

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Extracellular polymeric substances (EPS) are the major structural and functional components of microbial biofilms. The aim of this study was to establish a method for EPS isolation from biofilms of the thermoacidophilic archaeon, *Sulfolobus acidocaldarius*, as a basis for EPS analysis. Biofilms of *S. acidocaldarius* were cultivated on the surface of gellan gum-solidified Brock medium at 78°C for 4 days. Five EPS extraction methods were compared, including shaking of biofilm suspensions in phosphate buffer, cation-exchange resin (CER) extraction, and stirring with addition of EDTA, crown ether, or NaOH. With respect to EPS yield, impact on cell viability, and compatibility with subsequent biochemical analysis, the CER extraction method was found to be the best suited isolation procedure resulting in the detection of carbohydrates and proteins as the major constituents and DNA as a minor component of the EPS. Culturability of CER-treated cells was not impaired. Analysis of the extracellular proteome using two-dimensional gel electrophoresis resulted in the detection of several hundreds of protein spots, mainly with molecular masses of 25–116 kDa and pI values of 5–8. Identification of proteins suggested a cytoplasmic origin for many of these proteins, possibly released via membrane vesicles or biofilm-inherent cell lysis during biofilm maturation. Functional analysis of EPS proteins, using fluorogenic substrates as well as zymography, demonstrated the activity of diverse enzyme classes, such as proteases, lipases, esterases, phosphatases, and glucosidases. In conclusion, the CER extraction method, as previously applied to bacterial biofilms, also represents a suitable method for isolation of water soluble EPS from the archaeal biofilms of *S. acidocaldarius*, allowing the investigation of composition and function of EPS components in these types of biofilms.

Keywords: Archaea, thermoacidophile, *Sulfolobus acidocaldarius*, biofilms, extracellular polymeric substances, proteins, enzymes

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Introduction

Microorganisms in the biofilm mode of life predominate, numerically and metabolically, in a wide variety of natural, technical, and medical environments (Costerton et al., 1995). Microbial biofilms are single- or multi-species communities that accumulate at interfaces (solid–liquid or solid–air), where the microorganisms live at high cell densities in a matrix of hydrated extracellular polymeric substances (EPS) (Hall-Stoodley et al., 2004). EPS are mainly polysaccharides, proteins, extracellular DNA (eDNA), and lipids. They mediate biofilm adhesion to surfaces and form a cohesive, three-dimensional polymer network interconnecting and immobilizing biofilm cells, and they provide mechanical stability to biofilms (Flemming and Wingender, 2010). The biofilm matrix acts as an external digestion system, keeping extracellular enzymes close to biofilm cells, capable of metabolizing dissolved, colloidal and solid biopolymers. The EPS matrix constitutes the immediate environment and conditions of life for biofilm organisms and thus, is regarded as a key component for the understanding of the biofilm mode of life. Bacterial biofilms have been intensively studied not only because of their ecological importance, application in biotechnology, and waste water treatment but also due to their potential role in human infections and function as environmental reservoirs for pathogens (Hall-Stoodley et al., 2004). Members of the Archaea have gained special research interest due to their adaptation to extreme environments. Though the potential of this domain especially for biotechnological applications of their enzymes is well documented, only recently fundamental studies about archaeal biofilms have been initiated (Schopf et al., 2008; Baker-Austin et al., 2010; Koerdt et al., 2010, 2012; Orell et al., 2013). It has become evident that environmental biofilm communities often contain both bacterial and archaeal species; in addition, laboratory investigations have shown that single archaeal species are able to form biofilms on diverse biotic and abiotic surfaces (Fröls, 2013; Orell et al., 2013).

Investigations of EPS involve sampling of biofilms, extraction, and in some cases, purification of EPS, and finally analysis of composition and identity of the EPS (Nielsen and Jahn, 1999; Denkhaus et al., 2007). The extraction efficiency for EPS varies depending on the origin, composition, and constituent microorganisms of the biofilm as well as on the extraction method used. There is no universal extraction method for the quantitative recovery of EPS from biofilms, and in some cases, complementary methods may have to be applied in order to obtain all fractions of the EPS matrix (Park and Novak, 2007). Different physical and chemical methods, including centrifugation, filtration, heating, blending, sonication as well as treatment with sodium hydroxide alone or in combination with formaldehyde, use of a complexing agent, or cation-exchange resin (CER), have been described for the extraction of EPS from monospecies biofilms and mixed population biofilms as well as flocs from natural environments and technical water systems (Nielsen and Jahn, 1999). Heating or addition of chemicals, such as sodium hydroxide and formaldehyde for the extraction process can result in the disruption of macromolecules, and chemicals like EDTA may also interfere with subsequent EPS analysis (Comte et al., 2006). In contrast, the CER method has been accepted as a mild EPS extraction method for many types of biofilms, causing limited cell lysis and no disruption and interference with EPS analysis (Sheng et al., 2010). Thus, an appropriate method has to be chosen and adapted to the properties of the biofilm under study with the aim to give an effective recovery of EPS, to cause minimal cell lysis, to avoid destruction of EPS, and to be compatible with EPS analysis methods.

Extracellular polymeric substances have often been studied in natural and laboratory biofilms of mixed-populations or single species grown under non-extreme conditions. There is some evidence that microbial biofilms in extreme environments also produce EPS as, for example, has been demonstrated for pellicle biofilms, composed of bacteria and archaea, from acid mine drainage solutions (pH 0.83–1.0), where extracted EPS components were found to be carbohydrates, proteins, DNA, and lipids (Jiao et al., 2010). Staining of monospecies archaeal biofilms with fluorescently labeled lectins and fluorescent DNA-binding dyes allowed the microscopic visualization of extracellular glycoconjugates (polysaccharides) and eDNA, indicating the presence of an EPS matrix. This was shown for archaeal extremophiles, including halophilic organisms (*Halobacterium*, *Haloferax,* and *Halorubrum* species; Fröls et al., 2012) and thermoacidophilic *Sulfolobus* species (Koerdt et al., 2010). However, detailed information on the composition of EPS from archaeal biofilms is still lacking, since EPS extraction and subsequent biochemical analysis have not been applied to these biofilms in contrast to the intensively studied EPS from biofilms of single bacterial species.

As outlined above, in Archaea – constituting the third domain of life with unique cellular and metabolic properties – the biofilm mode of life is evidently as ubiquitous and therefore comparably important as in Bacteria (Fröls, 2013). Although widely distributed in mesophilic habitats, most so far cultivable archaeal species are adapted to extremes of temperature, pH, salinity, or a combination thereof. With optimal growth requirements of 78°C and pH 2–3.5, the crenarchaeal members of the order Sulfolobales are adapted to both high temperature and acidic conditions, a property so far only found in Archaea but not in Bacteria. *Sulfolobus* spp. are easy to grow on minimal and complex media and several *Sulfolobus* genome sequences as well as comprehensive biochemical and functional genomics data are available (Zaparty and Siebers, 2011). *S. acidocaldarius* was first isolated from acid hot springs at Yellowstone National Park (Brock et al., 1972), and has become a well-established model strain for the archaeal domain. In contrast to the physiologically more versatile *S. solfataricus*, its genome size is 30% smaller and more genetically stable due to no mobile genetic elements. A robust and versatile genetic system is available for *S. acidocaldarius*, which enables the construction of in-frame markerless deletion mutants, ectopic integration of foreign DNA, and an effective homologous expression system (Wagner et al., 2012), which is important for future analyses on gene functions in biofilm formation. For biofilm investigations, the thermoacidophilic growth requirements represent a special challenge. The focus of the present study was to get first insights into the EPS composition of this archaeon by (i) cultivation of *S. acidocaldarius* as unsaturated biofilms yielding sufficient amounts for EPS isolation and analyses, (ii) by selecting a method suitable for EPS extraction from *S. acidocaldarius*

biofilms, and (iii) by subsequent biochemical characterization of the isolated EPS.

Materials and Methods

Growth Conditions

S. acidocaldarius DSM 639 was grown to the mid-exponential growth phase in liquid Brock medium (Brock et al., 1972) supplemented with 0.1% (w/v) N–Z-amine and 0.2% (w/v) dextrin at 78°C for 2 days with shaking (180 rpm) up to an optical density at 600 nm of 0.6–0.8. For biofilm cultivation, culture fluid was densely streaked in lines on plates of Brock medium (pH 3.5) solidified with gellan gum (6 g L^{-1}; Gelzan™ CM, Sigma-Aldrich, Germany) and supplemented with 3 mM $CaCl_2$ and 10 mM $MgCl_2$. The plates were sealed in plastic bags and incubated at 78°C for 4 days.

Characterization of Biofilms

Determination of dry weight and residue on ignition as well as loss on ignition (volatile matter) of *S. acidocaldarius* DSM 639 biofilms was performed according to the standard DIN EN 12880 and DIN EN 12879, respectively. Briefly, samples of approximately 1 g (wet weight) biofilm mass were scraped from the surface of gellan gum plates after 4 days of incubation and successively dried to constant weight at 105°C and 550°C for determination of dry weight and residue on ignition, respectively. Loss on ignition was calculated by the difference between dry weight and residue on ignition values. For the determination of cation content, cells were disintegrated by acid digestion, using HNO_3/H_2O_2, in combination with microwave treatment, and the cations were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES) according to ISO 11885 (2007) at the IWW Water Centre (Mülheim an der Ruhr, Germany).

Determination of Total Cell Counts and Colony Counts

Total cell counts and colony counts were determined in biofilm suspensions. The total cell number was determined by staining with 4′,6-diamidino-2-phenylindole (DAPI; 25 µg mL^{-1} in 2% formaldehyde, 20 min) and enumeration at 1000-fold magnification, using an epifluorescence microscope. Viability of cells was determined in terms of colony forming units (CFU). Dilutions (1 mL) of the biofilm suspension as well as cells obtained after each EPS isolation procedure were spread onto gellan gum-solidified Brock medium for determination of the CFU after 4 days at 78°C. The impact of the phosphate buffer (pH 7.0) used for EPS isolation on cell viability was determined by comparing CFUs of cells suspended in either buffer or standard Brock medium (pH 3.5) under different conditions (shaking with/without CER). The statistical significance of differences between the CFUs was determined using a two-tailed paired Student's *t*-test.

EPS Extraction

Biofilm mass was scraped from the surface of gellan gum-solidified Brock medium using a spatula, and suspended in phosphate buffer (2 mM $Na_3PO_4 \times 12\ H_2O$, 4 mM $NaH_2PO_4 \times 1\ H_2O$, 9 mM NaCl, 1 mM KCl, pH 7.0) at a concentration of 0.1 g wet weight/10 mL. For EPS extraction, five methods were applied: shaking, CER extraction, EDTA extraction, crown ether extraction, and NaOH extraction. For the shaking procedure, biofilm suspensions were transferred into 50 mL polypropylene centrifuge tubes in 10 mL aliquots and shaken at highest capacity for 20 min on a shaker (VortexGenie®2, Scientific Industries, USA). For CER extraction, biofilm suspensions were transferred into 50 mL centrifuge tubes in 10 mL aliquots. To each tube, 2 g of hydrated CER (Dowex® Marathon® C sodium form, Sigma-Aldrich, Germany) washed twice with phosphate buffer (15 min; 10 mL g^{-1} Dowex) were added. The samples were shaken at highest capacity for 20 min on a shaker (VortexGenie®2, Scientific Industries, USA). For EDTA extraction, 30 mL 2% (w/v) EDTA (disodium salt) solution in deionized water were added to 30 mL biofilm suspension, and the mixture was stirred for 3 h at 4°C. For crown ether extraction, 30 mL of biofilm suspension were centrifuged, the pellet was suspended in 30 mL crown ether solution (30 mM dicyclohexyl-18-crown-6-ether in 50 mM Tris buffer, pH 8.0), and the suspension was stirred for 3 h at 4°C (Wuertz et al., 2001). For NaOH extraction, 12 mL 1 M NaOH was added to 30 mL biofilm suspension and the mixture was stirred for 3 h at 4°C. After the respective treatment, the pH values of the extracts were determined and the samples were centrifuged at 20,000 × *g* for 20 min (4°C). The cells were resuspended in one volume of deionized water. The supernatants were filter sterilized (0.22 µm pore size) and the filtrates were dialyzed against deionized water (molecular weight cut-off 3,500 Da, 2× 1 h and overnight) to obtain high molecular weight compounds (EPS fraction).

Chemical Analysis of EPS

Carbohydrates and proteins were quantified in the biofilm suspensions as well as in the cell suspensions after separation from EPS, the filter sterilized supernatant (total extracellular material), and the EPS solution after dialysis. Carbohydrate concentrations were determined with the phenol sulfuric acid method using D-glucose as a standard (Dubois et al., 1956). For determination of protein concentrations, a modified Lowry assay was applied using commercial reagents (Sigma) and bovine serum albumin as a standard (Wingender et al., 2001). DNA was quantified using the Quant-iT PicoGreen dsDNA reagent kit (Invitrogen, Molecular Probes) and λ-DNA as a standard. The measurement was carried out with a fluorimeter (SFM 25, Kontron instruments) set to an excitation wavelength of 480 nm and emission readings at 520 nm.

Two-Dimensional Gel Electrophoresis

For two-dimensional gel electrophoresis (2 DE) analyses, Tris buffer (pH 8.0), $MgCl_2$, and Benzonase (purity >99%; Novagen) to final concentrations of 50 mM, 10 mM, and 65 U mL^{-1}, respectively, were added to the EPS solutions, and samples were incubated for 1 h at 37°C. Subsequent dialysis using Spectra/Por dialysis tubing (MWCO 12–14 kDa) was performed in three steps, each against 5 L deionized water, with the first two changes after 1 h and the final step overnight. Determination of protein content in the isolated EPS fractions was carried out according to a modified Lowry procedure (Peterson, 1977) and aliquots with a protein amount of 100–400 µg were lyophilized. For two-dimensional gel electrophoresis, lyophilized samples were first resuspended in

380 µL IEF buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 5 mM tributylphosphine, 0.25% (w/v) Servalyt 3–10 ampholyte (Serva), a few crystals bromophenol blue prepared in ultrapure water; Ultrapur, Merck] and incubated at room temperature for 1 h. The samples were then applied to an IEF tray, an IPG strip was added (immobilized pH gradient with pH range of 3–10, linear or non-linear, Bio-Rad, Germany), and covered with mineral oil (3 mL). After overnight rehydration at 20°C, samples were subjected to IEF at a maximum of 75 µA in five steps at (i) 200 V (45 min), (ii) 500 V (45 min), (iii) 1,000 V (45 min), (iv) 10,000 V (4 h), and (v) finally at 10,000 V (5 h) using a Protean IEF cell (Bio-Rad, Germany). Afterwards, the strips were applied to the second dimension separation or stored at −20°C. For the second dimension, separation 20 cm × 20 cm 12% polyacrylamide gels were used. After polymerization (4 h), gels were stored at 4°C overnight. Following two 15 min equilibration steps [first step in 6 M urea, 30% (w/v) water free glycerol, 2% (w/v) SDS, 0.05 M resolving gel buffer, and 0.1 g dithiothreitol (DTT); second step in the same buffer containing 0.5 g iodoacetamide instead of DTT] focused IPG strips were washed with 1× electrophoresis buffer (Rotiphorese® 10× SDS-PAGE, Roth) and applied to the SDS gel together with a wick soaked with 5 µL of Mark12, Invitrogen, or PageRuler™ Plus Prestained Protein Ladder (Fermentas) and dried. Both IPG strip and marker wick were fixed using 2 mL 0.5% (w/v) agarose containing bromophenol blue. Using a Protean II Xi cell (Bio-Rad, Germany) with 1× SDS–Tris–glycine running buffer, electrophoresis was carried out at initially 20 mA for 45 min and subsequently at 35 mA for 4.5 h, and proteins were visualized by silver staining (Blum et al., 1987).

Identification of EPS Proteins via nanoRSLC-Orbitrap LC–MS/MS

Dialyzed EPS proteins were analyzed in the Core Facility for Mass Spectrometry at the Chemistry department of the Philipps-University Marburg (Germany). Samples were digested by the addition of Sequencing Grade Modified Trypsin (Promega) and incubated at 37°C overnight. The mass spectrometric analysis of the samples was performed using an Orbitrap Velos Pro mass spectrometer (ThermoScientific). An Ultimate nanoRSLC system (Dionex), equipped with a custom 20 cm × 75 µm C18 RP column filled with 1.7 µm beads, was connected online to the mass spectrometer through a Proxeon nanospray source. A total of 1–15 µL of the tryptic digest (depending on sample concentration) were injected onto a C18 pre-concentration column. Automated trapping and desalting of the sample was performed at a flowrate of 6 µL/min using water/0.05% formic acid as solvent. Separation of the tryptic peptides was achieved with the following gradient of water/0.05% formic acid (solvent A) and 80% acetonitrile/0.045% formic acid (solvent B) at a flow rate of 300 nL/min: holding 4% solvent B for 5 min, followed by a linear gradient to 45% solvent B within 30 min and linear increase to 95% solvent B in additional 5 min. The column was connected to a stainless steel nanoemitter (Proxeon, Denmark) and the eluent was sprayed directly toward the heated capillary of the mass spectrometer using a potential of 2300 V. A survey scan with a resolution of 60,000 within the Orbitrap mass analyzer was combined with 10 data-dependent MS/MS scans with dynamic exclusion for 30 s using HCD combined with orbitrap detection at a resolution of 7,500. Data analysis was performed using Proteome Discoverer 1.4 (ThermoScientific) with SEQUEST and MASCOT (version 2.2; Matrix science) search engines using either SwissProt or NCBI databases.

Only proteins with two or more unique identified peptides were taken as significant. The identified proteins were further analyzed and functionally categorized bioinformatically using the arCOG database (archaeal cluster of orthologous groups of proteins) and the updated *S. acidocaldarius* genome annotation (Esser et al., 2011). The subcellular localization was predicted by PSORTb (Yu et al., 2010).

Fluorimetric Assays of Extracellular Enzyme Activities

The pH of the cell-free, dialyzed EPS solutions were adjusted to pH 3.5 (corresponding to the pH value of the biofilm growth medium) and screened for enzyme activities of seven different enzyme groups. The fluorogenic 4-methoxy-β-naphthylamide and methylumbelliferyl (MUF) substrate analogs (Sigma-Aldrich, Germany) were used as 2 mM stock solutions in 2-methoxyethanol, i.e. L-alanine-4-methoxy-β-naphthylamide, 4-MUF-α-D-glucopyranostide, 4-MUF-β-D-glucopyranoside, 4-MUF-*N*-acetyl-β-D-glucosaminide, 4-MUF-stearate, 4-MUF-butyrate, and 4-MUF-phosphate. A total of 190 µL of the isolated EPS fractions were supplemented with 10 µL of the respective substrate stock solutions. A total of 10 µL 2-methoxyethanol was used as negative control. Incubation was carried out in 96-well plates at 70°C. Before fluorescence measurements, plates were agitated using the shaking function of the plate reader at 1.5 mm amplitude for 5 s. Fluorescence was determined using the Infinite Pro 200 microplate reader (Tecan) with 360 nm and 450 nm (MUF substrates) as well as 330 nm and 420 nm (4-methoxy-β-naphthylamide) as emission and excitation wavelength, respectively.

Non-enzymatic substrate hydrolysis was measured mixing 10 µL of the respective substrate solution with 190 µL phosphate buffer pH 3.5 (without isolated EPS) as negative controls. 4-methylumbelliferone (0–200 mM) and 4-methoxy-β-naphthylamide (0–100 mM) were utilized for calibration.

Zymographic Analysis of Protease and Esterase Activity

For one dimensional zymographic analyses native, SDS free discontinuous 10% polyacrylamide gels supplemented with 0.1% casein (C3400 Sigma-Aldrich, Germany) or gelatin (48723 Sigma-Aldrich, Germany) were used. EPS samples (5 µg protein) were supplemented with non-reducing Roti®-Load 2 (4×) sample buffer (Roth, Germany). After electrophoresis [125 V, 90 min, Rotiphorese SDS running buffer (Roth, Germany)], gels were washed twice (30 min) in Novex® Zymogram renaturing buffer (Invitrogen) and incubated in developing buffer pH 3.8 or 7.0 (Novex® Zymogram, Invitrogen) overnight at 37°C or 70°C with one buffer exchange after 60 min. Protease activity was detected as halo formation upon Simply Blue Safe Stain (Invitrogen) for 1 h and subsequent destaining in water (2× for 1 h). Imaging was carried out with the Bio-Rad Densitometer GS-700.

For 2D zymographic analyses, gels were prepared as described above omitting SDS and adding casein. Samples corresponding to a total protein amount of 400 µg were applied. Washing (2× 1 h), developing (1× 1 h, 1× 48 h), and staining (1× 2 h) were performed using the same buffers as for the 1 DE analyses. 2 DE gels were also analyzed for esterase activity. Therefore, gels were washed in 100 mM Tris–HCl, pH 8, 25% (v/v) isopropanol (2× 30 min) and incubated in 100 mL 5 mM 4-methylumbellyferyl-butyrate (Sigma, in renaturing buffer) at pH 3.0 and 8.0, respectively (10 min). The Molecular Imager Gel Doc XR System (Bio-Rad) was used for visualization.

Results

Composition of *S. acidocaldarius* Biofilm

Biofilms of *S. acidocaldarius* grown at 78°C for 4 days on gellan gum-solidified Brock medium contained a total biomass of approximately 0.6 g (wet weight) per Petri dish (100 mm in diameter). The dry weight of these biofilms accounted for $24.9 \pm 1.4\%$ (0.249 ± 0.014 mg/g wet weight; $n = 3$), of which approximately 97% (0.242 ± 0.013 mg/g wet weight) and 3% (0.008 ± 0.002 mg/g biofilm wet weight) were organic and inorganic matter, respectively, as determined by loss of ignition and residue on ignition. The total cell count was $1.2 \pm 0.4 \times 10^{12}$ per gram of biofilm wet weight ($n = 3$). As major multivalent cations in the biofilms, magnesium > iron > calcium > copper (in decreasing order) were observed (**Table 1**).

Efficiency of EPS Extraction Procedures

Extracellular polymeric substances were isolated from biofilms of *S. acidocaldarius* scraped from the medium plates using five different procedures, including shaking, shaking in the presence of CER, stirring with added NaOH, EDTA, and crown ether, respectively. Cells were separated from the extracellular material by centrifugation and the cell-free supernatant was dialyzed against deionized water to obtain the EPS. The different fractions (biofilm suspensions, cells after separation from extracellular material, total extracellular material, EPS solution) were analyzed for total carbohydrates, proteins, and DNA. Independent of the extraction method, the EPS preparations were always found to contain carbohydrates, proteins, and minor amounts of DNA (**Figure 1**; **Table 2**).

TABLE 1 | Concentration of multivalent cations in *S. acidocaldarius* biofilms cultivated on Brock medium plates at 78°C for 4 days.

Cation	Concentration (µg g^{-1} biofilm wet weight)
Mg^{2+}	385.0 ± 7.1
$Fe^{2+/3+}$	162.0 ± 56.6
Ca^{2+}	125.0 ± 7.1
$Cu^{1+/2+}$	5.6 ± 0.8
Sr^{2+}	0.265 ± 0.064
Zn^{2+}	0.235 ± 0.007
Al^{3+}	0.147 ± 0.095
Ba^{2+}	0.039 ± 0.004
B^{3+}	0.037 ± 0.001
$Mo^{4+/6+}$	0.031 ± 0.001
$Cr^{3+/6+}$	0.004 ± 0.001
$Co^{2+/3+}$	0.003 ± 0

Cations were analyzed by ICP-OES.

Shaking, CER, and EDTA extraction resulted in similar carbohydrate content in the analyzed fractions, i.e., cell fraction (35–40 fg/cell), total extracellular material (8–9 fg/cell), and EPS fraction (3–4.7 fg/cell). The protein concentrations of 121–141 fg/cell in the cell fraction, 2.8–18 fg/cell in the total extracellular material, and 2.7–4.2 fg/cell in the EPS were also similar between these three isolation methods.

The CER method tended to yield the highest EPS carbohydrate and protein concentrations among the three methods. Preliminary analysis of CER-extracted EPS by acid hydrolysis and thin-layer chromatography revealed D-glucose as the main neutral carbohydrate component (data not shown).

The highest concentrations of carbohydrates and proteins in the EPS were observed after crown ether (carbohydrates 11.4 fg/cell, proteins 35.3 fg/cell) and NaOH (carbohydrates 29 fg/cell, proteins 122 fg/cell) extraction, respectively. However, crown ether treatment appeared to interfere with the analytical measurements as indicated by the inconsistent amount of carbohydrates determined for the total biofilm, which is exceeded by the sum of the total extracellular carbohydrates and the carbohydrate content of the cell fraction by 60%. Also, the EPS analysis after NaOH extraction appeared inaccurate because of the high discrepancy between carbohydrate (29 fg/cell) and protein (122 fg/cell) content of the EPS compared to the cell fraction (carbohydrate 10.5 fg/cell, protein 14.8 fg/cell), which suggests cell damage or lysis during preparation. This was also visually observed as transition of the suspended biofilms from brownish/turbid to clear/viscous upon NaOH treatment.

In addition to carbohydrates and proteins, 0.09–1.5 fg/cell DNA was detected in the EPS fraction with highest amounts for crown ether followed by NaOH, CER, and shaking in the presence of EDTA (**Table 2**). The protein to carbohydrate mass ratio of the total biofilm of *S. acidocaldarius* was determined to be 2.96. Only for crown ether extraction, a similar value was obtained (3.07) in EPS. All other EPS isolation methods resulted in different protein/carbohydrate ratio with a slightly higher carbohydrate content in the shaking (0.86) and CER (0.90) isolation, whereas the protein and carbohydrate content was the same in the EDTA prepared EPS (1.07). Significantly, more proteins than carbohydrates were observed in the NaOH extracted EPS (4.22; **Table 2**).

Determination of the pH in the solutions after EPS extraction revealed a constant neutral pH for shaking and CER and a slightly acidic pH for EDTA (5.14 ± 0.03). pH values in the alkaline range were observed for NaOH and crown ether with values of 13.28 ± 0.13 and 8.12 ± 0.07, respectively.

Effect of EPS Extraction on Total and Viable Cell Counts

The impact of the EPS isolation method on cell integrity was analyzed by determining the total and viable cell counts after isolation and comparison to the biofilm cells before isolation. As shown in **Figure 2**, shaking, CER, and EDTA had nearly no effect on both total and viable cell counts compared to the untreated

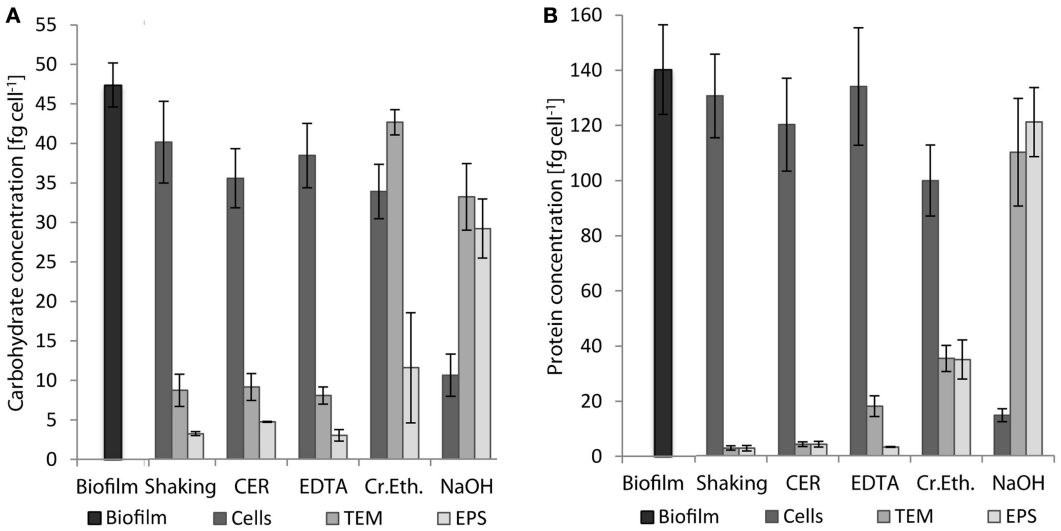

FIGURE 1 | Concentrations of carbohydrates (A) and proteins (B) in whole biofilms of *S. acidocaldarius* and different biofilm fractions after EPS extraction with shaking, CER, crown ether (Cr.Eth.), EDTA, or NaOH. EPS were obtained from biofilms recovered from Brock medium plate cultures after incubation for 4 days at 78°C ($n = 3$). TEM, total extracellular material.

TABLE 2 | Carbohydrate, protein, and DNA content of EPS extracted from *S. acidocaldarius* biofilms with shaking, CER, EDTA, crown ether, and NaOH.

Extraction method	Carbohydrates		Proteins		Protein to carbohydrate ratio	DNA
	(fg cell^{-1})	(%)[a]	(fg cell^{-1})	(%)[a]		(fg cell^{-1})
Shaking	3.2 ± 0.3	6.7	2.7 ± 0.9	2.0	0.86	0.09 ± 0.02
CER	4.7 ± 0.1	9.8	4.2 ± 1.1	3.0	0.90	0.91 ± 0.48
EDTA	3.0 ± 0.7	6.2	3.2 ± 0.2	2.3	1.07	0.40 ± 0.29
Crown ether	11.4 ± 6.9	24.2	35.2 ± 7.2	25.1	3.07	1.50 ± 0.34
NaOH	28.9 ± 3.7	61.0	122.0 ± 12.6	87.0	4.22	0.99 ± 0.29

[a] *Percentage of carbohydrate or protein fraction with respect to total biofilm ($n = 3$).*

suspended biofilm cells. Culturable cells and total cell counts were in the same order of magnitude. However, the crown ether method resulted in a significant loss of culturability, whereas the total cell count was not changed. Use of NaOH caused a complete loss of total cells below the detection limit as well as of culturability.

The pH value of the isolation medium for biofilm suspension before EPS extraction, either phosphate buffer pH 7.0 or Brock medium pH 3.5, did not significantly influence the culturability ($p > 0.05$, two-tailed paired Student's *t*-test). Also, the additional stirring (4°C, 3 h) under both pH conditions in the NaOH, EDTA, and crown ether isolation methods was not harmful for the culturability, indicating that even for the thermoacidophilic *S. acidocaldarius*, a pH 7.0 is suitable in the EPS isolation procedure.

Electrophoretic Analysis of EPS Proteins

The proteins obtained after EPS extraction using the different methods were analyzed by 2 DE using linear IPG strips and visualization by silver staining (Supplementary Material; **Figure 1**). Most protein spots (approximately 600) were obtained after crown ether isolation, followed by CER (~500 spots) and shaking (~300 spots). Utilization of NaOH for EPS extraction led to poor resolution of the protein spots particularly in the low molecular weight range. With EDTA, no separation could be achieved at all presumably due to elevated electrical resistance and accompanying high voltage during IEF. With application of linear IPG strips, the majority of proteins observed had a molecular mass between 25 and 115 kDa and a isolelectric point between 5 and 8. A refined analysis using non-linear IPG strips with an enhanced resolution in the pI range of 4–7 carried out with EPS proteins obtained by the CER method led to an increased number of protein spots (~1000) compared to the linear IPG strip separation (**Figure 3**).

Identification of EPS Proteins via nanoRSLC-Orbitrap LC-MS/MS

NanoRSLC-Orbitrap LC–MS/MS analyses of the EPS proteins after tryptic digestion gave first insights into the extracellular proteome and resulted in the identification of 85 proteins, which could be assigned to 15 functional arCOG categories. (**Figure 4**; Table S1 in Supplementary Material). The majority of proteins (53) were categorized into the four arCOG clusters energy production and conversion (arCOG C), amino acid transport and metabolism (arCOG E), lipid transport and metabolism (arCOG I), and translation, ribosomal structure, and biogenesis

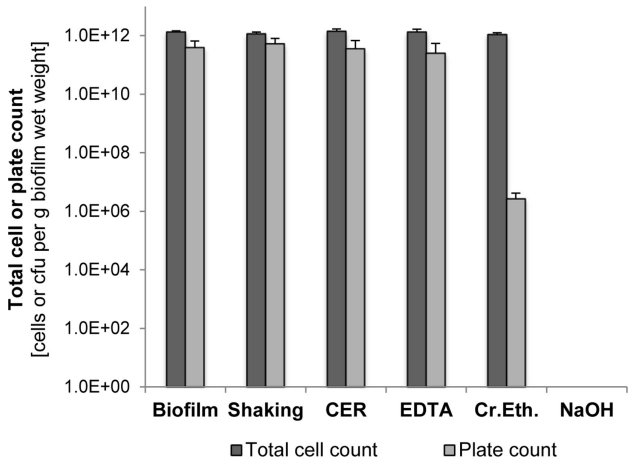

FIGURE 2 | Total cell counts and plate counts of *S. acidocaldarius* after EPS extraction using shaking, CER, NaOH, crown ether (Cr.Eth.), and NaOH. Measurements were performed using suspensions of intact biofilm and treated cells in phosphate buffer (pH 7.0). Total cell counts were determined via counting of DAPI-stained cells. Colonies formed on Brock medium plates were counted after incubation for 4 days at 78°C ($n = 3$).

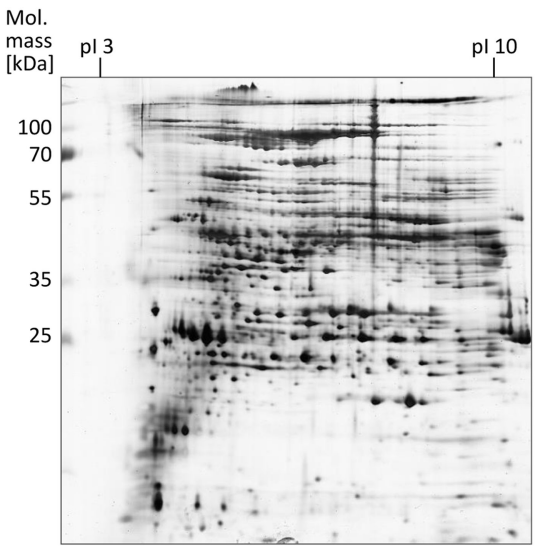

FIGURE 3 | EPS proteins of *S. acidocaldarius* biofilms separated by 2 DE and visualized by silver staining. EPS were isolated from biofilms collected from Brock medium plates after 4 days of incubation at 78°C. A total of 200 µg EPS protein were applied for IEF (non-linear IPG strips). Molecular mass marker: PageRuler Prestained Protein Ladder (Fermentas).

(arCOG J). Most proteins (73) were predicted to be of intracellular localization, one, a putative ATPase involved in archaella (archaeal flagella) biosynthesis, is associated with the cytoplasmatic membrane and for the remaining 11 proteins no subcellular localization could be assigned. However, none of the identified proteins possessed a predictable signal peptide for secretion.

Enzyme Activities

Hydrolytic enzyme activity was determined in cell-free dialyzed EPS solutions at pH 3.5 corresponding to the pH value of the

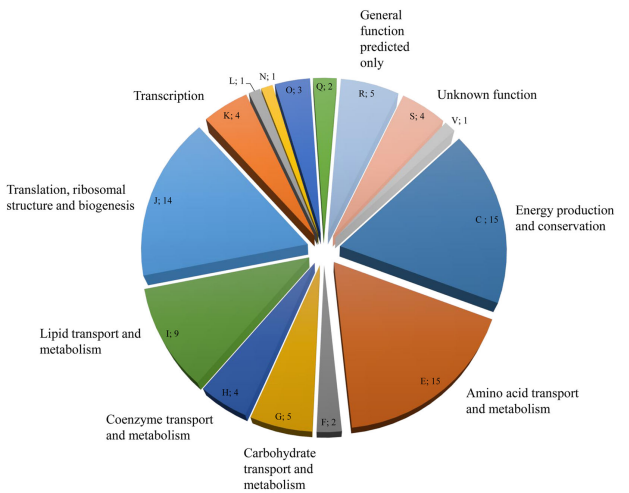

FIGURE 4 | Distribution and number of *S. acidocaldarius* biofilm EPS proteins in the different arCOGs functional categories. For identification, EPS isolated from unsaturated *S. acidocaldarius* biofilms (4 days, 78°C) was digested with trypsin and applied for nanoRSLC-Orbitrap LC-MS/MS analysis. Proteins were assigned to arCOG functional categories (capital letters). F, Nucleotide transport and metabolism, L: Replication, recombination, and repair, N: Cell motility, Q: Secondary metabolites biosynthesis, transport, and catabolism, V: Defense mechanism.

growth medium, and thus should reflect the external environment of extracellular enzymes in the *S. acidocaldarius* biofilms. Esterases were demonstrated to be most active followed by lipases, phosphatases, *N*-acetyl-β-D-glucosaminidases, β-D-glucosidases, and α-D-glucosidases, while peptidase activity was not observed (**Figure 5**).

However, in zymogram gels, protease activity could clearly be detected as single halo bands corresponding to a molecular mass of 47 kDa in EPS samples (5 µg protein) with casein and gelatin, respectively, as a substrate. Protease activity was higher at pH 3.8 than at 7.8 and casein was more actively degraded than gelatin as indicated by the band intensities (**Figure 6**). These results could be confirmed by 2 DE zymography with casein as substrate. The protein spot detected at pH 3.8 and less pronounced also at 7.8, corresponded to a molecular mass of 47 kDa and pI of 3–4. In 2 DE zymogram gels also, two weaker spots with lower molecular mass (27 kDa) and higher pI could be found. The observed 47 kDa signal in the 1 DE zymography showed pronounced heat stability at 100°C for 1 h. Preincubation of the EPS solution under these conditions did not result in a significant fading of the corresponding band. However, autoclaving at 121°C for 20 min resulted in a complete loss of activity under both pH conditions. Protease inhibitors (23 mM AEBSF, 2 mM bestatin, 100 mM EDTA, 0.3 mM E-64, 0.3 mM pepstatin A, Sigma-Aldrich) had nearly no effect on activity.

Also, the presence of esterases showing the major activities in the EPS fraction (see above) could be confirmed by 2 DE with MUF-butyrate as substrate at both pH 3.5 and 8.0. A spot cluster was detected corresponding to pI 3 and 28 kDa as well as two further signals at 20 and 35 kDa, respectively, and higher pI. Only at pH 8.0, an additional esterase signal was obtained at pI 10 and 25 kDa.

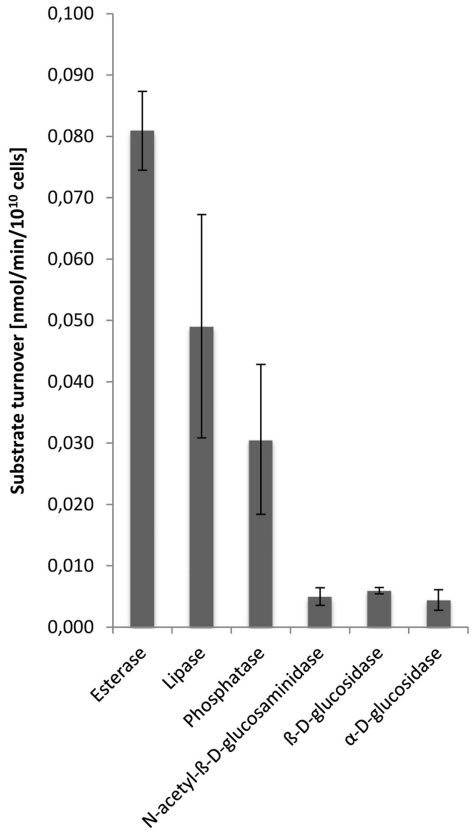

FIGURE 5 | Enzyme activities in the EPS from *S. acidocaldarius* biofilms. EPS were extracted with the CER method from unsaturated biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Enzyme activities in EPS solutions were determined at pH 3.5, using a microtiter plate assay as described in the Section "Materials and Methods."

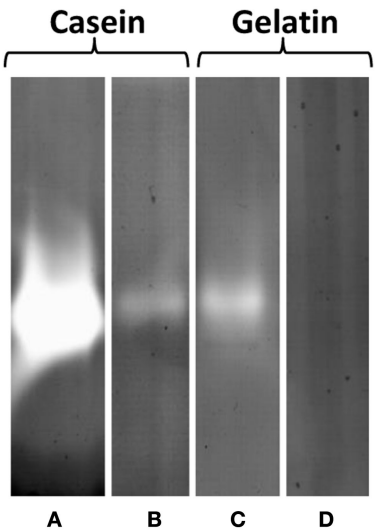

FIGURE 6 | Protease activity of EPS prepared from *S. acidocaldarius* biofilms, visualized in zymogram gels. EPS (5 µg protein/lane) were electrophoresed in gels containing 0.1% casein or 0.1% gelatin and incubated at either pH 3.8 **(A,C)** or 7.8 **(B,D)** for 24 h at 78°C. EPS were isolated with the CER method from biofilms collected from Brock medium plates after 4 days of incubation at 78°C. Molecular mass marker: PageRuler Prestained Plus Protein Ladder (Fermentas).

Discussion

Efficiency of EPS Isolation Methods

For the quantitative and qualitative EPS analyses, an appropriate cultivation technique of the thermoacidophilic Crenarchaeon *S. acidocaldarius* was required capable of high biofilm yields enabling the isolation of sufficient EPS amounts. However, so far only limited information about the cultivation of *Sulfolobus* spp. biofilms was available. In previous studies, *Sulfolobus* biofilms were grown on surfaces of glass slides, carbon-coated grids, µ-dishes or polystyrene microplates, and Petri dishes with the focus on the investigation of biofilm formation and analysis of attached biofilms with microscopic techniques, such as scanning electron microscopy or confocal laser scanning microscopy (Koerdt et al., 2010; Zolghadr et al., 2010; Henche et al., 2012). For quantitative and qualitative EPS analysis, destructive methods are usually employed, including dispersal of biofilm cells and extraction of EPS by physical and/or chemical procedures (Nielsen and Jahn, 1999). So far, the production of elevated biofilm mass on solid surfaces necessary for the recovery and subsequent biochemical analysis of EPS from *Sulfolobus* biofilms has never been reported. In the current study, *S. acidocaldarius* was grown as an unsaturated biofilm on the surface of gellan gum-solidified Brock medium plates, resulting in the reproducible formation of sufficient biomass suitable for subsequent EPS isolation and analysis. A similar approach proved useful for the cultivation of unsaturated bacterial biofilms grown on the surface of agar-solidified media with substantial production of EPS as has been shown, e.g., for *Pseudomonas aeruginosa* (Wingender et al., 2001) and *Pseudomonas putida* (Metzger et al., 2009).

For EPS analyses, the establishment, evaluation, and choice of suitable EPS isolation procedures and conditions are crucial, since all components should be obtained as entirely as possible without damaging the cells yielding to contaminations of the EPS extracts with intracellular components. Previous studies showed that there is no universal method applicable and possibly several methods are required to obtain all different EPS fractions (Park and Novak, 2007). As shown by Aguilera et al. (2008) by comparison of five EPS extraction methods applied to benthic eukaryotic biofilms from an acidic river, different methods potentially resulted in divergent yields of EPS and heavy metals. They also showed that most efficient EPS isolation, in that case with NaCl, resulted in highest contamination with the exclusively intracellular enzyme glucose-6-phosphate-dehydrogenase (G6PDH), indicating cell lysis. This again highlights the importance also known from other reports to carefully balance both EPS yield and cell damage. Thus, appropriate methods to detect cell lysis like protein/carbohydrate ratios and eDNA are most important in the establishment of EPS extraction methods. However, it is well established that eDNA can be a crucial component of the EPS matrix of several microorganisms and sometimes even be required for initial cell attachment and biofilm formation (Whitchurch et al., 2002; Flemming and Wingender, 2010; Jakubovics et al., 2013). Moreover, the ratio of proteins to carbohydrates can vary

significantly between EPS of different microorganisms or different growth conditions and does not necessarily indicate cell lysis. The activity screening of strictly intracellular enzymes, like G6PDH for bacteria, is not applicable for EPS isolations with chemicals inhibiting enzyme activity like EDTA, which complexes cations, or NaOH and crown ether, which significantly increase the pH value, thus denaturing proteins (Frølund et al., 1996). The application of viability stains, for instance, using the Live/Dead staining method, is common to detect membrane disintegration (Wu and Xi, 2009). This method, however, could so far not be adapted to the type of biofilms used here for the archaeon, *S. acidocaldarius*. In this study, cell lysis was excluded by monitoring the impact on total and viable cells counts. Total (DAPI-stained, and thus, DNA-containing) cell counts and colony counts indicated that *S. acidocaldarius* cells agitated in the presence or absence of CER at pH 3.5 (Brock medium) or 7.0 (phosphate buffer) are largely insensitive toward mechanical shear forces and pH stress.

After establishing efficient culturing of *S. acidocaldarius* biofilms on solidified Brock medium plates (4 d, 78°C), in this study, five different EPS extraction procedures were analyzed and the influence on cell viability, EPS yield, and composition was determined. First, biofilms were suspended in phosphate buffer (biofilm suspension) followed by the actual EPS extraction. The five applied EPS isolation methods included shaking used as a simple reference method, shaking with the addition of CER as well as treatment with EDTA, crown ether, and NaOH. Finally, after removal of cells by centrifugation, subsequent filter sterilization, and dialysis of the supernatant, the final cell-free EPS solution was obtained.

Application of all five extraction methods provided EPS that contained carbohydrates, proteins, and minor amounts of DNA. However, the EPS yield and the ratio of the EPS components were strongly dependent on the extraction procedure. Thus, these archaeal EPS from *Sulfolobus* biofilms resemble the common situation for bacterial biofilms, where carbohydrates, proteins, and DNA are consistently found, with varying extraction efficiencies obtained when different isolation methods were compared (Jahn and Nielsen, 1995; Comte et al., 2006; Park and Novak, 2007; Aguilera et al., 2008). Comparison of the yield of total EPS in terms of carbohydrate, protein, and DNA content obtained by the five extraction methods applied in this study revealed the following order NaOH > crown ether > CER > EDTA > shaking. In agreement with this, the 2 DE analyses of the extracellular proteins showed the fewest proteins (approximately 300 spots) in the EPS isolated by shaking and no reduction of cell viability was observed. Hence, shaking represents a gentle physical procedure that isolates only not tightly bound, soluble EPS components without cell lysis, and effects on further EPS analysis.

Addition of CER improved the EPS yield via shaking. The stabilizing effect of multivalent cations, permitting electrostatic crosslinking interactions between polysaccharides and/or proteins, on the EPS is already known. The CER extraction procedure acts partly mechanically due to shear forces and partly chemically by the removal of divalent cations, with the destabilizing of the EPS matrix and the release of water soluble EPS (Jahn and Nielsen, 1995; Frølund et al., 1996). In this study, substantial removal of calcium and magnesium ions was confirmed for the *S. acidocaldarius* biofilms, showing an abstraction of at least 60% for magnesium to 80% for calcium from the biofilms (data not shown), similar to the values stated by Park and Novak (2007) for CER extraction of EPS from activated sludge. No removal of other cations was observed, such as iron and copper (this study) and iron and aluminum in the study of Park and Novak (2007). This observation indicates that the CER procedure seems to be selective for targeting calcium and magnesium ions and their associated EPS within the biofilm matrix. CER isolation of the EPS did not affect viability of *S. acidocaldarius* cells indicating that intracellular or membrane-derived contaminations in course of the isolation procedure are negligible. This is in accordance with studies on EPS isolation from bacterial biofilms, which also demonstrated that CER is suitable for mild extraction without elevated cell damage (Frølund et al., 1996; Chen et al., 2013).

However, both NaOH and crown ether led to substantial loss of culturability, probably due to cell lysis. Compared to the physical methods, the chemicals used for isolation remained in the EPS solution and interfered with certain subsequent analyses. EDTA interferes with protein determination according to the Lowry method by chelating copper ions and also with the isoelectric focusing applied in the 2 DE procedure. Crown ether interfered with the carbohydrate assay leading to unrealistic high concentrations. NaOH led to the loss of culturability and the reduction of total cell counts to below the detection limit, probably caused by the elevated pH resulting in cell lysis and denaturation of proteins, thus precluding any enzyme assays. In contrast to this, an advantage of CER apart from the low costs and easy handling is its complete removal from the EPS via centrifugation or settling of the resin while the chemical methods remain in the EPS and have to be removed via dialysis if possible.

Analysis of EPS Protein

The analysis of EPS proteins via 2 DE revealed an unexpected large number of proteins and first assays using fluorogenic substrates as well as zymography showed activities of diverse classes of hydrolytic enzymes, such as proteases, lipases, esterases, phosphatases, and glucosidases usually involved in extracellular degradation of polymers into assimilable mono- or oligomers (for review, see Wingender and Jaeger, 2002). A genomic analysis in *S. solfataricus*, a close relative of *S. acidocaldarius*, identified a total of more than 4% (corresponding to >100 proteins) of all encoded proteins in the genome to contain a signal sequence for protein export among them, e.g., the protease thermopsin and several endoglucanases (Albers and Driessen, 2002). In *Sulfolobus* species, few enzymes have previously been reported to be extracellular including the thermoacidophilic protease thermopsin (Lin and Tang, 1990, 1995; Rawlings, 2013), a carboxylesterase (Huddleston et al., 1995) and several endoglucanases (Limauro et al., 2001; Girfoglio et al., 2012), which coincides well with the respective enzyme activities reported here for the biofilms of *S. acidocaldarius*. Furthermore, the zymography signal of the protease observed in the EPS corresponded well to the molecular mass and pI reported for the glycosylated form of the thermopsin in *S. acidocaldarius*. However, in contrast to the enzyme activity assays, in the mass spectrometry analysis of the EPS fraction, none of

these extracellular proteins could be identified and all of the proteins found were predicted to be cytoplasmic not harboring any predictable signal peptide sequence for secretion. Furthermore, the enzyme activities found could not be clearly correlated to detectable protein spots or bands in the gels, indicating that the enzyme amount present in the EPS allows for activity measurement, but is not sufficient for protein detection. Also, in the extracellular proteome of planktonic cell cultures of *Sulfolobus*, none of the known extracellular enzymes could be identified by SDS-PAGE separation and subsequent MS analyses of the excised bands. It has been concluded that these proteins are excreted directly to the medium, but only in very small quantities, not detectable by electrophoretic methods (Ellen et al., 2010a). The high number (several hundred) of proteins in the 2 DE gels was unexpected, since such high numbers of proteins were not identified in supernatants of planktonic cell cultures of *Sulfolobus* spp. (Ellen et al., 2010a). However, in contrast to the EPS proteins, most of the proteins isolated from culture supernatants contained a signal peptide for excretion. These signal peptide containing proteins were mainly argued to remain associated with the cell membrane, e.g., through C-terminal membrane anchors and only a small number appears to be directly released into the medium. Also, the S-layer appears to operate as a barrier for protein secretion. Accordingly, only limited protein secretion into the medium has been proposed for *Sulfolobus* spp. (Ellen et al., 2010a). The high number of cytoplasmic proteins in the EPS could be excluded to occur due to cell disruption caused by the EPS isolation procedure (no decrease of plate counts), and thus points either to membrane vesicles or to biofilm-inherent cell lysis: *Sulfolobus* cells were shown to form membrane vesicles involving an endosomal sorting complex required for transport (ESCRT) III-dependent budding process similar to the endosomal sorting pathway of exosomes in eukaryotes (Ellen et al., 2010b). For *S. islandicus*, growth inhibition of other *Sulfolobus* spp. by a protein factor isolated from these vesicles was demonstrated (Prangishvili et al., 2000). Membrane vesicle structures are also known from several bacteria and have been described in planktonic cultures, but also as a component of the matrices of biofilms (e.g., *P. aeruginosa*) (Schooling and Beveridge, 2006), where a role of these vesicles in antimicrobial defense, quorum sensing, providing hydrolytic activity, and stress response has been discussed. In the EPS isolation procedure reported here, such membrane vesicles may retain in the preparation, thus causing elevated numbers of cytoplasmic proteins. This is supported by the finding that 6 out of 32 proteins found in membrane vesicle proteomes from *Sulfolobus* spp. (Ellen et al., 2009) were also identified in the EPS. On the other hand, cell lysis within biofilms is established as inherent element of biofilm development in Bacteria (Jakubovics et al., 2013) and may also contribute to the large amount of cytoplasmic proteins in the EPS fraction. For bacterial biofilms, a major function of cell lysis in DNA exchange and nutrition has been discussed. However, in order to elucidate the reason for cytoplasmic protein enrichment in the matrix of *S. acidocaldarius* biofilms, further studies are required.

Conclusion

In this study, a suitable and efficient method for the EPS extraction from *S. acidocaldarius* biofilms was achieved. Evaluation of the yield of each method together with the suitability for further analysis showed that CER is superior to the other applied methods leading to high EPS yields with no apparent cell lysis and no impact on subsequent biochemical analysis. The main component of *S. acidocaldarius* EPS was carbohydrates followed by proteins and DNA. Determination of the culturability for the different methods at a wide pH range demonstrated the high tolerance of the thermoacidophilic *S. acidocaldarius* toward pH stress at low temperature. Significant hydrolytic enzyme activity was observed in the EPS fraction. The high number of cytoplasmic proteins in the EPS fraction points to either the formation of extracellular membrane vesicles or cell lysis during biofilm development.

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Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fbioe.2015.00123

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Biopolymers from lactic acid bacteria. Novel applications in foods and beverages

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Lactic acid bacteria (LAB) are microorganisms widely used in the fermented food industry worldwide. Certain LAB are able to produce exopolysaccharides (EPS) either attached to the cell wall (capsular EPS) or released to the extracellular environment (EPS). According to their composition, LAB may synthesize heteropolysaccharides or homopolysaccharides. A wide diversity of EPS are produced by LAB concerning their monomer composition, molecular mass, and structure. Although EPS-producing LAB strains have been traditionally applied in the manufacture of dairy products such as fermented milks and yogurts, their use in the elaboration of low-fat cheeses, diverse type of sourdough breads, and certain beverages are some of the novel applications of these polymers. This work aims to collect the most relevant issues of the former reviews concerning the monomer composition, structure, and yields and biosynthetic enzymes of EPS from LAB; to describe the recently characterized EPS and to present the application of both EPS-producing strains and their polymers in the fermented (specifically beverages and cereal-based) food industry.

Keywords: exopolysaccharides, homopolysaccharides, heteropolysaccharides, lactic acid bacteria, fermented foods, fermented beverages, sourdough

Introduction

Since ancient times lactic acid bacteria (LAB) have been empirically exploited as starter cultures to improve the preservation, nutritional value, and sensorial characteristics of a variety of fermented foods and products derived from animal and vegetable origins (Wood and Holzapfel, 1995; Wood, 1997; Leroy and De Vuyst, 2004). LAB have been rationally applied as lactic starter cultures in the fermented food industry since the 1930s–1940s. In addition, certain LAB strains have been used as probiotics because of their health-promoting effects in the host (Foligné et al., 2013; Martín et al., 2013; Borges et al., 2014). Due to their long history of safe use in human consumption, some LAB strains have the Qualified Presumption of Safety (QPS) or Generally Recognized As Safe (GRAS) status (EFSA, 2010).

In addition to their main feature, which is lactic acid production from the carbon source present in the matrix where they grow, several LAB strains form other compounds such as vitamins, bioactive peptides, antibacterial compounds, aroma compounds, low-calorie sugars, exopolysaccharides (EPS), etc. All these traits confer desirable attributes to specific fermented foods and products (Hugenholtz, 2008; LeBlanc et al., 2013; Ortiz et al., 2013). In this respect, efforts have been made to use LAB as microbial cell factories for the production of industrially interesting metabolites either to be used as purified compounds or to be produced *in situ* in fermented foods (Hugenholtz, 2008; Gaspar et al., 2013; Boguta et al., 2014).

In this work, we aimed to collect the most relevant issues of former reviews on EPS from LAB such as their monomer composition, structure, yields and biosynthetic enzymes; to describe the recently characterized EPS and to present the application of both EPS-producing strains and their polymers in the fermented (specifically beverages and cereal-based) food industry.

Several reviews on EPS produced by LAB have been published dealing mainly with the physiology, biosynthesis, chemical and structural characteristics of the EPS molecules. To our criteria, the most relevant and detailed works covering these topics through the last decade include those from Ruas-Madiedo and de los Reyes-Gavilán (2005), Badel et al. (2011), Patel et al. (2012), Leemhuis et al. (2013b). Regarding the health-promoting benefits of these polymers two reviews on their immunomodulatory activity and prebiotic effects were recently addressed by Ryan et al. (2015) and Salazar et al. (2015).

EPS Classification, Biosynthesis, and Yields

As the majority of bacteria, LAB can synthesize cell-wall structural polysaccharides such as peptidoglycan and lipoteichoic acids, and exocellular polymers. The latter include both capsular polysaccharides (CPS), covalently bound to the cell surface, and EPS, which may form a loosely bound layer that can also be secreted into the environment (Chapot-Chartier et al., 2011).

Exopolysaccharides from LAB are highly diverse and can be classified following different criteria. The most classical one is based on their monomer composition, which allows classifying them into two major groups: homopolysaccharides (HoPS) and heteropolysaccharides (HePS).

Homopolysaccharides

The most notorious advance in the research on EPS from LAB in the last decade has been related to HoPS. Indeed, the isolation of HoPS-producing strains (mainly belonging to the *Weissella* genera), the molecular and structural characterization of these EPS, studies on their biosynthetic enzymes, and the HoPS application in food have been described (Galle and Arendt, 2014; Lynch et al., 2014; Tingirikari et al., 2014; Wolter et al., 2014a,b).

Homopolysaccharides contain one neutral monosaccharide type either glucose (glucans), fructose (fructans), or galactose (polygalactan) (Monsan et al., 2001; Mozzi et al., 2006; Ruas-Madiedo et al., 2008). Among LAB, a sub-classification has been established depending on the linkage type and the position of the carbon involved in the bond. Thus, glucans can be sub-classified into (i) α-glucans [dextran: α-D-Glc(1,4); mutan: α-D-Glc(1,3); alternan: (α-D-Glc(1,6)/α-D-Glc(1,3); and reuteran: α-D-Glc(1,4)/α-D-Glc(1,6) with α-D-Glc(1,4)/α-D-Glc(1,6) branching points], and (ii) β-glucans [β-D-Glc(1,3) with side chain linked (1,2)]. Fructans can be classified into (i) levan-type: β-D-Fru(2,6), and (ii) inulin-type: β-D-Fru(2,1), being both β-fructans. Finally, polygalactans, which contain a pentameric repeating unit of galactose; these polymers being more rare and were only described for the strain *Lactococcus. lactis* subsp. *lactis* H414 (van Kranenburg et al., 1999) and two strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (CRL 406 and 142; Mozzi et al., 2006). Recently, a polygalactan-containing CPS from the strain *Lactobacillus plantarum* 70810 was analyzed by different techniques (GC-MS, gas chromatography–mass spectrometry; FTIR, fourier transform infrared spectroscopy; and NMR, nuclear magnetic resonance) resulting in a low molecular weight (MW) polymer of 1.7 kDa with a repeating unit of α-D-(1,6)-linked galactcosyl, β-D-(1,4)-linked galactcosyl, β-D-(1;2,3)-linked galactcosyl residues and a tail end of β-D (1,)-linked galactcosyl residue (Wang et al., 2014).

Traditional fermented foods can be a rich source of HoPS-producing LAB as well as of different polymers. Recently, several dextran-producing LAB strains from sourdoughs and indigenous Asian foods were isolated (**Table 1**).

In addition Park et al. (2013) have isolated dextran-like polymer-producing strains belonging to the genera *Leuconostoc* and *Weissella* from kimchi. The MW of these HoPS being about 1.1×10^6 Da; FTIR analysis showed that these polymers had a similar structure to that of commercial dextran from *Leuc. mesenteroides* B-512F. *Leuconostoc* strains produce putative alternan polymers being those α-(1,2) branched polymers particularly high (Passerini et al., 2015). Interestingly, the ^{1}H NMR spectra of the EPS produced by *L. curvatus* 69B2 and *Leuc. lactis* 95A showed that the HoPS formed was constituted by a single repeating glucopyranosyl unit linked by an α-(1,6) glycosidic bond in a dextran-type carbohydrate (Palomba et al., 2012).

Patel et al. (2012) showed that some strains of *L. fermentum*, *L. sakei*, and *L. hilgardii* produced α-(1,6) glucans ramified by glucose residues at position 3, and at lesser extent at positions 2 and 4 (dextran). The degree of branching involving α-1,2, α-1,3 and α-1,4 linkages varying according to the origin of the biosynthetic enzymes.

Some species such *L. reuteri,* a predominant HoPS producer, is also able to generate different types of EPS including dextran (Rühmkorf et al., 2013), levan, inulin-type fructan, mutan, and reuteran with a broad MW range. Also, the single strain *L. reuteri* 121 synthesizes several HoPS when grown under the same culture conditions (van Geel-Schutten et al., 1999; van Leeuwen et al., 2009; Pijning et al., 2012).

Respect to HoPS biosynthesis, these polymers are mainly synthesized extracellularly from an existing sucrose molecule, which acts as donor of the corresponding monosaccharide by action of a single type of extracellular enzyme belonging to the glycosyl hydrolase (GH) family. Thus, α-glucans and β-fructans are formed by glucansucrases (GS; GH family 70) and fructansucrases (FS; GH family 68), respectively (http://www.cazy.org; Cantarel et al., 2009). Generally, these enzymes catalyze the polymerization of the HoPS out of sucrose as donor of the corresponding monosaccharide and transfer the molecule to the reducing end of the glucan or fructan, respectively (Korakli and Vogel, 2006; van Hijum et al., 2006; Chapot-Chartier et al., 2011; Leemhuis et al., 2012). Both enzymes are typical transglycosylases or glycansucrases displaying a dual mode of action as they can cleave the glycosidic bond in sucrose (hydrolysis reaction) and

TABLE 1 | Dextran-producing LAB strains and properties of their polymers.

Microorganisms	Source	Dextran characteristics[&]	Structural studies*	Molecular studies	Reference
Leuconostoc mesenteroides NRRL B-1149	Sugar-cane juice	Insoluble (medium used: sucrose, 10.0%; maltose, 5.0%; 20 mM sodium acetate buffer (pH = 5.4) containing 0.1% sodium azide and purified dextransucrase (0.2 mg/mL, 13 U/mg) Fibrous structure	FTIR, ^{1}H NMR, ^{13}C NMR spectroscopy and SEM technique	Purification and characterization of dextransucrase (enzyme activity in SDS-PAGE)	Shukla et al., 2010, 2011
Pediococcus pentosaceous CRAG 3	Fermented cucumber	MM 2.9 × 10^5 Porous structure	FTIR, NMR, SEM	Purification and characterization of dextransucrase (SDS-PAGE)	Shukla and Goyal, 2013, 2014
P. acidilactici M76	Korean fermented rice wine (*makgeolli*)	MM 6.7 × 10^4	SEC, FTIR		Song et al., 2013
Lactobacillus hilgardii TMW 1.828	Water kefir			Purification and sequence analysis of glucansucrase (GS). Characterization of GS activity in SDS-PAGE. Identification and sequence of glucansucrase gene [FN662554]	Waldherr et al., 2010
L. acidophilus ST76480.01	Fermented vegetables	Insoluble (medium used %: sucrose, 15.0; bacto-peptone, 0.5; yeast extract, 0.5; K_2HPO_4, 1.5; $MnCl_2.H_2O$, 0.001; NaCl, 0.001; $CaCl_2$, 0.005 and pH 7.0)	Rheology, ^{13}C-NMR spectroscopy.	Characterization of dextransucrase activity (SDS-PAGE)	Abedin et al., 2013
L. plantarum DM5	Indian traditional fermented beverage (*Marcha*)	MM 1.1 × 10^6 Smooth porous structure	SEC, FTIR, NMR, SEM, rheology	Purification and characterization of GS (SDS-PAGE)	Das and Goyal, 2014
Weissella confusa MBF8-1	Indonesian fermented soybean foods (*Pamulang, Tangerang*)			Two *gtf* genes: *gtf8-1A* [FJ436354] and *gtf8-1B* [FJ460018]	Malik et al., 2009
Weisella. sp. TN610	Pear		NMR	Characterization of dextransucrase activity (SDS-PAGE). Sequence of *gtf* gene [HE818409]	Bejar et al., 2013
W. cibaria MG1	Sourdough	3 x 10^9, 5 x 10^6–4 x 10^7 GOS is formed in addition to dextran when maltose is present	FFF, SEC, HPAEC-PAD	Genome sequence including the *gtf* gene (JWHU00000000)	Galle et al., 2010, 2012a,b; Zannini et al., 2013; Lynch et al., 2014; Wolter et al., 2014a,b
W. cibaria JAG8	Apple peel	Porous structure	DLS spectroscopy (monodisperse), SEM	Purification of dextransucrase; *in vitro* synthesis of dextran	Rao and Goyal, 2013

Information published by Ruas-Madiedo and de los Reyes-Gavilán (2005) and Badel et al. (2011) was omitted in this Table to avoid overlapping.

[&]*MM, molecular mass expressed in Daltons; GOS, gluco-oligosaccharides.*

**FITR, fourier transform infrared; ^{1}H NMR and ^{13}C NMR, nuclear magnetic resonance spectroscopic; SEM, scanning electron microscopy; SEC, size-exclusion chromatography; MALLS, multiangle laser light scattering; HPAEC-PAD: high performance anion exchange chromatography with pulsed amperometric detection; DLS, dynamic light scattering.*

with the energy released they can transfer the glucosyl or fructosyl moiety (transferase reaction) to the growing reducing end of the polymer (Chapot-Chartier et al., 2011).

Glucansucrases can synthesize a variety of α-glucans with different physicochemical characteristics such as solubility, viscosity, and other properties by altering the type of glycosidic linkage, degree of branching, length, mass, and conformation of the polymers. So far, α-glucan formation by GS has been reported for the genera *Leuconostoc, Lactobacillus, Streptococcus, Pediococcus,* and *Weissella*. Depending on the reaction catalyzed and the specificity, GS are classified by the Nomenclature Committee of the International Union of Biochemistry and

Molecular Biology (NC-IUBMB) in four groups: dextransucrase (EC 2.4.1.5), mutansucrase (EC 2.4.1.5), alternansucrase (EC 2.4.1.140), and reuteransucrase (EC 2.4.1.5), which catalyze α-(1,6), α-(1,3), α-(1,3 and 1,6), and α-(1,4 and 1,6) glycosidic linkages, respectively (Leemhuis et al., 2013b).

The *in vitro* synthesis of glucooligosaccharides (GOS) and fructooligosaccharides (FOS) by using selected GS and FS and electron acceptors reaction (i.e., maltose, raffinose) was reported in *Lactobacillus* (Tieking et al., 2005a; Ozimek et al., 2006; Katina et al., 2009; Galle et al., 2010, 2012b; Shukla and Goyal, 2014), *Leuconostoc* (Leemhuis et al., 2013a), and *Weissella* (Malang et al., 2015). This approach constitutes one of the most interesting perspectives to design short tailor-made HoPS with prebiotic activity.

Glucansucrases expression is involved in the constitutive production of HoPS as reported in the strains *L. reuteri* 121, 180 and *L. parabuchneri* 33. On the contrary, the GS expression by *Leuc. mesenteroides* is induced by sucrose (Kralj et al., 2004), these enzymes are high MW extracellular proteins exclusively encoded by GS (*gtf*) genes. Variations in EPS structures, even belonging to the same group (i.e., dextran, mutan, etc.) are due to different enzymes intervention. For example, the product of the gene *gtf* Kg3 from *L. fermentum* Kg3 is responsible for the synthesis of a dextran which is 89% linked by α-(1,6) linkages, while *gtf* 180 (from *L. reuteri* 180) encoded for a protein involved in the synthesis of a dextran with 51% of α-(1,6) glucosidic bonds. In addition, biosynthesis of glucans by a wine strain of *Lactobacillus* sp. leads to a linear dextran whereas *L. reuteri* 180 synthesizes a branched EPS (Uzochukwu et al., 2001; van Leeuwen et al., 2009). Branching is catalyzed by GS involved in the HoPS backbone synthesis; the degree of branching depending on the enzyme conformation. Some experiments accomplished on GS from the strain *L. reuteri* 121 showed that mutations of the gene encoding for this enzyme increased α-(1,6) and decreased α-(1,4) linkages.

The production of β-glucan has mainly been described in LAB isolated from alcoholic fermented beverages and its synthesis occurs intracellularly by a membrane-associated glucosyltransferase (GTF). Although its mechanism of action is not fully yet understood, it does not need sucrose as substrate (Werning et al., 2006). LAB strains belonging to the genera *Pediococcus*, *Lactobacillus*, and *Oenococcus* isolated from cider and wine are able to produce a 2-substituted (1, 3)-β-D-glucan (Llaubères et al., 1990; Dueñas-Chasco et al., 1997, 1998; Ibarburu et al., 2007; Dols-Lafargue et al., 2008) by a single transmembrane GTF that polymerizes glucosyl residues from UDP-glucose (Werning et al., 2006, 2008; Velasco et al., 2007; Garai-Ibabe et al., 2010). Considering the requirement of sugar nucleotide intermediates as precursors, the synthesis of β-glucans resembles HePS's synthesis and it is linked to the growth and the central carbon metabolism of the producer organism. Also, GTF are part of the enzyme machinery responsible for HePS formation (De Vuyst and Degeest, 1999). The (1,3)-β-D-glucans are attractive for the pharmaceutical and functional food industries because of their beneficial effects on human and animal health (Zekovic et al., 2005). The 2-substituted (1,3)-β-D-glucan producers *P. parvulus* 2.6 and *L. paracasei* NFBC 338 have been tested for the production of oat-based products, yogurt and various beverages increasing significantly their techno-functional properties (Mårtensson et al., 2002; Kearney et al., 2011). Moreover, the synthesis of the 2.6 β-glucan confers higher survival to the producing-strain during the gastrointestinal passage or technological process (Stack et al., 2010).

The enzyme FS or fructosyltransferase (FS, EC 2.4.1.10) cleaves the glycosidic bond of the fructosyl-donor molecule (substrate, i.e., sucrose, raffinose, stachyose, verbascose) and uses the released energy to couple a fructose moiety to a growing fructan chain but also to sucrose or to another acceptor such as raffinose (Meng and Fütterer, 2003, 2008; van Hijum et al., 2006; Teixeira et al., 2012). As mentioned earlier, two fructan types are known, levan [with β-2,6 glycosidic bonds, produced by levansucrases (Lev, E.C. 2.4.1.10; van Hijum et al., 2004)] and inulin (with β-2,1 bonds) synthesized by inulosucrase (Inu, E.C. 2.4.1.9; van Hijum et al., 2003, 2006). Fructan production has been reported for *W. confusa* strains isolated from Malaysian soy, Malaysian coconut milk beverage (Malik et al., 2009), wheat sourdough (Tieking et al., 2003), fermented cassava (Malang et al., 2015), and for *L. reuteri* Lb121 (van Geel-Schutten et al., 1999) and a *L. pontis* strain (Tieking et al., 2003). Inulin biosynthesis is rare in LAB and only individual strains of *Lactobacillus* and *Leuconostoc* as well as a few streptococci were reported to produce inulin or to possess inulosucrase-encoding genes (van Hijum et al., 2002; Olivares-Illana et al., 2003; Schwab et al., 2007; Anwar et al., 2008, 2010). Recently, Malang et al. (2015) reported the first structural characterization of a fructan-type polymer produced by a *Weissella* strain as well as the first *Weissella* strain to produce inulin in addition to oligosaccharides. By means of NMR, the soluble EPS formed by nine strains were identified as low α-1,3-branched dextran, levan and inulin-type polymers. Besides, six isolates synthesized a highly ropy polymer together with CPS formation composed of glucose, *O*-acetyl groups and two unidentified monomers when using glucose as carbon source.

The fact that GTF cannot use sucrose as an acceptor but GS can, and that FS can also use raffinose, stachyose, and verbascose as substrates, are clear differences among these enzymes. Indeed, the reactions formed by FS and GS are similar with respect to the use of sucrose as a substrate (Galle and Arendt, 2014), but the proteins involved do not share a high amino acid sequence similarity and differ strongly in protein structures (Vasileva et al., 2009; Leemhuis et al., 2013b).

Due to the interest in the food industry, the number of isolations of GS-producing LAB has been rapidly increasing. The strains are typically identified on solid or liquid media supplemented with sucrose by the appearance of slimy/ropy colonies or viscous solutions. Gene knock-out studies have demonstrated that the slimy colony morphology is caused by GS activity in wild-type strains (Fujiwara et al., 2000; Waldherr et al., 2010). However, also β-fructan-producing strains form slimy colonies in the presence of sucrose. Replacing sucrose by the tri-saccharide raffinose used by FS but not by GS enzymes, allows easy identification of β-fructan-producing strains. By this means, many α–glucan-(and β-fructan-)-forming strains have

been identified in fermenting cabbage, fruits, and vegetables, sourdoughs, beverages, dairy, cereals, dental plaque, intestines, environmental spills of sugar plants, and in fish gastrointestinal tract (van Geel-Schutten et al., 1998; Tieking et al., 2005b; Van der Meulen et al., 2007; Kang et al., 2009; Malik et al., 2009; Bounaix et al., 2010a,b; Shukla and Goyal, 2011; Aman et al., 2012; Hongpattarakere et al., 2012). Nevertheless, the number of well-characterized GS remains behind with respect to available *gtf* sequences as only three-dimensional structures of GS complexes (from *Leuc. mesenteroides* NRRL B-1299, *Streptococcus mutans* and *L. reuteri* 180) are available (Vujičić-Žagar et al., 2010; Ito et al., 2011; Brison et al., 2012). Expanding the structural knowledge to a wider range of GS and FS, either alone or complexed with different molecules (substrates or products) may pave the way for the rational application of HoPS. Specific information on the structure-function relationships in GS- and FS-producing HoPS, and on their structures, reactions, and GS mechanisms has been reported by van Hijum et al. (2006) and Leemhuis et al. (2013a), respectively. The GS from strains *L. reuteri* 121 and TMW 1.106, *L. curvatus* TMW 1.624, *L. animalis* TMW 1.971, and *L. hilgardii* TMW 1.828 were biochemically characterized (Kralj et al., 2004; Waldherr et al., 2010; Rühmkorf et al., 2013).

The yields of HoPS produced by LAB are low as compared with other bacterial EPS (i.e., xanthan from *Xanthomonas campestris*) already used in the food industry. It has been reported that EPS formation by *Weisella* sp. and *L. sanfranciscenis* strains reached levels up to 16 and 5 g EPS/kg dough, respectively, thus showing their potential to replace hydrocolloids (Galle and Arendt, 2014). Also, HoPS production values up to 10 g/L by the strain *L. reuteri* Lb121, which simultaneously synthesizes α-glucan and β-fructan has been reported (van Geel-Schutten et al., 1999). The MW of HoPS varies according to the producing-strain (Ruas-Madiedo et al., 2009a,b). The average MW of soluble dextran is in a range between 6.2 and 7.1 $\times$ 10^6 Da, bacterial inulins' MW range between 1 and 9 $\times$ 10^7 g/mol (Shiroza and Kuramitsu, 1988; van Hijum et al., 2002), while the MW of levans are within the range of 10^5–2 $\times$ 10^6 g/mol for lactobacilli and *Leuconostoc* sp. strains and 10^8 g/mol for *S. salivarius* (Tieking et al., 2003). Of note, the MW of *Weissella* levans (1.4 $\times$ 10^5–1.7 $\times$ 10^7 g/mol) depend on whether the strains grow on sucrose or raffinose as carbon source (Malang et al., 2015). The most remarkable difference among HoPS, specially inulin-like fructan and FOS or inulin is precisely the size, which directly correlates to their degree of polymerization (DP); FOS and inulin are short oligosaccharides (DP $<$ 30, MW ∼5 kDa) while HoPS are branched polymers of high MW (∼1,000 kDa; Salazar et al., 2015).

Heteropolysaccharides

Heteropolysaccharides from LAB were extensively studied at structural, genetic, and functional levels by Jolly and Stingele (2001) and Ruas-Madiedo and de los Reyes-Gavilán (2005).

In contrast to HoPS, HePS are complex polymers composed of a backbone of repeating subunits, branched or unbranched, that consist of three to eight monosaccharides, derivatives of monosaccharides or substituted monosaccharides. HePS usually contain D-glucose, D-galactose, and L-rhamnose although at different ratios. To a lesser extent, *N*-acetylated monosaccharides (*N*-acetyl-glucosamine and *N*-acetyl-galactosamine), other monosaccharides (fucose, ribose) as well as organic and inorganic (glucuronic acid, acetyl groups, glycerol, phosphate, etc.) substituents (De Vuyst and Degeest, 1999; De Vuyst et al., 2001; Mozzi et al., 2006) can be found in some HePS. Inversely to HoPS, the HePS reapeating units are intracellularly synthesized, and polymerized outside the cell. More than 45 different repeating units have been described by NMR spectroscopy (De Vuyst et al., 2001; Broadbent et al., 2003; Ruas-Madiedo et al., 2009a), mostly of these corresponding to EPS synthesized by LAB and a few by bifidobacteria strains (Ruas-Madiedo et al., 2012). In accordance with this chemical diversity the enzymatic machinery involved in the synthesis of HePS as well as the genes encoding these enzymes, which are organized in *eps* clusters showing an operon-like structure, are much more complex than those of HoPS (Jolly and Stingele, 2001; Broadbent et al., 2003; Ruas-Madiedo et al., 2009a). These genes are organized in four functional regions involved in chain length determination, polymerization, export, and regulation of the gene cluster (van Kranenburg et al., 1997). Because of their different compositions and MW, HePS may vary in charge, spatial arrangements, rigidity, and the ability to interact with proteins (Duboc and Mollet, 2001). All these characteristics strongly affect the EPS physico-chemical properties, such as solubility, viscosity, etc., (Monchois et al., 1999). Noticeable, a common property of the HePS from LAB is the high thickening power displayed by some of them even at very low concentrations (Vaningelgem et al., 2004). This fact seems to be linked to the molar mass (MM) of the EPS rather than the biopolymer charge as suggested by Mende et al. (2013). Indeed, when equal amounts of EPS or CPS from *S. thermophilus* ST-143 were added to milk prior to acidification (induced by a chemical acidulant), the stiffness of the acidified milk gels increased almost linearly with the EPS concentration whereas the CPS concentration did not affect the rheology of the acidified gels when the same amounts of CPS and EPS were added. Both EPS fractions were determined to be uncharged but differed distinctly in their MM, which was 2.6 $\times$ 10^6 Da, and 1.4 $\times$ 10^5 and 7 $\times$ 10^3 Da for the EPS and CPS, respectively (the latter one being composed of two fractions). In contrast, Pachekrepapol et al. (2015) found that the EPS charge positively influenced the rheology of acidified milk gels when using dextran and dextran-sulfate.

Regarding the HePS MW a broad range, varying from 10^4 to 10^6 Da, has been reported. Interestingly, simultaneous occurrence of more than one chromatographic peak of different size in polymers synthesized by a given strain is often frequent in these HePS (Mozzi et al., 2006; Ruas-Madiedo et al., 2009a; Salazar et al., 2009). The EPS MW is a property of special relevance for the technological functionality of the polymers that strongly influence the viscosity and texture of the matrix in which they are present. An increased use of specific methodologies such as Multi-Angle Laser Light Scattering (MALLS) coupled to a refractometer and NMR have been applied to obtain concluding

results on EPS MW and structure in the last years (Shao et al., 2015). By this way, Laws et al. (2008) confirmed the presence of EPS showing three (4, 1.5, and 1.3 × 10^5 Da) and two (2.5 and 3.5 × 10^5 Da) peaks isolated from cultures of *L. acidophilus* 5e2 growing in skim milk or skim milk plus glucose, respectively. Both HePS consist of a heptasaccharide repeating unit containing D-glucose, D-galactose and either *N*-acetyl-D-glucosamine, or D-glucosamine. Simultaneous occurrence of more than one chromatographic peak of different size in HePS synthesized by *L. rhamnosus* KL37B was also reported (Górska-Frączek et al., 2013b). In this case, different repeating units (penta- and nona-saccharide, respectively) were found for each peak, both containing D-glucose and D-galactose (1:2 molar ratio). Pentasaccharide repeating units are present in HePS from *L. johnsonii* 151 (1 × 10^5 Da) and *L. helveticus* sp. *Rosyjski* (1 × 10^6 Da) containing D-glucose and D-galactose (1:1.5 molar ratio) and D-glucose, D-galactose, and *N*-acetyl-D-mannosamine (2:2:1 molar ratio), respectively, (Górska-Frączek et al., 2013a; Patten et al., 2014).

In general, the HePS production levels reported for LAB are lower than those for HoPS. It has been observed that EPS-production is strain-dependent and is strongly affected by the microbial culture conditions such as the composition of the culture medium, pH, temperature, incubation time, etc., (Ruas-Madiedo and de los Reyes-Gavilán, 2005; Torino et al., 2005). Nevertheless, the use of different methods to isolate and quantify the HePS production makes difficult to compare yields accurately. To overcome this issue, methodologies for improving the purity of EPS and thus, to minimize variability in the methods were applied (Leemhuis et al., 2013b; Polak-Berecka et al., 2013). HePS yield values reported in the literature oscillate from 25 to 600 mg/L (Ruas-Madiedo et al., 2009b) while only a few strains have been reported to produce higher amounts under optimized growth conditions being the EPS yield (2 g/L) obtained with *L. rhamnosus* RW-9595M the highest reported to date (Bergmaier et al., 2005). Within the *S. thermophilus* species, Vaningelgem et al. (2004) reported HePS yields varying from 20 to 600 mg/L in milk-based medium under optimal culture conditions. Zisu and Shah (2003) reported the highest EPS production (1029 mg/L) for the strain *S. thermophilus* ASCC 1275 (ST 1275) in milk medium supplemented with 0.5% (w/v) whey protein concentrate (Zisu and Shah, 2003); this strain producing both CPS and ropy EPS (Zisu, 2005).

Methodological Approaches

The structural analysis and yields of any EPS start with the isolation of pure polymers. In this regard, one of the most important issues is to avoid EPS contamination with components from microbial culture medium, usually mannan-containing yeast extract. Different EPS isolation protocols have been reported all of them including: (i) cell removal by centrifugation or filtration, (ii) polymer precipitation from the cell-free supernatant by the addition of chilled ethanol or acetone (the amounts needed to achieve the recovery of the EPS depended on the type of polymer released; two or three volumes are usually used), (iii) dialysis and drying of the precipitated polymer, and eventually (iv), a new reprecipitation and dialysis step (Ruas-Madiedo and de los Reyes-Gavilán, 2005). Purification of EPS can also include membrane-filtration, anion-exchange and/or gel permeation chromatography (Sanz and Martinez-Castro, 2007). In general, EPS isolation from high-protein content culture media (e.g., dairy products), proteins are typically removed by (i) precipitation with trichloroacetic acid, (ii) hydrolysis with proteases, (iii) or a combination of both.

Exopolysaccharides concentration is estimated as neutral carbohydrate content usually determined by the phenol–sulphuric acid method (Dubois et al., 1956) or by weighting the polymer dry matter (Vaningelgem et al., 2004). In addition, EPS concentration can be determined by means of high-performance size exclusion chromatography coupled with refractive index (RI) detection (HPSEC-RI); EPS concentration is calculated by the integration of the RI signal using calibration curves obtained with known MW dextrans (Sánchez et al., 2006).

Recently, Polak-Berecka et al. (2013) compared EPS yields using different extraction procedures. The authors found that a sample heat treatment as first step in the EPS isolation is critical for its complete recovery, especially when CPS or EPS-degrading enzymes are present. In addition, they assumed that centrifugal forces over 10,000 × *g* are too high being likely that part of the EPS is discarded together with the cell pellet. Considering the precipitation step, better results when prolonging the ethanol incubation (24 h at 4°C) under stirring were obtained. Goh et al. (2005) found that 70% (v/v) was sufficient to precipitate all dextran (2 × 10^6 Da) present in a sample while centrifugation regimes after the ethanol precipitation step should last 30–40 min at 27,000–28,000 × *g*.

Capsular polysaccharides can be visualized by light microscopy after negative staining with Indian ink as described by Mozzi et al. (1995); thus, a white polysaccharide layer surrounding the cells can be observed. In addition, Malang et al. (2015) used crystal violet to stain the cells blue facilitating the differentiation of CPS negative cells on dark background. CPS isolation can be done by harvesting 24 h-cells, washing and resuspending them in 1/10 of the initial volume. Then, cell suspensions are heated at 90 °C for 15 min to detach cell bound polysaccharides (Mende et al., 2013). Cells are removed by centrifugation (15 min, 12,000 × *g*) and CPS are isolated from supernatants as described earlier for EPS.

As mentioned before, the major improvements in EPS characterization have been done related to techniques used for MW determination. Polymer MW was measuread based on the retention time of the polysaccharide eluted by HPSEC-RI; later, an increased use of HPSEC-MALLS was observed (Picton et al., 2000). In a higher degree of specificity or complexity, field flow fractionation (FFF) and hydrodynamic chromatography (HDC) can be used for determining the average MW of ultrahigh-MM polysaccharides (Cave et al., 2009; Isenberg et al., 2010; Galle et al., 2012b). Moreover, asymmetrical flow field fractionation (AF4) was successfully applied for the separation of starch-like glucans. Both, HPSEC and AF4, coupled with multiple inline detection of scattering intensities and mass profiles, provide

distributions of apparent MW and radius of gyration with respect to the separated fractions (Rolland-Sabate et al., 2007; Juna et al., 2011).

The EPS monomer composition can be determined by total acid hydrolysis followed by monomer detection using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD; Cataldi et al., 2000). Alternatively, methanolysis and pertrimethylsilylation provide samples that can be analyzed by gas chromatography (GC). In addition, the monosaccharide analysis is used to determine the carbohydrate content, verifying the purity of the sample. The D or L configuration of the monosaccharide residues can be established by GC of the corresponding (−) 2-butyl glycosides (Gerwig et al., 1979). The linkage pattern of the monosaccharide constituents is determined after methylation of all free hydroxyl groups, followed by polymer hydrolysis, and further reduction of the monomers to alditols by sodium borodeuteride. Subsequent acetylation provides deuterated partially methylated alditol acetates that are analyzed by GC coupled with EI-MS (Ciucanu and Kerek, 1984). The percentages (ratio) of the terminal, internal, and branched glucose units as determined by the methylation analysis provides an idea of the polymer structure. Additional information about the structural features of the EPS can be obtained by ^{1}H and ^{13}C NMR spectroscopy. High-resolution NMR spectroscopy is the most powerful method for the unambiguous identification of carbohydrate chains (Damager et al., 2010). This method provides information of the type of constituent monosaccharides, ring size, and anomeric configuration, and the position of glycosidic linkages.

Role of EPS-Producing LAB and their Polymers

Generally, LAB divert a small percentage of their sugar substrates toward the EPS synthesis, whose main biological function *in vivo* has not been exactly established. However, it has been reported that EPS may help to protect bacterial cells against harsh environmental conditions (i.e., desiccation, heat or acidic stresses, phage attack) and the presence of adverse compounds (i.e., antibiotics, human gastric and pancreatic enzymes, bile salts; Ozturk et al., 2009; Stack et al., 2010; Lebeer et al., 2011). In addition, EPS are involved in biofilm formation, host–pathogen interactions, and cellular recognition (Kumar et al., 2007; Gänzle and Schwab, 2009; Chapot-Chartier et al., 2011). Moreover, it has been claimed that EPS may play a role in the adhesion to surfaces such as eukaryotic cells (plants, human intestinal cells) and in the host immune system modulation (Russo et al., 2012; Salazar et al., 2015).

In biofilm formation, EPS production enhances local accumulation of microbes enmeshing them in the insoluble biofilm matrix (Koo et al., 2013). Dental plaque represents the best-known example of microbial biofilm whose formation begins with the adhesion of oral bacteria to the tooth surface. In the complex microbiome, *S. mutans* is the major EPS producer when sucrose and starch are present in the diet, favoring the *in situ* polymer (glucans and fructans) biosynthesis (Paes Leme et al., 2006; Bowen and Koo, 2011).

Krinos et al. (2001) demonstrated that like CPS, EPS can affect the surface antigenicity of different strains resulting in maintenance or elimination of specific strains in the gut ecological niche (Ruas-Madiedo et al., 2009b; Pessione, 2012). Welman and Maddox (2003) stated that EPS may create biofilms aiding in the colonization of the gut by increasing the gut-transit time of LAB. On the other hand, some EPS-producing LAB involved in alcoholic beverage spoilage causing 'ropy' textural alterations have been shown to be resistant to lysozyme treatment (Coulon et al., 2012).

Other studies failed to reveal a significant phage resistant phenotype in EPS and CPS producer strains (Deveau et al., 2002; Rodriguez et al., 2008); in contrast, McCabe et al. (2015) recently reported on the targeted recognition of *Lactococcus lactis* phages to their polysaccharide receptors.

In addition, it has been hypothesized that EPS may play a role as extracellular energy/carbon reserve; however, most EPS producer species lack the genes involved in their own EPS degradation (Badel et al., 2011). EPS could be useful as carbon reserve in the case of sintrophyc/symbiotic life with other bacteria, considering the overall population. Thus, glucans and fructans formed by oral streptococci and lactobacilli have major influences on the formation of dental plaque since they are involved in adherence of bacteria to each other and to the tooth surface serving as extracellular energy reserves to the non-lactic biota (Russell, 1994; Colby et al., 1995). However, Russo et al. (2012) observed a positive effect on the growth of *L. plantarum* WCFS1 and *L. acidophilus* NCFM in a glucose-containing chemically defined medium by the β-D-glucan isolated from *P. parvulus* 2.6, although the EPS alone did not serve as carbon source.

Interestingly, while EPS are synthesized by certain LAB species (i.e., *S. mutans*) under quorum-sensing control (related with biofilm formation and adhesion to solid surfaces; Kumar et al., 2007; He et al., 2015), in *L. reuteri* strains EPS formation has been demonstrated to be induced by environmental stress (Hüfner et al., 2008).

Applications of EPS from LAB in the Food Industry

Exopolysaccharides from LAB have received special attention as valuable compounds because of their potential economic applications that include natural, safe-food additives or natural functional food ingredients increasing the possibility to replace or reduce the use of external hydrocolloids (Giraffa, 2004; Tieking et al., 2005a; Leemhuis et al., 2013a).

The large diversity of LAB species frequently isolated from raw materials and traditionally fermented foods (Van der Meulen et al., 2007; Endo et al., 2009; Robert et al., 2009; Ayeni et al., 2011; Shukla and Goyal, 2011; Di Cagno et al., 2013) has been the source of strains with interesting functionalities. In this sense, a wide diversity of EPS and genes encoding biosynthetic enzymes from naturally occurring LAB in fermented

foods and beverages have been extensively studied for their role in the physicochemical (viscosifying, stabilizing, or water-binding capacities) and sensorial (palatability) characteristics in the final food products. A remarkable structural diversity of EPS produced by *Lactobacillus, Leuconostoc,* and *Weissella* strains has been reported (Uzochukwu et al., 2001; Olivares-Illana et al., 2003; Di Cagno et al., 2006; Mozzi et al., 2006; van Hijum et al., 2006; Van der Meulen et al., 2007; Bounaix et al., 2010a,b; Amari et al., 2012; Dimopoulou et al., 2014; Grosu-Tudor and Zamfir, 2014). With exception of dextran synthesized by *Leuc. mesenteroides*, EPS from LAB have not yet been commercially exploited as food additive due to their low yields (Monsan et al., 2001); nonetheless, the structural characteristics of EPS and the GRAS status of most of the LAB EPS producer strains allow considering the *in situ* production of texturing and/or biologically active EPS (Badel et al., 2011).

Fermented Milks and Beverages

The use of EPS-producing cultures in the elaboration of fermented milks and beverages has been applied to reduce the amount of added milk solids, to improve the viscosity, texture, stability, and mouthfeel of the final products as well as to avoid syneresis (whey separation) during fermentation or upon storage (De Vuyst et al., 2001). Rheological problems like low viscosity, gel fracture or high syneresis are frequently encountered during fermented milk manufacture and can be solved by using EPS-producing LAB strains.

The contribution of the HePS to structure/function relationships is very complex. It has been postulated for example, that for high intrinsic viscosities stiffer chains, as the case of β-(1,4) linkages, are required, which in turn lead to higher consistency of the EPS solutions. In addition, the degree of branching may contribute to the stiffness of the polymer and while the complexity of the primary structure (namely size, monomer composition, and side groups, α- and β-linkages, branching) influence the viscosity of EPS solutions (Tuinier et al., 2001). As no clear-cut relationships of EPS yields and functional properties exist, a rational selection of the EPS-producing LAB strains for the production of yogurt and other fermented milks should be considered to increase the viscosity and texture of the final product through *in situ* EPS production. This was the case for the yogurt *S. thermophilus* EPS-producing strains (Vaningelgem et al., 2004).

Certain fermented beverages owe their organoleptic and sensorial characteristics to the presence of LAB and the *in situ* EPS production during the elaboration of the fermented drinks as *kefir* and *Pulque* (**Table 2**).

Kefir is a viscous, slightly carbonated and alcoholic dairy beverage, traditionally consumed in Eastern European countries. Kefir is produced by bacteria and yeasts contained in kefir grains that have unique taste and properties. During fermentation, peptides, and EPS showing bioactive characteristics are formed. Kefiran is a water-soluble EPS produced in kefir grains, which consist of a complex population of LAB and yeasts firmly embedded (Cheirsilp et al., 2003). The principal kefiran producers in kefir grains is *L. kefiranofaciens* and several other unidentified species of lactobacilli. Kefiran is a branched glucogalactan composed of hexa- or heptasaccharide repeating structure with almost equal amounts of glucose and galactose

TABLE 2 | Fermented beverages and foods containing EPS-producing LAB and effect of their polymers on their rheological and technological properties.

Fermented foods and beverages	EPS-producing microorganisms	Sensorial attributes	Reference
Fermented beverages			
Yogurt	*S. thermophilus; L. delbrueckii* subsp. *bulgaricus*	Increase in viscosity and texture improvement of fermented milks and beverages	De Vuyst et al., 2003; Vaningelgem et al., 2004
Kefir	*L. kefiranofaciens*		Maeda et al., 2004; Wang et al., 2008
Mexican *Pulque*	*Leuc. mesenteroides*		Escalante et al., 2008
Cheeses			
Low-fat Italian *Cacciotta* type	*S. thermophilus*	Pleasant to taste and chew, flavor, overall acceptability	Di Cagno et al., 2014
Egyptian *Karish*		Enhanced acceptability, spreadability, and creaminess	Hassan et al., 2004
Reduced-fat	*S. thermophilus* TM11	Increased moisture content and high yield	Wang et al., 2012
Mexican *Chihuahua*	EPS-producing starter culture	Improved cheese yield and texture	Trancoso-Reyes et al., 2014
Reduced-fat Cheddar		Improved body and texture	Agrawal and Hassan, 2008
Fermented Breads			
Gluten-free breads	*W. cibaria; W. confusa; Leuc. mesenteroides; L. sanfranciscensis*	Enhanced texture and quality	Schwab et al., 2008; Galle et al., 2012a; Rühmkorf et al., 2012; Wolter et al., 2014a,b
Gluten-free sorghum	*L. casei FUA3185* and *FUA3186, L. buchneri FUA3154*	Improved rheology of sorghum sourdoughs	Galle et al., 2011
Wheat	*Leuc. lactis*, *L. curvatus*	Improved viscoelasticity and quality	Palomba et al. (2012)

(Maeda et al., 2004; Medrano et al., 2009). The MW of kefiran obtained from kefir grain has been reported as higher than 10^7 Da by Piermaria et al. (2008) while Maeda et al. (2004) found a MW value of 7.6×10^5 when culturing the single strain *L. kefiranofaciens* WT-2B(T) isolated from kefir grain in liquid medium containing a rice hydrolysate.

Pulque is a traditional Mexican, non-distilled alcoholic fermented beverage consumed mainly in Central Mexico. It is obtained from the fermentation of a fresh sap known as *aguamiel*, which is extracted from different Agave species. Freshly collected *aguamiel* is deposited in open containers where previously fermented *pulque* acts as seed for a new batch. Fermentation time varies from a few hours to several days. The viscosity resultant from EPS synthesis and the alcoholic content of the beverage are features used to determine the extent of fermentation and the sensorial properties of *pulque* (Escalante et al., 2008). Among LAB, *Leuc. mesenteroides* has been traditionally considered one of the most important microorganisms during *pulque* fermentation due to its ability to synthesize dextrans from sucrose present in *aguamiel* and *pulque*. The structure of an EPS produced by the strain *Leuc. mesenteroides* IBT-PQ isolated from *pulque* revealed the presence of a soluble linear dextran with glucose molecules linked by α-(1,6) bonds with branching from α-(1,3) bonds in a 4:1 ratio, respectively. A great LAB diversity in *aguamiel* and *pulque* samples from different geographical origins, composed mainly of *Lactobacillus* and *Leuconostoc* species, has been reported (Escalante et al., 2004). *Leuc. citreum* and *L. kimchii* were reported to be the most abundant LAB species present in *aguamiel* during the early stages of *pulque* fermentation (Escalante et al., 2008).

Cheeses

It has been reported that EPS-producing LAB improve the sensory attributes of various cheeses, especially those reduced- and low-fat varieties where the decrease in the fat content negatively affects the textural and meltability properties of cheese (**Table 2**). In this sense, the application of EPS-producing strains for improving the texture and technical properties of reduced-fat cheese has been very promising. EPS produced by non-ropy strains have drawn the attention of the dairy industry since their ability to produce CPS and EPS could improve the texture of dairy products without causing the undesirable slippery mouthfeel produced by the ropy strains (Hassan, 2008).

Recently, Di Cagno et al. (2014) used an EPS-producing *S. thermophilus* strain to produce low-fat Italian *Caciotta*-type cheese. The sensory attributes of cheeses containing the EPS-producing strain were pleasant to taste and to chew, showed intensity of flavor, and overall acceptability. Based on these observations, 14-day ripened low-fat *Caciotta*-type cheese had promising features to be further exploited as a suitable alternative to the full-fat variant.

Costa et al. (2010) found that reduced-fat *Cheddar* cheese made with an EPS-producing *Lactococcus lactis* strain displayed an 8.2% increase in yield (per 100 kg of milk), 9.5% increase in moisture content, and increase in water activity and water desorption rate. Interestingly, the presence of EPS did not negatively affect the flavor profile of the cheese.

Agrawal and Hassan (2008) studied the technological characteristics of reduced-fat *Cheddar* cheese made with ultrafiltrated (UF) milk and an EPS-producing culture. The authors observed that UF did not affect the hardness, cohesiveness, adhesiveness, chewiness, and gumminess of the EPS-containing cheese while the springiness of the EPS-containing cheese made from UF milk was much lower than that of the non-EPS cheeses. Texture of the EPS-negative cheese was more affected by UF than the EPS-positive cheese. While UF increased the elastic modulus in the 6-month old EPS-positive cheeses, higher body, and texture scores were given to EPS-positive cheeses than to EPS-negative ones.

Hassan et al. (2004) reported that the inclusion of EPS-producing cultures in the traditional Egyptian cheese *Karish* enhanced consumer acceptability by improving their spreadability and creaminess. This product is conventionally produced by adding fat, sugar, protein or stabilizers like pectin, starch, alginate or gelatin; thus, the addition of EPS cultures could be an interesting and viable alternative to the use of exogenous polysaccharides. Moreover, EPS from LAB may prolong the retention time of the milk product in the mouth, enhancing its delicacy.

More recently, Trancoso-Reyes et al. (2014) observed that the addition of EPS alone improved the Mexican *Chihuahua* cheese yield by increasing water and fat retention, causing, however, a negative effect on the texture and flavor of the cheese. When authors used the EPS-producing bacteria in combination with a phospholipase-A1 (PL-A1)-producing strain, an improvement on cheese yield, moisture, and fat content was observed. The cheeses showing the best flavor and texture were those manufactured with PL-A1 and with the combination of PL-A1 and the EPS-producing culture.

In addition, the EPS-producing strain *S. thermophilus* TM11 was evaluated for the production of reduced-fat cheese using reconstituted milk powder (Wang et al., 2012). The physicochemical analysis of fresh and stored cheeses showed that this strain slightly increased moisture content resulting in cheese with higher yield and lower protein content compared to the direct acidified cheese.

In summary, EPS provide functions that benefit reduced-fat cheeses since they bind water and increase the moisture in the non-fat portion, interfere with protein–protein interactions, reduce the rigidity of the protein network, and increase viscosity of the serum phase (Hassan, 2008).

Fermented Breads

Grinding of cereals and addition of water results in the formation of a dough that after some time will turn into a sourdough with characteristic acidic taste, aroma and increased volume due to gas formation. Cereal fermentation goes back to ancient times as one of the early microbial processes employed by man leading to the use of sourdough for bread-making (Hammes and Gänzle, 1997). The sourdough process was rediscovered because of the effects on the sensory, structural, nutritional and shelf life properties of leavened baked goods (Arendt et al., 2007; Gobbetti et al., 2014). In sourdough fermentation LAB and yeast communities are involved; while LAB dominate the microbial community

and are responsible for acid production, yeasts are responsible for dough leavening. LAB are involved in both decreasing the α-amylase activity and improving dough texture. In addittion, sourdough LAB may synthesize a large variety of EPS through GS activity. Since these bacteria are used as starters in cereal fermentations, these polymers are available for food applications through the *in situ* biosynthesis during processing (Tieking and Gänzle, 2005; Bounaix et al., 2009).

Lactic acid bacteria able to produce HoPS are already applied in the elaboration of conventional bread (Lacaze et al., 2007); however, their use is more promising in gluten-free baking since EPS can potentially act as hydrocolloids improving their rheological properties (Schwab et al., 2008; Galle et al., 2012a; Rühmkorf et al., 2012).

It has been reported that microbial *in situ* production of EPS during sourdough fermentation is more effective than the addition of comparable amounts of EPS to the bread formulation. The ability to produce HoPS by LAB strains during sourdough fermentation depends on the metabolic activity of the fermentation microbiota (Gänzle et al., 2007), and contributes to the sourdough ability to influence bread quality (Katina et al., 2009; Galle et al., 2012a; Palomba et al., 2012). More recently, Gobbetti et al. (2014) have observed that the *in situ* EPS formation was responsible for the significant decrease of dough strength and elasticity; however, dextran produced showed the best shelf life improvement.

Dextran production from sucrose is a phenotypic identification characteristic of the *W. confusa* and *W. cibaria* species. *Weisella* strains have been used for *in situ* dextran and GOS synthesis to improve the texture and quality of wheat and gluten-free breads (Schwab et al., 2008; Katina et al., 2009; Galle et al., 2010, 2012a; Wolter et al., 2014a,b). In this sense, high MW dextrans are used in sourdough baking to produce good quality bread (Maina et al., 2011). Dextrans have displayed prebiotic potential (Olano-Martin et al., 2000) and have been already approved by the European Commission to be used as additive in bakery products.

Exopolysaccharides produced by certain lactobacilli, such as the β-(2,6) levan synthesized by the sourdough strain *L. sanfranciscensis,* positively influence bread textural properties by facilitating water absorption, softening the gluten content of dough, improving the structure build-up, retarding bread staling and prolonging shelf life (Tieking and Gänzle, 2005).

In the traditional Italian sweet bread panettone, dextran produced by a *Leuc. mesenteroides* strain was responsible for the long storage stability (Decock and Cappelle, 2005). In this product, dextran production was optimized by transferring the doughs seven times. In sweet wheat milk bread, a remarkable reduction in bread firmness along the storage period was obtained (Lacaze et al., 2007).

As mentioned above, EPS isolated from sourdough include mainly HoPS; however, Van der Meulen et al. (2007) reported the production of HePS from a sourdough isolate strain of *L. curvatus*, which synthesized an EPS composed of galactosamine, galactose, and glucose in a ratio of 2:3:1, respectively. In addition, Galle et al. (2011) have investigated the influence of HePS-producing LAB in sourdough fermentation and found that the use of this type of EPS positively affected the rheological properties of sorghum sourdough, expanding the diversity of EPS and the variety of cultures used for baking.

Concluding Remarks

Exopolysaccharides-producing LAB hold great potential for the functional food sector either as starter and adjunct cultures or as *in situ* supplier of bioactive polymers with positive impact on the rheology of fermented products or on the human health. The increased knowledge concerning the enzymes involved in HoPS synthesis and their regulation open new possibilities for their use to improve the texture of sucrose-supplemented products. Most of the research done on EPS during the last decade was focused on the evaluation of HoPS structures as well as the novel functionalities of HePS. In this concern, the requirement of specific methodologies (FTIR, NMR, MALLS, FFF, AF4, etc.) for the EPS structural analysis as well as the *ex vivo* assays (cell tissues) to determine their biological activity was essential. Nevertheless, the structure/function relationships of EPS should be *in situ* proved in foods or other matrices of interest. The improvement of EPS production to reduce their industrial costs and the design of tailor-made EPS with desired/specific functionalities remain challenging.

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Exopolysaccharides enriched in rare sugars: bacterial sources, production, and applications

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Microbial extracellular polysaccharides (EPS), produced by a wide range of bacteria, are high molecular weight biopolymers, presenting an extreme diversity in terms of chemical structure and composition. They may be used in many applications, depending on their chemical and physical properties. A rather unexplored aspect is the presence of rare sugars in the composition of some EPS. Rare sugars, such as rhamnose or fucose, may provide EPS with additional biological properties compared to those composed of more common sugar monomers. This review gives a brief overview of these specific EPS and their producing bacteria. Cultivation conditions are summarized, demonstrating their impact on the EPS composition, together with downstream processing. Finally, their use in different areas, including cosmetics, food products, pharmaceuticals, and biomedical applications, are discussed.

Keywords: bacterial extracellular polysaccharides, rare-sugars, fucose, rhamnose, glucuronic acid

Introduction

Bacterial extracellular polysaccharides (EPS) presenting a wide range of physicochemical properties have emerged as promising polymers for many commercial applications in different industrial sectors like food, pharmaceuticals, cosmetics, oil drilling, and paper manufacturing (Kumar and Mody, 2009; Freitas et al., 2011a). Despite this potential, bacterial EPS currently represent a very small fraction of the global polymer market, mostly because of their production costs. However, in some cases, they might address very specific niche markets, where the production cost does not become a limiting step for their commercialization (Freitas et al., 2011a).

Rare sugars are monosaccharides that are not commonly found in nature, where D-glucose, D-galactose, D-fructose, D-xylose, D-ribose, and L-arabinose are more abundant. Rare sugars such as L-fucose, L-rhamnose, or uronic acids present many interesting properties, making them attractive for various fields of applications, such as anti-inflammatory substances, antioxidant, or as building blocks to synthesize the nucleoside analogs which are used as antiviral agents, and justifying the effort to produce them synthetically (Kumar et al., 2007). Today, some of them might be produced biochemically by the use of specialized enzymes belonging usually to the classes of keto–aldol isomerases, epimerases, and oxidoreductases using glucose as main precursor (Granström et al., 2004; Beerens et al., 2012). Their scarceness makes them highly valuable and, consequently, bacterial EPS containing rare sugars represent an interesting source for their isolation and production. Although polysaccharides containing rare sugars are also found in plants, seaweeds, and animals, microbial production of such polymers is advantageous for several reasons, namely, production is

TABLE 1 | Production and composition details of some bacterial polysaccharides rich in rare sugars.

EPS	Producing strain	Composition	Substrates	Production (g/L)	Productivity (g/L·day)	Reference
Gellan gum	*Sphingomonas paucimobilis*	Rhamnose, glucose, and glucuronic acid	Sucrose	19.9–22.6	6.6–9.0	Zhang et al. (2015)
			Glucose	12.4	7.4	Giavasis et al. (2003)
			Whey	1.6–3.1	0.6–1.2	Dlamini and Peiris (1997)
		Acetyl and glyceryl groups	Soluble starch	37.5–43.6	14.5–18.8	Bajaj et al. (2006)
			Molasses	13.8	n.a.	Banik et al. (2007)
Welan	*Alcaligenes* sp.	Rhamnose, glucose, and glucuronic acid	Glucose	25.0–26.3	8.3–8.8	Li et al. (2011)
			Corn starch	22.8	7.6	Li et al. (2010)
Clavan	*Clavibacter michiganensis*	Fucose, glucose, and galactose Acetyl, pyruvyl, and succinyl groups	Glucose	0.7	0.06	van den Bulk et al. (1991)
FucoPol	*Enterobacter* A47	Fucose, glucose, galactose, and glucuronic acid	Glycerol	7.5–8.0	2.0–2.5	Torres et al. (2012)
			Glucose	13.4	3.4	Freitas et al. (2014)
			Xylose	5.4	1.4	Freitas et al. (2014)
		Acetyl, pyruvyl, and succinyl groups				
Hyaluronic acid	*Streptococcus* sp.	Glucuronic acid and acetylglucosamine	Glucose	0.4–6.9	0.8–1.5	Huang et al. (2008)

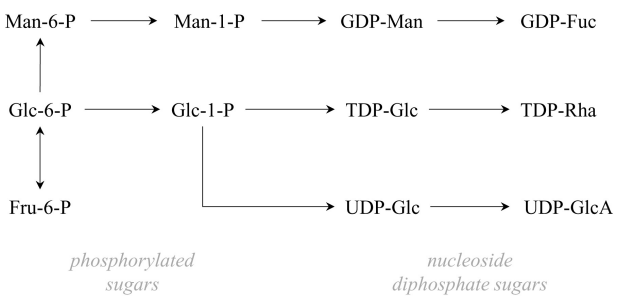

FIGURE 1 | Schematic diagram of the biosynthetic pathways in Gram-negative bacteria involved in the intracellular synthesis of nucleoside diphosphate sugars that are precursors of some rare sugar monomers (Fuc, fucose; Glc, glucose; GlcA, glucuronic acid; Man, mannose; Rha, rhamnose; Fru, fructose; GDP, guanosine diphosphate; TDP, tyrosine diphosphate; UDP, uridine diphosphate).

not affected by environmental factors, the bioprocesses are easily controlled and manipulated, and they guaranty fast and reproducible production.

Some EPS containing rare sugars have been extensively studied (**Table 1**), not only because of their composition, but also because of their physical and bioactive properties. These include polymers containing fucose, such as colanic acid (Ratto et al., 2006), fucogel (Paul et al., 1996), clavan (Vanhooren and Vandamme, 2000), or FucoPol (Freitas et al., 2011b), or rhamnose, such as rhamsan (Xu et al., 2014), gellan (Zhang et al., 2015), or welan gum (Wang et al., 2014; **Figure 1**).

Extensive work has been performed to discover new strains able to produce such rare sugar-rich EPS and to improve their production and purification processes. Many of those polymers are being studied for new applications in pharmaceuticals cosmetics, food products, among others. Rare sugar-rich EPS can also be used as sources of rare sugar monosaccharides. Such EPS can be subjected to hydrolysis (either enzymatic or chemical) and the resulting monomers can be separated by chromatographic methods to yield pure monosaccharides. The rare sugars thus obtained can then be used as precursors for the synthesis of high-value molecules for use in high-value applications. The exploitation of EPS as sources of rare sugars still remains a rather unexplored and unexploited aspect. The economic viability of such strategy depends on the EPS content on a given rare sugar but the high demand for these molecules will be a strong driving force.

EPS Containing Rare Sugars and Their Producers

Even though not all rare sugars can be found in microbial polysaccharides, some EPS, secreted by bacteria in specific limited conditions, contain rare sugars, offering an opportunity to produce them in sustainable and controlled conditions. These include polymers containing fucose, such as colanic acid, fucogel, and clavan, or rhamnose, such as rhamsan, gellan, diutan, or welan gum, very often in combination with uronic acids such as glucuronic or galacturonic acids (Kumar and Mody, 2009).

Similarly to most bacterial EPS, rare sugar enriched EPS synthesis occurs intracellularly (Freitas et al., 2011a,b). Energy-rich monosaccharides, mainly nucleoside diphosphate sugars (NDP-sugars), are synthesized from phosphorylated sugars formed from primary metabolites (**Figure 1**). Polymerization commonly occurs at the cytoplasmic face of the inner membrane or at the periplasm, and the polysaccharides are exported to the extracellular.

The presence of rare sugars on bacterial EPS is not well understood yet, but they may provide the cells with an extra biological protection in addition to the physical barrier provided by the EPS.

Rhamnose Containing EPS

Most rhamnose containing EPS belong to the family of sphingans are secreted by strains of the *Sphingomonas* genus (Fialho et al., 2008). Sphingans, as rhamsan, gellan, diutan, or welan gum present a common linear tetrasaccharide repeating unit Glc-GlcA-Glc-Rha or Man (Pollock, 1993). The presence of rhamnose or mannose at the end the repeating unit is responsible for the main structural variations between sphingans. For instance, diutan has exactly the same backbone as gellan (Glu-GlcA-Glu-Rha) but is substituted with Rha-Rha side chain. *Klebsiella* genus also presents EPS producers. For instance, *Klebsiella* I-714 produces an EPS with a high rhamnose content, composed of hexasaccharide units of L-rhamnose, D-galactose, and D-glucuronic acid in a molar ratio of 3:2:1 (Serrat et al., 1995).

Fucose Containing EPS

Clavan, composed of tetrasaccharide repeating units of D-glucose, D-galactose, L-fucose, and pyruvic acid in the molar ratio of 1:1:2:1, is one of the richest polymers in fucose. It is produced by *Clavibacter* strains, in particular *C. michiganensis* (Vanhooren and Vandamme, 2000). In comparison, Fucogel, commercialized by Solabia (France) is a linear anionic polymer produced by *K. pneumoniae*, constituted of trisaccharide repeating units of galacturonic acid, L-fucose, and D-galactose (Vanhooren and Vandamme, 2000). A marine bacterium *Enterobacter cloacae* was also reported to produce a polymer composed fucose, galactose, glucose, and glucuronic acid in a molar ratio of 2:1:1:1 (Iyer et al., 2005). The strain *Enterobacter* A47 synthesizes FucoPol, a fucose-containing polysaccharide with nearly equimolar amounts of fucose, glucose, and galactose, and minor content of glucuronic acid (10–15 mol%; Freitas et al., 2011b).

Other Bacterial EPS

Even rarer sugars that rhamnose and fucose might be encountered in bacterial EPS. For instance, strains of the rumen bacterium *Butyrivibrio fibrisolvens* produce significant amounts of EPS containing L-altrose and L-iduronic acid (Ferreira et al., 1997). Due to its scarcity, reports on altrose production and applications remain still limited today. *Pseudomonas viscogena* produces a polysaccharide containing another scarce sugar, allose, the C2 epimer of altrose, but only in small proportion (around 2.5% of sugar content; Takemoto and Igarashi, 1984). A much more studied rare sugar containing EPS is hyaluronic acid (HA), because of its proven properties (Collins and Birkinshaw, 2013). HA is a linear polymer composed of *N*-acetylglucosamine and glucuronic acid, as disaccharidic repeating units produced by *Streptococcus zooepidemicus* (Liu et al., 2011)

Bioproduction

Cultivation Conditions

Bacterial EPS are industrially produced in single strain systems (Kumar and Mody, 2009). As for other bacterial EPS production bioprocesses, the production of EPS rich in rare sugars rely on the use of carbohydrates as carbon sources because they allow for high productivities and yields (**Table 1**; Kumar et al., 2007). Sucrose and glucose are the most commonly used substrates, while xylose, galactose and lactose are less frequently used because many bacteria are unable to use them or they result in reduced polymer productivities (Singh et al., 2008; Zhang and Chen, 2010). Since the substrate cost accounts for up to 40% of the total production costs of microbial polymers (Kumar and Mody, 2009), there is an intensive search for alternative raw materials to reduce the overall production costs. For that reason, several inexpensive agricultural and industrial wastes and by-products have been proposed as substrates for microbial cultivation, including lignocellulosic materials, cheese whey, molasses, and glycerol-rich product (**Table 1**). On the other hand, the use of some agricultural and industrial wastes/by-products is held up by the difficulty in guarantying their supply in terms of both quantity and quality, and they may require costly pretreatments prior to use. Moreover, non-reacted components may accumulate in the broth and eventually be carried-over to the final product. Hence, for applications wherein high-purity and high quality products are needed, the use of wastes or by-products may not be an option or, otherwise, higher investment must be put in downstream procedures (Freitas et al., 2011a).

Media composition and cultivation conditions highly influence the amount of polysaccharide synthesized, as well as the final product's composition, molecular structure, average molecular weight, and, consequently, their functional properties (Nicolaus et al., 2010; Poli et al., 2011). Hence, it is possible to manipulate polysaccharides' characteristics by altering the growth conditions of the producing strains, thus obtaining biopolymers with tailored properties to fit a given application. Enrichment of the polysaccharides in rare sugar monomers is of particular interest since it may enhance their bioactive properties, thus increasing their market value. However, the impact of media and cultivation conditions on EPS composition is strain-dependent and only a few bacteria can be manipulated to alter the composition of the EPS they synthesize. In fact, for most bacteria, EPS sugar composition is a genetically determined trait, which is not significantly altered by the cultivation conditions (López et al., 2003; Celik et al., 2008). An exception to this is, for example, the bacterium *Enterobacter* A47 that was shown to be able to synthesize EPS with different fucose contents by cultivation as a function of the carbon source used and the operating conditions (Torres et al., 2012; Freitas et al., 2014). High fucose content EPS (36–38 mol%) were synthesized by *Enterobacter* A47 using glycerol or xylose as sole carbon sources, while glucose, methanol or citrate led to lower contents (22–29 mol%; Freitas et al., 2014). On the other hand, the composition of the EPS produced by *Enterobacter* A47 could also be manipulated by cultivation at different pH and/or temperature ranges: maximum fucose content (>30 mol%) was obtained for cultivation at 25–35°C and pH = 6.0–8.0 (Torres et al., 2012).

Downstream Processing

The recovery of bacterial EPS from the culture broth commonly involves cell removal, usually by centrifugation or filtration, polymer precipitation from the cell-free supernatant by the addition of a precipitating agent consisting of a water-miscible solvent in which the polymer is insoluble (e.g., methanol, ethanol, isopropanol, acetone) and drying of the precipitated polymer (e.g., by freeze drying, drum drying; Freitas et al., 2011a, 2013). For

some applications that require high purity grade products, the polymer is subjected to one or several additional methods, such as re-precipitation of the polymer from diluted aqueous solution, deproteinization by chemical or enzymatic methods, and membrane processes, such as dialysis, ultrafiltration and diafiltration (Kumar et al., 2007; Wang et al., 2007; Ayala-Hernández et al., 2008; Bahl et al., 2010; Freitas et al., 2011a). The most appropriate downstream procedure is selected to guaranty the required products' purity. Additionally, the impact of the purification procedures on the polysaccharides' properties must also be taken in consideration.

Industrial and Biomedical Applications

Most polysaccharides' applications are related to their behavior in aqueous media. Their physical and chemical characteristics, such as water binding capacity, high average molecular weight, polyelectrolyte behavior (in some cases), molecular structure, and the possibility of being chemically modified, enables this type of molecules to present diverse functional properties (e.g., thickening, film forming, gelling, emulsion stabilizing, flocculating, and nano/microstructures production abilities; Dumitriu, 2004). Furthermore, in the case fucose and rhamnose-rich bacterial polysaccharides, they also present interesting biological activities that render these molecules potential to be used in a wide range of applications, particularly in added value products like cosmetics, pharmaceuticals, medical devices, and functional food products (Péterszegi et al., 2003a; Ravelojaona et al., 2009). As examples, rhamnose is commonly used as precursor for the production of aroma and flavors and, together with fucose, has attracted more attraction as fucose and rhamnose-rich oligo- and polysaccharides (FROP and RROP, respectively) have been found to counteract several of the mechanisms involved in skin aging (Robert et al., 2009).

Gellan gum is a linear anionic EPS, and its native form contains two acyl substituents, L-glyceryl and acetyl, being known as high acyl gellan (HA-gellan). The substituents may be removed by alkaline hydrolysis to give deacetylated gellan, also called low acyl gellan (LA-gellan). HA-gellan usually produces elastic, soft, non-brittle, and opaque gels while LA-gellan enables the formation of non-elastic, hard, brittle, and transparent gels (Sworm, 2009). Therefore, a wide range of structures, with varied rheological properties and appealing textures may be designed by controlling the acyl content. Gellan produced by cultivation of a pure culture of *Sphingomonas elodea* using a sugar as carbon source, and commercialized by CPKelco (with the trade name KELCOGEL®), has been used in the food industry as additive that functions, not only as gelling, but also as texturizing, stabilizing, suspending, film-forming, and structuring agent. Types of food products that contain gellan gum include bakery fillings, dairy products, low-fat spreads, dessert gels, jams and jellies, sauces, and structured foods (Freitas et al., 2013; Prajapati et al., 2013). It has also been used to develop edible coatings for the improvement of fruits shelf life (Rojas-Graü et al., 2007; Tapia et al., 2008).

The applications of gellan gum in pharmaceutical technology and medicine were recently reviewed by Osmałek et al. (2014). In pharmaceuticals, it has been studied as carrier material in drug delivery, in the form of tablets, capsules, beads, and hydrogels. Formulations based on gellan gum for oral, ophthalmic, and nasal applications have been developed. In medicine field, the use of gellan has been investigated for tissue engineering (e.g., for cartilage reconstruction and guided bone regeneration) and wound healing (e.g., in wound dressings to inhibit postsurgical adhesion and scar formation). Gellan sulfate derivatives are promising materials for rheumatoid arthritis treatment, as they have a tendency for selective binding of fibronectin molecules (Miyamoto et al., 2001), and for the development of cell-hybrid materials for artificial veins design due to the anticoagulant activity of such derivatives (Miyamoto et al., 2002).

Regarding welan gum, the main applications studied so far are in the field of cement production. The use of low viscosity welan gum in cement compositions reduces fluid loss of those compositions, increases the suspension properties of cement suspensions, being effective at low concentrations (Kaur et al., 2014). The rheological properties of welan gum in aqueous media were compared to that of xanthan gum, well known for its good thickening capacity. For the same biopolymer concentration, welan solutions presented higher apparent viscosity and higher viscoelastic moduli (loss and storage moduli). In addition, the welan gum solution is able to maintain high viscosity at high temperature while the molecular aggregation of xanthan gum is more sensitive to temperature variations (Xu et al., 2013). As such, it is envisaged the application of welan gum in the same areas as xanthan gum, such as in food products (e.g., jellies, beverages, dairy products, and salad dressings); as well as in oil drilling fluids. As welan gum is a rhamnose containing polysaccharide, attention should be driven to the study of its biological activity and applications in the cosmetic, pharmaceutical, and medicine fields.

Native rhamsan gum is non-gel forming polysaccharide, but originates thermostable highly viscous solutions even at temperatures above 100°C. It possesses good stability under shearing, great compatibility with high salt concentrations and excellent suspension properties, even superior to that of xanthan gum. Furthermore, gelation was observed in deacetylated rhamsan gum solution at concentrations of 0.3%wt at low temperature (Tako et al., 2003). This polymer finds applications in the same fields of the other polysaccharides of the gellan family, namely in the oil field, concrete, food products, cosmetics, pharmaceuticals, and medicine (e.g., plastic surgery), referred by Xu et al. (2014).

Fucose is another rare sugar that is today used in the composition of anticarcinogenic and anti-inflammatory drugs, in the preparation of creams for the acceleration of wound healing and as hydrating and anti-aging additives (Péterszegi et al., 2003a,b; Cescutti et al., 2005). Fucogel, a fucose containing EPS, has been used extensively in skin care cosmetic formulations (Bauer et al., 2012; Tecco and Sanders, 2012). This fact is mainly due to its moisturizing properties and to the scientific evidence of the action of fucogel and fucogel oligosaccharides as skin anti-aging agents, namely for stimulation of fibroblast proliferation and survival (Péterszegi et al., 2003a). Another recently reported fucose-rich EPS, FucoPol, has demonstrated interesting functional characteristics, such as thickening, emulsion stabilizing, film forming, and flocculating capacities (Cruz et al., 2011; Freitas et al., 2011b; Ferreira et al., 2014). These properties envisage its application in

several areas, namely in emulsion-based cosmetics, drug delivery systems, biodegradable films for packaging, oil drilling fluids, and as biodegradable bioflocculant (e.g., in wastewater treatment).

Hyaluronic acid is abundantly found in mammalian skin where it constitutes a high fraction of the extracellular matrix of the dermis. Its physical and biochemical properties, either in solution or as hydrogel, are attractive for various technologies related to body repair. As such, it is a biomaterial of increasing importance finding applications in cosmetics, pharmaceuticals, and medicine (Collins and Birkinshaw, 2013). Either in a stabilized form or in combination with other polymers, it is used in cosmetic surgery as a component of commercial dermal fillers (e.g., Dermalive® and Hylaform®). It is reported that injection of such products into the dermis, can reduce facial lines and wrinkles in the long term (Brown and Jones, 2005). HA-based materials have been applied in scaffolds for wound healing, bone and cartilage tissue repair and regeneration, nerve and brain tissue repair, soft tissue repair, and smooth muscle engineering (Collins and Birkinshaw, 2013). In addition, this biopolymer has been extensively explored as drug carrier, including target specific and long-acting delivery of protein, peptide, and nucleotide therapeutics (Oh et al., 2010).

Conclusion

Only a minority of microbial EPS are today used as commodity products mostly because of their production and purification costs. However, the presence of rare sugars in specific EPS may confer them high added value, to be used in highly specialized applications such as biomedicine, pharmaceuticals, or cosmetics. The physical and chemical properties of the polymers determine their potential applications as thickeners, film producers, emulsion stabilizers, flocculants or materials for nano/microparticles, and scaffolds for tissue engineering among others. In addition to that, their biological activity, potentiated by the occurrence of rare sugars, may offer new market opportunities. EPS microbial production presents the advantage of being simple, robust, reproducible, and easily controllable by growth conditions, such as type and concentration of carbon source, pH, or temperature. However, despite the fact it is possible to produce a polymer with constant characteristics (e.g., composition, purity, and homogeneity), a better understanding of EPS synthesis, in particular EPS with high rare sugars content, is still needed to maximize production and obtain an economical viable process.

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Microbial production of scleroglucan and downstream processing

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Synthetic petroleum-based polymers and natural plant polymers have the disadvantage of restricted sources, in addition to the non-biodegradability of the former ones. In contrast, eco-sustainable microbial polysaccharides, of low-cost and standardized production, represent an alternative to address this situation. With a strong global market, they attracted worldwide attention because of their novel and unique physico-chemical properties as well as varied industrial applications, and many of them are promptly becoming economically competitive. Scleroglucan, a β-1,3-β-1,6-glucan secreted by *Sclerotium* fungi, exhibits high potential for commercialization and may show different branching frequency, side-chain length, and/or molecular weight depending on the producing strain or culture conditions. Water-solubility, viscosifying ability and wide stability over temperature, pH and salinity make scleroglucan useful for different biotechnological (enhanced oil recovery, food additives, drug delivery, cosmetic and pharmaceutical products, biocompatible materials, etc.), and biomedical (immunoceutical, antitumor, etc.) applications. It can be copiously produced at bioreactor scale under standardized conditions, where a high exopolysaccharide concentration normally governs the process optimization. Operative and nutritional conditions, as well as the incidence of scleroglucan downstream processing will be discussed in this chapter. The relevance of using standardized inocula from selected strains and experiences concerning the intricate scleroglucan scaling-up will be also herein outlined.

Keywords: scleroglucan, fermentation, bioreactor, optimization, non-conventional substrates, downstream processing

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THE DISCOVERY OF A CHALLENGING POLYSACCHARIDE

Around 180-million-tons of polymers are produced per year, which play a relevant role in our modern society. Petroleum-based polymers and polymers of plant origin have the disadvantage of their limited resources in addition to the well-known environmental impact of the former ones. In this context, microbial polysaccharides represent a valuable alternative, with the benefit of lower-cost, standardized and sustainable production, along with high quality. In addition, exopolysaccharides (EPSs) from microbial sources usually exhibit shortened production times (e.g., in a matter of days), the possibility of using industrial wastes, no competition with production lands, and their frequent ease of recovery. Production values in the microbial EPS field can be so varied as 0.0022–86.3 g/L (Donot et al., 2012).

Among microbial polysaccharides, the general term scleroglucan is commonly used to designate a class of EPSs with similar structure, which are mainly -but not exclusively- produced by filamentous fungi of the genus *Sclerotium* (Halleck, 1967). Upon complete hydrolysis, only D-glucose is released from this water-soluble homopolysaccharide. The Pioneering Research Division of the US Army Quartermaster Corps Research and Engineering Centre provided, in Reese and Mandels (1959), the first insights into the scleroglucan structure by enzymatic approaches. Thereafter, in Halleck (1967) patented the scleroglucan basic structure as elucidated by means of enzymatic hydrolysis. This patent also included information about some producing strains, the production processes and the methods of purification thereof (Halleck, 1967). Based on Halleck's work, Pillsbury Co. (Minneapolis, MN, USA) began scleroglucan commercialization under the name Polytran®. Since then, different companies entered into the scleroglucan market under different trademarks (Clearogel, Polytetran, Polytran FS, Sclerogum, and Actigum; Coviello et al., 2005).

SCLEROGLUCAN CHEMICAL STRUCTURE AND CONFORMATIONAL FEATURES

Scleroglucan is a high molecular weight (MW), non-ionic branched glucan. It consists in a backbone of (1,3)-β-linked D-glucopyranosyl residues bearing a single (1,6)-β-linked D-glucopyranosyl unit every three sugar residues of the main chain (Rinaudo and Vincendon, 1982; Fariña, 1997). The structure of this repetitive unit determines a degree of branching (DB) around 0.33 (**Figure 1**). Besides being a common feature among most biologically active β-(1,3)-glucans (Rinaudo and Vincendon, 1982; Bohn and BeMiller, 1995; Kim et al., 2000), this high branching frequency would also be responsible of the great water solubility of this polysaccharide. When dissolved in water at room temperature and low concentrations of alkali, usually below 0.15 M NaOH, it can be assumed that scleroglucan adopts a highly ordered, rigid, triple helical tertiary structure (**Figure 1**). Under this macromolecular conformation, protruding (1,6)-β-glycosidic side branching prevents the intermolecular approach by extensive H-bonding, which otherwise would lead to aggregated forms and precipitation (Fariña et al., 2001, 2009; Laroche and Michaud, 2007). Meanwhile, interstrand hydrogen bonding at the center of the triplex stabilizes the macromolecular structure (Atkins and Parker, 1968; Bluhm et al., 1982; Sletmoen et al., 2009). However, at higher NaOH concentrations, where drastic changes in viscosity are commonly observed, the triple-strand helices probably undergo the ionization of hydroxyl groups which thus disrupts hydrogen bonds and prompts the subsequent polysaccharide denaturation (Fariña et al., 2001; Viñarta et al., 2013a).

To deepen into the knowledge of this denaturation–renaturation process, Virgili Alemán (2011) monitored, by fluorescence resonance energy transfer (FRET) spectroscopy, the conformational changes of scleroglucan triplexes when exposed to different NaOH concentrations. This study revealed that triple helix denaturation would take place by a partial opening mechanism, with a degree of opening related to both the NaOH concentration and the EPS conformational features (degree of expansion) of the triplexes.

The length of polymer chain, and therefore the molecular mass of scleroglucan, may differ depending on the microbial strain, fermentation process (culture media, fermentation time, etc.) and the recovery method used, with an average MW ranging from 1.3–3.2 $\times$ 10^5 to 0.3–6.0 $\times$ 10^6 Da (Rodgers, 1973; Fariña et al., 2001). Within this range, *Sclerotium rolfsii* ATCC 201126 scleroglucan exhibits an average MW of about 5.2 $\times$ 10^6 Da for the triplex and 1.6–1.7 $\times$ 10^6 Da for the random coil, in association to high intrinsic viscosities ($[\eta] = 9510–9610$ mL/g, for the triplex in water; Fariña et al., 2001). With reference to the degree of polymerization (DP), the reported values are variable from 110 for *Sclerotium glucanicum* scleroglucan (Bielecki and Galas, 1991), 800 for a commercial scleroglucan (Bluhm et al., 1982), 500–1600 for related glucans (Sandford, 1979), 2400–2500 for *S. rolfsii* ATCC 201126 scleroglucan (Fariña et al., 2009) and up to 5600 for another cited scleroglucan (Rice et al., 2004).

A tendency to adopt a highly ordered, triple-helical conformation and semi-rigid structure in neutral aqueous solution, in association to high DP and MW ($\sim$5 $\times$ 10^6 Da) values, may account for the marked viscosifying ability, outstanding rheological behavior and the emerging scleroglucan successful applications (Yanaki et al., 1981; Brigand, 1993; Falch et al., 2000; Fariña et al., 2001; Viñarta et al., 2006, 2007; Giavasis, 2014).

A BRIEF PANE ON SCLEROGLUCAN PROPERTIES AND APPLICATIONS

Scleroglucan exhibits a range of distinctive physico-chemical properties that provide an advantage to itself over other polysaccharides, especially for the development of certain products and processes. Nevertheless, slight to great variations of these properties may be seen depending on the producing strain, the polymer production process and the downstream processing, facts that might modify the MW, DP, DB, conformational parameters, and/or the polymer purity grade, and so will determine its final potential applications. For instance, we reported that low concentrations (e.g., 2 g/L) of pure ($\sim$90–98% EPS) *S. rolfsii* ATCC 201126 scleroglucan in water are able to yield highly viscous solutions with non-Newtonian, non-thixotropic and pseudoplastic behavior (Fariña et al., 2001, 2009; Viñarta et al., 2007).

Solutions of scleroglucan are notably stable over temperature up to 100–120°C, and within a broad range of pH (1–13). Additionally, the EPS neutral nature allows keeping pseudoplasticity even in the presence of a variety of salts, such as NaCl, KCl, $CaCl_2$, $MgCl_2$, and $MnCl_2$ (Fariña et al., 2001; Viñarta et al., 2013a). In contrast, slightly refined solutions (2 g/L) of commercial scleroglucans and crude polymer isolates from fermentation broths produce lower viscosity solutions with a lesser ability to retain stable rheological features when exposed to alkali, high temperatures, or salts (Wang and McNeil,

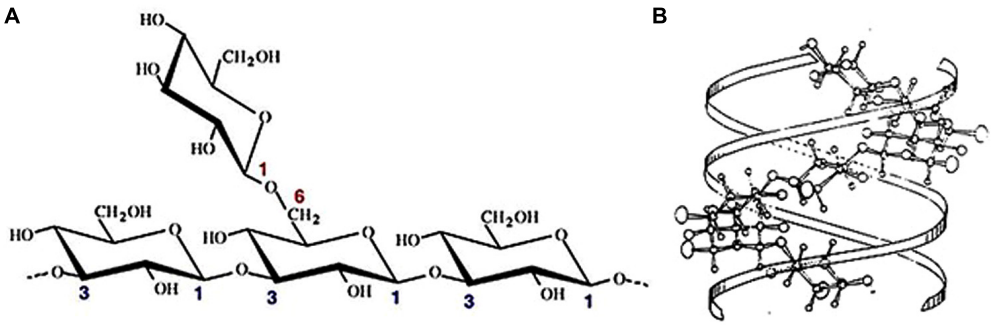

FIGURE 1 | (A) β-(1,3)-β-(1,6) glucan structure exhibiting the (3:1) side branching ratio of scleroglucan (Martin et al., 2007). **(B)** Tridimensional conformation of scleroglucan triplex. Projection perpendicular to the fiber axis (Crescenzi et al., 1988).

1996; Viñarta et al., 2007, 2013a). Differences depending on the EPS purity grade were also found between Biopolymer CS6 (60–70% scleroglucan) and Biopolymer CS11 (85–90%; Survase et al., 2007a). Based on these and other outstanding scleroglucan properties, a wide spectrum of biotechnological and industrial applications has been proposed and evaluated up to date (**Table 1**).

Scleroglucan as triplex exhibits the tendency to form thermo-reversible gels at low temperatures (close to 7°C), due to a weakly interacting triple-helix cross-linking mechanism (Bluhm et al., 1982; Biver et al., 1986). On the other hand, mimicking the behavior of other closely related β-(1-3)-D-glucans, scleroglucan triple helices can also be affected by denaturing conditions (e.g., pH $\geq$ 13), where destabilization of the interstrand H-bonding leads to the dissociation into single stranded random coils (Deslandes et al., 1980; Norisuye et al., 1980; Bluhm et al., 1982; Yanaki and Norisuye, 1983; Ensley et al., 1994). Denaturation of triplexes may occur in alkaline solutions ($\geq$0.25 M NaOH), in dimethylsulfoxide (DMSO; water weight fraction, WH $<$ 0.13), or by increasing the temperature above the triplex melting temperature ($T_m \cong 135°C$; Fariña et al., 2001; Sletmoen et al., 2009; Viñarta et al., 2013a). Typically, denatured solutions show much lower or nil viscosity as compared to the triplex-containing solutions. Nevertheless, under certain conditions, if denatured samples are taken to conditions that favor the restoration of the triple helical structure, circular structures might be observed by ultramicroscopy techniques among the "renatured triplexes" (Stokke et al., 1991, 1993; Sletmoen et al., 2009).

With regard to the scleroglucan biological properties, it was reported that its administration by diverse routes in rats and dogs did not induce toxicity, tissue pathology, or blood abnormalities. Neither eye nor skin irritation was detected in pigs, rabbits, and humans. Furthermore, scleroglucan role as an immune stimulant and a non-digestible dietary fiber for humans has been reported (Rodgers, 1973; Rapp, 1989; Giavasis, 2014). A wide range of physico-chemical, nutritional, and biological properties have been extensively described in the literature, and certainly are worth to mention. Relevant activities for health involve hypocholesterolemic, hypoglycemic, health-promoting effects, antioxidant and anti-obesity properties, many of them applicable for developing functional foods or nutraceuticals (Giavasis, 2014). A general overview of relevant polymer features and their actual or potential implications are depicted in **Table 1**.

REVIEWING THE KNOWLEDGE AND ADVANCES ON SCLEROGLUCAN PRODUCTION

To date, all scleroglucan production processes take place with a selected producing strain and under submerged aerobic conditions. This process is generally carried out in stirred-tank reactors using a sterile medium under aseptic management of the culture. Scleroglucan synthesis proceeds along with mycelial growth, so that the culture broth develops with time a gel-like consistency (Rodgers, 1973). A sharp drop in pH (∼2–2.5) is normally observed during the first 12–24 h of cultivation, mainly due to the accumulation of oxalic acid (Maxwell and Bateman, 1968; Fariña et al., 1998; Lee, 1998).

As aforementioned, changes in culture medium composition, process parameters or even the downstream processing may lead to dissimilar scleroglucan recovery and quality, with eventual variations in its chemical, physical, and/or biological properties. Therefore, in order to obtain high yields of a consistent polymer, it becomes essential to standardize a large-scale production process with a given strain under controlled conditions (Fariña et al., 1998; Survase et al., 2007a; Fazenda et al., 2008; Seviour et al., 2011a). A quite relevant step consists in selecting an appropriate producing strain, whose preservation procedure should be assessed and standardized, and its production ability must be monitored over time (Fariña et al., 1996; Survase et al., 2006; Schmid, 2008).

The nutritional requirements and culture conditions are commonly evaluated at minor scale (i.e., shake flasks) at the beginning of optimization, in order to maximize scleroglucan production and simultaneously reduce the accumulation of unwanted by-products, such as oxalic acid (Fariña et al., 1998; Schilling et al., 2000; Valdez, 2013). Following these essential studies, the scaling-up to bioreactor becomes a critical but difficult step, and this issue will be discussed later.

From our research on scleroglucan production during the last two decades, *S. rolfsii* ATCC 201126 (**Figure 2**) became

TABLE 1 | Summary of the main scleroglucan applications, according to its refined grade and its physico-chemical, biological, and biotechnological properties.

Scleroglucan source	Highlighted scleroglucan properties	Proposed applications	Reference
Actigum CS-11* (formerly known as Polytran® from Ceca S.A., France)	• Water solubility • Viscosifying activity • Salts tolerance	Scleroglucan aqueous fluid, used for petroleum recovery	Doster et al., 1984a,b
Sclerotium rolfsii ATCC 15206 Actigum CS-11* and Actigum CS6**, by Sanofi Bio Industries, France	• Thickening power • High carrying capacity and lubricating power	Scleroglucan gels for enhanced oil recovery (EOR)	Holzwarth, 1984; Donche et al., 1994; Pirri et al., 1996
Actigum CS-11* and CS-6**, by Sanofi Bio Industries, France	• Viscosifying ability • Rheological stability vs. temperature, salinity, and pH	Viscosity control of a bituminous binder for road repair and construction, soil stabilization, and sealing in civil engineering	Chaverot et al., 2001
Polytran®, by Pillsbury Company, USA	• Stimulation of murine macrophage activity • Increase in murine macrophage tumor cytotoxicity • Enhancement of murine bone marrow proliferation • Increase in survival of carcinoma-challenged mice • Increase in mice resistance against pathogenic bacteria and virus, w/o toxicity or hepatomegaly	Immune stimulating	Pretus et al., 1991
Not specified	• Apt matrix for controlled drug delivery • Drug protection at gastric level • Bioadhesive properties • Biodegradability	Edible films and tablets for nutraceuticals and pharmaceuticals Capsule granulates for controlled release of active substances	Lovrecich and Riccioni, 1991, 1993
Actigum CS11*, by Mero Rousselot - Satia, France	• Biocompatibility • Thermal and chemical stability		Grassi et al., 1996; Coviello et al., 1999
Not specified	• Antitumour, antiviral, and antibacterial activity (native or derivatized)	Drugs, vaccines, and immuno-potentiators (combinable with chemotherapy)	Jong and Donovick, 1989; Giavasis, 2014
Scleroglucan, by Sanofi Bio Industries, France	• Hypocholesterolemic, hypolipidemic, and hypoglycemic	Nutraceuticals	Mastromarino et al., 1997
Scleroglucan purified from fermentation broth, provided by Statoil/Norferm	• Stabilizer-texturizer at low pH and high temperature • Edible-film forming properties	Functional foods Low-calorie foods (since non-digestible)	Falch et al., 2000
Actigum CS6** and CS11*, by Sanofi Bio Industries, France			Vaussard et al., 1997
Actigum CS-11*, by Sanofi Bio Industries, France Amigel, by Alban Muller International	• Homogeneity and thickening enhancer • Softness developer	Component for washing keratinous materials (e.g., shampoo, shower gel, conditioner)	Dubief, 1996; Dubief and Cauwet, 2000
Actigum CS-11*, by Satia, France	• Remains elastic at saliva viscosity (1.5–3 mPa.s), at a physiological pH (5–9), and at physiological ionic strength (15–80 mM)	Saliva substitution agent Artificial tear water Mouth rinse Toothpaste	Van Nieuw Amerongen et al., 1999
Actigum CS-11* and CS-6**, by Sanofi Bio Industries, France	• Viscosifying agent of polyol base solvents • Long term stability at high temperatures and against metal ion contaminants	Rheological modifier for thermal insulation fluids	Skaggs and Swazey, 1999
S. rolfsii ATCC 15205	• Forms flexible, insoluble in water films when dried, but swell readily • Dispersing agent • Smoothing agent • Lubricating agent • Emulsifier or co-emulsifier agent • Improvement of fixing of dyes or UV-absorbers in shampoo/conditioners • Skin anti-inflammatory effect	Useful as active and/or excipient ingredient in cosmetic formulations (shampoo, conditioner, after-sun preparations, skin care compositions) and ophthalmological preparations	Maier et al., 2000, 2002

(Continued)

TABLE 1 | Continued

Scleroglucan source	Highlighted scleroglucan properties	Proposed applications	Reference
Not specified	• Compatible thickening agent	Component of a cosmetic mixture for the oxidation tinting of keratin fibers	Lang and Cotteret, 2003
BIOVIS, by SKW, Germany	• Suspending agent	Formulation of an aqueous storable cement used for cementing an area of a borehole	Fanguy et al., 2006
S. rolfsii ATCC 201126	• Retrogradation preserving agent in cooked starch pastes • Synergic rheological improvement of starch-based pastes (in water and milk)	Food stabilizer	Viñarta et al., 2006
	• Gel and film forming properties • Particulate suspending properties • Emulsifying activity	Drug delivery Paper, painting, ceramic, cosmetic, food and pharmaceutical industries Bioremediation, agriculture, and detergents	Viñarta et al., 2007
Not specified	• Gelling and viscosifying properties • Resistance to degradation, even at high temperatures and after 500 days in seawater	Industrial and oil field operations where acidizing procedures are applied	Welton et al., 2009a,b
Not specified	• High flocculant capacity • Fast settling rate	Addition to liquor of a Bayer process fluid stream to improve the recovery of alumina trihydrate	Chester et al., 2012a,b, 2013

**Biopolymer CS-11 is a refined product and has a content of 85–90% polysaccharide.*
***Biopolymer CS-6 contains 60–75% scleroglucan.*

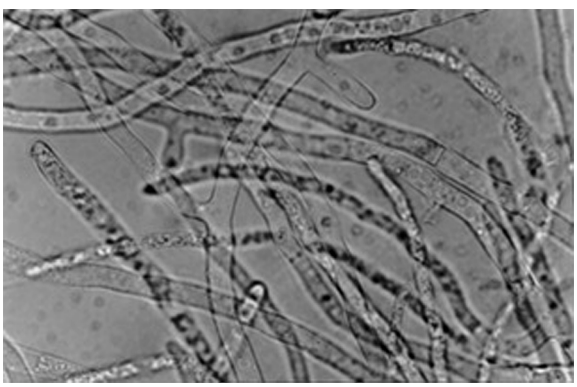

FIGURE 2 | Microscopic (40×) observation of *Sclerotium rolfsii* ATCC 201126 mycelium after growth for 3 days at 30°C in liquid PM_{20} (with 20 g/L sucrose, Fariña et al., 1998). Mycelium was disaggregated in 10% w/v KOH.

the selected strain to obtain vast amounts of EPS. This usually involved batch processes at different working scales and/or with varying fermentation strategies. This strain was isolated in the field as a phytopathogen from rotten red pepper. Based on our screening, it was later deposited and cataloged in the ATCC because of its marked ability to excrete biopolymer. There are several aspects that must be considered in order to achieve stable and maximum EPS productivity levels, many of them which are shared in common with other microbial polysaccharide production processes, and these will be herein revisited.

Strain Preservation

Although it may seem a minor issue, an adequate strain preservation technique is undoubtedly recognized as a crucial strategy in order to assure long-term viability as well as the maintenance of fungal properties (Smith, 1988). In the first reports, scleroglucan-producing strain conservation was performed by monthly transfers either on PDA or PDY slants (Christias and Lockwood, 1973; Griffith and Compere, 1978; Punja et al., 1982; Pilz et al., 1991). While this is a quite common methodology, an alternatively described technique consists in the preservation of mycelium in sterile distilled water (also known as Castellani's method; American Type Culture Collection, 1982). These above and other methods were tested with different *S. rolfsii* strains isolated from nature (Fariña et al., 1996). Early reports revealed that periodic sub-culturing on different culture media, followed by preservation at low temperature (4–7°C), may lead to a lack of viability and a critical decrease in scleroglucan synthesizing capacity. Conversely, preservation as 'sclerotia' (the resistance structures of the non-sporogenic *S. rolfsii*) in sterile distilled water at 4°C or even at room temperature (**Figure 3**), allowed the retention of the glucanogenic ability at similar and even higher levels than those observed for the abovementioned methods, and even after years of preservation (Fariña et al., 1996).

Inoculum Standardization

The success of scleroglucan production at bioreactor scale also closely depends on the inoculum proportion and its quality, which must be standardized (van Wollingen and Seviour, 1986). It is known that the preparation of a standard inoculum is usually problematic since the genus *Sclerotium* does not produce spores, and because of the non-homogeneous nature of mycelial suspensions. However, *Sclerotium* genus has the ability of forming resistance structures called sclerotia, which can be thereafter used for strain activation and inocula preparation (Fariña et al., 1996; Survase et al., 2006). It has been demonstrated that activation of water-stored sclerotia followed

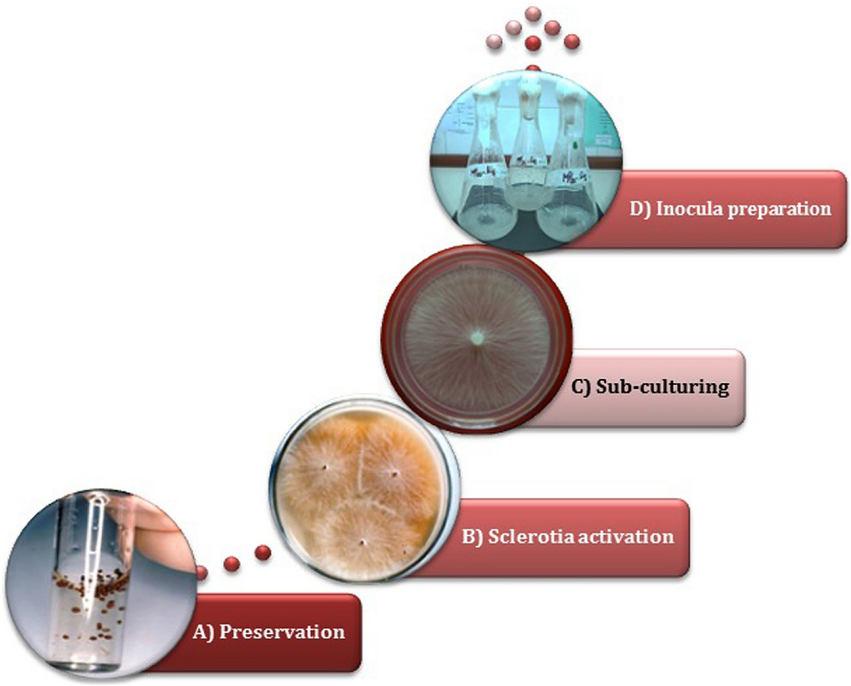

FIGURE 3 | Inocula preparation starting from sclerotia of *S. rolfsii* ATCC 201126. Sequence order:⟶ **(A)** Sclerotia preserved in sterile distilled water ⟶**(B)** Sclerotia germinated in Czapek malt agar ⟶**(C)** Sub-culturing in PM_{20} agar ⟶**(D)** Cultivation in PM_{20} liquid medium, at 220 rpm and 30°C (Fariña et al., 1998).

by sub-culturing in liquid production medium (PM) allows the proper preparation of relatively homogeneous suspensions for inoculation (**Figure 3**), being possible to achieve scleroglucan concentrations similar to those obtained with the strain periodically activated (Fariña et al., 1996).

Another approach that greatly contributes to the preparation of homogeneous inocula is the inclusion of a homogenization step of mycelium-covered agar plugs, suspended in the appropriate volume of culture medium. Standardized proportions should then be used to assure reproducibility, and the inoculum preparation can be safely performed with the aid of a hand blender at controlled speed and for a given time, under aseptic conditions (Fariña et al., 1998).

Cultivation Conditions

As for any other microbial process, scleroglucan production requires some specific culture conditions which become critical in order to achieve maximum productivity. These not only involve nutritional requirements of the producing strain but also operative conditions such as pH, temperature, aeration, agitation, foam control and inoculum size, among the most representative ones. For scleroglucan production with *S. rolfsii* ATCC 201126, many of these conditions were first experimentally adjusted at flask scale and then scaled-up to bioreactor. Additionally, other groups working on scleroglucan production have also evaluated most of these parameters and showed agreement or not with our findings with *S. rolfsii* ATCC 201126. These results will be subsequently discussed.

Nutritional Requirements

Several reports state that broth concentrations of fungal EPSs can be both affected by the nature of carbon and nitrogen sources, as well as by their initial concentration in culture broth (Deshpande et al., 1992; Seviour et al., 2011b). It was also described that highest amounts of biomass do not always lead to optimal EPS production (Giavasis et al., 2005). In the case of *S. rolfsii* ATCC 201126, as also found for other scleroglucans, a high carbon to nitrogen ratio in culture medium would be required to enhance EPS biosynthesis (Fariña et al., 1998; Survase et al., 2007a). Additionally, concerning the nature of nutritional sources, higher polymer concentrations would be associated to the preferential use of sucrose as C-source and $NaNO_3$ as N-source. On the other hand, N-sources such as $(NH_4)_2SO_4$ and other NH_3-based N-sources led to a significant decline in scleroglucan production, a fact likely related to a negative metabolic regulation on the EPS biosynthetic machinery by ammonium (Fariña et al., 1998, 1999). Similar findings were observed for the EPS formation by *Phoma herbarum* CCFEE 5080 (Selbmann et al., 2002b). As also described for pullulan production (by *Aureobasidium pullulans* ATCC 9348), the initiation of scleroglucan production by *S. rolfsii* ATCC 201126 did not coincide with nitrate exhaustion (Campbell et al., 2003). In addition, carbohydrates other than sucrose and glucose, or eventually non-conventional substrates, could alternatively be used as C-sources for scleroglucan production (Wang and McNeil, 1996; Selbmann et al., 2002a; Survase et al., 2007b; Fosmer et al., 2010; Schmid et al., 2011; Valdez, 2013).

Among culture medium characteristics, the C-source concentration has normally exhibited a remarkable influence on scleroglucan production (Fariña et al., 1998; Survase et al., 2006). As emphasized above, a high C:N ratio usually favors EPS production, and different reasons were speculated for this observation. One rational explanation would be the preferential use of the C-source to produce a carbonaceous product (polysaccharide) with lower osmotic effects than the original sugar substrate (sucrose), which would be available for growth under future starvation conditions (Nelson et al., 2008). On the other hand, an increase in scleroglucan production growing under high-osmotic pressure conditions (e.g., 150 g/L sucrose) could reasonably be part of an osmoregulation mechanism (Fariña et al., 1998), as discussed below (see Effect of Other Factors). Scleroglucan production has been mentioned to occur in parallel with cell rescue processes and alternative energy-generating pathways, which may explain the EPS overproduction as a mechanism for survival under anoxic or other stressing conditions (Schmid et al., 2011).

In the case of *S. rolfsii* ATCC 201126, a 7.5-times increase in carbon concentration as compared to the basal medium (150 g/L vs. 20 g/L of sucrose, respectively), led to a fivefold increase in EPS concentration after 72 h of cultivation at shake-flask scale, with a similar behavior at fermenter scale (Fariña et al., 1998, 2001). The detection of glucanases (β-1,3-glucanase, β-1,6-glucanase, and β-glucosidase) under C-source limiting conditions along with the ability to degrade scleroglucan was early reported for *S. glucanicum* and *S. rolfsii* (Rapp, 1989; Martin et al., 2007). In culture broths of *S. rolfsii* ATCC 201126, reduced extracellular β-glucanase activity could be detected at the end of cultivation with low sucrose concentrations (e.g., 20 g/L sucrose for inoculum preparation). However, after transferring the culture to the fermenter with a high-sucrose PM (MOPT with 150 g/L), glucanases became undetectable. The measured high residual sucrose concentrations, even at the end of cultivation in MOPT (ca. 80–100 g/L), may repress β-glucanase activity (Rau, 2004). Nevertheless, high titers of EPS would be more likely related to an osmotically induced β-glucan synthesis than to this glucanase catabolic repression (Fariña et al., 1998, 2009; see Effect of Other Factors). In addition, previous reports on fungal glucan synthetase activity demonstrated that the concentration of sucrose proved to be crucial for enzyme stability at 30°C (Leal et al., 1984; Finkelman and Vardanis, 1986).

Culture media with concentrations of glucose or sucrose (30–35 g/L) lower than that one (150 g/L) being optimal for *S. rolfsii* ATCC 201126 have been reported by different authors, with a maximum production of 8.5–10 g EPS/L (Wang and McNeil, 1995d; Schilling et al., 2000). Nevertheless, the C-source requirements seem to be strain-specific. In this sense, it has been found for example that growth of *S. glucanicum* is completely inhibited by sucrose concentrations above 45 g/L, which further limits the scleroglucan production (Wang and McNeil, 1994). On the other hand, similar to the effects of high sucrose concentrations in *S. rolfsii* ATCC 201126 (Fariña et al., 1998), Survase et al. (2006) reported a maximum production of 16.5 g EPS/L with a sucrose concentration of 80 g/L.

Other components of culture medium exhibited lesser influence on scleroglucan production. In *S. rolfsii* ATCC 201126, a change in phosphate concentration from 1.3 to 2 g/L led to higher scleroglucan concentrations. Meanwhile, additional constituents such as L-threonine and ascorbic acid significantly decreased scleroglucan production (Fariña et al., 1998). With regard to nitrogen and phosphate sources, Taurhesia and McNeil (1994a) found that higher titers of scleroglucan from *S. glucanicum* were produced in a P-limited medium (18.9 g/L of scleroglucan) than in a N-limited medium (11.4 g/L EPS). High scleroglucan concentrations have also been commonly supported by the presence of yeast extract and casein hydrolysates in culture medium (Taurhesia and McNeil, 1994a).

To conclude, with regard to the scleroglucan production by *S. rolfsii*, there seems to be much agreement in the culture medium composition over the latest years, using optimized culture medium formulations similar to MOPT, which contain a high C:N ratio with sucrose as C-source (e.g., 150 g/L), $NaNO_3$ as N-source (in the order of 2.25 g/L), $K_2HPO_4{\cdot}3H_2O$ as P-source (∼2 g/L) and other minor components (in g/L): KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; yeast extract, 1; citric acid·H_2O, 0.7; $FeSO_4{\cdot}7H_2O$, 0.05 (initial pH adjusted to 4.5). Cultivation of *S. rolfsii* ATCC 201126 at eight L-fermenter scale by using this culture medium led to the highest EPS production kinetic parameters (i.e., 26 g scleroglucan/L, theoretical yield factor, $Y_{p/c} = 0.68$ and volumetric productivity, $Pr = 0.542$ g/L·h) at 48 h of fermentation (Fariña et al., 1998).

Culture Conditions

The control and maintenance (or modulation) of operative parameters becomes fundamental for a process optimization. Some typical aspects for the particular case of scleroglucan production will be discussed below. Along the scleroglucan research history, many researchers and engineers have ventured different alternatives in a continuous effort to improve EPS production and purification, and some of these will be herein revisited.

Effect of temperature

This parameter typically affects both culture growth and polysaccharide production. However, it has been reported that in batch cultures, maximum EPS biosynthesis is achieved at temperatures somewhat lower than the one for optimal growth rate. When the organism growth rate is decreased by reducing cultivation temperature, this may increase the availability of isoprenoid lipid carrier for non-growth functions, thus stimulating polysaccharide production (Wang and McNeil, 1996; Fosmer and Gibbons, 2011). Optimal biomass production usually occurs at temperatures above 28°C, while "optimum" temperature for scleroglucan formation was found to be ∼28°C (Giavasis et al., 2005). Instead, below 28°C, by-product (oxalic acid) formation is gradually increased, so that at 20°C acid production may exceed biomass and EPS biosynthesis (Wang and McNeil, 1995b). In the case of *S. rolfsii* ATCC 201126, the production process is commonly carried out at 30°C, obtaining optimal EPS yields.

Effect of pH

This factor frequently influences the microorganism physiology by affecting both nutrient solubility and uptake, enzyme activity, cell membrane morphology, by-product formation, redox reactions, etc. (Giavasis et al., 2005). As described for temperature, the appropriate pH for maximum polysaccharide production can differ from that for optimal growth. Kang and Cottrell (1979), pointed out that the optimum pH for fungal polysaccharide synthesis usually lies between 4.0 and 5.5.

First studies of pH effects on scleroglucan production by *S. rolfsii* ATCC 201126 were performed at shake flask scale where, after initial adjustment, pH was left uncontrolled. Comparison of such results to those obtained in stirred-tank reactors is not very different, as most of the cultivations up to date gave the best EPS values when pH was not fixed to a given set-point. It naturally reaches values of 2–2.5 at around 18–24 h of fermentation with a slow and slight increase afterward (Fariña, 1997; Fariña et al., 1998; Valdez, 2013). However, other authors recommended the control of pH during scleroglucan fermentation with *S. glucanicum* NRRL 3006 in order to achieve higher polysaccharide titers (Wang and McNeil, 1995c). As the automatic pH control implies extra costs and further handling of the batch, this aspect will require a proper examination depending on the species and/or the strain to be employed.

Effect of dissolved oxygen (DO)

For aerobic organisms, particularly for fungi, oxygen plays a vital role in many aspects of cellular metabolism. Changes in dissolved oxygen often impact respiration rate, enzymatic synthesis and activity, formation of metabolic products, etc. (Forage et al., 1985). Rau et al. (1992) investigated the effect of DO on polymer biosynthesis by *S. glucanicum*. They reported that a high oxygen supply resulted in increased cell growth rate along with decreased scleroglucan production. In contrast, when oxygen partial pressure in the liquid phase was almost reduced to zero, the fungal response was a limited growth but the specific stimulation of scleroglucan formation (Rau et al., 1992; Schilling et al., 1999). Wang and McNeil (1995a) also suggested that, when oxygen becomes limiting, growth undergoes a severe restriction for the C usage, which in turns can be derived toward different metabolic activities such as polymer and/or oxalate production.

The effect of limiting DO tension on the stimulation of EPS production may be difficult to understand; biopolymer synthesis is an energy-demanding process and the generation rate of energy in aerobic microorganisms always relates to the oxygen supply. A reduction in culture DO, however, may disturb fungal morphology, broth rheology or C assimilation, in a favorable way for EPS biosynthesis (Olsvik and Kristiansen, 1994). Nevertheless, since EPS production has been found to start before the DO levels reach limiting values, it becomes unlikely that oxygen limitation triggers scleroglucan biosynthesis by itself (Wang and McNeil, 1995a).

A previous report suggested that at high respiration rates (high DO tension) more carbon would be converted into carbon dioxide, thus leaving less available C-source for scleroglucan production (Sutherland, 1977). Reaffirming this assumption, Wang and McNeil proposed that low DO levels would lead to restricted *Sclerotium* fungal growth (which is an aerobic process) and consequently, more C-source could be driven to scleroglucan biosynthesis. This is in agreement with the statement that EPS production and cell growth are alternative fates of the C-source. Therefore, it sounds very realistic the rationale that, under certain conditions, the improved availability of C is governing polysaccharide production (Wang and McNeil, 1995a). Moreover, low DO levels might decrease by-product (oxalate) formation by repressing the synthetic enzyme glycolate oxidase (Schilling et al., 2000), thus reducing the diversification of the C-source to undesirable products, and simultaneously favoring the C-flow toward β-glucan production.

Effect of aeration and agitation rates

Optimizing aeration and agitation rates represents a critical tool to control cell growth and scleroglucan production. These parameters are related to an adequate culture mixing and the sufficient mass and heat transfer rate, which can therefore increase the transference of nutrients and oxygen from liquid medium to the cells, and also modulate the rate of metabolite release from cells to the surroundings, this latter including biopolymers, by-products, and carbon dioxide (Giavasis et al., 2005).

As aforementioned, EPS production is characterized by the development of a very viscous culture broth that commonly exhibits Non-Newtonian, pseudoplastic rheological behavior, emphasized by the production of high biomass concentrations. Furthermore, at moderate to low stirring rates, the fungus forms mycelial pellets. These both phenomena result in the development of a very heterogeneous culture, where nutrient and oxygen transfer into the pellets, as well as metabolites and toxic products release, become very limited or even nil (Forage et al., 1985; Brown et al., 1987). Under these conditions, the shear rate within the bioreactor varies extraordinarily, from the highest value close to the impeller to the lowest one next to the vessel wall, even in lab-scale stirred-tank reactors.

Fungal morphology in submerged cultures (pelleted vs. dispersed filamentous growth) is affected by a variety of cultural parameters like inoculum strength, medium composition, fermenter configuration (see below) and particularly, agitation rates (Papagianni, 2004; Fazenda et al., 2008). It was early suggested that a pelleted fungal morphology in *Sclerotium* sp. may produce higher β-glucan yields than diffuse mycelial cultures (Gibbs et al., 2000; Papagianni, 2004; Wucherpfennig et al., 2010; Schmid et al., 2011; Seviour et al., 2011a). Systematic studies on the relationship between aeration rate, mycelial morphology and EPS production were previously performed in *Cordyceps militaris*, finding that DO starvation at low airflow levels led to pellet autolysis and looser mycelial clumps. However, high aeration rates were neither beneficial. Intermediate values (2 vvm) led to compact mycelial pellets which resulted optimal for cell growth and EPS production (Park et al., 2002). In the case of *S. rolfsii* ATCC 201126, frequently working at 0.5 vvm and 400 rpm at fermenter scale, loose lenticular pellets are frequently associated to an optimal scleroglucan production (Valdez, 2013).

In stirred tank reactors (STRs), the average shear rate in the impeller zone is known to be a function of the impeller diameter

and the impeller tip velocity. In pneumatic contactors such as bubble column and air-lift reactors (ALRs), it depends on the superficial gas velocity (Forage et al., 1985; Wecker and Onken, 1991). Bulk mixing may be improved by either increasing the impeller speed, increasing the gas velocity or altering the design of fermenter and/or impellers (Wang and McNeil, 1996).

A critical balance should be maintained concerning stirring rates, since scleroglucan would not be released from cell walls at sub-optimal shear stress. Low shear stress has been linked to the release of very low MW scleroglucan, whereas large biopolymer molecules remained adhered to mycelial walls (Rau et al., 1992). Poor bulk mixing may also add problems in terms of process monitoring and control (Wang and McNeil, 1996). However, very high stirring rates can usually lead to increased damage of hyphal cells along with the degradation of biopolymer macromolecular structure and the consequent reduction in broth viscosity (Nielsen, 1992; Rau et al., 1992).

Similarly, Schilling et al. (1999) confirmed that under high stirring rates, the produced scleroglucan showed a low MW as compared with that obtained after moderate agitation. A combination of moderate agitation plus high aeration rate allowed them to attain a maximum-MW scleroglucan. Although good agitation may be essential for mixing, if surpassed, it may adversely affect culture viability or physiology or even the EPS quality. Aeration, on the other hand, contributes to good mixing in a milder manner with no disturbance of the culture or polysaccharide molecular size (Giavasis et al., 2005). Additionally, foam control at high agitation rates requires special care since while some antifoams may considerably enhance EPS production in certain fungi, others (e.g., some vegetable oils) are known to be inhibitory (Stasinopoulos and Seviour, 1990; Fariña et al., 1998; Yang et al., 2000; Hsieh et al., 2006).

There is limited literature concerning the production of scleroglucan under different airflow and stirring rates. Rau et al. (1992) carried out batch cultivations with *S. glucanicum* CBS 52071 at different airflow rates using draft-tube propeller systems at a constant shear rate of 600 rpm. They reported that high airflow rates, in the order of 0.3 m^3/h, were associated to larger growth rates and decreased scleroglucan formation.

Effect of other factors

Some additional effectors have been tested in order to increase scleroglucan biosynthesis. Among these, it is worth to mention the positive influence of high-osmotic-pressure conditions on scleroglucan biosynthesis. For instance, in *S. rolfsii* ATCC 201126, the influence of high osmolarity on scleroglucan production was partially evidenced by means of complementing the osmotic effects of culture media containing either 50 or 75 g/L-sucrose with additional NaCl or KCl, in order to mimic the 150 g/L-sucrose osmotic pressure (Fariña et al., 1998). Normally, in media with 150 g/L sucrose as C-source (e.g., MOPT medium), where EPS reaches the highest values, much of the sugar remains at the end of the batch. This finding was already associated to an EPS protective role and its increased production under high osmotic pressure environments, where scleroglucan might be part of an osmoregulation mechanism (Seviour et al., 1992; Fariña et al., 1998; Schmid et al., 2010).

In natural environments, EPS formation has been linked to substrate adhesion (e.g., biofilm formation) or desiccation prevention, among other roles (Donot et al., 2012). The production of EPSs has been also described as an adaptation strategy for microbial survival under stressing conditions such as freezing. Ice crystal formation, osmotic pressure variations, and water availability are factors normally related to cell stress during freezing. This would agree with the current use of polysaccharides (e.g., alginate) in cryopreservation techniques and would explain the protective properties of EPSs in Antarctic microbes to overcome lethal effects of freeze-thaw cycles, which are harsher than a continuously frozen environment (Selbmann et al., 2002b).

As it was noted in a previous report (Fariña et al., 1996), *S. rolfsii* ATCC 201126 was shown to be relatively halotolerant. Although osmosity exhibited a significant influence on β-glucan production, the highest EPS values reached with 150 g/L sucrose could not be equaled just by increasing culture medium osmotic pressure by means of salts addition. The osmotically active salts added to the medium did showed to increase EPS production but, the normal scleroglucan concentration obtained in 150-g/L-sucrose culture medium (MOPT) could not be achieved, probably because of the high ionic strength exerted by the NaCl or KCl surplus (Fariña et al., 1998). Similar evidences were found while examining the effects of osmotic pressure on the erythritol production by *Trigonopsis variabilis* (Kim et al., 1997).

Instead of salts, other non-metabolizable water activity (a_w) adjusters like polyethylene glycol (PEG) 200 have been already cited in the literature, for example to study the osmotically modified enzymatic production in *Aspergillus niger* (Taragano et al., 1997) or to relate water activity depletion and the stimulation of EPS production in *Ganoderma lucidum* (Papinutti, 2010). In the latter case, and similarly to *S. rolfsii* ATCC 201126, an incomplete utilization of reducing sugars was found at high malt extract concentrations, suggesting a high C-source concentration as a positive effector for EPS production by decreasing water activity (Papinutti, 2010). Likewise, high solute concentrations in culture medium have been related to a reduced oxygen solubility and diffusion coefficient (Büchs, 2001), which may also stimulate EPS biosynthesis.

On the other hand, supplementation with sunflower oil and ascorbic acid, and particularly with L-threonine, led to a diminished EPS production in *S. rolfsii* ATCC 201126 (Fariña et al., 1998). Although some proteomic approaches already described the activation of heat-shock proteins and the increased expression of ATP citrate lyase (decreased TCA cycle activity) with increased EPS production by *Pleurotus tuber-regium* when stimulated by Tween 80 (Zhang et al., 2012), this supplementation to culture medium did not exerted stimulation toward EPS production in *S. rolfsii* ATCC 201126 (Fariña et al., 1998). In addition to the influence of culture medium composition, and with regard to external factors other than those already discussed, some authors also found that white or blue light facilitated glucan formation in *S. rolfsii* (Miller and Liberta, 1976), but no further information is currently available on this aspect.

In the particular case of *S. rolfsii* ATCC 201126, several studies performed at shake-flask scale during the last decades allowed to establish optimal conditions for scleroglucan production (Fariña et al., 1996, 1998). Subsequently, when scaling-up to bioreactor scale, stirred-tank reactors were used under the following operative conditions: airflow rate, 0.5 vvm; stirrer speed, 400 rpm; temperature, 30°C and initial pH 4.5 (uncontrolled throughout the fermentation process). These conditions allowed to achieve a 1.5-fold increase in volumetric productivity (*Pr*, from 0.365 up to 0.542 g/L·h) in association to a significant shortening of the required cultivation time (from 72 to 48 h) and a final EPS concentration of 26 g/L (Fariña et al., 1998). Considering that typical batch cultures for scleroglucan production were usually described with a length of around 100 h (McNeil and Harvey, 1993), these latter results would be quite promising. A scheme depicting the optimized and standardized method for production and recovery of scleroglucan by *S. rolfsii* ATCC 201126 is shown in **Figure 4**.

Concerning scleroglucan production optimization, our first studies in this field were performed by using the one-factor-at-a-time method, which consists in changing one variable (nutrients, pH, temperature, etc.) while fixing the others at certain arbitrary levels (Fariña et al., 1998). This technique was also successfully applied to other EPSs (Xu et al., 2003). Nevertheless, because of the great number of factors that are involved in the process, this method usually implies a large number of experiments, and thus results very laborious and time consuming, and not always guarantees the disclosure of optimal conditions (Survase et al., 2006). Different methods have been proposed in the literature for the empirical modeling and optimization of EPS production which may behave more efficiently and/or accurately.

With reference to scleroglucan from *S. rolfsii* MTCC 2156, two main methods were proposed, one consisting in a statistical-based approach (response surface methodology) and the other one, in an artificial intelligence-based approach (artificial neural network-genetic algorithm; Desai et al., 2008). Both methods allowed a significant increase in scleroglucan titers (from 7.8 ± 0.54 g/L in non-optimized medium to 16.42 ± 0.68 g/L and 16.22 ± 0.44 g/L for artificial neural network-genetic algorithm and response surface methodology, respectively) with the requirement of a minimum number of experiments. Both methods provided a deep knowledge (e.g., interactions between different components) on the scleroglucan production system. Moreover, artificial neural network-genetic algorithm allowed predicting more accurate values of optimal conditions and optimum titers as compared to response surface methodology.

Fermenter Configuration

Relatively little information is available on the influence of fermenter configuration on α- or β-glucan yields in fungi (McNeil and Harvey, 1993; Gibbs and Seviour, 1996; Wang and McNeil, 1996). Bioreactor architecture is mainly involved in the efficient homogeneous mixing of the culture, especially promoting heat, oxygen and other substrates mass transfer to the cells (Rau et al., 1992; McNeil and Harvey, 1993). STRs are the workhorse in the fermentation industry, and they are the most utilized at both research and industrial scale (Lawford and Rousseau, 1989; Kang et al., 2000).

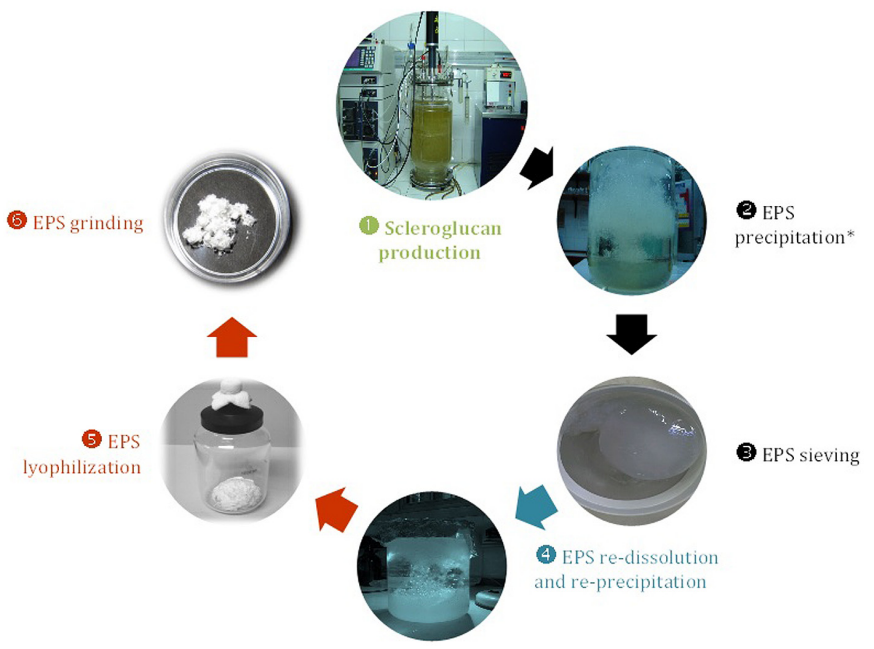

FIGURE 4 | Flowchart illustrating the main stages during production and downstream processing of scleroglucan from *S. rolfsii* ATCC 201126 (Fariña et al., 1998; Viñarta et al., 2013a). Chain starts with ❶ production at fermenter scale with MOPT culture medium under the following operative conditions: 400 rpm, 0.5 vvm and 30°C, in a BioFlo 110 fermenter (New Brunswick Sci.) with an 8 L-working volume. ❷❸ Preliminary EPS recovery; ❹ EPS purification; ❺❻ Final EPS treatments for storage and usage. ❷* After biomass separation by centrifugation.

Two configurations are the most commonly used for fungal fermentations: the continuous STR and the ALR, whose different principles of mixing represent a high- and low-shear regime, respectively (Seviour et al., 1992; Gibbs et al., 2000; Papagianni, 2004). Even though data are available in the public domain, it is difficult to separate the complex individual effects of shear/mixing/mass transfer or DO levels and biomass morphology on β-glucan production, mainly, because of the performed experiments were not conceived to differentiate between each effect.

A stirrer system that imparts a high shear stress upon the medium normally uses Rushton turbine impellers, which pump out the medium radially from the turbine (McNeil and Harvey, 1993; Wang and McNeil, 1996; Gibbs et al., 2000; Papagianni, 2004; Fazenda et al., 2008). Radial flow (turbine) impellers are efficient at achieving oxygen transfer by virtue of their ability to increase turbulence. Their efficiency is, however, counteracted by the negative effect of this shear intensive system on the "quality" of the isolated exopolymer. Product quality is a relative term that can only be properly defined in terms of the end-use application (Lawford and Rousseau, 1991). As stirrer speed augments in a high-shear configuration, oxygen and heat mass transfer rates increase whilst the mixing times decrease (McNeil and Kristiansen, 1987). Fungal morphology is often quite different from that seen in low-shear systems (Gibbs et al., 2000; Papagianni, 2004; Fazenda et al., 2008; García-Ochoa and Gómez, 2009).

In smaller laboratory fermenters, and particularly for polysaccharide fermentations, wall effects become significant. It is not unusual to see impellers turning at high rpm and stagnant broth a few inches away. Poor mixing, particularly near the walls, is worsened by the presence of excessive baffling, cooling devices, pH and dissolved oxygen probes, and sampling lines (Wernau, 1985). These difficulties have been frequently observed during scleroglucan production by *S. rolfsii* ATCC 201126 (see below, **Figures 5A,B**).

The most common configuration to work under low-shear conditions is the ALR, which uses differences in hydrostatic pressure or density in order to achieve a fluid mixing. Air is injected through a sparger into the bottom of a riser tube, decreasing the effective density of the medium there. As bubbles rise to the top, they are released into the headspace; the medium becomes denser and it then descends to the vessel bottom *via* a downcomer or an external loop (McNeil and Harvey, 1993; Gibbs et al., 2000; Chisti and Jauregui-Haza, 2002; Papagianni, 2004; García-Ochoa and Gómez, 2009).

On the other hand, although less used, low-shear configurations in continuous STRs rely on modifications of the stirring systems and the impellers. Available low-shear impellers include axial flow and helical ribbon stirrers. The operation in both cases implies the pumping of the fluid from the top to the bottom of the fermenter at reduced liquid stress and against the airflow (Rau et al., 1992; McNeil and Harvey, 1993). For schizophyllan, a β-glucan similar to scleroglucan, EPS production reached higher values in fermenters with axial flow impellers than with helical ribbon stirrers (Rau et al., 1992).

The use of ALRs is being increasingly considered in fermentation industries instead of the traditional mechanically agitated bioreactor. Their design is mechanically simpler than the observed in STRs, and because of the absence of mechanical stirring, they are also less expensive to operate. Main advantages include low power inputs, relatively low shear, simple construction, and no moving mechanical parts, which additionally reduces contamination risks. Despite this, the comparatively low shear regime and lower oxygen transfer rates may represent difficulties at the time of cultivating filamentous fungi (Barker and Worgan, 1981; Blenk, 1985; Merchuk and Siegel, 1988; Allen and Robinson, 1989). These difficulties can be satisfactorily solved by the introduction of internal or external loops (Seviour et al., 2011b).

Higher EPS scleroglucan concentrations could be achieved with *S. glucanicum* NRRL 3006 (Wang and McNeil, 1995d) in a 120-L ALR with an external loop in comparison with the classical STRs, probably by satisfying a low oxygen demand when using the ALR fermenter architecture. Similarly, Kang et al. (2000), investigated scleroglucan production in an ALR with an internal loop. They found that scleroglucan productivity obtained in this system was comparable to those achieved in ALRs with an external loop or in stirred-tank reactors, presenting the additional advantage of low equipment investment and operational costs (Kang et al., 2000). Despite the clear economic advantages that ALRs offer for scleroglucan industrial or lab-scale production, these systems are not commonly used, perhaps because the lack of knowledge in this bioreactor configuration of

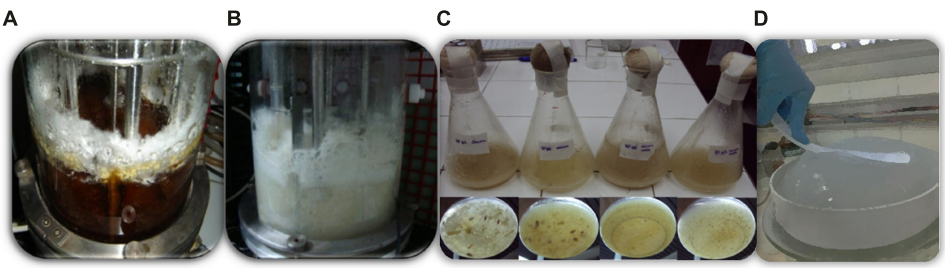

FIGURE 5 | Scleroglucan production by *S. rolfsii* ATCC 201126 with alternative C-sources: (A) Sugarcane molasses, (B) Corn starch or, (C) Ground lemon peel. Aspect of EPS precipitate **(D)** after settling overnight at 5°C the corn starch-broth supernatant with an equivalent volume of ethanol 96°. **(A,B)** 2 L-working volume fermenter scale. **(C)** 100 mL-working volume shake-flask scale. Other culture medium components, as in PM_{20} (Fariña et al., 1998).

both, process rheology and hydrodynamics (Wang and McNeil, 1996).

In conclusion, the technology of scleroglucan production still seems to be an aspect deserving further investigation, since the choice of fermenter design will help to determine the ultimate economic attractiveness of any proposed industrial process.

Utilization of Alternative or Non-Conventional Substrates

At present and mainly for economic reasons, most of the commercialized polysaccharides are derived from plants, with the exception of xanthan and curdlan gums which are from bacterial origin (Clementi, 1997; Sutherland, 2008). The introduction of fungal EPSs like scleroglucan in the market is usually limited by low yields and high production costs. Therefore, unraveling these shortcomings may help to expand the scleroglucan market to relevant areas such as pharmaceuticals, cosmetics, food and agriculture (Singh et al., 1974; Pretus et al., 1991; McNeil and Harvey, 1993; Wang and McNeil, 1996). A tactic to reduce the costs and boost EPS production may involve developing fermentative processes that strategize the use of cheap and/or easily available substrates. Scleroglucan production commonly considers the use of conventional substrates like sucrose or glucose as C-source (Fariña et al., 1998; Survase et al., 2006; Schmid, 2008; Schmid et al., 2010), along with small amount of salts, yeast extract and nitrate as N-source. These latter are not quite expensive, however, their use might have more impact on production costs at industrial scale. Therefore, some authors have highlighted the importance of evaluating the employment of certain wastes or agro-industrial sub-products as potential scleroglucan production substrates.

Survase et al. (2007b) reported high scleroglucan production with *S. rolfsii* MTCC 2156 by using in culture medium either sugarcane molasses (∼19 g EPS/L) or coconut water (∼13 g/L). On the other hand, a reduction in production costs could also be achieved with *S. glucanicum* NRRL 3006 in a culture medium containing a sub-product coming from corn-based ethanol production [*Condensed Corn Soluble (CCS)*; Fosmer et al., 2010]. Similarly, other researchers found high scleroglucan yields by growing *S. glucanicum* NRRL 3006 in culture media based on starch-derived raw materials (Selbmann et al., 2002a). The use of alternative substrates may represent an attractive proposal not only from the economic point of view, but also at environmental level. It would allow reducing production costs at the same time of giving added value to non-valuable wastes or sub-products with scarce commercial value, what entails a revalorization of these alternative substrates. Additionally, their conversion into high-added-value bio products (EPS) would also contribute to the mitigation of the environmental impact generated by their eventual disposal in nature (Fernandes Silva et al., 2009; Taskin et al., 2011; Abdul Razack et al., 2013; Muhammadi and Afzal, 2014).

In recent years, with the aim of reducing production costs for scaling-up and based on the *S. rolfsii* ATCC 201126 ability to metabolize diverse C-sources, our group evaluated the possibility of using different available agro-industrial sub-products with relevance in the region (Valdez, 2013). Under-utilized biomasses such as vegetable and fruit processing residues constitute a promising source, being generated in huge amounts every year and representing an environmental and economic problem of worldwide concern. Among these residues, peels, seeds and pulps constitute a 30–50% of input materials contributing with high organic matter to the environment. These agro-wastes might then be envisaged as suitable C-sources for revalorization through the production of high added-value biotechnological products ("biorefinery" strategy) such as EPSs (Poli et al., 2011). Simultaneously, this would aid to cope with the depletion of natural resources and environmental concerns.

Among alternative substrates, the use of sugarcane molasses and lemon peel have been tested for scleroglucan production by *S. rolfsii* ATCC 201126 (Valdez, 2013; Montes de Oca, 2014). The former one is a liquid by-product of the sugar industry with a dark brown color, highly viscous and homogeneous appearance, where the major sugar component is sucrose. Some ions like Mg^{2+}, Mn^{2+}, Al^{3+}, Fe^{3+}, and Zn^{2+} may also be present in variable proportions (Prescott and Dunn, 1987).

Molasses (≡20 g/L reducing sugars) with no pre-treatment could be used in culture medium which led to proper growth and scleroglucan production in *S. rolfsii* ATCC 201126 (Valdez, 2013). Conversely, Roukas and Liakopoulou-Kyriakides (1999) proposed a pre-treatment of this substrate prior to pullulan production, due to the presence of heavy metals with inhibitory effects on microbial growth and EPS-linked enzymatic activities. In our case, the maximal scleroglucan production at bioreactor scale with molasses as C-source (5.11 g/L; **Figure 5A**) was moderately lower than the one normally achieved in control medium with 20 g/L sucrose (6.87 g/L). The crude EPS obtained under these conditions exhibited an undesirable brownish coloration, likely related to the presence of melanoidins as the main pigment in sugarcane molasses (Chandra et al., 2008). However, this pigmentation could be significantly reduced once EPS downstream processing was completed, thus reflecting a higher purity grade.

As the quality and EPS structural features may vary depending on the C-source, the obtained EPS was evaluated on its purity degree and rheological characteristics (Castillo et al., 2014). The EPS coming from molasses medium showed high purity values (93% w/w total sugars, 9.1% w/w reducing sugars, with minimal protein contamination). Aqueous solutions containing this EPS showed non-Newtonian pseudoplastic behavior with better rheological properties (higher consistency coefficient, *K*, and lower flow behavior index, *n*) as compared to the EPS obtained from conventional sucrose, at identical EPS concentration.

These results allowed us to confirm the feasibility to produce high-quality scleroglucan from sugarcane molasses, which may represent a more economic option, especially for regions where this sugar industry by-product is abundant (Koller et al., 2012). That would be valuable at industrial level at the time of facing large-scale production and taking into account the wide potential uses of this EPS. Applications may include from the utilization of the whole culture broth, as for enhanced oil recovery (EOR; Sandford, 1979; Holzwarth, 1984), to the use of a refined grade EPS (e.g., for cosmetic, pharmaceutical or medical applications; Pretus et al., 1991; Falch et al., 2000; Mueller et al., 2000; Coviello

et al., 2005; Laroche and Michaud, 2007; Viñarta et al., 2007; Giavasis, 2014).

The use of commercial corn starch (≡20 g/L reducing sugars) has also been tested as C-source for scleroglucan production by *S. rolfsii* ATCC 201126 at bioreactor scale (Valdez, 2013). Under these conditions, EPS production (7.95 g/L; **Figure 5B**) exceeded the obtained values with the same concentration of either sucrose or molasses as C-source. The obtained EPS was more easy to precipitate (**Figure 5D**) and the efficiency of the recovery process thus ascended from the typical ∼30% (28.5% with sucrose and 33.2% with molasses, respectively) to above 50%. Purity analyses of EPS revealed similarities in total and reducing sugars, and protein contents as in the EPSs obtained with sucrose or molasses. Rheological properties, however, for an equal EPS concentration, indicated a lower *K* value in comparison to the molasses-EPS but higher than the traditional sucrose-EPS. In this sense, our results have oriented us to the study on the use of low-cost amylaceous materials or residues, as potato washing wastewater. The alternative use of cheese whey (Fernandes Silva et al., 2009) and olive mill wastes (Dermeche et al., 2013) is also being considered.

Another C-source more recently tested with *S. rolfsii* ATCC 201126 was the ground- and acid-treated lemon peel obtained from a local citrus-processing factory (Montes de Oca, 2014). The use of this substrate (≅15 g/L reducing sugars; **Figure 5C**) led to interesting EPS production values (∼13 g/L), even surpassing the final EPS concentrations (5–8 g/L) in sucrose-, molasses-, or corn starch-containing media (Valdez, 2013). However, the maximal EPS concentration in pre-treated citrus-peel-containing media could just be achieved at 7 days of cultivation instead of the 2–3 days required for culture media with sucrose, molasses or corn starch as C-sources. An additional aspect to consider is that pectin (released from citrus peel to culture broth) may co-precipitate along with EPS when alcohol is added for the recovery process. This hitch could be partially solved by previously treating the samples (48 h at 40°C) with commercial pectinase (Montes de Oca, 2014). The practicability of using citrus peel for EPS production should then be carefully weighed taking into account that factors such as the pre-treatment of raw material, the longer cultivation time and the remaining pectin interference may deleteriously influence the production process costs. On the other hand, despite the cited limitations for EPS production, the finding of an appropriate fungal growth along with the secretion of a wide variety of enzymes with this substrate would allow to suggest these cultivation conditions for the production of other biotechnologically relevant products, such as hydrolytic and ligninolytic enzymes (Montes de Oca, 2014).

As a final point, it is already described in the literature that *S. rolfsii* and *S. glucanicum* behave as phytopathogens with the natural ability to produce a wide enzymatic spectrum including cellulases, phosphatases, arabinases, exogalacturonases, polygalaturonases, galactosidases, and exomannanases (Survase et al., 2007b). It can be therefore expected that some plant residues such as sugarcane bagasse (Koller et al., 2012), solid olive-mill by-product called "alperujo" or olive mill wastewater ("alpechin"; Dermeche et al., 2013) may also be evaluated as potential unconventional raw materials for the low-cost scleroglucan production within an eco-sustainable framework. Nevertheless, in spite of all the work performed up to date and their prospects, scleroglucan production at industrial scale still remains limited to glucose or sucrose, which makes the finding of alternative substrates as well as the reduction of costs a continuing challenge to keep working on.

DOWNSTREAM PROCESSING

As relevant as improved cultivation conditions are for achieving high polymer yields, the downstream processing also represents a crucial step in order to ensure a high recovery yield of a refined-grade polysaccharide. This stage may affect critical macromolecular features such as polymer MW, DP, DB, purity and composition, and also constitutes a significant part of the total production costs (Wasser, 2002; Wang et al., 2010; Viñarta et al., 2013a,b). This processing stage optimization becomes imperative and, depending on the desired purity or the intended use (which usually requires given physico-chemical properties), different extraction and purification techniques should be developed.

In the case of extracellular slimy polysaccharides, this process generally involves the sterilization or pasteurization of the fermentation broth in order to kill microbial cells, inactivate undesirable enzymes (e.g., glucanases) and facilitate EPS detachment from cells. The subsequent removal of biomass is frequently carried out by filtration or centrifugation. The polysaccharide in the cell-free filtrate or centrifugate is then precipitated with alcohol, followed by further purification steps (if required) such as ultrafiltration, gel permeation/ion-exchange chromatography, or diafiltration. The end product is finally obtained after drying with either air/inert gas under vacuum, spray drying, or lyophilization, plus a final milling to the desired mesh size (Giavasis, 2013).

Referring to the *S. rolfsii* ATCC 201126 scleroglucan downstream processing, different strategies were evaluated in order to finally establish an optimized purification protocol. That led to an industrially acceptable recovery of EPS with high purity grade (∼98%; Fariña et al., 2001; Viñarta et al., 2013b). This protocol commonly involves a step of homogenization to facilitate the EPS release from mycelium and to weaken the intermolecular polysaccharide association, so that it is completely dispersible in water. This is followed by a threefold dilution of culture broth with distilled water, which otherwise is extremely viscous and difficult to process, and a final neutralization with NaOH. Thereafter, the diluted/neutralized broth is heated (80°C for 30 min) to inactivate eventually produced glucan-degrading enzymes and to enhance glucan solubilization in water, and finally centrifuged (27500 × *g*, 15°C, 20 min). The EPS from clear supernatant is then precipitated by adding an equivalent volume of an organic solvent, commonly a lower alcohol.

Generally speaking, when a polysaccharide is present, fungal mycelia are easier to remove from diluted broths; but the additional cost of re-concentration normally imposes an economic drawback (Wernau, 1985). The possibility to achieve

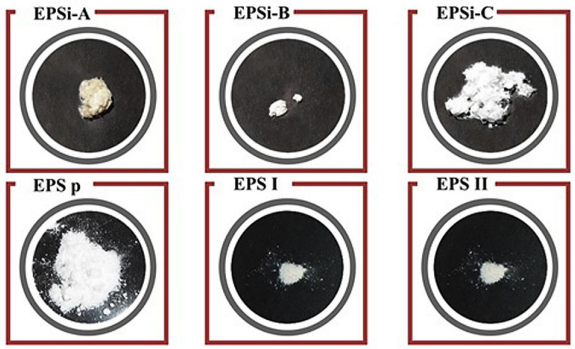

FIGURE 6 | Appearance of EPSs produced by *S. rolfsii* ATCC 201126 at fermenter scale in MOPT culture medium and subsequently downstream processed. EPSi corresponds to scleroglucan precipitated with isopropanol after 1 (A), 2 (B), or 3 (C) re-dissolution/re-precipitation steps. EPSp illustrates the EPS precipitated with PEG. EPS I and EPS II correspond to ethanol-precipitated scleroglucans after 48 or 72 h of cultivation (Fariña et al., 1998; Viñarta et al., 2013b).

a high recovery of pure scleroglucan from diluted supernatants of *S. rolfsii* ATCC 201126, from ∼30% (with sucrose or molasses media) up to 50% (with corn starch medium), attenuates this disadvantage. Concerning the precipitation step for EPS recovery, different alcohols (ethanol 96°, isopropanol, and PEG) were tested (Johal, 1991; Fariña, 1997; Fariña et al., 2001; Viñarta et al., 2013b). Among them, we found that ethanol 96° and isopropanol allowed obtaining the highest recovery, high purity degree, finest appearance, optimal water solubility and remarkable rheological properties of EPS (**Figure 6**). In addition to the first precipitation step at the end of centrifugation, the inclusion of a three-step re-precipitation/re-dissolution cycle (**Figure 4**) with either ethanol or isopropanol, was the best methodology to achieve a refined-grade scleroglucan, suitable for example for biomedical testing (Fariña et al., 2001; Viñarta et al., 2013b).

OPERATIONAL STRATEGIES

As scleroglucan represents a growth-associated metabolite, it has been early recognized that those conditions favoring growth will also do so on EPS production. However, it was noted that some required conditions that seem to promote polysaccharide production do not always imply the stimulation of growth but the opposite. This gave place to bi-staged processes, where optimal conditions for biomass production are supplied during the first stage and then, once a critical biomass has been produced, the conditions are changed in order to stimulate EPS production (Sutherland, 1982).

The commonly selected method for EPS production is the *batch process*, where the producing microorganism is inoculated into the culture medium which contains all the required nutrients. Under these conditions, growth and EPS production take place until the exhaustion of a given critical substrate (which usually turns limiting). On the other hand, the *continuous culture* strategy, which consists in the uninterrupted addition of fresh culture medium whilst spent broth (containing part of the biomass and the product of interest) is simultaneously harvested, is not frequently used for EPS production (Rosalam and England, 2006). Nevertheless, this latter methodology has been eventually employed to study biochemical and physiological aspects related to some polysaccharide-production processes (Sutherland, 1982). In the case of scleroglucan, an alternative continuous culture process at lab and industrial-scale has been patented years ago (Maier, 2004).

Unfortunately, in contrast to batch-wise cultivation, continuous culture resulted not feasible under non-aseptic conditions, being less effective with regard to yield and product quality, as compared to batch cultures (Schilling, 2000). It may also be worthwhile to highlight that for some microorganisms such as the xanthan-producer *Xanthomonas campestris*, the continuous culture strategy might lead to the undesirable selection of poorly EPS-producing strains (Sandford, 1979).

In order to improve scleroglucan production by *S. glucanicum* NRRL 3006, some researchers developed a *bi-staged process*. During the first phase of cultivation, pH was controlled at 3.5 with the aim of promoting optimal growth, and thereafter pH was raised up to 4.5 to favor polysaccharide biosynthesis. The second stage allowed achieving a 10% reduction of by-product (oxalic acid) formation, simultaneously with an increased scleroglucan concentration. This fact may reflect that pH levels (i.e., 4.5) higher than those for optimal growth prompt the carbon flux toward biopolymer synthesis (Wang and McNeil, 1995c). In a similar way, the chosen process temperature is often a compromise between the optimal temperature for growth and the one for EPS production. A bi-staged process of temperature could also be adopted for the improvement of polysaccharide synthesis at a second stage (Wu et al., 2010). The use of dual-stage production processes have been also successfully applied to other microbial polysaccharides in order to achieve different optimal conditions, either for growth or biopolymer synthesis (Zheng et al., 2013).

Although batch cultures are usually adopted on an industrial scale, a *fed-batch process* with a stepwise addition of the C-source (and further nutrients) may often improve the final product concentration, thus eliminating any eventual substrate inhibition. The feeding of concentrated medium to the culture is commonly performed at a rate that prevents the carbon source from reaching the threshold value for catabolite repression (Spohr et al., 1998). In the case of scleroglucan from *S. glucanicum* NRRL 3006, this methodology has been reported to avoid the inhibitory effects of high (≥45 g/L) initial sucrose concentrations (Taurhesia and McNeil, 1994b). Supplemented batch cultures (at around 72 h) with additional sucrose after the initial growth phase overcame these difficulties while improving EPS production and its yield on C-source. The feeding strategy has also been reported for polysaccharides such as curdlan (Lee et al., 1997), gellan (Wang et al., 2006), scleroglucan (Survase et al., 2007a; Fosmer et al., 2010), ganoderan (Tang and Zhong, 2002), and *Saccharomyces cerevisiae* glucan (Kim and Yun, 2006).

METABOLIC AND GENETIC ENGINEERING AS A TOOL FOR INCREASING EPS PRODUCTION

A better understanding of the EPS biosynthesis regulation will be crucial to face a rational and not empirical optimization of polysaccharide production. Acquiring this information might be challenging, but essential to produce tailor-made EPSs with enhanced bioactivity and more attractive physicochemical properties (Angelova and Hunkeler, 1999). The advancement in practical tools for genetic manipulation of fungi would be the main ally for elucidating EPS biosynthetic pathways and their regulation (Seviour et al., 2011a).

At the moment, much is known about the physicochemical properties of scleroglucan and its applications. Conversely, up to a few years ago, there was a lack of information regarding its biosynthetic pathway and regulation, at both genetic and enzymatic levels. First discernments in this field were recently published by Schmid et al. (2010) who established the first sequence database for *S. rolfsii* ATCC 15205. These authors compared the gene expression and transcriptomes of *S. rolfsii* under conditions of either maximum or minimum scleroglucan production. Obtained data allowed them to predict the pathways for scleroglucan and oxalate synthesis and degradation, and led to the knowledge that metabolic pathways for scleroglucan and oxalate synthesis were not coupled to each other, as it was believed, but oxalate synthesis may be rather linked to biomass formation (Schmid et al., 2011).

The same group of researchers could also identify important unigenes putatively involved in determining scleroglucan yields, and found that almost all the genes supposed to be involved in scleroglucan synthesis, glycolysis, TCA, and glyoxylate cycles were not differentially transcribed under high- or low scleroglucan producing conditions. Their results further suggested that the regulation of polymer synthesis would be rather linked to mechanisms ensuring fungal survival under anoxic and other stress conditions (Schmid et al., 2011). The complete genome sequencing of scleroglucan-producing strains, along with metabolomics and proteomics inputs, would provide a real boost toward the optimization of scleroglucan production.

CONCLUDING REMARKS

With the advent of modern biotechnology, a new perspective for the use of fungi as generators of innovative products was completely opened up. Scleroglucan biopolymer belongs to these original products of fungal origin, and due to its versatility and unique properties it may find its way into numerous industries, such as oil, cosmetics, food, and pharmaceuticals. Microbial production of scleroglucan, both at lab and industrial scale, remains as one of the most multifaceted processes currently known. The optimization of scleroglucan production as well as solving its related obstacles will ensure the economic success of this development and its competitiveness. That will involve integrating the inputs from multiple disciplines such as microbiology, biochemical engineering, process engineering, statistics and genetics.

In this chapter we have reviewed much of the knowledge on scleroglucan production to date, particularly regarding the process design and optimization, and by examining both the available literature and our experience. There are still several hitches mainly regarding to the bioreactor design and process development for large-scale scleroglucan production, which are expected to be solved during the following years. Additionally, a deeper insight into the scleroglucan biosynthesis and its regulatory networks will also be crucial for the process optimization. This information, together with genetic engineering and transformation techniques, may allow modifying the expression of scleroglucan-biosynthesis related genes in order to divert more carbon flux toward polysaccharide production.

AUTHOR CONTRIBUTIONS

JF conceived the idea and established the general outlines. NC wrote the table draft and a major part of the manuscript. AV wrote a sub-section of the manuscript. JF prepared illustration panels and critically revised the intellectual content of the work. All authors read and approved the final version of the manuscript.

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REVIEW
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Present and future medical applications of microbial exopolysaccharides

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Microbial exopolysaccharides (EPS) have found outstanding medical applications since the mid-20th century, with the first clinical trials on dextran solutions as plasma expanders. Other EPS entered medicine firstly as conventional pharmaceutical excipients (e.g., xanthan – as suspension stabilizer, or pullulan – in capsules and oral care products). Polysaccharides, initially obtained from plant or animal sources, became easily available for a wide range of applications, especially when they were commercially produced by microbial fermentation. Alginates are used as anti-reflux, dental impressions, or as matrix for tablets. Hyaluronic acid and derivatives are used in surgery, arthritis treatment, or wound healing. Bacterial cellulose is applied in wound dressings or scaffolds for tissue engineering. The development of drug controlled-release systems and of micro- and nanoparticulated ones, has opened a new era of medical applications for biopolymers. EPS and their derivatives are well-suited potentially non-toxic, biodegradable drug carriers. Such systems concern rating and targeting of controlled release. Their large area of applications is explained by the available manifold series of derivatives, whose useful properties can be thereby controlled. From matrix inclusion to conjugates, different systems have been designed to solubilize, and to assure stable transport in the body, target accumulation and variable rate-release of a drug substance. From controlled drug delivery, EPS potential applications expanded to vaccine adjuvants and diagnostic imaging systems. Other potential applications are related to the bioactive (immunomodulator, antitumor, antiviral) characteristics of EPS. The numerous potential applications still wait to be developed into commercial pharmaceuticals and medical devices. Based on previous and recent results in important medical-pharmaceutical domains, one can undoubtedly state that EPS medical applications have a broad future ahead.

Keywords: exopolysaccharides, medical applications, pharmaceuticals, perspectives

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Introduction

Exopolysaccharides (EPS) are extracellular carbohydrate polymers produced and secreted by microorganisms, which accumulate outside the cells. They are capable to be released into the surrounding environment. Despite their monomeric composition, similar to well-known plant or animal products, the EPS of different microbial origin (bacteria and fungi) display a large variety of structural combinations, which mediates them their unique properties. Microbial production

shows several advantages over plant- or macro algae-derived products, such as defined and reproducible production parameters to circumvent environmental influences, and obtain a high quality of the final product. Additionally, much higher production titers can be obtained as compared to polysaccharides extracted from plants.

Obtained from easily available, renewable resources, biocompatible and apparently non-toxic, microbial EPS have found a very large field of applications, within which their medical uses play an important role.

This review study aims at achieving a useful insight in the domain, and at clearly distinguishing, in their historical development, the current commercial applications of EPS – officially acknowledged by worldwide accepted documents of medical authorities, from their promising potential applications – discussed in numerous publications. Such a valuable knowledge was updated and organized according to modern research directions in pharmaceutical science and therapy. The presented data highlight a real outlook and the necessary steps to enhance the efficiency and maximal exploitation of the scientific progress recorded in the EPS field up to date.

Current Commercial Applications

Only a few microbial polysaccharides have found up to date commercial applications. Amongst them, **dextran**, a neutral polymer with α-(1⟶6) and α-(1⟶4) glucopyranosyl linkages was, discovered in wine in mid of the 19th century. Dextran could be considered the first remarkable example for a microbial EPS used in pharmaceutical applications (Nwodo et al., 2012). It was used as a plasma volume expander for controlling wounds shock since 1953 (Amspacher and Curreri, 1953; United States Pharmacopeia [USP] – National Formulary [NF], 2012; European Pharmacopeia [EP], 2014).

Further microbial EPS were employed in medical applications as pharmaceutical excipients, after they were approved as food additives. **Xanthan**, a bacterial branched anionic heteropolysaccharide composed of a five sugar repeating unit and different amounts of acetate and pyruvate, was discovered in 1950 (Born et al., 2002). Firstly it was used in large quantities for enhanced oil recovery, and later on was approved as a food additive in the USA (1969), by FAO/OMS (1974). In Europe, xanthan was approved as food additive as E415 (198 2), with subsequent inclusion in US and EU pharmacopeias (Born et al., 2002; United States Pharmacopeia [USP] – National Formulary [NF], 2012; European Pharmacopeia [EP], 2014). Similarly with food products, its properties as a thickener and suspension stabilizer are useful in pharmaceutical creams and suspensions and, recently, it has been used as a drug controlled release carrier (Morris and Harding, 2009).

Alginate, originally obtained by extraction from seaweeds, was discovered as a bacterial product in 1964, only differing from the seaweeds extraction product by the presence of acetyl groups in the linear structure of β-(1⟶4) mannuronic and α-(1⟶4) guluronic acid (Cyber Colloids Ltd.). This anionic polysaccharide is a good disintegrating agent in tablets (better than starch), a thickening and stabilizing agent in pharmaceutical suspensions and emulsions, as well as an antiacid stomach protector in capsules, as a sodium salt (Mc Hugh, 1987). Bacterial alginate has been also employed in cell microencapsulation, as microsphere vectors for drug delivery (Mukherjee and Atala, 2005; Nwodo et al., 2012). Five alginates were approved by FDA in 1973. Sodium alginate found its monographs in US and EU pharmacopeias (United States Pharmacopeia [USP] – National Formulary [NF], 2012; European Pharmacopeia [EP], 2014). Dental impression compounds are based on alginate cold-setting gels. Alginate fibers can be used as wound dressings and bandages with hemostatic properties, approved by FDA for human use (Mc Hugh, 1987; Mukherjee and Atala, 2005; Nwodo et al., 2012).

Gellan was discovered in 1978, as a bacterial anionic linear heteropolysaccharide, with a repeating unit of α-rhamnose, two residues of β-D-glucose and β-D-glucuronate. The native form contains acyl (acetyl and glyceryl) substituents. The acetyl groups can be easily removed by alkaline hydrolysis to lower the acyl content if necessary. Nineteen oral, 16 ophthalmic and six nasal drug formulations with a very large spectrum of therapeutic action are cited between 1993 and 2013 (Osmalek et al., 2014). Some of them became commercial medical products, determining the inclusion of low and high acyl forms of gellan in US pharmacopeia (United States Pharmacopeia [USP] – National Formulary [NF], 2012). Gellan has been FDA approved as a stabilizer and thickener in food since 1990. A low acyl form is used in solid dosage formulations, as a disintegration agent in immediate release tablets, or, in higher concentrations, as a matrix-forming excipient in sustained release, based on its swelling behavior (Osmalek et al., 2014; CP Kelco). In physiological ion concentrations, it forms *in situ* strong gels (Hagerstrom, 2003; Osmalek et al., 2014). In ophthalmic preparations, core gellan gum hydrogel showed a prolonged contact time (ocular residence) and enhanced bioavailability. Thus, ophthalmic controlled release anti-glaucoma preparations containing commercial low acetyl gellan are marketed under the trade name of Timoptic XE, or Blocadren depot (Merck, Co.; Felt et al., 2002; Hagerstrom, 2003).

Pullulan seems to be the single commercially produced EPS of fungal origin, with marketed pharmaceutical applications. It was discovered in 1938, but mostly studied after its description in 1959. This biopolymer is a neutral linear homopolysaccharide, consisting almost of regularly repeating α-(1⟶4) – maltotriosyl units (3-D-glucopyranosyl) joined through α-(1⟶6). Its present applications as a pharmaceutical ingredient are based on its distinct binding and film-forming properties, as well as on its strong oxygen impermeability. Such properties make it very suitable for granulation and coating tablets, non-animal capsules (Plantcaps-Capsugel, Inc.), oral and wound care products (e.g., Listerine – Pfizer; Mocanu et al., 2011b; Nagase Group; Tianjin SF–Bio). Approved firstly

as a food ingredient in Japan (1976), USA-FDA (2002), Europe, as E1204, and China (2006), pullulan has been lately included in US, EU (United States Pharmacopeia [USP] – National Formulary [NF], 2012; European Pharmacopeia [EP], 2014) and Japan Pharmacopeias (Japan Pharmacopeia [JP], 2011).

Hyaluronic acid was discovered in 1934 and with its chemical structure established in the 1950s. Hyaluronic acid is an anionic linear polysaccharide belonging to the glycosaminoglycans, composed of disaccharide units consisting of (1⟶4) – β-linked-D-glucuronic acid and (1⟶3) – β-linked *N*-acetyl-D-glucosamine residues (Chong et al., 2005; Kogan et al., 2007; Necas et al., 2008). As sodium hyaluronate (hyaluronan), it plays important physiological roles in living organisms, including the human body. Its first medical application, maintained until now, was as a vitreous substitution/replacement during eye surgery in the late 1950s (Necas et al., 2008). Originally obtained by animal tissue extraction, especially from rooster coombs, it is now produced by recombinant bacteria. The bacterial product was firstly approved only for topical issues: chronic, difficult wound healing, e.g., Hyiodine, produced by Contipro (Contipro Pharma), and cosmetic applications. Lately, medical devices containing bacterial hyaluronan have been approved for use in eye surgery and intraarticular injections in osteoarthritis – e.g., Biolon – approved by Medical Device Certification-European Commission in 1995, FDA-USA in 1998, EUFLEXXA – approved by FDA-USA in 2011 (both produced by Ferring Pharmaceuticals – BTG), DUROLANE (EC-2004), produced by Bioventus (Bioventus). A sustained-release formulation of recombinant human growth hormone (SR-rhGH, DeclageTM, LG Life Sciences, Korea) using sodium hyaluronate microparticles was developed for administration on a weekly basis, being approved by Korean FDA in 2007. Recent clinical studies confirmed its efficacy and safety (Kim et al., 2014). Sodium hyaluronate has got a monograph in European Pharmacopeia (European Pharmacopeia [EP], 2014). A summary of the EPS mentioned above, including their medical applications is given in **Table 1**.

Potential Applications

An impressively large volume of publications have been dedicated to the potential medical applications of EPS, considering not only their number, but also their diversity. The present paper aims at grouping such works in an accessible

TABLE 1 | Microbial exopolysaccharides with acknowledged commercial medical applications.

EPS	Monomer composition	Main producing microorganism	Applications	Reference
Bacterial				
Dextran	Glucose	*Leuconostoc mesenteroides*	Blood plasma volume expander (controlling wound shock)	Amspacher and Curreri, 1953; Nwodo et al., 2012; United States Pharmacopeia [USP] – National Formulary [NF], 2012; European Pharmacopeia [EP], 2014
Xanthan	Glucose (2), mannose (2), glucuronic acid, acetate, pyruvate	*Xanthomonas campestris*	Thickener, suspension stabilizer in pharmaceutical creams and suspensions Controlled release carrier	Born et al., 2002; Morris and Harding, 2009; United States Pharmacopeia [USP] – National Formulary [NF], 2012; European Pharmacopeia [EP], 2014
Alginate	Mannuronic acid, guluronic acid, acetate	*Azotobacter vinelandii, Pseudomonas aeruginosa*	Disintegrating agent in tablets; thickener, stabilizer in pharmaceutical suspensions and emulsions; dental impressions; antiacid (anti-reflux) stomach protector; microspheres for drug delivery; fibers in wound hemostatic dressing and bandages	Mc Hugh, 1987; Mukherjee and Atala, 2005; Nwodo et al., 2012; United States Pharmacopeia [USP] – National Formulary [NF], 2012; European Pharmacopeia [EP], 2014; Cyber Colloids Ltd.
Gellan	Glucose, rhamnose, glucuronic acid, glycerate, acetate	*Sphingomonas paucimobilis* (formerly *Pseudomonas elodea*)	Excipient in oral, ophthalmic and nasal drug formulations, for: tablet disintegration, sustained/controlled release	Felt et al., 2002; Hagerstrom, 2003; United States Pharmacopeia [USP] – National Formulary [NF], 2012; Osmalek et al., 2014; CP Kelco
Hyaluronic acid/hyaluronan	Glucuronic acid *N*-acetyl-glucosamine	*Streptococcus equisimilis/zooepidemicus; Bacillus subtilis* (recomb.*Str. equisimilis*)	Chronic, difficult wound healing; osteoarthritis treatment (intraarticular injection); eye surgery (vitreous substitution/replacement)	Kogan et al., 2007; Necas et al., 2008; Food and Drug Administration-Premarket Approval Application [FDA-PMA], 2011; European Pharmacopeia [EP], 2014; Bioventus; Contipro Pharma
Fungal				
Pullulan	Maltotriose	*Aureobasidium pullulans*	Tablet granulation and coating, binder, and oxygen impermeable film forming, non-animal capsules, oral, and wound care products	Japan Pharmacopeia [JP], 2011; Mocanu et al., 2011b; United States Pharmacopeia [USP] – National Formulary [NF], 2012; European Pharmacopeia [EP], 2014; Nagase Group; Tianjin SF–Bio

manner, to obtain a relevant and suggestive image of the field.

Drug/Hydrogel Controlled Release Systems

Micro- and Nanosystems for Sustained Delivery

Polysaccharides and, among them, EPS, have become highly promising materials in the field of "intelligent drug delivery systems" or "intelligent therapeutics" (Caldorrera-Moore and Peppas, 2009; Patel et al., 2011). The ability of such hydrophilic polymers to form hydrogels, cross-linked 3D network structures retaining a large amount of water while remaining insoluble, makes them very useful as drug carriers. Thus, as macro- and especially nanoparticles, such gels can entrap drugs or biomolecules into their inner structures and/or adsorb the therapeutic molecules onto their external surfaces and penetrate cells and tissue gaps to arrive at target organs. Having reached them, the drug delivery systems can show different drug rate-release properties due to their various characteristics, including bioadhesion, biodegradability, pH, ion and/or temperature sensitivity. In this way, they can prolong drug residence and, therefore, its therapeutically usable fraction, thus increasing its bioavailability and permitting lower doses with consequently reduced toxic effects (Liu et al., 2008). Natural polysaccharides have a large number of reactive groups, a wide range of molecular weights and different chemical composition, leading to highly diverse structures and properties; additionally, they are biodegradable, non-toxic and safe.

Hydrogels with thermo-, pH-, and cation-sensitive drug-delivery properties

Pullulan has been intensively studied as a drug carrier in pharmaceutics, particularly because of its neutral nature with nine hydroxyl groups on the repeating unit conferring to it a special availability to chemical derivatization. By cross-linking and grafting on the backbone, poly-(*N*-isopropyl-acrylamide)-co-acrylamide and ether succinic carboxyl groups, thermo- and pH-sensitive microspheres were obtained. With lysozyme as the protein drug model, both simple and electrostatic (ionic) entrapment and pH/thermo sensitive release were achieved (Fundueanu et al., 2008). Pullulan/silver nanoparticles composite nanospheres with controlled spherical structure showed to enhance antibacterial activity (Kumar et al., 2012). Silver-containing nanoparticles have been also obtained by partial oxidation of pullulan (carboxylation; Coseri et al., 2015). Carboxymethyl pullulan, grafted with polyether-amine, as the thermo sensitive part of the copolymer, exhibited a sol-gel transition at body temperature, assuring sustained drug-delivery (Dulong et al., 2012). Such polymers, containing carboxymethyl pullulan, cross-linked to thermo- and pH-sensitive hydrogels, gradually retained the antioxidant biomolecules – lutein and α-tocopherol – and showed scavenging activity (Mocanu et al., 2012). *In situ* rapid cation-induced gelation of gellan favored, the epithelial uptake (at a residence time shown *in vivo* as long as 4 h), and transfer of a model substance demonstrated the use of gellan as a promising strategy for nasal drug delivery, thus avoiding a slow and reduced absorption of some drugs by oral administration (Hagerstrom, 2003).

Amphiphilic EPS, as controlled delivery systems

Grafting of hydrophobic segments onto the hydrophilic polymeric backbone leads to amphiphilic polymers which form self-associate thermodynamically stable nanogel structures, with an inner hydrophobic core. Stable in size over time, such polymeric micelles have been recognized as promising drug carriers, the hydrophobic core-shell structure being able to trap hydrophobic drugs (Liu et al., 2008; Bataille et al., 2011; Jiang et al., 2013). In this respect, the most cited paper was an early work from 1993 on the self-assembly of cholesteryl-bearing pullulan (CHP), forming stable hydrogel nanoparticles, which represented the starting point for future perspective drug-release complexes (Bataille et al., 2011). Thus, self-aggregated nanoparticles of cholesterol-modified pullulan with succinyl linkages were loaded with mitoxantrone (as model anticancer) and exhibited sustained release (Yang et al., 2010).

Other cross-linking microspheres obtained from carboxymethyl pullulan became more adsorptive by their ionic and hydrophobic affinity for lysozyme. After hydrophobization with palmitoyl the release rate was also controlled (Mocanu et al., 2004).

Interpenetrating polymer networks (IPNs)

Interesting potential medical applications may be realized by the hydrogels of polysaccharides. Such "smart" systems, consisting of penetrating polymers at molecular scale, promise to be perspective drug-carriers. Thus, interpenetrating polymer network (IPN) microspheres of alginate and synthetic or natural polymers showed entrapping and sustained release properties of different drug substances: anticancer 5-fluorouracyl, anti-inflammatory indomethacin, antibiotic gatifloxacin, anticoagulant heparin, NSAID sulidac, as well as intestinal release of the poorly soluble anti-hypertensive pindolol (Matricardi et al., 2013).

Beads of gellan gum/pectin mixture, obtained by ionotropic gelation, using Al^{3+} as crosslinker, showed suitable entrapment and mucoadhesive properties for enteric *in vitro* controlled release of anti-inflammatory ketoprofen (Prezotti et al., 2014).

Poly (ε-caprolactone)-grafted dextran nanoparticles entrapped and exhibited *in vitro* sustained release of amoxicillin antibiotic (Saldias et al., 2015).

Steroid hormones, as important drug-substances, whose administration may be improved by sustained delivery, are almost absent in latest EPS research. Micro- and nanoparticles containing such compounds in transdermal drug delivery systems can avoid many of the side effects associated with first-pass metabolism, gastrointestinal absorption and high plasma levels after oral administration, reaching clinically significant concentrations at lower doses. Studies on their pharmacokinetics, bioequivalence, local tolerance, and adhesion performance are necessary.

Another suggestion involves grafted block copolymers of only EPS, not considered in recently published research results.

Drug-Targeting, Macromolecule, and Cell Carriers

Drug-targeting

Polysaccharide conjugates, especially the amphiphilic ones, with different hydrophobization degrees, showed particular binding and penetrating features to cellular receptors, appearing as promising drug-targeting carriers.

Thus, cholesteryl-pullulan nanoparticles can hydrophobically interact with the beta oligomer forms of beta-amyloid, significantly reducing its toxicity, which appears as a possible complementary approach in neurologic disorders with formation of soluble toxic aggregates, e.g., in Alzheimer disease (Boridy et al., 2009).

Anticancer drug-targeting

Self-assembled nanoparticles of carboxymethyl curdlan, a known (1⟶3)-β-glucan, hydrophobized by deoxycholic residues, physically loaded with anticancer epirubicin, increased the drug uptake in tumors and decreased it in kidney and heart on tumor-bearing mice, showing a sustained release pattern and a tumor volume reduced by 70% (Gao et al., 2010). Other hydrophobized polysaccharide – bile acid conjugate (hyaluronic-cholanic acid) nanoparticles showed a receptor-mediated *in vitro* and *in vivo* preferential accumulation, in murine carcinoma SCC7 cells (Choi et al., 2009). Hyaluronic acid-coated solid lipid (freeze-dried) nanoparticles showed *in vitro* and *in vivo* a receptor-mediated, sustained and targeted delivery of anticancer vorinostat to SCC7 carcinoma, human breast adenocarcinoma (MCF-7) and human lung epithelial adenocarcinoma (A549; Tran et al., 2014).

Recently, a novel gellan nanohydrogel system was developed, for simultaneously carrying two poorly water soluble drugs (physically entrapped paclitaxel and chemically linked prednisolone) to achieve target delivery of such anticancer and anti-inflammatory drugs. Promising *in vitro* results – induced by their synergistic effect – on three types of cancer cells, were noticed (D'Arrigo et al., 2014).

Transdermal drug delivery is an important pharmaceutical alternative for drug-targeting in skin diseases. Hyaluronic acid nanoemulsions showed an efficient selective *in vitro* and *in vivo* controlled-release of methylene-dioxycamptothecin, an inhibitor of keloid dermal tumors (Gao et al., 2014).

Curcumin-loaded hydrogels of xanthan-plant galactomannan mixture were effective in skin-diseases, as anti-inflammatory and antioxidant drugs, which may recommend them as a promising alternative in the treatment of skin-cancer and psoriasis (Koop et al., 2015).

Biolabile prodrug compounds (releasing the drug by micro flora enzymatic glycosidase hydrolysis of the gels selected from dextran, pullulan, and alginates), or cross-linked dextran hydrogels showed colon-specific drug delivery (Vandamme et al., 2002). Another prodrug conjugate, the hyaluronic acid-methotrexate, an anti-inflammatory substance, optimized the treatment of osteoarthritis, showing *in vivo* effect on rats (Homma et al., 2010).

A very promising, still non-explored field is the use of properly functionalized EPS as drug-carriers, to cross the blood-brain barrier for treating tumors or other neurological diseases.

Recombinant macromolecular biopharmaceuticals

Proteins, peptides, small-interfering RNA (siRNA), vaccines and hormones – represent a rapidly growing class of modern therapeutics, superior to small drugs for serious and deadly diseases, as well as for diagnostics. However, they have difficulties in crossing mucosal surfaces and biological membranes, due to their susceptibility to lose the native structure and to be rapidly cleared in the liver or other body tissues. Polymeric hydrogels used as carriers could diminish their instability and improve bioavailability, permitting other routes of administration, apart from the frequently injectable: pulmonary, oral, nasal ones (Ganguly et al., 2014).

In this respect, special importance is paid to insulin, a long-term treatment drug of *diabetes mellitus*. Nanoparticles of cholesterol-bearing pullulan preserved insulin from enzymatic degradation, its activity being unchanged after i.v. injection (Akiyoshi et al., 1998). Insulin was 85% electrostatically associated to nanoparticles of dextran sulfate/chitosan, with a 24 h sustained release in a simulated intestinal medium, suggesting a possible oral delivery (Sarmento et al., 2006). Vitamin B12-derivatives-coated dextran nanoparticles encapsulated more than 65% insulin and demonstrated a prolonged hypoglycemic action on diabetic mice and rats, after oral administration (Chalasani et al., 2007).

Recombinant human growth hormone-Zn^{2+}/dextran nanoparticles preserved 99% of hormone bioactivity (Yuan et al., 2012). Lysozyme, as a model protein, was highly retained and well-released on pH and thermo sensitive (Poloxamer grafted) carboxymethyl pullulan micro particles (Mocanu et al., 2011a).

The hyaluronic acid-gold nanoparticle/interferon α complex showed target specificity and prolonged delivery in the murine liver tissue, leading to superior immune responses than the known interferon α preparation for the treatment of hepatitis C virus infection (Lee et al., 2012). Nowadays, successful non-interferon therapeutics diminishes the importance of such systems.

Vaccines

Exopolysaccharide could be useful as antigen-carriers or as antigens themselves in vaccine preparations. Tetanus toxoid (anatoxin) was entrapped and efficiently released on Poloxamer hydrophobized carboxymethyl pullulan micro particles (Mocanu et al., 2011a). Intranasal immunization with alginate-tetanus toxoid microparticles resulted in a strong immunoresponse in rabbits. Esters of the hyaluronic acid loaded with hemagglutinin influenza H1N1 were also effective after intranasal administration in mice, rabbits, micro-pigs (Sharma et al., 2009). Curdlan sulfate enhanced antigen-specific immunity in mice immunized with human recombinant hepatitis B protein, appearing as a promising vaccine adjuvant (Li et al., 2014). Purified by ultrafiltration, type B capsular polysaccharide produced by *Haemophilus influenzae*, which was linked to a protein, became a component of polyvalent vaccines against severe infections in children (e.g., meningitis; Albani et al., 2015; De Oliveira Cintra and Takagi, 2015).

As novel adjuvant systems, EPS could enhance vaccine-induced protection, providing a strong tailored and immune

response, especially targeting challenging pathogens, such as new influenza pandemic strains (e.g., H1N1, parasites (malaria), highly variable viruses (hepatitis C, AIDS), or resistant mycobacteria (tuberculosis). Another direction is represented by antigen-specific cancer immunotherapy, stimulating an immune response to reject the tumor.

Gene delivery

Non-viral vectors, e.g., polymers, are preferred to deliver nucleic acid materials, to improve the transport and avoid degradation by lysosomal enzymes. Plasmid encapsulation in pullulan nanoparticles demonstrated a successful internalization (Gupta and Gupta, 2004). Cationic dextran derivative nanoparticles, loaded with gene silencing siRNA, achieved an effective transfection in hepatoma Huh-7 cells, by association with a photosensitizer (Raemdonck et al., 2010). The same genetic material was also transferred by PEG-ylated dextran to Huh-7 and A 431 human epithelial carcinoma cells (Naeye et al., 2010). Cationic siRNA loaded 6-amino-deoxicurdlan efficiently transfected human lung H727 and human colon HCT116 cancer cell lines, human leukemia monocyte THP-1 cell-derived macrophages, as well as mouse primary and stem cells (Han et al., 2015). Other gene-delivery studies, including pDNA with EPS derivatives, mostly amino-modified, e.g., gellan, alginate, schizophyllan and scleroglucan, fungal β-(1⟶3)-glucans with β-(1⟶6)-glucose side chains, were also cited (Khan et al., 2012; Zhang et al., 2013).

Using EPS as gene delivery vectors is a recent potential application which should be confirmed by *in vivo* studies, as future research depends on gene therapy development in cancer.

Cell encapsulation

This technology is considered protecting transplanted cells from hostile immune reactions of the body, assuring at the same time the permeation of nutrients and secreted proteins. The transplantation of encapsulated cells has been considered a promising treatment for a variety of diseases (e.g., diabetes, liver failure; Rokstad et al., 2014). To make able the 3D structure of bacterial cellulose (BC) nanofibers to form a cross-linked alginate-based composite with improved mechanical and chemical stability, BC, produced by *Gluconacetobacter xylinus* fermentation, was oxidized at C-6 in the NaBr/NaClO/TEMPO (tetramethyl-piperidine-1-oxyl) system as a catalyst. The C-6-carboxyl-cellulose-sodium alginate beads successfully encapsulated fibroblast cells. Such composite is considered a candidate to encapsulate cells forming islets to activate insulin secretion (Park et al., 2015).

Obtaining encapsulated cells within a three-dimensional hydrogel layer is considered as a new direction in microscale tissue engineering (Evans et al., 2006).

Wound Healing and Tissue Engineering

Wound healing-skin repair

Bacterial cellulose

Bacterial cellulose is produced by bacterial fermentation with controllable 3D structure, based on strains selection or cultivation parameters (Lin et al., 2013). The resulting non-water soluble nanofibril network shows high similarity to that of collagen and has an outstanding biocompatibility (Torres et al., 2012). The applications of BC are in the field of wound healing-skin repair, as absorbent of exudates as well as permeable material. Compared to plant cellulose, BC possesses a high crystallinity, a high water absorption capacity, as well as high resistance to microbial or enzymatic degradation. Its properties as a wound healing and tissue engineering scaffold could be diversified and improved according to specific aims, by chemical derivatization or association of other synthetic, mineral (e.g., hydroxyapatite) substances, biopolymers (e.g., hyaluronan, alginate, gellan, carrageenan, chitosan, collagen, gelatin, elastin), or cell-growth factors (Torres et al., 2012; Fu et al., 2013). Clinical trials with BC-dressings on acute and chronic wounds (e.g., diabetic foot ulcers) showed superior results than similar dressings from plant-cellulose (Fu et al., 2013). BC-glycerin membranes with a clinically proved moisturing effect could be relevant for dryness induced by skin diseases, such as psoriasis and atopic dermatitis (Almeida et al., 2014). Electrospun acetylated BC presented a more symmetric nanopore structure than that of the casting films, suitable for cell integration and adhesion (Costa et al., 2012). Dressings with hydrogels of BC/acrylic acid synthesized by electron beam-irradiation accelerated burn wound healing in rats (Najwa et al., 2014).

Tissue engineering

Heart tissue engineering aims at designing structures to support, repair, replace, or enhance the function of injured or diseased myocardial tissues, especially as caused by infarction. In this respect, alginate, pullulan, dextran, hyaluronan have been intensively studied (Silva et al., 2015). Photopolymerizable hyaluronic acid-methacrylic anhydride (HA-MA) hydrogels mimicked the extracellular matrix in heart valve applications (Aravamudhan et al., 2014). Alginate-based hybrid copolymers with poly(propylene) fumarate, morphologically modified by covalent linking with acrylates, acquired improved mechanical properties. One of them was described to promote cardiomyoblast growth, recommending it for potential applications in cardiac tissue engineering (Thankam and Muthu, 2014).

Schiff-base formation between oxidized dextran (aldehyde) and poly-L-lysine led to a potential bioadhesive in surgery (Matsumura et al., 2014). Carboxymethyl pullulan-heparin conjugates were developed and studied for tissue engineering applications (Mishra et al., 2011). Electrospun gelatin nanofibers cross-linked with oxidized dextran (aldehyde) demonstrated good scaffold properties for L-926 fibroblasts (Jalaja et al., 2014). Alginate scaffolds were effective for chondrocyte culture stem cells, as a promising solution for cartilage regeneration, combating osteoarthritis and arthroplasty. If they contain chitosan, calcium, or are impregnated with antibiotics, they could enhance the antibacterial and wound healing effect of bandages (Ivanova et al., 2014). Alginate materials have been successfully used in tissue-engineering of bioartificial pancreas, bone, vasculature, and liver cell cultures (Aravamudhan et al., 2014). Some new EPS produced by bacteria isolated from

extreme marine environments showed promising properties for tissue engineering in bone healing (Mancuso Nichols et al., 2005).

In tissue engineering, special interest is paid to stem cells and the design of bioactive nanopatterned scaffolds of different polymeric materials, including EPS, with specific ligands that direct and enhance cell function and differentiation of embryonic stem cells (Evans et al., 2006).

Diagnostics

Polysaccharide-coated nanoparticles used in diagnostics (e.g., quantum dots, magnetic materials, such as iron oxide) could play a key role in medical imaging and also in theranostics (diagnosis and therapy). Specific ligand groups could be attached to the biopolymer, such as amino. Cholesterol pullulan modified by amino groups showed a higher intensity of fluorescence in tumor cells, comparatively with conventional quantum-dots-liposomes (Bataille et al., 2011; Mishra et al., 2011; Prajapati et al., 2013). A complete platform of super paramagnetic iron oxide nanoparticles with cross-linked dextran coating (CLIO) – containing large series of multifunctional imaging agents for diagnostic magnetic resonance (DMR), magnetic resonance imaging (MRI), positron emission tomography (PET) imaging, fluorescence molecular tomography (FMT) – has been developed. As a theranostic agent, CLIO could be used with a photosensitizer in photodynamic therapy, killing atheroma cells in carotid arteries through irradiation (Tassa et al., 2011). A theranostic effect was obtained with acetylated pullulan-coated magnetic nanoparticles, killing 80% of the KB tumor cells by magnetic field-induced hyperthermia (Bataille et al., 2011; Mishra et al., 2011).

Theranostic application of such diagnostics implies repeated dose administration, depending essentially on the metabolism and elimination of iron particles.

Bioactive EPS as Potential Therapeutics

Fungal β-Glucans

Some EPS showed biological activities which promote them as potential therapeutics. A special interest in this view has attracted β-glucans. Generally, the immunomodulation effect of β-glucans is due to their interactions with macrophage receptors, activating these cells as basic effectors in host defense against bacteria, viruses, parasites, and tumor cells (Novak and Vetvicka, 2009). Therefore, from the early 90s on, the backbone of β-(1⟶3) glucans has been considered as essential for their antitumor activity, based on the Sarcoma tumor inhibition by sulfoalkyl-curdlan (Demleitner et al., 1992), as well as for their immunopotentiating, antibacterial, or antiviral activity (Kulicke et al., 1997). β-glucans have shown varying activity against sarcomas, mammary cancer, some chemically induced cancers, colon cancer, and some leukemia (Laroche and Michaud, 2007).

Schizo- and scleroglucans

The most important representatives of fungal beta-glucans are schizophyllan (SPG) and scleroglucan (SG), neutral EPS produced by *Schizophyllum commune* (Zhang et al., 2013) and *Sclerotium rolfsii*, respectively (Survase et al., 2007). They are composed by a β-(1⟶3)-D-glucopyranose backbone which is branched with a single β-(1⟶6)-D-glucopyranose residue at every third glucose unit. Single or associated with chemotherapeutics, these EPS and their derivatives showed promising antitumor (in sarcoma, carcinomas, bladder tumor, fibro sarcoma, mammary carcinoma, leukemia) and immunopotentiator activities. SPG has been approved for clinical studies in Japan (Daba and Ezeronye, 2003; Zhang et al., 2013). SSG, produced by the *Sclerotinia sclerotiorum* fungus, whose structure is similar to that of schizophyllan, yet more branched (a glucose residue occurs at every two β-1, 3-glucosyl units) showed antitumor activity on Sarcoma 180 in mice (Ohno et al., 1986).

The β-(1⟶3) backbone, as mentioned above, but the β-(1⟶6)-glycosidic linked branches structures, too, appear as important for antitumor activity.

Botryosphaeran

Represents another β-(1⟶3, 1⟶6)-glucan (1⟶3 backbone, 1⟶6 branched glucose and gentiobiose) and is produced by *Botryosphaeria rhodina*. Botryosphaeran showed anticlastogenic activity *in vivo* (mice) after cyclophosphamide (Miranda et al., 2008). It also exhibited antidiabetic (reducing plasma glucose level in streptozocin-induced diabetic rats by 52%) and hypocholesterolemic activities (total and LDL cholesterol reduced until 27% in hyperlipidemic rats; Miranda-Nantes et al., 2011). Chemical modifications increased biological activity (Kagimura et al., 2015). The sulfonated derivative induced *in vitro* a dose-dependent anticoagulant and antithrombotic activity (Mendes et al., 2009).

Lasiodiplodan

Exopolysaccharide, a β-(1⟶6)-D-glucan produced by *Lasiodiplodia theobromae*, showed anti-proliferative activity in breast cancer MCF-7 cells (Alves da Cunha et al., 2012), whereas its sulfonated derivative exhibited anticoagulant and antithrombotic activity similar to that of heparin (Vasconcelos et al., 2013).

A hetero-EPS (mainly mannose, glucose, galactose, xylose) produced in submerged cultivation of the *Pycnoporus sanguineus* fungus exhibited *in vitro* antioxidant activity (Cao et al., 2014). Similar results were obtained with a *Hirsutella* sp. fungus (neutral EPS containing mannose, glucose, galactose), proving the importance of the mannose content (Meng et al., 2015). A fraction of α- and β-EPS (mainly composed of mannose, glucose, and talose), produced in a submerged culture by the Chinese medical fungus *Inocutis tamaricis,* exhibited *in vitro* antioxidant and antitumor (Hep G2 cells) activities (Zheng et al., 2014).

Antitumor activity of EPS is immune-mediated (immunotherapy) by the activation of the T and B defender cells against cancer cells. Although their action is slower than that of traditional therapies (chemotherapy, radiation), it is nevertheless specific, adaptable and durable. Thus, immunotherapeutic EPS could be valuable prophylactic and synergic anticancer agents.

Bacterial EPS

Lactic bacterial EPS

One group of bioactive EPS is represented by those produced by lactic bacteria (LAB). A 2-substituted β-(1⟶3)-D-glucan produced by *Pediococcus parvulus* 2,6 and a recombinant *Lactococcus lactis* showed immunomodulation by human macrophage activation *in vitro*, promoting the production of anti-inflammatory cytokines (Notararigo et al., 2014). A similar effect was noticed with a neutral hetero-EPS (glucose, rhamnose, galactose) produced by a *Lactobacillus paraplantarum* strain in an *in vitro* study as a probiotic agent (good adherence to intestinal mucosa; Nikolic et al., 2012). Other EPS of LAB species (e.g., *Weissella cibaria*), isolated from the gastrointestinal tract of marine fish, showed a prebiotic *in vitro* activity, stimulating probiotics (e.g., bifidobacteria; Hongpattarakere et al., 2012).

A β-D-(1⟶4) with 1:2 β-D-(1⟶6) branched glucan produced by an isolated Chinese *Rhizobium* showed immunopotentiating and antitumor activities in mice with sarcoma 180 (S180), hepatoma 22 (H22) and Ehrlich ascites carcinoma (EAC; Zhao et al., 2010).

Bioactive xanthan derivative

Xanthouronan, obtained by C-6 oxidation of xanthan with a NaOCl/NaBr/TEMPO catalyst, exhibited *in vitro* antioxidant activity (Delattre et al., 2015).

Hetero-EPS produced by nature isolated *Enterobacter* bacteria strains, composed of fucose, glucose, galactose, glucuronic acid, pyruvate, succinate, acetate (Freitas et al., 2011b; Huang et al., 2015) showed hypoglycemic and hypolipidemic activities in type 2 diabetic mice (Huang et al., 2015). The signaling pathways of action and the corresponding biomarkers – still under study – are probably similar to plant extraction active polysaccharides.

Levans represent an EPS group of β-(2⟶6)-D-fructans with some β-(2⟶1)-branches synthetized from sucrose by levansucrase, produced by several bacteria, including species of *Bacillus*, *Zymomonas*, *Halomonas*, *Pseudomonas*, *Rahnella*, *Aerobacter*, *Erwinia*, *Streptococcus*, *Microbacterium* (Freitas et al., 2011a; Esawy et al., 2012; Öner, 2013; Srikanth et al., 2015). The levan produced by a *Halomonas smyrnensis* strain, especially its oxidized (aldehyde) derivative, showed *in vitro* anti-cancer activity against human lung (A549), liver (Hep G2/C3A), gastric (AGS), breast (MCF-7) cancer cell lines (Sarilmiser and Öner, 2014). Acetyl, phosphoryl, and benzyl esters of levan EPS, produced by a strain of *Paenibacillus polymyxa,* exhibited antioxidant and anti-proliferative activities against human gastric BGC-823 cancer cells *in vitro* (Liu et al., 2012; Zong et al., 2012). The levan produced by *Bacillus licheniformis* exhibited hypoglycemic and antioxidant activities, enhanced enzymatic defense, protecting the main organs in alloxan-induced diabetic rats (Dahech et al., 2011). The levans produced by honey isolated *Bacillus subtilis* strains showed antiviral activity on avian influenza and type 40 adenovirus (Esawy et al., 2012). The anti-AIDS activity of levan was also noticed (Srikanth et al., 2015).

Antiviral activity, enhanced by sulfation, of other bacterial EPS, was also noticed earlier (Matsuda et al., 1999; Laroche and Michaud, 2007), as due to the interference with viral attachment and penetration/infection of cells, probably by interacting with viral envelope glycoproteins. Recent results, obtained by surface plasmon response technology, confirmed the interaction, curdlan sulfate binding the recombinant human hepatitis B viral protein, with increased affinity toward the sulfation degree (Li et al., 2014).

Perspectives/Conclusion

Microbial EPS offer a very large field of medical applications, increasingly exploited, yet at a too slow rate, if considering their obvious advantages, some of them induced by their unique properties.

As to the solid dosage forms and rate-controlled release systems, they compete with cheaper natural polymers, as plant cellulose and starch derivatives, specific niches (intelligent therapeutics) being still to be established, along with their cost-effectiveness, by means of new, performant technologies.

As drug-targeting and carriers, EPS nanoparticulate systems present certain advantages over those of chemical synthetic origin, such as biocompatibility and apparent lack of toxicity. However, they are not excluded from the mandatory non-clinical pharmacotoxicology studies, as the chemical modifications undergone by natural EPS still have to be carefully analyzed. In this stage of research, the most important challenge, common to all nanopharmaceuticals, is to demonstrate their *in vivo* bioavailability for assuring a suitable drug exposure, by passing from the discovered particular effects to well-established research protocols of absorption, distribution, metabolism and elimination (ADME), drug metabolism and pharmacokinetics (DMPKs). In this respect, recent developments in EPS nanoparticle medical imaging systems could be very useful, having also higher chances to be approved as diagnostics. Obviously, based on all the above-mentioned considerations, the external administration forms with topic action (e.g., wound healing, skin-repair) are likely to be more rapidly approved for and to pass clinical trials, as well as to enter the market, followed by EPS-nanopharmaceuticals which have already shown *in vivo* promising results, as well as by those which avoid the digestive way (e.g., transdermal, nasal).

The EPS nanoparticulate systems should exploit some of their advantages over liposomes (e.g., higher stability and versatile functionalization), which have already advanced in approval of clinical trials. More research effort in specific challenging areas of high interest, e.g., brain-targeting in neurological disorders (stroke, tumors, Alzheimer's), is expected.

Regarding bioactive EPS as potential therapeutics, their challenge is to prove, necessarily *in vivo,* therapeutic advantages over the drugs available on the market, and to observe the above-mentioned well-established protocols for new drugs. Research on associative formulations with already known therapeutics for synergetic effects should be also considered. New studies on the molecular biology mechanisms of action will highlight structure-activity relationships.

Nevertheless, the important therapeutic domains having recorded promising results (e.g., cancer, diabetes, vaccines) still expect further intense world-wide research work and a higher involvement of microbial EPS in health improving resources.

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Insight into the Functionality of Microbial Exopolysaccharides by NMR Spectroscopy and Molecular Modeling

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Microbial polysaccharides represent an important class of microbial polymers with diverse functions such as biofilm formation, thickening, and gelling properties as well as health-promoting properties. The broad range of exopolysaccharide (EPS) functionalities has sparked a renewed interest in this class of molecules. Chemical, enzymatic as well as genetic modifications by metabolic engineering can be used to create large numbers of analogous EPS variants with respect to EPS functionality. While this top–down approach is effective in finding new candidates for desired functionality, there seems to be a lack of the corresponding bottom–up approach. The molecular mechanisms of the desired functionalities can be established from Nuclear Magnetic Resonance (NMR) and molecular models and it is proposed that these models can be fed back into the biotechnology by using a quantitative structure–property approach. In this way it will be possible to tailor specific functionality within a given design space. This perspective will include two well-known commercial microbial EPS examples namely gellan and diutan and show how even a limited use of multiphase NMR and molecular modeling can increase the insight into their different properties, which are based on only minor structural differences.

Keywords: gellan, diutan, bacterial exopolysaccharides, NMR spectroscopy, molecular modeling, molecular hydration

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INTRODUCTION

Exopolysaccharides (EPSs) is a very diverse group of polysaccharides produced by a broad range of microbes, plants, and vertebrates. In natural environments EPS may constitute the main part of, e.g., biofilms, consisting of DNA, proteins, and several EPSs (Flemming and Wingender, 2010), but under controlled settings bacterial strains can produce highly specific types of polysaccharides (Coleman et al., 2008; Schmid et al., 2014). According to a recent review (Freitas et al., 2011), applications of EPS are increasing within the food, pharmaceutical, and petroleum industries. In the food industry several EPS are used as food ingredients, serving as emulsifiers, thickeners or gelling agents due to their ability to form hydrocolloids in aqueous solutions. Moreover, some EPS such as those produced by *Bifidobacteria* have probiotic characteristics (Salazar et al., 2008). EPS production may also have negative impact with their role in the formation of biofilms and biofouling, where the EPS provide a matrix on which bacteria can adhere and grow.

Modern biotechnology enables the use of genetically modified bacteria to produce large numbers of potentially different EPS samples. Obtaining a desired or new functionality can be achieved using high throughput functionality screening systems, but will normally not include molecular knowledge on how to correlate structure and function for analogous series of EPS with smaller and bigger differences. Accordingly, too little is known about the EPS and there is thus a need to develop an understanding of structure–function relationships, i.e., to relate EPS structure with rheological properties of their aqueous solutions, in order to exploit the use of EPS in new applications as well as during the production of fermented products. Detailed knowledge of the structures of EPS will furthermore help in determining the pathways by which the EPSs are synthesized *in vivo*.

One of the main challenges in systematic production of EPS with new functional characteristics is to find suitable rapid analytical solutions to probe the molecular structure. In this context, EPS present two major challenges: firstly the extreme flexibility of carbohydrates often makes them difficult to characterize experimentally, and secondly the high molecular weight (MDa range), high viscosity and low water solubility associated with EPS are major obstacles for the majority of analytical methods and in particular liquid-state ^{1}H Nuclear Magnetic Resonance (NMR) which in many other respects would be the ideal method to study carbohydrate conformation in solution. Other analytical techniques can provide useful information about the secondary structure of EPS in solution such as small angle x-ray scattering (Yuguchi et al., 1996; Dogsa et al., 2008) and multi-angle light scattering (Tuinier et al., 2001) from which parameters such as radius of gyration and hydrodynamic ratio can be inferred. However, these techniques also suffer from the requirement of careful analytical sample preparations (purification, dilution, etc.). In fact only vibrational spectroscopy based techniques such as for example near infrared (NIR) spectroscopy has been reported to provide information that is largely independent of the molecular weight and state of the polymer, but unfortunately with less molecular details than more advanced analytical methods. Nevertheless the NIR method has proven to be a very efficient screening tool in phenomics and for detecting differences in biopolymer structure (Khakimov et al., 2014), and is thus an ideal pre-screening method for investigating if an EPS has been produced, and if so, if it is different from previous analyzed forms. An overall strategy to screen and test new EPS for a desired functionality is outlined in **Figure 1**.

When a new EPS is detected detailed knowledge about the biopolymer structure is required and NMR spectroscopy is irreplaceable for two reasons. Firstly, it can provide detailed information about the composition, connectivity, and conformation of the carbohydrate biopolymer, and secondly it can do so for aqueous solutions (Duus et al., 2000). No other analytical technique is capable of this and for carbohydrates and particularly carbohydrate polymers, it is their detailed hydrations that determines their conformation and therefore their resulting functionality (Kirschner and Woods, 2001). Liquid-state ^{1}H NMR is for example the "golden reference method" for determining alginate functionality. The alginate polysaccharide is a simple linear polymer with repeating units of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) arranged in a blockwise pattern along the polymer chain as homopolymeric (MM or GG) or heteropolymeric (MG) regions. The alginate functionality depends on the average molecular weight and on the distribution of the M- and G-units and on cations present. A low M/G-ratio typically results in a brittle gel, while a high M/G-ratio results in a softer and more flexible gel (Salomonsen et al., 2009). In this respect the linear ABABA-type alginate polymer is not very different from the complex hetero-polysaccharides, made from assembled repeating units. The only downside of liquid-state NMR is the requirement of

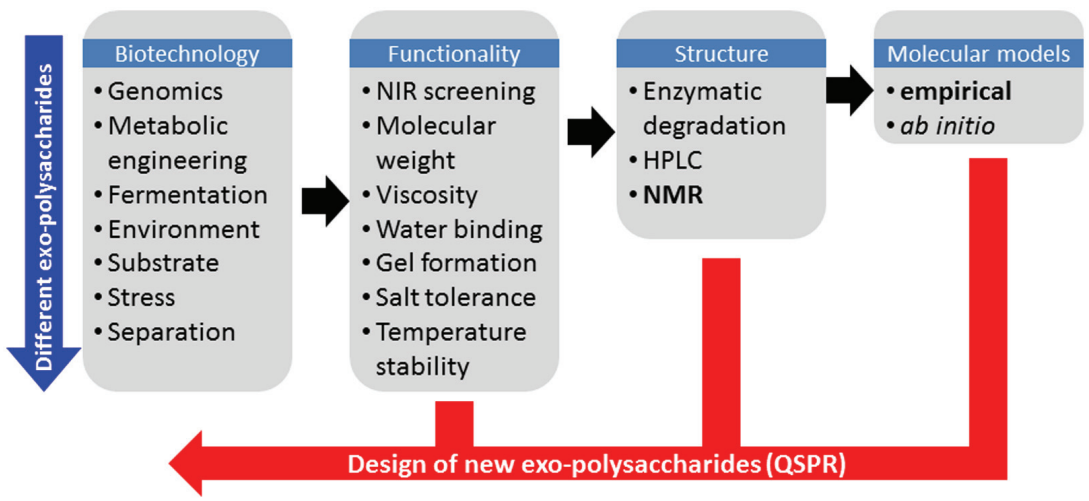

FIGURE 1 | Schematic overview of the work flow in tailoring exopolysaccharide functionality by first principles and by Quantitative Structure –Property Relationships (QSPRs).

depolymerised EPS samples using a stepwise hydrolysis. Secondly liquid-state NMR cannot be used as a stand-alone technique to study the detailed hydration of the polysaccharide. In order to achieve this, a combination of molecular modeling and multiphase NMR is required.

The combination of NMR and molecular modeling has proven a powerful tool for detailed characterization of the polymer structure and hydration for plant polysaccharides such as starch and pectin (Braccini and Perez, 2001; Damager et al., 2010; Larsen et al., 2011). However, also when it comes to molecular modeling, carbohydrates are a special case because of their extreme flexibility and the differences in their electronic arrangements such as the anomeric, exo-anomeric, and gauche effects (Pérez et al., 1996). Because of these inherent complex carbohydrate properties a pragmatic approach is required for modeling of polysaccharide structures. For this reason, software tools for structural calculation and visualization approaches of polysaccharides have been developed (Engelsen et al., 1996, 2014). This type of software can be used to study the packing of complex branched homo-polysaccharides such as starch (Damager et al., 2010) and for studying the helical structure of complex linear bacterial EPSs based on heterologous repeat units (Robijn et al., 1996). Evidently, the elucidation of structural and functional properties of polysaccharides are intimately linked to their interactions with water and NMR techniques provide an effective tool for to investigate the hydration of complex solutes such as aqueous EPS solutions.

This perspective aims to demonstrate the utility of a combined multiphase NMR and molecular modeling approach for the characterization of two microbial sphingan EPSs: gellan and diutan gum. Gellan has been by far one of the most studied EPS and include X-ray as well as NMR analysis (Jansson et al., 1983; Grasdalen and Smidsrod, 1987; Chandrasekaran et al., 1988; Bosco et al., 2000), whereas only few studies included NMR analysis of diutan (Evans et al., 2000). Even though X-ray analysis provides very detailed information about crystalline samples, multiphase NMR enables structural characterization of molecules present in crystalline and amorphous solids as well as gels and solutions. Since applications of hydrocolloids are often related to their gel or semi-solid state the molecular characteristics in these states are very important. Therefore, the combination of multiphase NMR with molecular modeling represents a powerful tool for the characterization of EPS's in their true state of application.

MATERIALS AND METHODS

Samples

Samples were kindly provided by Jochen Schmid, Technische Universität, München, Germany, and used without further purification. The gellan gum was Kelcogel F and the diutan gum was Kelco-Crete DG. Both were manufactured by CP Kelco.

NMR Analysis

Samples for liquid-state NMR spectroscopy were prepared by mixing 1.6–2.3 mg of powder with 1200 μL D_2O (containing TSP-d4). Liquid-state NMR was performed on a Bruker Avance DRX 500 spectrometer operating at 500.13 MHz for ^1H using a double-tuned BBI probe equipped for 5 mm (o.d.) sample tubes. All experiments were performed at 80°C using a single-pulse experiment (90°degree flip angle), a recycle delay of 5 s, an acquisition time of 1.64 s and a spectral width of 10 kHz. All spectra were referenced to TSP-d4 at 0.0 ppm.

Solid-state NMR experiments were performed on a Bruker Avance 400 spectrometer operating at 400.13 and 100.62 MHz for ^1H and ^{13}C, respectively; using a double-tuned solid-state NMR probe equipped with 4 mm (o.d.) rotors. Both ^{13}C CP/MAS NMR spectra were recorded at room temperature using a spin-rate of 9 kHz, a contact time of 1 ms, a recycle delay of 8 s, 1024 scans and an acquisition time of 49.3 ms during which high power ^1H decoupling was applied. The spectra were referenced to α-glycine (external sample) at 176.5 ppm.

Molecular Modeling

Models of the two EPSs gellan and diutan were constructed using the molecular modeling software POLYS (Engelsen et al., 1996, 2014). This program constructs the polysaccharides from their primary structure by combining information about the constituting monosaccharides with information about the glycosidic linkage geometries. In molecular models of polysaccharides, the two torsional angles (ϕ, Ψ) describing the glycosidic linkage (three for 1-6- linkages) are the most important structural degrees of freedom and the conformational parameters that determine the overall geometry of the polysaccharide. The glycosidic linkage torsional angles ϕ and Ψ, which have the definition ϕ = O5-C1-O-C′, and Ψ = C1-O-C'_X-$C'_{(X-1)}$ for a (1-X) linkage. In this study the data for the glycosidic linkages was adapted from the fiber-diffraction data of Chandrasekaran et al. (1988). Using these data the structures of the two EPS gellan and diutan were constructed using a simple nomenclature and subsequently optimized to the nearest threefold helical geometry (Pérez et al., 1996).

```
PRIMARY
[   <bDGlcp>       (1:4; −85.7; −139.5)    /* A */
    <bDGlcpA>      (1:4; −149.6; −133.5)   /* B */
    <bDGlcp>       (1:4; −167.5; 75.2)     /* C */
    <aLRhap>
  <<aLRhap>        (1:3; −85.0; −140.0)
    <aLRhap>       (1:3; −85.0; −140.0)
    <#4>>  ]  6    (1:3; −139.6; 105.5)    /* D */
STOP
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RESULTS AND DISCUSSION

Determination of the 3D-structures of macromolecules can be very time consuming. Often it requires preparation of crystalline materials which may not always be possible for polysaccharides. Two different EPS were analyzed in this study. Gellan, which has a backbone of [→4) α-L-Rha (1→3) β-D-Glc (1→4) β-D-GlcA (1→4) β-D-Glc (1→], and diutan with a similar backbone

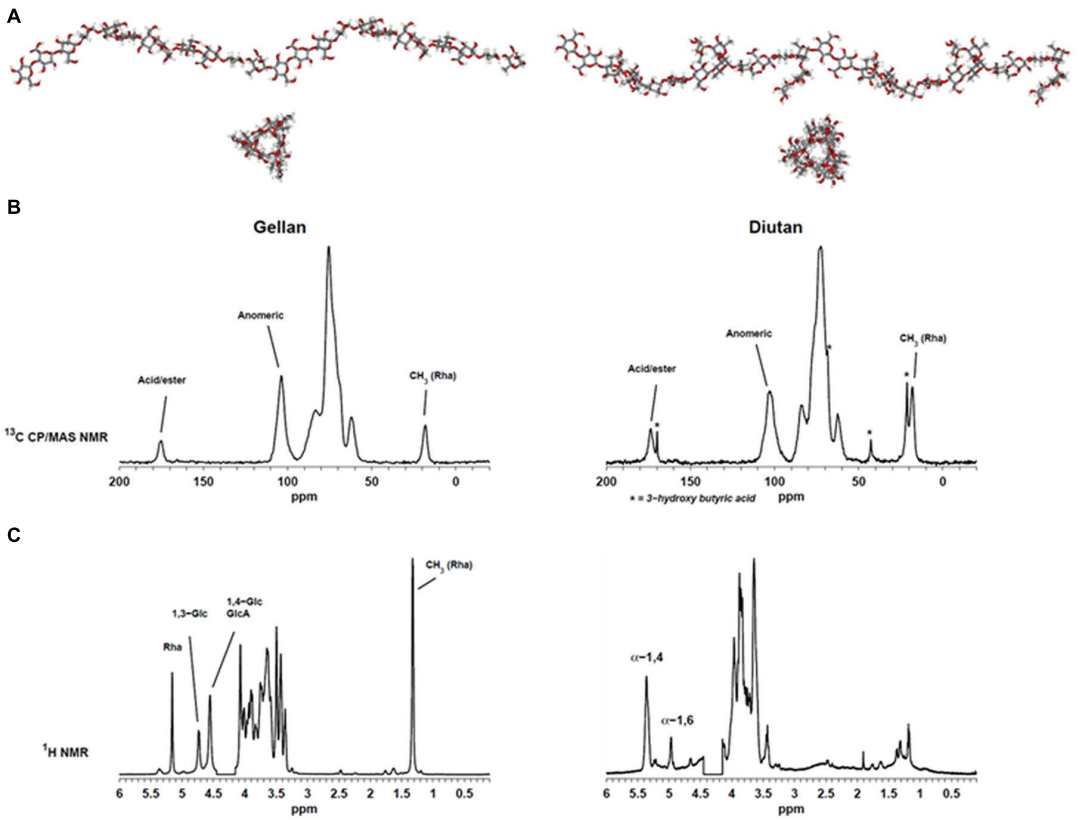

FIGURE 2 | (A) Molecular modeling of gellan (left) and diutan (right). **(B)** Solid-state ^{13}C CP/MAS spectra of gellan (left) and diutan (right). **(C)** ^{1}H Liquid-state Nuclear Magnetic Resonance (NMR) spectra of gellan (left) and diutan (right). The partly suppressed water resonance (4.15–4.45 ppm) was removed from the ^{1}H Liquid-state NMR spectra.

except that the β-(1→4)-linked glucopyranose has a sidechain of a α-(1→4)-linked L-Rha disaccharide attached to C3. Gellan was characterized by X-ray diffraction (Chandrasekaran et al., 1988) and shown to be organized (in solid state) in two left-handed threefold helices. Construction of molecular models of the two polysaccharides gellan (heterologous linear repeat polysaccharide) and diutan (heterologous branched polymer) can efficiently generate visual models (**Figure 2A**) which can be used to explain NMR data and/or molecular functionality. It is for example obvious that the side chain in diutan do not extend the highly probable threefold structure of the gellan backbone and thus do not entangle in complex condensed phase which may indicate that the diutan molecules will still be able to align and create chain–chain interactions (Pérez et al., 1996). However, on the other hand the sidechain may suffice to prevent stronger junction zones (double helical interaction) and thus more likely to create thickening properties, but does not show the same strong gel-behavior like gellan.

Figure 2 shows the ^{1}H liquid-state and ^{13}C CP/MAS NMR spectra of the two EPS. The solid-state NMR spectra (**Figure 2B**) display resonances from the methyl group in rhamnose at 18.1 ppm, resonances from CHOH and CH_2OH carbons in the carbohydrate residues in the region 50–110 ppm and resonances from acid carbonyls in GlcA at 175.2 and 173.6 ppm, respectively. Moreover, a series of narrow resonances (21.2, 42.7, 68.4, 169.7 ppm) in the sample of diutan suggests the presence of 3-hydroxy butyric acid (Orgambide et al., 1991). The absence of an intense resonance at ∼21 ppm indicates that the β-1,3-linked glucose in the backbone was not O-acetylated and also no significant presence of O-glycerate was detected in the gellan sample.

The corresponding liquid-state NMR spectra (**Figure 2C**) provide a much higher spectral resolution compared to the solid-state NMR spectra, but only the mobile parts of the samples (i.e., no junction zones) are detected. With regard to the gellan spectrum it is similar to previously reported spectra and besides the resonance from methyl in rhamnose at 1.32 ppm, three resonances in the region 4.5–5.5 ppm could be assigned to anomeric hydrogens from rhamnose (5.16 ppm), glucose (4.73 (1→3); 4.55 (1→4) ppm) and galacturonic acid (4.55 ppm) (Bosco et al., 2000). Structurally, diutan is very similar to gellan and only differ by the addition of a rhamnose disaccharide side chain on the β-1,4-linked glucose in the repeating unit, however, the corresponding liquid state spectrum is very different. In the liquid-state NMR spectrum of diutan two main anomeric resonances are observed, neither of which belong to diutan. These correspond to α-1,4-linked (5.37 ppm) and α-1,6-linked (4.97 ppm) glucose in amylopectin/pullulan and their assignment

is supported by ^{13}C HSQC data (not shown). Apparently, the diutan sample is not only polluted, but it is also a good example on an EPS which do not dissolve well, presumably because of a high molecular weight (see also alginate case above). However, from the high similarity between the ^{13}C CP/MAS NMR spectra of gellan and diutan it is evident that the molecular structures are very much alike and the diutan is not polluted to a high degree.

The discrepancies between the observations from ^{1}H liquid-state NMR and the ^{13}C solid-state NMR reflect the low water solubility of the EPS. Only for the gellan sample a good correlation between the liquid-state and solid-state analysis was obtained. This indicates that the relatively short side chain of diutan has a major impact on the hydration properties. In the solid-state NMR spectra of gellan and diutan the integrals of the methyl resonance of rhamnose relative to the anomeric resonances (∼103 ppm) are 1:4 and 1:1.7. For gellan the ratio is just as expected, whereas the ratio is slightly lower than anticipated (1:1.33) for diutan, which supports the presence of starch/amylopectin impurities.

When comparing gellan and diutan NMR results, it seems like the presence of dimeric rhamnose side chains lowers the solubility of the EPS. Part of the reason for the lower solubility might be a higher molecular weight of diutan compared to gellan or a different packing (tertiary structure). At the demonstrated level of analysis, this is speculative, but may be further explored using a more comprehensive combined NMR and molecular modeling approach. In such a study the interaction with water could be monitored at different temperatures using both D_2O and $H_2{}^{17}O$ as sources of hydration. Due to the presence of GlcA, addition of cations may also have a great impact on the structural and function properties of EPS and the effects of cations should therefore be included in the comprehensive study as well. Previously, the impact of cations in homogalacturonans has been explored by solid-state NMR (Renard and Jarvis, 1999).

CONCLUSION

Besides structure elucidation NMR also has a role in describing the functionality and in particular the hydration of the EPS. In this work, two closely related commercial EPS were characterized by preliminary multi-phase NMR spectroscopic analysis and molecular modeling with special emphasis on the hydration properties. Previously a similar approach was used to characterize the hydration behavior of native and enzymatically modified pectin (Larsen et al., 2011) which showed that the ratio of arabinan to galactan side chains were very important for the hydrophobicity of the pectin. A similar effect was observed when comparing the results for gellan and diutan. While gellan was hydrated sufficiently for analysis by ^{1}H liquid-state NMR, only resonances from an amylopectin-like impurity were observed for diutan in the liquid-state NMR spectra. This illustrates that addition of the rhamnose disaccharide side chain significantly increases the hydrophobicity.

The combination of NMR and molecular modeling has great potential for providing the structural information required for relating EPS structures with rheological properties. Due to the inherent difficulties with establishing the EPS structure with the current analytical approaches we are still in need of a high throughput structure elucidation pipeline approach of novel EPS. We propose a sequential approach where NIR spectroscopy is used for identifying new EPS structures and NMR and molecular modeling for establishing the primary structure and key features of the tertiary structure of the selected new EPS. In a first approach, the EPS structure can be represented by the primary sequence and simple molecular descriptors such as persistent length and radius of gyration which can easily be derived from modeling packages such as the POLYS program. This can be achieved in semi-automated manner and when enough data becomes available EPS structures can be interfaced to databases containing related biological and physical properties. This will allow for automatic searches for correlations between structure and function through exploratory data analysis. Quantitative Structure–Activity Relationships (QSAR) or rather Quantitative Structure–Property (QSPR) relationships can then be used to explore functional changes arising from structural alterations. POLYS can thus be considered as a prototype program for opening and exploring new possibilities in the field of bacterial EPS engineering.

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Novel imaging technologies for characterization of microbial extracellular polysaccharides

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Understanding of biology is underpinned by the ability to observe structures at various length scales. This is so in a historical context and is also valid today. Evolution of novel insight often emerges from technological advancement. Recent developments in imaging technologies that is relevant for characterization of extraceullar microbiological polysaccharides are summarized. Emphasis is on scanning probe and optical based techniques since these tools offers imaging capabilities under aqueous conditions more closely resembling the physiological state than other ultramicroscopy imaging techniques. Following the demonstration of the scanning probe microscopy principle, novel operation modes to increase data capture speed toward video rate, exploitation of several cantilever frequencies, and advancement of utilization of specimen mechanical properties as contrast, also including their mode of operation in liquid, have been developed on this platform. Combined with steps in advancing light microscopy with resolution beyond the far field diffraction limit, non-linear methods, and combinations of the various imaging modalities, the potential ultramicroscopy toolbox available for characterization of exopolysaccharides (EPS) are richer than ever. Examples of application of such ultramicroscopy strategies range from imaging of isolated microbial polysaccharides, structures being observed when they are involved in polyelectrolyte complexes, aspects of their enzymatic degradation, and cell surface localization of secreted polysaccharides. These, and other examples, illustrate that the advancement in imaging technologies relevant for EPS characterization supports characterization of structural aspects.

Keywords: AFM, high resolution AFM, superresolution optical microscopy, SHG

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Introduction

Polymers are abundant constituents of microbes and host a variety of specific functions. Among these, extracellular polysaccharides, or exopolysaccharides (EPS), constitute a group of carbohydrate based polymers, secreted from the bacteria that represent an interface to the environment 1. Exopolysaccharrides are generally thought to be released from the bacterial surface at variance with surface bound polysaccharides. Various types of microorganisms produce exopolysaccarides. These organisms exist in nature as environmental microbes and/or pathogens for humans. Additionally, they are also utilized industrially where their successful biotechnological fermentation yields efficient production of large quantitities of EPS. Bacterial exopolysccharides

can be grouped into homo- and heteropolysaccharides. Cellulose is one example of a homopolysaccharide. Xanthan, gellan gum, succinoglycan, and alginates are examples of heteropolysaccharides. EPS posess various functionalities in their natural habitat, whereas fermented microbial EPS are applied in a wide range of application, where the functionality are indeed underpinned by their structure, but the exploited function is not necessarily coinciding with their original functionality. Application of imaging strategies to EPS is directed toward elucidation of their structure at various length scales and determination of aspects of functionalproperties.

The paper is organized as outlined in the following, we summarize briefly imaging modalities developed based on scanning probe platforms, mainly the atomic force microcsopy (AFM), their operation modes and associated contrast mechanisms. Recent developments of the AFM is imaging platform include novel operation modes to increasing data capture speed toward video rate, exploitation of several cantilever frequencies, and advancement of using specimen mechanical properties as contrast. In addition, the discovery of tip-enhanced Raman spectroscopy (TERS) bolster the development of probe based scanning technologies utilizing molecular vibration signals from nanoscale domains of the specimens. This review then summarizes advancement in optical microscopy with resolution beyond the far field diffraction limit, optical non-linear methods, and combinations of the various imaging modalities. The overview illustrate applications of these imaging modalities with reference to examples of results either reported for EPS or being relevant for future applications within the EPS field. Issues related to preparation of the specimens are briefly presented within the examples. The capability of AFM and optical based techniques to aquire high resolution information on specimens under hydrated conditions is different from the sample preparation requirements for electron microscopy (e.g., Vu et al., 2009, related to preparation of EPS). The selection of focus in this review on AFM and optically based imaging modalities is due to the combination of novel developments in these field and their capabilities to realize high resolution imaging also under aqueous conditions.

Scanning Probe Based Microscopy and their Applications to Microbial Extracellular Polysaccharides

The imaging principle of the atomic force microscopy (AFM; Binnig et al., 1986) is based on raster scanning of a sharp tip at the end of a small cantilever with sub-nanometer resolution of the position control (Ellis et al., 2006). The deflection of the cantilever as perturbed by the localized interaction of the specimen and the tip is recorded and used as basis for the image generation. There are various operating principles of the AFM, where the most common ones are contact, intermittent contact, and non-contact mode. In the contact mode, the tip is scanned over the sample surface using a piezoelectrical scanner for positioning control and the same time the localized induced deflection of the cantilever is recorded, most commonly by monitoring the laser position of a reflected laser beam with a quadrant photodiode. Contact mode can in principle be operated in constant height or constant force, the latter invoking the feedback loop to maintain a constant net force at a user set value. Possible adverse effect associated with image acquisition induced sample relocation and deformation, in particular for soft samples like extracellular polysaccharides, has led to development of alternative imaging modes. The AFM tip is put into forced oscillations in both non-contact and intermittent contact mode. In the non-contact mode, the AFM tip is oscillated sufficiently close to the sample surface that mainly attractive sample-AFM tip interactions, but not steric repulsive interactions, affect the characteristic oscillation parameters of the cantilever. In the intermittent contact mode, the oscillating AFM tip is in contact with the sample surface at a certain fraction of the oscillation cycle (e.g., 5–15%) and thereby affects the oscillation parameters in a characteristic way. The feedback system is for most operations also engaged in the oscillatory mode, using, e.g., changes in oscillation amplitude as the feedback signal. Thus, topographs representing various features of the sample are obtained. For instance, the topographs obtained for the contact mode operation using the deflection of the cantilever as the feedback signal, can be understood as height isocontours representing the same force due to the AFM tip – sample interactions. There are analogous interpretations of the AFM topographs for the oscillatory modes. Irrespective of the scanning operation mode being contact or oscillation mode, the interpretation of EPS structures observed by AFM appears mainly to focus on the structures seen as polymer chain trajectories or overall conformation if the chain trajectory is not resolved.

One recent example of application of AFM imaging to EPS is the AFM topographs reported for sacran (Okajima et al., 2009). Sacran is a high molecular weight extracellular polysaccharide of the cyanobacteria *Aphanothece sacrum*. It is a heteropolysaccharide reported to consist of various monosaccharides Glc, Gal, Man, Xyl, Rha, Fuc, GalA, and GlcA and trace amounts of additional ones. Sacrans dominating anionic character is originating from 17 mol% of the sugar residues bearing carboxylate and 12 mol% bearing sulphate groups (Mitsumata et al., 2013). Samples of sacran were characterized by AFM to obtain information related to the physical structure of the polysaccharide chains. The AFM topograph of sacran dried from a 1 μg/ml (1 ppm) aqueous solution (**Figure 1A**) reveal rather elongated and apparantly interconnected structures, and for some regions also bundle-like appearance. The structural indication of an intertwined chain organization was further investigated using transmission electron microscopy, providing pictorial evidence that was interpreted in terms a two-chain bundle similar to a coil-coil structures well known for certain proteins. The possible variable pitch was observed to be in the range 20–120 nm. Increasing the sacran concentration in the aqueous solution to 0.1 mg/ml, drying an aliquot and imaging, yields an appearance with tendency for alignment of the chains (**Figure 1C**), but apperantly not increasing extent of bundling by further lateral associations as compared to the specimen dried from the less concentrated case.

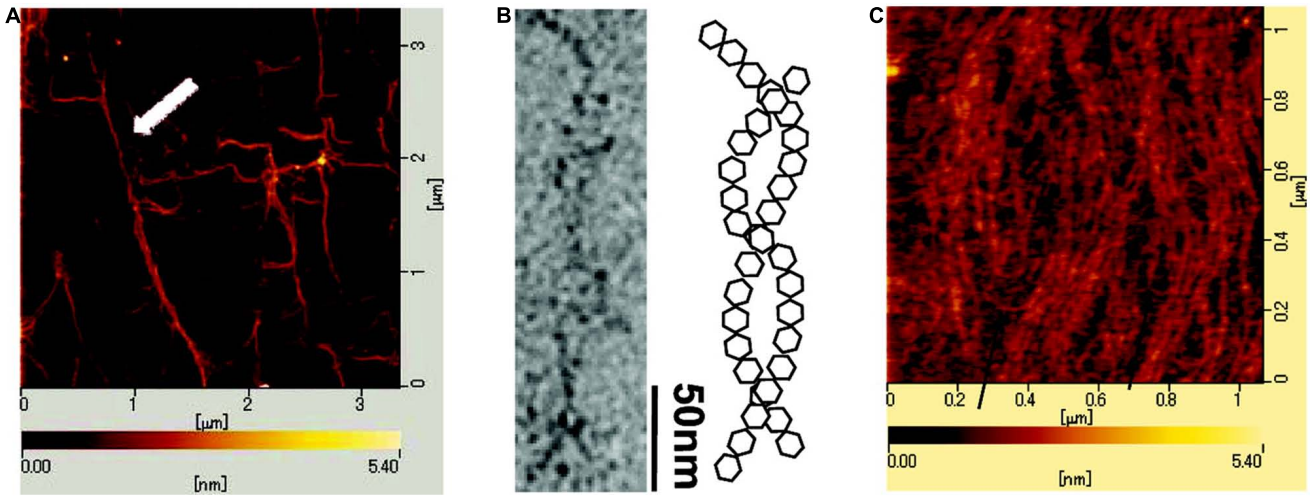

FIGURE 1 | Atomic force (A,C) and electron microscopic (B) images of cyanobacterial polysacaccharide sacran. The atomic force microcsopy (AFM) topograph **(A)** was obtained from a 1 ppm sacran aqueous aliquot dried on mica. The white arrow highlights a bundle of sacran chains. **(B)** Transmisssion electron micrograph of sacran dried on carbon-coated Cu-grid and schematic illustration of intertwined (helical) structure adopted by sacran chains as a possible interpretation of the TEM micrograph. **(C)** AFM topograph of an aliquot of 0.1 mg/ml sacran aqueous solution dried on mica. Scale bars for lateral dimensions **(A–C)** and height **(A,C)** are indicated on the topographs. Reprinted with permission from Okajima et al. (2009). Copyright (2009) American Chemical Society.

Provided the polysaccharide chain trajectories are resolved in the topographs as obtained by AFM, one can extract quantitative information of chain stiffness by applying a statistical model. The persistence length, L_p, of the polymer is a parameter reflecting the chain stiffness, and can be extracted from observed chain trajectories based on changes in tangent direction φ, as a function of the segment length l. Under the assumption that the chain is equilibrated in proximity of imaging surface and 2-dimensional chain statistics can be applied, the mean of the square of φ increases with the segment length in a way that allow determination of the persistence length (Frontali et al., 1979):

$$\langle \varphi^2(l) \rangle = l/L_p \quad (1)$$

or alternatively:

$$\langle \cos(\varphi(l)) \rangle = e^{-l/2L_p} \quad (2)$$

The mean (as depicted by the angle brackets) here imply accumulation of sufficient basis in terms of independent observations of φ at the various l for the statistics to be fulfilled, and equations supporting such a test have been provided (Frontali et al., 1979; Stokke and Brant, 1990). The model assumes homogeneous stiffness along the chain. To discern whether the observed trajectories correspond to the 2-dimensional chain statistics or not is a demanding task. In general, surfaces that interact with the macromolecules with different strengths have shown to affect the overall extension (Lamour et al., 2014), thus providing a practical handle to this issue. Data from the application of such a procedure have been reported for the bacterial polysaccharides xanthan (Camesano and Wilkinson, 2001) and succinoglycan (Balnois et al., 2000), which also include studies at different salt concentrations. Application of such a procedure appears to be difficult to the AFM topographs of sacran (**Figure 1**) due to the not clearly resolved chain trajectories. We have previously reviewed ultramicroscopy of polysaccharides using this approach (Stokke and Elgsaeter, 1994). There are a number of issues of the approach briefly described here to extract conformational parameters in a quantitative way based on observed trajectories. We refer the interested reader to the recent review (Gallyamov, 2011) for a more thorough discussion on the various assumptions and limitation of this approach.

Additional examples of application of AFM to interrogate EPS physical structure include effect of fermentation medium composition on the structure of EPS produced by *Lactobacillus rhamnosus* (Polak-Berecka et al., 2015), and effects of solvent and derivatization on the higher order structures of curdlan (Jin et al., 2006a,b). Cholesterol modified pullulan, the unmodified polymer being produced by the fungus *Aureobasidum pullulans* (Zalar et al., 2008) have a globular appearance in the AFM (Lee and Akiyoshi, 2004). The latter represent an example where the imaging technology was not able to provide direct information of the chain trajectory, nevertheless, the globular appearance is valuable qualitiative information in interpretation of other physical observables.

Atomic force microscopy applied to determine overall structure of polyelectrolyte complexes (PECs) of xanthan, an anionic, semiflexible extracellular polysaccharide, have contributed to the understanding of chain stiffness of PEC morphologies and their possible folding path toward a stable state. Xanthan is an EPS from *Xanthomonas sp.*, and biotechnological production of xanthan by fermentation of *Xanthomonas campestris*, is one of the success stories of

industrial scale EPS production. The primary structure of xanthan is a pentasaccharide repeating unit with two 1,4 linked β-D-Glcp residues making up cellulosic polymer backbone while β-D-Man, β-D-Glu, β-D-Man trisaccharides are linked to every second backbone residue (Jansson et al., 1975; Melton et al., 1975) thus making it appear with a comblike brushed architecture. Historically, there has been much controversy related to the molecular detail of the secondary structure of this polysaccharide, while there today appears to be consensus toward a duplex structure in the chiroptically detected ordered state prevailing at high ionic strength and low temperature (Stokke et al., 1998; Matsuda et al., 2009). In this ordered state, the xanthan behaves as a semiflexible polymer with persistence length of about 120 nm. Xanthan–chitosan PECs prepared at low concentration (1–2 μg/ml), prepared for AFM imaging on freshly cleaved mica and imaged by tapping mode AFM revealed various types of structures (**Figure 2**; Maurstad et al., 2003; Maurstad and Stokke, 2004). For complexes prepared using high molecular weight xanthan, toroidal species were present, although not all complexes were conforming to this well known structure observed for condensed DNA (Martin et al., 2000; Hud and Downing, 2001; Hud and Vilfan, 2005). The xanthan–chitosan AFM topographs were subjected to an image analysis process to quantitate fraction of species in dominating morphologies such as toroids, topologically straight (i.e., including also curved ones) and globular appearance. Thus, the asphericity index A (Eq. 3) was calculated for each of the identified PECs (Zifferer and Olaj, 1994; Zifferer, 1998; Maurstad et al., 2003):

$$A = \frac{\left(\lambda_1^2 - \lambda_2^2\right)^2 + \left(\lambda_2^2 - \lambda_3^2\right)^2 + \left(\lambda_1^2 - \lambda_3^2\right)^2}{2\left(\lambda_1^2 + \lambda_2^2 + \lambda_3^2\right)^2} \quad (3)$$

where λ_i, $i = 1,3$, are the eigenvalues of the tensor moment of inertia:

$$X_{\alpha,\beta} = \frac{1}{N}\sum_{i=1}^{N} S_i^{\alpha} S_i^{\beta}; \qquad \vec{S_i} = \vec{r_i} - \vec{R}_{CM}; \quad \alpha, \beta = x, y, z \quad (4)$$

Parameters $\vec{r_i}$ and $\vec{R}_{CM}$ in Eq. 4 depicts the positional coordinate of the i'th pixel element and the center of mass of the PEC, respectively. Parameter A is reported to adopt values of 0, 0.25, and 1.0 for the idealized sphere, an infinitely thin circle and rigid rod, respectively (Noguchi and Yoshikawa, 1998), and thus serve as a morphological sensitive parameter. Distributions of A extracted from a large number of xanthan–chitosan complexes prepared from a high molecular weight xanthan revealed a peak centered at $A = 0.25$ reflecting the toroidal like structure, and a tail in the distribution of A's toward 1 reflecting rod-like PECs with different tortuosity (Maurstad et al., 2003; Maurstad and Stokke, 2004). Reduction of the molecular weight of xanthan below that needed for a toroidal morphology resulted in rod-like PECs with a concomitant suppression of the peak in the distribution of A around 0.25, and increase in the fraction of species with A closer to 1. Thus, the inclusion estimates of the asphericity indeces A provide quantitative characterization of distribution of species that (co-)exist in topographs.

Additionally, AFM imaging of heat-treated complexes formed between xanthan and chitosan provided indirect evidence of the folding pathway toward the toroid. Toward this end, the AFM topographs were considered as snap-shots of a particular state on its path toward a more stable state, stimulated either by increasing the temperature during a given duration of thermal treatment, or increasing duration at a given, elevated temperature. The fraction of species consisting of more than one loop, and often dangling ends, were observed to decrease, while the fraction of toroids was found to increase. Thus, the torus was suggested to represent the energetically favorable structure and that other morphologies observed at room temperature were metastable states driven toward the more stable state of a torus by the increased temperature. These observations have more recently been put in context with a numerical account of the collapse pathway occurring in polymers in poor solvents (Lappala and Terentjev, 2013).

While the above examples provide important information as deduced from the static observation of various chain trajectories arrested at various conformation during the immobilization process (preparation for AFM imaging), there is a growing interest in dynamics within EPS. Historically, AFM has been inherently slow in image acquisition, with capture time for individual frames in the order of minutes or larger. Although this depends on the scan size, these inherent slow frame rates limit the access to information on dynamics related to EPS. Progress in increasing the frame rates of AFM imaging is based on detailed insight, optimization, and re-engineering of key components of the AFMs, like smaller moving mass, reducing the size of the cantilever to increase resonance frequency, and improved feedback circuit. The technicalities of these AFM hardware issues, despite its vital importance for successful higher frame rates capture, is considered beyond the topic of this overview and we refer the interested reader to relevant literature on these topics (Ando et al., 2001, 2008; Ando, 2012).

One intriguing example of the application of high speed AFM to EPS is the characterization of cellulase induced degradation of crystalline cellulose specimen (Igarashi et al., 2011). Although the employed cellulose I_α crystal was obtained from processing of a sample from green alga *Cladohora* sp. is not a truly microbial EPS, the similarity of the specimen with bacterial cellulose is considered sufficiently close to include this example here. This cellulose crystal specimen was incubated on a graphite sample disk, and following addition of cellulase Cel7A from *Trichoderma reesei*, in buffered solution, sequential AFM imaging was performed at frame rates of 1–4 Hz. The movement of the Cel7A was observed only on the top of the crystalline cellulose specimen. Example data from their seminal report (**Figure 3**) shows relocation of individual cellobiohydrolase enzymes unidirectionally along the crystalline specimen, with a migration speed that is uneven and differs between the individual enzymes, but spread around the mean value as estimated from other average data. The data also revealed that individual enzymes does not exhibit a constant migration velocity, some enzymes were migrating without stopping within the observation areas, whereas others were apparently halting, suggested to arise from the Cel7A molecules exhibiting intermittently halt and go

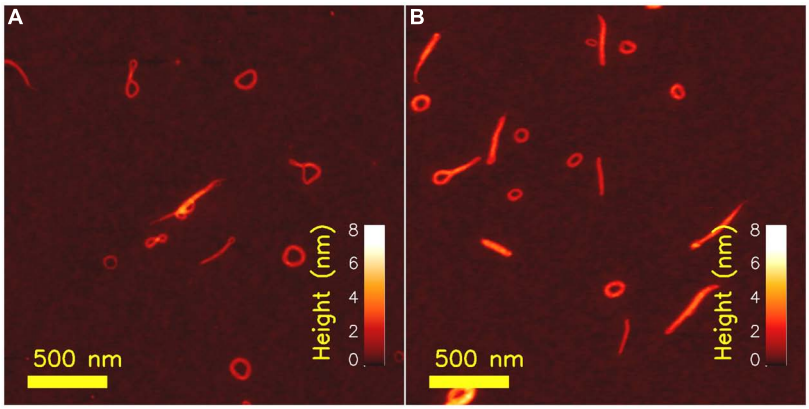

FIGURE 2 | Atomic force microcsopy topographs of chitosan–xanthan polyelectrolyte complexes (PECs) as prepared at low concentrations (xanthan concentration of 2 μg/ml and chitosan with degree of acetylation 0.49 at a concentration of 10 μg/ml) at room temperature and treated at 44°C for 30 min (A) and following annealing for 30 min at a temperature of 90°C (B). Reproduced with with permission from Maurstad and Stokke (2004). Copyright (2004) Wiley.

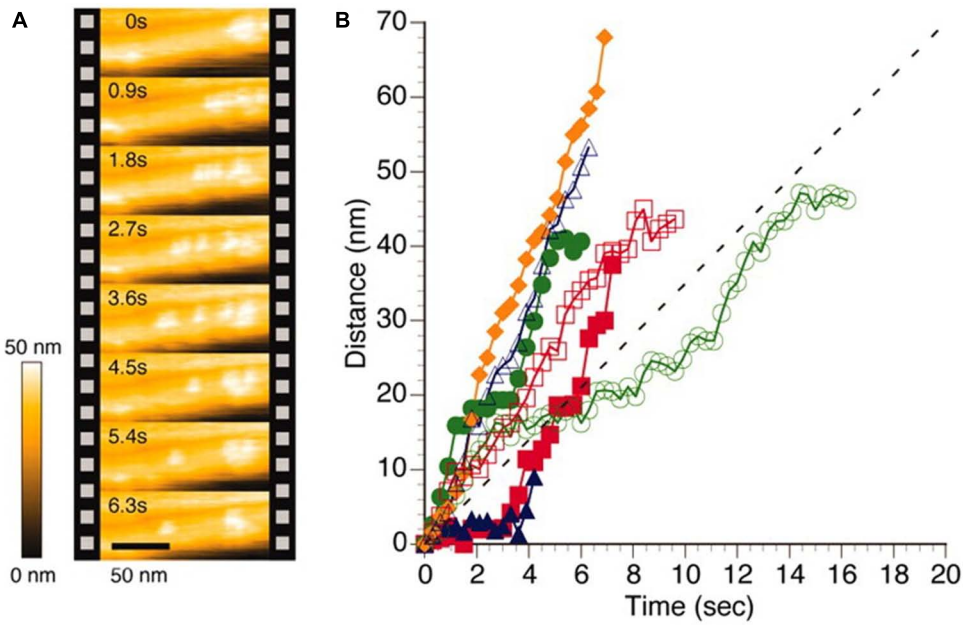

FIGURE 3 | High speed AFM imaging of *Trocoderma reesei* Cel7A cellulase acting on crystalline I_α cellulose. (A) AFM topographs of the same area of the cellulose crystal imaged at intervals of 0.9 s revealing different localization of individual cellulases. **(B)** Time dependence from the initial position of individual cellulase positions when acting on cellulose crystal as deduced by image processing of HS-AFM topographs. The colored symbols reflect data extracted from individual enzymes whereas the dotted line depict an average velocity as reported (Igarashi et al., 2009). Reprinted with permission from Igarashi et al. (2011). Copyright (2011) AAAS.

movements. The results were also discussed in terms of Cel7A interacting with the cellulose crystal in a productive and non-productive adsorption mode. Furthermore, detailed analysis of the dynamics revealed congestion between individual cellulases when moving on the crystalline specimen. As part of this, the inability of a particular enzyme to circumvent a stalled enzyme on the crystalline specimen was identified. Detailed molecular studies using high speed AFM imaging, such as that highlighted above, provide additional microscopic details on the statistical nature and congestion effects on the bioconversion of cellulose crystals that are not easily captured by other approaches. Application of HS-AFM in biological systems has recently been reviewed (Ando et al., 2014).

Direct imaging of the enzymatic polymerization of hyaluronan using an *in vitro* approach was recently reported (Mori et al., 2012). A sample of hyaluronic acid synthase isolate from *Pasteurella multocida* (pmHAS) was physiosorbed to mica or immobilized on an anionic lipid bilayer spread on

mica. The immobilized pmHAS sample was imaged at various intervals when incubated with both UDP-GlcA and UDP-GlcNAc monomers, being the UDP forms of the monomers of the repeating structure of the hyaluronan structure. The spatiotemporal imaging captured at intervals of the order of 10 s between frames, revealed structural evidence of polymerizing hyaluronan chains with increasing length with time. The imaging approach supported determination of hyaluronan polymerization rate at the individual enzyme level, and also determination of rate at various extent of polymerization. Such an approach indicates the high level of detail of information that can be extracted from such imaging studies.

While there are examples of application of recent development of high speed AFM for understanding biological functions relevant for EPS, the utilization of the more recently developed higher resolution imaging modalities to EPS is so far essentially lacking. The resolution in the obtained AFM topographs depend on a number of factors such as radius of curvature of the scanning tip, force control during the data acquisition, mechanical properties of the specimen. Development of higher resolution strategies within the scanning probe platform relevant for imaging of EPS include, e.g., the use of alternative feedback signals like the maximum force (PeakforceTM®, and others), exploitation of cantilever drive oscillations with more than one frequency (Garcia and Herruzo, 2012; Herruzo et al., 2013) and multimodal intermodulation AFM (Borysov et al., 2013), and ultra-low noise with improved force sensitivity. It should, however, be noted that not all imaging modalities are yet implemented for operation in liquid environment. Although not being an example of observation of an EPS, the recent report on imaging localized protrusion along the DNA double helical structure consistent with localization of phosphate groups is intriguing (Ido et al., 2013; **Figure 4**), and could also stimulate the use of such high resolution AFM techniques to EPS. In their study, Yamada and coworkers, deposited their nucleotide samples on freshly cleaved muscovite mica and rinsed with a solution containing Ni^{2+}, one of the divalent ions that has been reported to substitute with the K^{+} site in the surface of the muscovite mineral, and thereby mediate a cationic bridge interaction to polyanions like DNA when imaged in aqueous solution (Hansma and Laney, 1996). The AFM employed was modified from a commercial one focusing on improving the force detection and also operating at minute oscillation amplitudes (0.4–0.5 nm). The obtained AFM topographs (**Figure 4**) resolve minor and major grooves of the dsDNA similar similar to other reports (Leung et al., 2012; Pyne et al., 2014), but even more impressing is the additional identification of the protrusions identified to arise from the phosphate groups. Application of such high resolution force modulation AFM to EPS could potentially contribute to resolving structural detail beyond that currently available. In addition to the general quest for higher resolution also relevant for EPS, particular issues related to, e.g., direct localization of acetyl groups along microbial alginate (Davidson et al., 1977) and gellan gum (Giavasis et al., 2000) could add to current approaches elucidating localization of substituents.

Scanning probe imaging tools as the AFM can also be operated to obtain information on the mechanical properties of a specimen, either as topographically resolved information of the biological entity of interest like a baceterial polysaccharide, or within constructs designed to probe molecular conformation. Pulling experiments on isolated macromolecules have developed to a large field providing novel, fundamental information on structure of macromolecules (Noy, 2008; Bizzarri and Cannistraro, 2010; Noy and Friddle, 2013; Eghiaian et al., 2014). The forces applied and the molecular extensions range typically from tens to hundreds of pico-newtons (pN) and a few to tens of nanometers, respectively, in these approaches. For polysaccharides, the force-extension relationships are conventionally obtained by stretching chains adsorbed between a substrate and an AFM canitelever (Rief et al., 1997; Marszalek et al., 1998; Sletmoen et al., 2010; Marszalek and Dufrene,

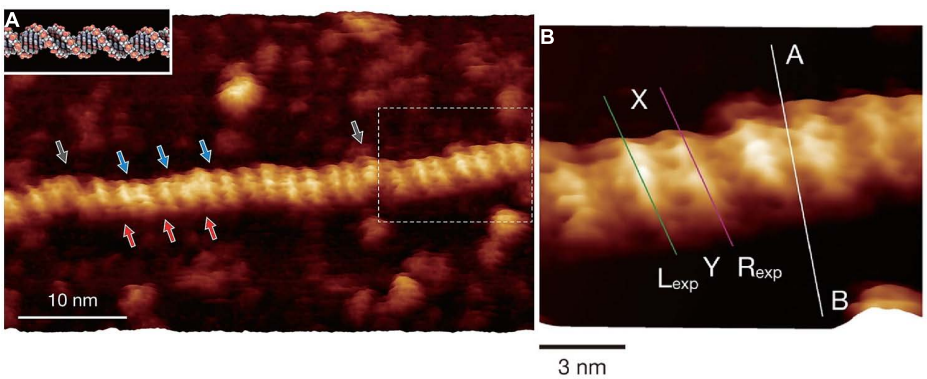

FIGURE 4 | Force modulation AFM topographs of plasmid pUC18 DNA (2686 basepairs) in aqueous solution. The aqueous solution contained Ni^{2+} to stabilize the interaction between DNA and mica. **(A)** High resolution force modulation AFM topograph of a section of the duplex DNA with major (red arrows) and minor (blue arrows) grooves identified along the B-DNA double-helical structure. The white dotted area of the topograph in **(A)** are shown at higher magnification in **(B)**. **(B)** Height variations along the **(A–B)** cross-sectional line are shown in the original publication. Height profiles along the L_{exp} and R_{exp} lines are compared with simulated data in the original publication. Reprinted with permission from Ido et al. (2013). Copyright (2013) American Chemical Society.

2012). Probing the mechanical properties of EPS are expected to provide data related to their mechanical and viscoelastic properties extending also to elastic responses associated with deformation of individual sugar residues. Although of interest and relevance for the functional properties of EPS, details within this topic is considered beyond the scope of the present review. The reason for this is that possible fingerprint signatures arising from stretching individual EPS have not yet been implemented as a possible contrast signature in AFM based imaging modalities.

While the above examples on the application of scanning probe based imaging modalities was mainly highlighting examples where the EPS is either isolated from its parent organism, or studied in combination with other macromolecules, there is also increasing applications of this toolbox in conditions closer to the natural habitat.

Dufrêne (2008) has recently summarized application of AFM based strategies to obtain image based information related to structure, chemical composition, nature of interaction forces, and specific molecular recognition as part of analysis of microbes at the nanoscale. Various examples of the application of more recent application of such strategies have been summarized (Sletmoen et al., 2010). Combined, the examples include studies of effect of inhibition of the biosynthetisis of arabinans (a major cell wall component), differences in EPS topology in bacterial mutants, and effects of EPS in cellular adhesion. Despite that individual EPS chains are not clearly resolved in such studies consequences of their changes are clearly emerging. The presence and overall outline of the polysaccharide capsules of the bacteria Zunongwangia profunda SM-A87 was recently characterized by tapping mode AFM (Su et al., 2012). In this report, the AFM clearly revealed the overall outline of the EPS containing capsules, the fibrils, but not individual, dispersed polysaccharide. The authors compared the recently implemented ScanAsyst mode from one commercial supplier and compared physical paramenters obtained by this mode to tapping mode. The recently reported mechanical and adhesive properties mediated by capsular EPS of *Lactobacillus johnsonii* FI9785 (Dertli et al., 2013) and changes in the EPS functionalities assocated with genetically modified variants affecting the biosynthesis of one of the EPS's, illustrate the applicability of the imaging technology also for such studies. Specific adhesion studies were realized using a lectin from *Pseudomonas aeruginosa* (PA1) covalent attached to the AFM probes using a flexible linker, and used for adhesion mapping of the native and genetically modified bacteria. Reduced adhesion in the mutated bacteria probed under aqueous solution showed the reduction of D-Gal in the strains genetically modified to reduce the expression of one of the two EPS. Such a study indicate the capability of the scanning probe tools to provide structural related information of capsular polysaccharides based also on the specific compostion. More recently, detailed surface characterization of the human pathogen *Sterotcoccus agalectiae* using AFM combined with electron microscopy has been reported (Beaussart et al., 2014).

While the force based scanning probe modalities outlined above provide important structural and functional information, they are not directly providing information on chemical compostion. There are additional imaging tools sensitive to chemical information that is of potential interest for application to EPS. TERS has attracted interest for application in life sciences because it potentially can be used to obtain Raman spectra localized to certain domains. Application of TERS to EPS appears so far to be limited. Although not being a microbial alginate, we summarize some features of alginate fibers obtained by application of TERS (Schmid et al., 2008). Toward this end, an aliquot of a 2% alginate solution was drop-coated on cleaned glass surface and allowed to dry and characterized by TERS in a customized scanning probe – Raman spectrometer set-up. Commercial contact mode AFM tips coated with AlF_3 (30 nm) and subsequently with Ag (30 nm) were prepared in a vapor coating system and used for the TERS characterization. The observed most prominent Raman bands determined by TERS were correlated with that of literature data and strategies for specta correction were suggested. The more recent overview of application of TERS for characterization of biological molecules provides guidelines considered relevant also for the EPS (Blum et al., 2012).

Optical Based Imaging Modalities and their Applications to Microbial EPSs

While scanning probe techniques provide resolution which is far superior to optical techniques, they are limited to interrogating only the surface properties of the sample. Optical techniques have the advantage of being able to volumetric images without sectioning and also necessitates less sample preparation implying that samples can be imaged in their native environment. Over the past century it was believed that the resolution limit of far-field optical microscopy was fundamentally limited by the diffraction limit which according to Abbe's theory limits the minimum focal area (Airy disk) to about half of the excitation wavelength. This paradigm has now been challenged. Over the last decade it has become clear that the diffraction limit is not an insurmountable barrier to resolution. Several concepts have evolved which provide resolution well beyond the resolution provided by conventional confocal microscopy.

These superresolution (or nanoscopy) techniques can be roughly divided into three categories. *Single molecule localization* techniques use the fact that the signal from a single molecule can be localized with a precision much greater than the size of the Airy disk. By using different concepts to sequentially turn fluorophores on and off, a superresolution image can be generated by combining multiple (often thousands) frames. Fluorophores can be turned on or off either stochastically (stochastical optical reconstruction microscopy –STORM Rust et al., 2006) or actively using light (Photoactivated light microscopy Betzig et al., 2006). The *RESOLFT* (Reversible Saturable OpticaL Fluorescence Transision) concept (Hofmann et al., 2005) is based on using a doughnut shaped beam which can turn off molecules reversibly [e.g., by stimulated emission (STED; Hell and Wichmann, 1994) or through ground-state depletion (GSD; Hell and Kroug, 1995)], reducing the

volume of molecules which can still fluoresce. Structured illumination microscopy (SIM) illuminates the sample with structured light at different angles and is able to extract superresolution information through subsequent computational techniques (Gustafsson, 2005). These superresolution techniques have in the last few years provided novel results in many areas of microbiology. Especially new information about organization of proteins, enzymes, and receptors have been gained through the use of these superresolution techniques (Chojnacki et al., 2012). In the field of microbial EPS the techniques show great promise but have not yet been widely applied. Part of the reason might be that the scientific questions in the field are focused at structural information which is still beyond the resolution of optical superresolution techniques, requiring, e.g., electron microscopy or AFM.

Another issue is that most optical techniques require labeling with fluorophores and the availability of these are much larger for proteins than carbohydrates. However, in the study of the dynamics and interactions between the EPS, linking proteins and enzymes, the techniques seem to hold much promise. This was nicely illustrated in the study of the EPS and linking proteins in *Vibrio cholera* (Berk et al., 2011, 2012; **Figure 5**). STORM revealed that polysaccharides were extruded as distinct spheroids from the cells. It was shown that in the extracellular matrix (ECM), the polysaccharides and crosslinking polymers appeared in distinct clusters which could be a reason for the rapid repair of the ECM after rupture. There are some studies of polysaccharides from non-microbial sources, e.g., plant cell walls using superresolution microscopy which demonstrates the application of the technique which could be extended to microbial sources of ECM (Moran-Mirabal, 2013).

While optical microscopy based on fluorescent labels is extensively used and can provide images with high specificity for a given molecule, the necessity of labeling will always to some degree perturb the biological system under investigation. In addition, since an attached label, rather than the molecule of

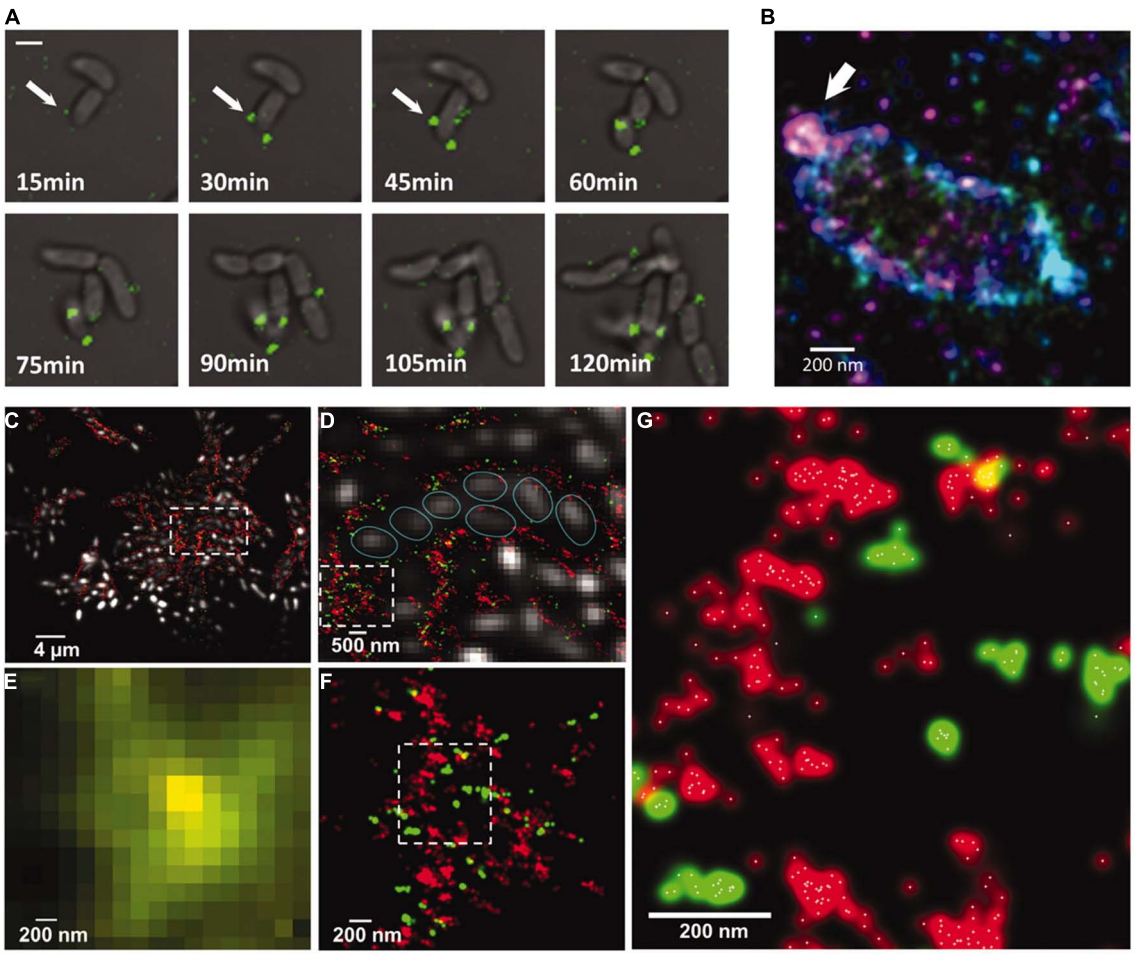

FIGURE 5 | (A) Sectretion of *Vibrio* polysaccharide (VPS) stained with Cy3 attached to wheat germ agglutinin (green). **(B)** 3D STORM superresolution image of a single cell showing sectretion of VPS around a single cell. The color represents height in the *z*-direction. **(C–F)** Multicolor microscopy images of VPS (red) and RbmC (green). **(C,D,F)** are STORM superresolution images while **(E)** is a conventional confocal of the same region as **(F)**, illustrating the increased understanding of the organization of VPS and RbmC in superresolution microscopy. **(G)** STORM image showing the individual localization points as white dots. Reprinted with permission from Berk et al. (2012). Copyright AAAS.

interest, is probed, it is not possible to extract further molecular information by analyzing the optical properties of the molecule. Label-free techniques, which directly probe the properties of the molecule of interest, show great potential for extracting subresolution information. Confocal Raman microscopy probes the vibrational levels of the molecules. This can provide detailed molecular information but is hampered by weak signals and long acquisition times. This limitation can to some degree be overcome by local enhancement effects as in the above mentioned TERS technique. Another class of label-free techniques is based on the non-linear optical properties of the sample. Non-linear optical microscopy (NLOM) has over the last decades evolved to become an important tool in biological research. Two-photon excited fluorescence (TPEF) was developed to overcome some of the limitations in conventional confocal microscopy (Helmchen and Denk, 2005), that is limited by sample penetration and out-of-focus photobleaching. NLOM is inherently confocal so that energy is only absorbed in the focal volume, reducing out of focus bleaching. Imaging down to 1 mm has been reported (Kobat et al., 2009). This feature could provide novel insight into the volumetric organization of EPS rather than only the surface, as is the case with, e.g., AFM.

In recent years several other non-linear interaction mechanismshave gained interest in biological research. The primary driver for these methods is that they provide label-free imaging of various molecules with relatively high specificity. They are therefore highly suitable for *in vivo* and even *in situ* imaging. In terms of studying EPS, the most important aspect of these techniques is the ability to image dynamic processes at very short timescales, without the necessity of introducing exogenous labels which might disturb the biological system, and where labeling efficiency is an unavoidable issue. Examples of mechanisms which have been studied dynamically are: secretion of cellulose from bacteria (Brackmann et al., 2010), hydration of starch (Slepkov et al., 2010) and digestion by cellulose (Brown et al., 2003).

Second harmonic generation (SHG) is a non-linear process where two photons combine to a single photon with twice the energy (half the wavelength) through the interaction with a molecule (Campagnola and Loew, 2003). The specificity of SHG arises from the fact that only molecules which are non-centrosymmetric and are ordered on the scale of the focal volume can generate SHG. Collagen is the most widely studied molecule with SHG owing to its non-centrosymmteric helical structure and that several collagen types order into fibrils (Lilledahl et al., 2007; Olderoy et al., 2014). Other molecules which can generate SHG are tubulin (Mohler et al., 2003; Stoothoff et al., 2008) and myosin (Plotnikov et al., 2006). Cellulose has also been shown to give a SHG signal. As SHG is very sensitive to the length scale on which the molecules are ordered it can provide the degree of crystalline ordering in the extracellular microbial cellulose. The extracellular cellulose of *Acetobacter* and *Valonia* was also studied by SHG (Brown et al., 2003; Nadiarnykh et al., 2007). By studying the polarization resolved SHG signal as well as the forward/backward scattering pattern of the SHG signal, the orientation and degree of ordering of the fibers in the lamella were analyzed.

Coherent anti-Stokes Raman scattering (CARS) is a technique which uses two femtosecond lasers which interacts with vibrational modes of the molecules, similarly to Raman microscopy (Zumbusch et al., 1999). The advantage of CARS is that for a moderately strong vibrational mode the signal is much stronger such that imaging of live samples at high framerates is possible (Evans et al., 2005). In addition, CARS also has the same 3D sectioning capability as other NLOM methods. CARS is, just like SHG, a coherent effect so that the total signal is highly dependent on the ordering of the molecules in the focal volume, thereby providing structural information below the resolution limit. Cellulose synthesis by *Acetobacter xylinum* was imaged using a combination of SHG and CARS (Brackmann et al., 2010). The SHG signal was used to image the synthesis of the cellulose while the bacteria where imaged using the (unspecific) CH_2 vibration detected by CARS. Development of the cellulose network was imaged non-invasively and label-free over 7 days. SHG was also used to study the interaction of human cells (Osteoprogenitor cells and smooth muscle cells) with the bacterial cellulose matrix as a potential scaffold in tissue engineering applications (Brackmann et al., 2011, 2012). CARS has only been used to image cellulose from plants and not microbial cellulose, but there is no reason why it should not be possible with microbial cellulose as well. It was shown that using polarization resolved CARS, distinct domains with different degrees of crystallinity was observed along the cellulose fibrils (Zimmerley et al., 2010).

To summarize, optical techniques can provide information from dynamic, living systems in 3D. Labeling techniques makes it possible to track multiple molecules simultaneously to study how the EPS interacts with other molecular species in the ECM. Non-linear, label-free, techniques provide an even less invasive imaging modality with the possibility of extracting sub-resolution structural information from the data.

Aspects of Preparation of EPS Samples for Imaging

Above, major advancement in the development of various imaging modalities, either scanning, probe or optically based, and their application to EPS or samples related to EPS, have been highlighted. For all these application, there is in most cases a need for the preparation of the sample in some way. The need for sample preparation depends on the operation environment of the imaging tool and the contrast mechanism exploited. For instance, in the case of AFM, the sample needs to be immobilized to withstand the actual forces imposed during the image acquisition. Although there is increasing control of the force, including reducing the magnitude of the force excerted by the tip on the specimen, there is still a need for adherence of the biological specimen to a sample surface, while being able to keep the sample in a hydrated environment to preserve a condition mimicking the natural state. The use of ionic bridges mediated by divalent ions, e.g., Ni^{2+}, between mica and biomolecules (DNA) have been reported (Hansma and Laney, 1996; Raigoza et al., 2013)

to provide sufficient strength in the non-covalent immobilization to allow scanning probe based imaging. Although there are instrumental developments that to a larger extent is based on inherent properties of biological specimens, and thus termed label-free, these approaches are not yet sufficiently established to replace all optically based imaging modalities. The use of fluorescence labels is therefore expected to be instrumental also in the further advancement for the characterization of EPS by optical imaging methods. In this context, the tools available for fluorescence labeling of EPS are considered not to be as versatile as the current state of the art for application in other families of biomacromolecules, and progress in imaging of EPS is also expected to benefit from advancement of novel labeling schemes.

Conclusion

Advancement of instrumentation and application of novel imaging strategies for determination of properties of extracellular polysaccharides has provided novel insight into this group of materials. Important examples include the possibility to capture information related to the dynamics in the processing of this group of biopolymers, as highlighted by the application of high speed AFM to capture relocation of a collection of cellobiohydrolase on their crystalline substrate while catalyzing the bioconversion (Igarashi et al., 2011). Despite the significant advancement in the AFM image capturing speed realized over the past decade, capturing key features of the dynamics associated with biomolecule movements are still not achievable by high resolution imaging (Raigoza et al., 2013). The situation for the high resolution AFM techniques is that the intriguing resolution reported for, e.g., B-DNA helix structures, should inspire migration of this technique to a number of interesting research questions within EPS. Added to this, is the advancement both in improved resolution beyond the diffraction limited barrier and label free tools, that is finding its way also into the EPS field. Moreover, there is also advancement in the imaging field bolstered by the capacity to observe identical locations of the sample using a combination of imaging tools, either as combined instruments, including spectroscopy (Moreno Flores and Toca-Herrera, 2009; Lucas and Riedo, 2012), or by the application other strategies. Thus, we conclude that the ultramicroscopy toolbox available for EPS are more richly developed than ever, and we expect further advancement in this field, with respect to improved dynamics, more precise structural, and localized chemical information potentially being unraveled to underpin improved understanding of the structure–function relationships.

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