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XXXIII SIMGBM CONGRESS 2019 - ENVIRONMENTAL AND INDUSTRIAL MICROBIOLOGY

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EDITORIAL
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Editorial: XXXIII SIMGBM Congress 2019 - Environmental and Industrial Microbiology

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Editorial on the Research Topic

XXXIII SIMGBM Congress 2019 - Environmental and Industrial Microbiology

The Italian Society for General Microbiology and Microbial Biotechnology (SIMGBM) was founded in 1982. At present, the society has more than 350 members from Academia, Research Institutions, and Biotech Companies with the aim to foster microbiological research in Italy and to promote collaborations and visibility of Italian microbiologists in the different areas of microbiology and biotechnology. Articles presented in this Research Topic come from attendants of Microbiology 2019, the 33rd Congress of SIMGBM, held in Florence, Italy, on 19th−22nd June 2019 and include selected contributions under the label "Environmental and Industrial Microbiology."

Microorganisms represent an environmental treasure in itself, playing a fundamental role in the sustainable development of ecosystems. Moreover, if exploited judiciously, they can be used to recover disturbed environments or for bioremediation processes. Both terrestrial and aquatic environments have been explored with this aim. In the papers from Vassallo et al. and Chamizo et al., microbial communities are studied aiming to obtain environmental benefits like the formulation of a synthetic bacterial community to improve phytodepuration processes, and for inoculation in soil restoration techniques, respectively. Vassallo et al. identified the dynamics of the microbiota composition of *Phragmites australis* roots as triggered by the presence of wastewater, identifying a direct correlation, linking the appearance of antibiotic- and synthetic wastewater-resistance with the time of exposure to wastewater. Cyanobacteria adapted to environmental stress can be used as inoculants to induce artificial biocrusts formation in degraded drylands. Exopolysaccharides (EPS) production is a key factor in biocrusts formation. In their study, Chamizo et al. investigated the differences in growth and polysaccharidic matrix features among three common biocrust-forming cyanobacteria proposed as soil inoculants. Although the proper care needs to be taken when releasing microorganisms into the environment, both studies highlight that a careful selection of microorganisms providing specific functions can be profitable for several environmental applications.

Selected microbial strains are, moreover, crucial to obtain specific biotechnological products. Three reviews are present in this Research Topic, focusing on three distinct applications of specific microbial groups, i.e., wild *Saccharomyces* strains for improved processes in industrial fermentations Di Paola et al., *Streptomycetes* for recombinant protein production (Berini et al. and calcium carbonate mineralizing bacteria for stone artwork consolidation (Marvasi et al.). The

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mini-review by Di Paola et al. outlines the history of fermentation and yeast domestication, leading to strain artificial selection by the breeding of wild species to obtain standardized yeast cultures. The authors highlight in particular the importance of social insects in yeast dispersion and in-gut breeding and discuss on their exploitation for the production of hybrid yeasts from environmental *S. cerevisiae* isolates suitable for industrial and biotechnological applications. The mini-review by Berini et al. focuses on the biotechnological exploitation of the streptomycetes by recapitulating recombinant protein production heterologously expressed in this genus in the last 40 years. The authors highlight *Streptomyces* as a promising, alternative, and versatile platform for recombinant protein production, discussing the pros and cons of using it as an expression chassis. Marvasi et al., finally, focus on the exploitation of bacteria to counteract the weathering threat to the stone cultural heritage given by pollution and global warming. Bacterial Calcium Carbonate Mineralization is indeed proposed as an environmentally friendly tool applicable *in situ* to protect calcareous stone artworks. The mini-review reports the milestones of the biomineralization approaches, discussing the challenging aspects and the perspectives of the different methods.

Marine Microbiology is a rapidly expanding branch of the science of Microbiology and thanks to latest generation molecular methodologies and technologies the nature, role, and biotechnological potential of marine microorganisms, the dominant life form on our planet, begin to be disclosed. In the work by Ottaviani et al. predatory bacteria belonging to the genus *Halobacteriovorax* were isolated from the Adriatic Sea and tested for their ability to prey pathogenic *Vibrio* spp. strains. Predator-prey interaction within the prokaryotic community was proposed to play a role in modulating the abundance of *V. parahaemolyticus strains* in the marine ecosystem through a top-down control of their bacterial prey community. Tamburini et al. investigate the structure and composition of the bacterial and archaeal communities in sediments from three Mediterranean ports. Using targeted metagenomic analysis of the 16S rRNA gene they were able to assess the effects exerted by multiple organic and inorganic contaminations on the benthic prokaryote community and designate bacterial community as a candidate tool for the monitoring of the sediment quality status in marine harbors.

The rise of extensive and widespread antibiotic resistance (AR) is one of the greatest threats to human health in environments exposed to antibiotic residues by means of wastewaters and animal manure. It has been recognized that some of the routes that cause AR spread in environments related to the agri-food system include the use of reclaimed water for irrigation purposes, and also the animal manure application to soils. In the study reported by Riva et al., the ability of an environmental *E. coli* strain, isolated form the crustacean *Daphnia* sp., to acquire exogenous DNA by natural competence with relatively high frequency was demonstrated. The protocol adopted was conceived to mimic conditions feasible in the environment, i.e., in natural and artificial water solutions considered as representative of environmental habitats. By also showing the capacity of this *E. coli* strain to colonize plant rhizosphere, using soil potted lettuce as a model system, the authors' results confirm the importance to investigate the possible spread of antibiotic resistant determinants through horizontal gene transfer in the environment and, particularly, in the rhizosphere of those employed plant species. Citterio et al. applied a PCR-based plasmid replicon typing to investigate the diversity and transferability of AR genes carrying plasmids in environmental *E. coli* strains, isolated from clams and marine sediments. Conjugative FIA, FIB, FII, plasmids (IncF group) were the most frequently found in AR strains isolated from the marine environment, suggesting a role played by these replicons in the spread of AR genes among environmental *Enterobacteriaceae* and, through the food chain, to human isolates.

Lastly, the use of antibiotics in the food animal industry is certainly considered among the main causes of propagation and dissemination of antibiotic residues, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the soil-water system. The review by Checcucci et al. highlights the most recent research on ARGs in farm environment and the strategies used to control their dissemination. This review analyzes the most recent research on antibiotics and ARGs environmental dissemination conveyed by livestock waste. Strategies to control ARGs dissemination and antibiotic persistence were reviewed with the aim to identify methods for monitoring DNA transferability and environmental conditions promoting such a diffusion.

AUTHOR CONTRIBUTIONS

All authors contributed to the critical discussion of this Editorial and wrote and approved the final editorial.

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ORIGINAL RESEARCH
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Plasmid Replicon Typing of Antibiotic-Resistant *Escherichia coli* From Clams and Marine Sediments

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Unlike human isolates, environmental *Escherichia coli* isolates have not been thoroughly investigated for the diversity and transferability of antibiotic-resistant plasmids. In this study, antibiotic-resistant strains from marine sediment (n = 50) and clams (n = 53) were analyzed (i) for their plasmid content using a PCR-based plasmid replicon typing (PBRT) kit and (ii) for the transferability of plasmid-associated antibiotic resistance (AR) traits by mating experiments. Fifteen of the thirty replicons targeted by the PBRT kit were detected in the isolates; 8/15 were identified in both sediment and clam isolates, although at different frequencies. The most frequent replicons in sediment (74%) and in clam strains (66%) alike, were FIA, FIB, or FII, which are associated with the IncF group, followed by the I1α replicon, which was more frequent in clam (24.5%) than in sediment (10%) strains. More than 50% of the strains contained multiple replicons; although 15 were untypable, S1-PFGE analysis demonstrated that 14/15 carried no plasmids. All cryptic strains were successfully typed and were positive for IncF or IncI replicons. Antibiotic-resistant strains accounted for 63% of all isolates and were significantly ($p < 0.05$) more frequent in phylogroup A. Most (35%) multidrug-resistant (MDR) strains belonged to phylogroup A, too. Although 25/26 MDR strains were positive for IncF plasmids (the exception being a clam strain), the FII-FIB rep combination was predominant (63%) among the sediment isolates, whereas most clam isolates (40%) carried the FII replicon alone. In mating experiments, selected MDR strains carrying FIB, FII, and I1α replicons, used as the donors, transferred multiple ARs together with the IncF or IncI plasmids at high frequency. Since IncI plasmids are common in *E. coli* and *Salmonella enterica* isolates from poultry, our findings suggest an animal origin to the *E. coli* clam strains carrying IncI plasmids. They also suggest a role for IncI plasmids in the spread of ARs among environmental *Enterobacteriaceae* and, through the food chain, to human isolates. In conclusion, the PBRT kit proved to be a useful tool to identify plasmids carrying antibiotic-resistant genes and to shed light on the factors underpinning their diffusion.

Keywords: PBRT kit, plasmid, Inc group, replicon, antimicrobial resistance, *Escherichia coli*

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INTRODUCTION

Antimicrobial resistance – a major and topical clinical issue – has also become an environmental concern owing to the growing spread and ubiquity of resistance genes and bacteria (Bengtsson-Palme et al., 2018). The human and animal microbiota appears to be the primary reactor where antibiotic resistance develops, due to exposure to the selective pressure of antibiotics administered for infection treatment or prophylaxis (Baquero et al., 2008; Rolain, 2013). Moreover, the gut may be a reservoir of resistance genes, where horizontal genetic transfer (HGT) among different microbial species may easily occur (Rolain, 2013). Human and animal antibiotic-resistant (AR) bacteria released into wastewater find their way to the soil and to water environments; in particular, water is a favorable habitat for interactions and gene exchanges among micro-organisms, the dissemination of resistance genes or bacteria, and the transmission of waterborne infectious disease (Finley et al., 2013; Jang et al., 2017). *Enterobacteriaceae* are found in a wide range of environments. The presence of *Escherichia coli* – a common bacterium of the human and animal intestinal microbiota – in natural water bodies has long been interpreted as indicating fecal contamination. More recently, *E. coli* has been demonstrated to be highly adaptable and to be able to survive and replicate outside the host, in water, soil, sediment and vegetables (Delaquis et al., 2007; Bergholz et al., 2011; Frank et al., 2011; Jang et al., 2017). Commensal as well as pathogenic *E. coli* strains resistant to several antibiotics have been recovered in the marine environment (Vignaroli et al., 2013, 2016; Charnock et al., 2014; Drali et al., 2018). Antibiotic resistance in *E. coli* species is of particular concern because of the growing prevalence of multidrug-resistant (MDR) strains involved in human and animal infections. Moreover, the incidence of AR commensal *E. coli* isolates in healthy humans is increasing worldwide (Broaders et al., 2013; Rolain, 2013), contributing to the emergence and spread of AR pathogens. AR clinical isolates of *E. coli* are also frequently related to those from animals, suggesting that both the food chain and food animals may be a source of MDR strains (Rolain, 2013; Lazarus et al., 2015; Berg et al., 2017).

Besides antibiotic exposure and HGT events, the acquisition of resistance genes by *E. coli* isolates is also affected by their genetic background. Some phylogenetic groups (i.e., A and D) are more prone to develop antibiotic resistances, and strains belonging to the same phylogroup and sequence type (ST) often share the same antibiotic resistance profile (Tenaillon et al., 2010). *E. coli* population structure can provide useful information on strain origin, since different phylogroups predominate in distinct ecological niches (Tenaillon et al., 2010). The acquisition of plasmids, which enhance resistance gene dissemination, is believed to play a key role in the growing prevalence of MDR *E. coli* strains (Mathers et al., 2015). In *Enterobacteriaceae*, some plasmids associated with specific resistance determinants are predominant in specific geographic areas; they are able to replicate in a wide host range (e.g., IncA/C and IncL/M plasmids) and their dissemination relies on antimicrobial selective pressure (Rozwandowicz et al., 2018). Other plasmids are only found in closely related hosts and are maintained by the bacterial cell because they encode virulence factors enhancing bacterial adaption and fitness (Carattoli, 2009).

This study was undertaken to determine: (i) the distribution and prevalence of major plasmid replicons in *E. coli* isolated from clams and marine sediment using the PCR-based replicon typing (PBRT) kit and (ii) the involvement of specific plasmids in the conjugal transfer of antibiotic resistance from environmental *E. coli*.

MATERIALS AND METHODS

Bacterial Strains

A total of 103 *E. coli* isolates (53 from clams and 50 from marine sediments) were used in the study. The 53 clam strains were selected from a collection of 141 strains isolated from Venus clams collected in Italy in the middle Adriatic Sea, which had previously been characterized for antibiotic resistance phenotype and phylogroup (Vignaroli et al., 2016). The 50 sediment strains were isolated from samples collected at three coastal sites (SE, PN, and API) at a depth of 4–12 m (from latitude 43°45.300′N, longitude 13°12.630′E, to latitude 43°39.0′N, longitude 13°22.0′E) near the areas where the clams had been harvested.

To detach bacteria from sediment, aliquots (5 g) of each sample were suspended in 20 mL sterile seawater, vortexed and sonicated (3 times, 1 min per cycle) as described previously (Luna et al., 2010; Vignaroli et al., 2013). The resuspensions were filtered by the membrane filter technique and *E. coli* strains were isolated in the selective medium mFC agar (BBL, Becton Dickinson & Co., Sparks, MD, United States) (Vignaroli et al., 2013).

Strain Identification and Typing

Presumptive *E. coli* colonies from sediment samples were identified by the molecular approach based on PCR amplification of the species-specific *uidA* gene (McDaniels et al., 1996). The PCR methods developed by Clermont et al. (2011, 2013) allowed each *E. coli* isolate to be assigned to a phylogenetic group or cryptic clade.

Antimicrobial Susceptibility and PCR Detection of Class 1 Integrons and Antibiotic Resistance Genes

Strain susceptibility to ampicillin (10 μg), cefotaxime (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), tetracycline (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg) and streptomycin (10 μg) was assessed by the disk diffusion method according to CLSI recommendation (Clinical and Laboratory Standards Institute (CLSI), 2017). Strains resistant to β-lactams were analyzed for extended spectrum β-lactamase (ESBL) production by screening and confirmatory tests (Clinical and Laboratory Standards Institute (CLSI), 2017). In brief, a standard disk diffusion test was performed in which the β-lactam antibiotic disks of both cefotaxime and ceftazidime, alone and in combination with clavulanate were used. A ≥5-mm increase

in a zone diameter for either antimicrobial agent tested in combination with clavulanate vs. the zone diameter of the agent when tested alone indicated the ESBL production by the strain (Clinical and Laboratory Standards Institute (CLSI), 2017).

E. coli ATCC 25922 was the reference strain in all antimicrobial susceptibility assays. Resistant strains were screened by PCR for the following determinants: bla_{TEM}, bla_{SHV}, and bla_{CTX-M} (ESBL-encoding genes) for β-lactam resistance; *tet*(A) for tetracycline resistance; *dfrA1* for trimethoprim/sulfamethoxazole resistance; and *strA*, *strB*, *aadA*, and *ant*(3″) for aminoglycoside resistance. Primers and PCR conditions were as reported previously (Vignaroli et al., 2012, 2013).

MDR strains were analyzed by PCR for the *intI1* integrase gene and the variable region of the class 1 integron using primers and PCR conditions described previously (Vignaroli et al., 2012). The resistance genes linked to class 1 integron were characterized by sequencing (GATC Biotech Cologne, Germany) the cassette amplicons and by nucleotide sequence analysis using the Basic Local Alignment Search Tool (BLAST)[1].

Plasmid Typing

The PBRT 2.0 kit (Diatheva, Fano, Italy) which has been used for plasmid identification and to type the major resistance plasmids found in *Enterobacteriaceae* (Carloni et al., 2017), was applied in our study. The amplicons recognized by the PBRT kit were analyzed by capillary electrophoresis with an AATI Fragment Analyzer (Agilent, Santa Clara, CA, United States).

Where necessary, amplicons were purified using MinElute PCR purification kit (Qiagen, Hilden, Germany) and directly sequenced by the Sanger method using BigDye Terminator v. 1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Vilnius, Lithuania) on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, United States). The consensus sequences obtained were submitted to the pMLST database for allele variant identification[2].

The *stx1*, *stx2*, and *eae* genes of shiga toxin-producing *E. coli* (STEC) strains were identified using STEC FLUO detection kit (Diatheva, Fano, Italy) according to the manufacturer's instructions; the strains that were positive for *stx* and/or *eae* genes underwent O-serogroup identification (O157, O111, O26, O103, and O145) using the STEC Serotypes FLUO kit (Diatheva).

S1-PFGE

S1-nuclease pulsed field gel electrophoresis (S1-PFGE) was performed to determine plasmid size and compare the plasmid profile of strains. Total DNA embedded in 0.8% agarose plugs was incubated for 30 min at 25°C with 100 U *Aspergillus oryzae* S1 nuclease (Takara Bio Inc., Shiga, Japan). The plugs were loaded on a 1% agarose gel using 0.5X TBE running buffer. Electrophoresis was performed in a CHEF-mapper system (Bio-Rad Laboratories, Inc., CA, United States) with the pulse time increasing from 1 to 25 s for 17 h at 14°C and 200 V (6 V/cm). The Low Range PFG Marker (0.1–200 kb) and Lambda Ladder PFG Marker (50–1,000 kb) from New England Biolabs (Ipswich, MA, United States) were used as molecular size markers.

Conjugation Experiments

Conjugal transfer of tetracycline and β-lactam resistance was performed by filter mating using the protocol previously described (Vignaroli et al., 2011). The *E. coli* 1816 (a mutant of *E. coli* C600, lactose-non-fermenting, resistant to nalidixic acid and rifampicin) was used as the recipient strain. Transconjugants were selected on Brain Heart Infusion agar (BHIA) (Oxoid, Basingstoke, United Kingdom) supplemented with tetracycline (20 μg/mL), rifampicin (50 μg/mL), and nalidixic acid (50 μg/mL). Transfer frequency was expressed as number of transconjugants per recipient. Transconjugants were first confirmed by three passages on MacConkey agar (Oxoid) containing all three antibiotics at the concentrations used for selection. Plasmid acquisition was assessed by comparing the S1-PFGE profiles of transconjugant and donor and confirmed using the PBRT kit.

Hybridization Assays

The plasmid location of the *tet*(A) gene was investigated in the donors, the transconjugants and the recipient *E. coli* 1816 by hybridization assays after S1-PFGE and Southern blotting. DNA was blotted onto positively charged nylon membranes (Bio-Rad Laboratories) and hybridized with a biotin-labeled *tet*(A) probe using North2South™ Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific, Rockford, IL, United States), according to the manufacturer instructions.

Statistical Analysis

Differences in the prevalence of the replicon types were analyzed by the χ^2 test. The significance of the association between a replicon type and resistance to a specific antibiotic or belonging to a specific phylogroup was analyzed by Fisher's test. A P-value < 0.05 was considered significant.

RESULTS

A total number of 103 *E. coli* isolates, obtained from clam and sediment samples collected at roughly the same three sites (API, SE, and PN) were analyzed in this study. The 53 *E. coli* clam strains belonged to different phylogroups; most (n = 31) were from clams harvested next to the API site, 10 came from site SE and 12 from site PN. Most isolates belonged to phylogroup A (n = 21), B1 (n = 7) or D (n = 6) whereas six were cryptic (Vignaroli et al., 2016; **Table 1**).

Analysis of the 50 isolates from sediment samples grown on mFC agar and identified as *E. coli* showed that most of them (n = 41) were from site PN and that they belonged to all phylogroups except E and F. There were only two cryptic strains, both of clade V, from site PN (**Table 1**).

All 103 strains were subjected to plasmid replicon typing; the results are reported in **Table 1**. Overall, 15 of the 30 replicons detected by PBRT kit were found in the strains;

[1] http://blast.ncbi.nlm.nih.gov/Blast.cgi

[2] https://pubmlst.org/plasmid

TABLE 1 | Prevalence of the replicons detected by the PBRT kit in *E. coli* isolates from clams and sediments.

Phylogroup	N. of strains	N. of strains positive for each replicon type																
		HI1	I1α	I1γ	X1	X3	X4	M	N	FIA	FIB	FII	FIB KN	K	B/O	R	More Rep	No Rep[a]
Clams																		
A	21	1	3	–	4	1	–	–	1	2	3	12	–	–	–	–	7	3
B1	7	–	1	–	–	–	–	–	–	–	3	4	2	–	–	–	3	–
B2	4	–	1	–	–	–	–	–	–	2	3	3	–	–	–	–	3	–
C	2	–	1	–	–	–	–	1	–	–	1	1	–	–	–	–	1	1
D	6	–	1	–	–	–	–	–	–	1	4	4	–	–	–	–	4	1
E	5	–	1	–	–	–	–	–	1	1	3	2	–	–	–	–	2	2
F	1	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
U[b]	1	–	–	–	–	–	–	–	–	1	1	1	–	–	1	–	1	–
clade III-IV-V	6	–	4	1	–	–	–	–	–	–	2	3	–	–	–	–	4	–
Total	53	1	13	1	4	1	–	1	2	7	20	30	2	–	1	–	25	7
Sediments																		
A	16	3	2	–	–	–	–	–	–	–	4	9	1	–	–	–	7	4
B1	13	–	3	–	1	–	–	–	–	1	6	7	2	–	–	1	6	2
B2	10	–	–	–	–	–	1	–	1	4	5	7	–	1	–	–	7	2
C	3	–	–	–	–	–	–	–	1	–	3	3	–	–	–	–	3	–
D	4	–	–	–	–	–	–	–	–	1	2	4	–	1	–	–	2	–
E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F	2	–	–	–	–	–	–	–	–	–	2	2	–	–	–	–	2	–
U[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
clade V	2	–	–	–	–	–	–	–	–	–	2	2	–	–	–	–	2	–
Total	50	3	5	–	1	–	1	–	2	6	24	34	3	2	–	1	29	8

[a]*Negative for all replicon types detected by the PBRT kit.* [b]*Unknown, PCR-negative for phylogroup determination (Clermont et al., 2013).*

8/15 were shared by clam and sediment strains, although at different frequencies. FIA, FIB, and FII, carried by IncF family plasmids, were the most frequent types, followed by replicon I1α, which is associated with IncI plasmids. IncF plasmids which were detected in 74% of sediment and 66% of clam strains carried single or multiple IncF replicons. In particular, the rep combination FII-FIB was significantly ($p = 0.03$) more frequent in sediment (54%) as well as in clam (28.6%) strains. More than 50% of strains contained multiple replicons, whereas 15 (8 from sediments and 7 from clams) were untypable by the PBRT kit. S1-PFGE analysis showed that 14/15 untypable strains did not contain plasmids. Interestingly, replicon I1α was more frequent among clam (24.5%) than among sediment strains (10%), but the difference was not significant. All cryptic clade strains (8) were typable by the kit and were positive only for IncF and/or IncI family replicons; 50% of these strains were positive only for FIB and FII replicons, whereas the remaining 50% carried I1α in addition to I1γ (clade III, *E. coli* ISZ 201), or FII (clade V, *E. coli* ISZ 272). In three *E. coli* clam strains (ISZ 45, ISZ 211, and ISZ 275), the PCR products of the FIA replicon were smaller (from ∼411 to ∼440 bp) than those of the control strain (462 bp). Sequencing and comparison with the pMLST database disclosed that all were FIA replicons carrying short deletions; in particular, *E. coli* ISZ 45 and *E. coli* ISZ 211 bore the FIA6 allele, whereas *E. coli* ISZ 275 carried the FIA5 allele; compared with the FIA2 allele of the control strain *E. coli* ISZ 35, the FIA6 and FIA5 alleles showed a deletion of 55 and 22 bp, respectively.

The STEC FLUO kit for the *stx1*, *stx2*, and *eae* virulence genes, to detect shiga toxin-producing *E. coli* showed that only *E. coli* PN37 from sediment was positive for gene *eae*, but that it did not belong to any of the serogroups detected by the kit.

The results of antimicrobial susceptibility testing showed that resistant isolates were significantly ($p < 0.05$) more numerous in phylogroup A (75.6%) than in the other phylogroups (56%). The prevalence of clam and sediment strains resistant to the different antimicrobials is reported in **Figure 1**. Among the 53 clam strains, resistance to tetracycline was the most frequent (62%; $n = 33$), followed by resistance to ampicillin (43%; $n = 23$) and streptomycin (26%; $n = 14$) (**Figure 1**), in line with a previous report (Vignaroli et al., 2016). Resistance to tetracycline was significantly more frequent ($p = 0.0015$) among clam than among sediment strains (**Figure 1**). Multidrug resistance was detected in 15 strains (28%), most of which belonged to phylogroup A (47%), B2 (20%) or E (13%) and was not significantly associated with any phylogroup. The percentage of resistant strains positive to the relevant resistance genes is shown in **Figure 2**. Of the 23 ampicillin-resistant strains, 21 (91%) were ESBL producers and 19 of them (83%) carried a gene (bla_{TEM}) encoding a TEM-type β-lactamase, whereas 24 out of 33 (73%) tetracycline-resistant strains were positive for *tet*(A). In the streptomycin-resistant strains ($n = 14$) the genes *strA* and *strB* were recovered at a

frequency of 86% (n = 12) and 57% (n = 8), respectively, whereas the *ant*(3″) gene was found in 21% (n = 3) of clam strains and *aadA* (50%; n = 7) was significantly (p = 0.03) more common in clam than in sediment strains (**Figure 2**).

Altogether, 30% (n = 15) of sediment strains were resistant to tetracycline, 30% (n = 15) to ampicillin and 22% (n = 11) to streptomycin, in line with the results from the clam strains, which come from the same areas (**Figure 1**). The association between the occurrence of antimicrobial resistance and specific Inc family plasmids was then investigated. The only detected association was between ampicillin resistance and the IncF family in the clam isolates (p = 0.007), as shown in **Figure 3**.

Multidrug resistance (to three or more antibiotic classes) was also detected in 11 sediment strains (22%); most of them (82%) belonged to phylogroup B1 (36%), B2 (28%) or A (18%); however, as in the clam strains, multidrug resistance was associated with none of the phylogroups. All but one of the ampicillin-resistant strains (93%; n = 14) were positive to the ESBL production test and carried bla_{TEM} gene. Two strains from the PN site were also positive for a gene encoding a SHV-type β-lactamase (bla_{SHV}) and an ESBL-encoding gene (bla_{CTX-M}), respectively. ESBL producers were recovered from all three sites, as displayed by the phenotypic screening. Moreover, 93% (n = 14) of tetracycline-resistant strains carried the *tet*(A) gene and all

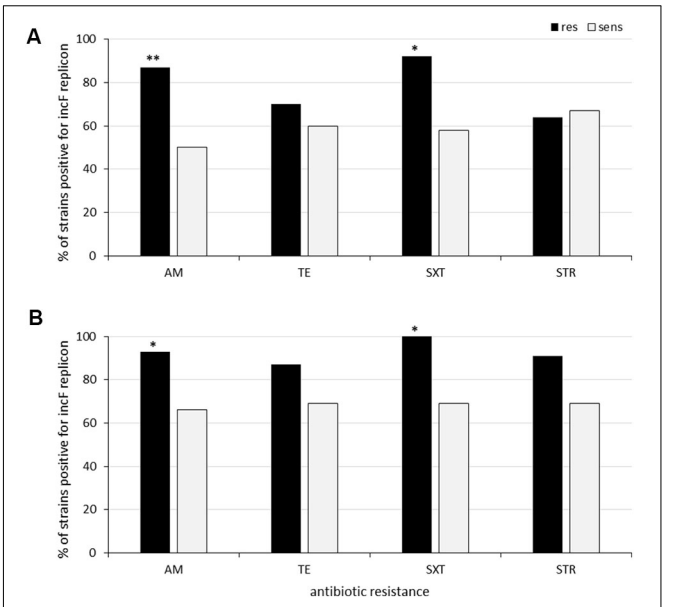

FIGURE 3 | Association between antibiotic resistance and IncF family replicons among clam **(A)** and sediment isolates **(B)**. **Statistically significant (p = 0.007). *Not quite statistically significant (p = 0.07–0.09).

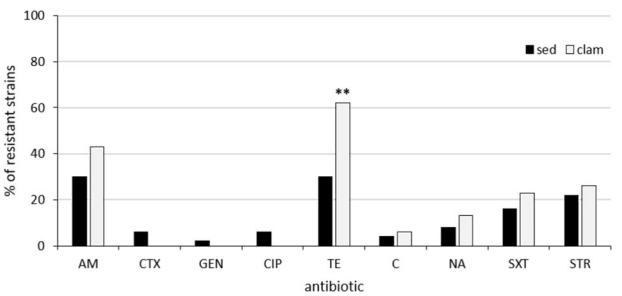

FIGURE 1 | Prevalence of resistant strains among clam and sediment isolates. AM, ampicillin; CTX, cefotaxime; GEN, gentamicin; CIP, ciprofloxacin; TE, tetracycline; C, chloramphenicol; NA, nalidixic acid; SXT, trimethoprim/sulfamethoxazole; STR, streptomycin. **Highly statistically significant (p = 0.0015).

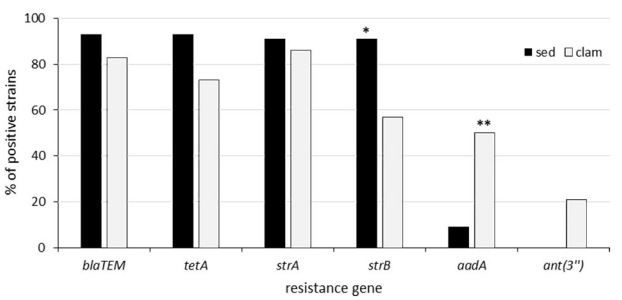

FIGURE 2 | Prevalence of resistance genes among resistant clam and sediment strains. **Statistically significant (p = 0.03). *Not quite statistically significant (p = 0.09).

streptomycin-resistant isolates were positive for *strA* and *strB* at a high frequency (91%; n = 10), like the streptomycin-resistant clam strains (**Figure 2**). The *ant*(3″) gene was never detected, whereas a single strain (*E. coli* PN56) carried both *aadA* and *dfrA1*. All MDR sediment strains (n = 11) carried multiple IncF replicons, with the FII-FIB combination being predominant (64%). All MDR clam strains but one (n = 14) were also IncF-positive. Most of them (n = 6) carried the FII replicon alone, whereas three strains carried FII and FIB.

Although 80% (n = 21) of MDR strains from both clam and sediment were positive for the integrase gene *intI1*, amplification of the variable region of the class 1 integron was obtained only from 20 and 9% of the clam and sediment strains, respectively. Sequence analysis of gene cassettes demonstrated three different arrangements: *dfrA1-aadA1* and *dfrA17-aadA5* in clam strains and *dfrA12-aadA2* in sediment strains.

The 14 *E. coli* isolates from clams (n = 9) and sediments (n = 5) showing positivity for the IncI1α group were subjected to S1-PFGE. Analysis of the number and size of their plasmids (**Table 2**) indicated that all isolates harbored 1–3 plasmids, ranging in size from 75 to 145 kb. To assess the involvement of the IncIα group in the transfer of antibiotic resistance, six tetracycline-resistant isolates carrying the *tet*(A) gene were used as donors in conjugation experiments. *E. coli* ISZ 220 from clams and *E. coli* PN30 from sediments transferred tetracycline resistance to *E. coli* 1816 at the highest frequency (respectively, 4.8×10^{-3} and 1.2×10^{-7}). Five transconjugants obtained from the two mating pairs were analyzed for their plasmid profile and resistance gene acquisition. In both mating assays the transconjugants acquired all the resistance determinants and the *intI1* gene, but not all the plasmids (**Table 2** and **Supplementary Figure S1**). In fact,

TABLE 2 | *E. coli* isolates from clams and sediments showing positivity for the IncI1α group: phylogroups, resistance genes and plasmid sizes.

E. coli strain	Phylo-group	Resistance genes	PBRT replicon	Plasmid size (∼kb)
Clams				
ISZ 220	C	bla_{TEM}, *tet*(A), *dfrA1*, *aadA*	I1α – FIB – FII – M	110, 95, 55
ISZ 276	D	*strA*, *strB*	I1α	90
ISZ 61	E	bla_{TEM}, *dfrA1*, *strA*, *aadA, ant*(3″)	I1α – FIB – FII – N	140, 80, 40
ISZ 80	F	–	I1α	85
ISZ 210	A	–	I1α – FIB	110, 95
ISZ 211	A	–	I1α – FIB – FIA	80, 40
ISZ 255	B1	–	I1α	80
ISZ 274	A	bla_{TEM}	I1α	90
ISZ 325	B2	*tet*(A)	I1α	90
Sediments				
PN41	A	*tet*(A)	I1α	95
PN44	A	*tet*(A)	I1α	95
PN16	B1	–	I1α	85
PN29	B1	bla_{TEM}, *tet*(A), *strA*, *strB*	I1α – FIB – FII	145, 75
PN30	B1	bla_{TEM}, *tet*(A), *strA*, *strB*	I1α – FIB – FII	145, 75

S1-PFGE showed that *E. coli* ISZ 220 transferred two (∼110 and 55 kb) of its three plasmids; PBRT confirmed that the transconjugants were positive for replicons I1α and M, but not for replicon F. In contrast, *E. coli* PN30 transferred only the IncF plasmid, although it also carried an IncI1 plasmid, as shown by S1-PFGE and by PCR amplification of the replicons. The hybridization assays confirmed the location of the *tet*(A) gene on the larger plasmids of the two donors (on the 110 kb plasmid in *E. coli* ISZ 220 and on the 145 kb plasmid in *E. coli* PN30) and of the relevant transconjugants (**Supplementary Figure S1**).

DISCUSSION

The rapid evolution and global diffusion of MDR *Enterobacteriaceae* (mostly *E. coli* and *Klebsiella pneumoniae*) is raising widespread concern. Specific successful bacterial clones such as *E. coli* ST131 and *K. pneumoniae* ST258 are major causes of hospital- and community-acquired infections (Mathers et al., 2015). For epidemiological purposes, epidemic clones are usually identified by their genetic background (e.g., ST determination), whereas plasmid DNA is ignored despite its important role in strain resistance traits. The association between high-risk clones and specific resistant plasmids involved in resistance gene dissemination has been demonstrated in clinical settings (Mathers et al., 2015; Roer et al., 2018). In contrast, environmental *E. coli* isolates have not been thoroughly investigated for the diversity and transferability of antibiotic resistance plasmids, despite the fact that assessment of their plasmid profile could help identify dangerous strains and their origin. Moreover, recurrent detection of some plasmid types could indicate their role in strain survival in specific habitats as well as in the spread of antibiotic resistance traits. In this study, 103 AR strains from clam and marine sediment samples, collected along the mid-Adriatic coast, were analyzed for their plasmid content and for the transferability of plasmid-associated resistance traits. The PBRT kit employed in the study proved useful to identify the most common plasmids. Even though 14.5% of strains were negative for all the replicons targeted by the kit, most (93%) were not typable because they did not contain plasmid DNA. IncF replicons were the most frequent in our isolates, in line with reports that IncF plasmids are the most common plasmids in *Enterobacteriaceae*, especially in *E. coli* species (Carattoli, 2009). In Europe IncF plasmids are described predominantly in human and animal isolates, not in environmental strains (Rozwandowicz et al., 2018). Consequently, their high prevalence in our clam and sediment isolates may reflect their human or animal origin.

IncF plasmids are typically multireplicon, often encoding FII together with FIA and/or FIB (Villa et al., 2010). The plasmids containing FII and FIA replicon types have been described in the epidemic *E. coli* strain ST131 and in *K. pneumoniae* ST258 clones (Mathers et al., 2015). In our *E. coli* strains, most IncF-positive isolates, particularly among sediment isolates, contained the FII-FIB combination or FII alone. In a recent study (Lambrecht et al., 2018), the FII-FIB combination was found to be predominant in commensal MDR *E. coli* from farm animals, mainly broilers. IncF plasmids are often associated with IncI plasmids, which are also common in *E. coli* and *Salmonella enterica* from poultry (Rozwandowicz et al., 2018). Moreover, IncF and IncI plasmids have been reported in association with MDR *E. coli* strains, mainly ESBL producers, from food animals (Xie et al., 2016). The prevalence of both IncF and IncI plasmids in our isolates, particularly from clams, strengthen the hypothesis that contamination of our clam harvesting areas came from animal sources.

Resistance to multiple antimicrobial classes is common in *E. coli* (ECDC, 2019) and the prevalence of resistance to β-lactams, tetracycline and aminoglycosides in our clam and sediment strains is in line with earlier reports (Vignaroli et al., 2016; Pormohammad et al., 2019). Notably, antibiotic resistances are frequently associated with conjugative IncF or

IncI plasmids (Rozwandowicz et al., 2018). Accordingly, in our study these Inc groups were detected at higher frequency in resistant than in susceptible strains; moreover, sediment strains exhibited a significant association between β-lactam resistance and the presence of IncF plasmids. This Inc group was also associated, besides the IncI group, with the conjugative transfer of tetracycline resistance from *E. coli* donors of both origin (clams and sediment). These plasmids were probably involved in multidrug resistance, since mating experiments resulted in the transfer of multiple resistance genes. Most MDR strains (∼80%) were positive for the class I integron and the co-transfer of the *intI1* gene and of the resistance genes suggest the plasmid location of the integron. In particular, donor *E. coli* ISZ 220 (from clams) carried an integron with the *dfrA1-aadA1* cassette that was probably linked to the ∼110 kb IncI plasmid transferred in mating assays. Plasmids containing this cassette array have been described more frequently in MDR *Salmonella* and MDR *E. coli* isolates from meat and food animals than in human isolates (van Essen-Zandbergen et al., 2009; Sunde et al., 2015). In contrast, the *dfrA17-aadA5* cassette, which was found in a single clam strain, has typically been reported in isolates of human origin (Povilonis et al., 2010; Musumeci et al., 2012; Sunde et al., 2015). This finding may also be ascribed to contamination of the sampling areas with fecal bacteria mostly of animal origin. Therefore, the presence of a class I integron on conjugative plasmids contributes both to the emergence of MDR strains and to the dissemination of antibiotic resistance.

CONCLUSION

In conclusion, to the best of our knowledge this is one of the few studies focused on the prevalence of specific Inc group plasmids in *E. coli* isolates from secondary habitats (like clams and sediments). The PBRT kit, which has been developed for human isolates *of Enterobacteriaceae*, proved a useful tool to type the plasmids conferring antibiotic resistance on environmental *E. coli* isolates, to predict their origin and to formulate hypotheses on the contamination source. Moreover, these data could help correlate a plasmid type to strain adaptation and survival strategies outside the host, and provide further information on the spread of antibiotic-resistant plasmid families among *Enterobacteriaceae* in different settings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

CV, BC, FA, and FB designed the experiments, analyzed the results, and drafted the manuscript. MM contributed to result interpretations. SS, EC, GM, and NC performed the sampling and the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01101/full#supplementary-material

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Impacts of Anthropogenic Pollutants on Benthic Prokaryotic Communities in Mediterranean Touristic Ports

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Ports and marinas are central nodes in transport network and play a strategic role in coastal development. They receive pollution from land-based sources, marine traffic and port infrastructures on one side and constitute a potential pollution source for the adjacent coastal areas on the other. The aim of the present study was to evaluate the effects of organic and inorganic co-contamination on the prokaryotic communities in sediments from three Mediterranean ports. The structure and composition of the bacterial and archaeal communities were assessed by targeted metagenomic analysis of the 16S rRNA gene, and the links of prokaryotic communities with environmental and pollution variables were investigated. The harbors presented pronounced site-specificity in the environmental properties and pollution status. Consistently, the structure of archaeal and bacterial communities in surface sediments exhibited a strong spatial variation among the three investigated ports. On the contrary, a wide overlap in composition of prokaryotic assemblages among sites was found, but local variation in the community composition and loss of prokaryotic diversity was highlighted in a heavily impacted port sector near a shipyard. We provided evidences that organic matter, metals and PAHs as well as temperature and salinity play a strong role in structuring benthic bacterial communities significantly contributing to the understanding of their responses to anthropogenic perturbations in marine coastal areas. Among metals, copper was recognized as strongly associated with the observed changes in bacterial assemblages. Overall, this study provides the first assessment of the effects exerted by multiple organic and inorganic contaminations on benthic prokaryotes in ports over a large spatial scale and designates bacterial community as a candidate tool for the monitoring of the sediment quality status in harbors.

Keywords: bacteria, archaea, next generation sequencing, metal, hydrocarbon, harbor, sediment, network

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INTRODUCTION

Over the last two centuries, different manufactured materials and hazardous substances have been introduced in marine ecosystems by human activities causing their anthropization (Waters et al., 2016). The Mediterranean Sea is an interesting case study for investigating the impacts of anthropogenic pressures on marine ecosystems as it combines numerous maritime activities and

demographic pressures (Piante et al., 2015). Moreover, its responsiveness to human pressures is accelerated by the oceanographic conditions of a semi-enclosed sea. The Mediterranean Sea is not only amongst the busiest routes of global maritime transport, but it is also the main tourism destination in the world, accounting for the 53% of EU passenger seaborne traffic in 2009 (Eurostat, 2011) and 30% of the total world tourists in 2012 (Plan Bleu, 2014). Therefore, the Basin is globally one of the main hotspots of vulnerability to pressures exerted by tourism activities. More specifically, marinas and recreational harbors are ubiquitous tourism infrastructures, with 940 marinas along the Mediterranean coasts in 2010 (Piante et al., 2015).

Ports and marinas are central nodes in the transport network and play a strategic role in coastal development. They receive pollution from land-based sources, marine traffic and port infrastructures on one side and constitute a potential pollution source for the neighboring coastal areas on the other. Port pollution may result from ship accidents, deliberate operational discharges from ships, land activities, ship bunkering, garbage, dust, dredging, port maintenance, ship air emission, sewage, and others (Vandermeulen, 1996). In the last decades, the negative effects on natural ecosystems caused by anthropogenic activities have received increasing attention by the EU environmental policies (Borja et al., 2010). In this context, port sustainability has become crucial for protection of coastal water quality, wildlife, and human health in port city destinations (Di Vaio and Varriale, 2018). On the other hand, port facilities are types of activities that can result in a water body designated as a Heavily Modified Water Body (HMWB, Water Framework Directive 2000/60/EC, WFD). Human activities to support specific uses (e.g., navigation) have indeed caused physical alterations (i.e., dredging, confinement) in the water body (i.e., port) basically modifying its hydromorphological properties (Ondiviela et al., 2012, 2013). The peculiar features of HMWBs justify the development of monitoring and remediation programs specific and adequate to port characteristics.

Among chemical contaminants, metals and polycyclic aromatic hydrocarbons (PAHs) are almost ubiquitous in anthropized coastal areas, especially in harbors, which usually exhibit higher concentrations than the adjacent zones (Angelidis and Aloupi, 1995; Merhaby et al., 2015; Schintu et al., 2015; Zakhama-Sraieb et al., 2016). The distribution of metals within the aquatic environment is controlled by complex processes of material exchange, which are altered by natural and anthropogenic factors (Christophoridis et al., 2009). Metals are natural components of metalliferous minerals, which are geographically distributed in a heterogeneous way. Therefore, the background values can vary widely in different geographic regions, even in non-anthropized environments depending on the abundance of such metalliferous minerals (Gadd, 2010). Anthropogenic activities alter the biogeochemical cycle by increasing the concentration of metals with respect to their natural background and modifying their speciation in the environment (UNEP, 2013). On the other hand, PAHs are derived from crude oil products (i.e., petrogenic PAHs) and incomplete combustion of organic matter (i.e., pyrolytic PAHs). Natural sources have been found to be marginal, while anthropogenic activities are generally considered to be the major source of PAHs into the marine environment (Baumard et al., 1998). Long-range aeolian PAH transport of fine combustion particles appears to dominate the oceanic PAH flux (Gustafsson et al., 1997). In ports and marinas, the predominance of pyrogenic emission sources has extensively been reported with marked differences in pollutant compositions among different sites and the coexistence of petroleum and pyrogenic PAHs in multi-sectoral harbors (McCready et al., 2000; De Luca et al., 2004; Sprovieri et al., 2007; Merhaby et al., 2015; Schintu et al., 2015; Vitali et al., 2019). Lastly, metals and PAHs entering the marine environments accumulate in sediments, which act as a long-term contaminant sink (Christophoridis et al., 2009). Sediments in anthropized coastal zones are therefore contaminated by complex mixtures of organic and inorganic pollutants exhibiting a range of multifaceted interactions with bacterial communities (Liu et al., 2017).

Bacteria play a pivotal role in determining the fate and distribution of contaminants in marine sediments by controlling the global PAH fluxes by degradation (Duran and Cravo-Laureau, 2016) and altering metal speciation (Gadd, 2010). More specifically, interactions with microorganisms can lead to either an increase (i.e., siderophore production, redox mobilization, acidification) or a decrease (i.e., exopolymer production, intracellular sequestration, redox immobilization, biomineral formation) in metal bioavailability (Gadd, 2010). On the other hand, essential and non-essential elements above threshold concentrations exert toxic effects on bacteria by different mechanisms, such as oxidative stress caused by reactive oxygen species (ROS, Lemire et al., 2013). The compounds with two or three aromatic rings (i.e., low-molecular-weight PAHs) are acutely toxic while those having four or more rings (i.e., high-molecular-weight PAHs) are generally genotoxic (Ghosal et al., 2016). Nevertheless, the simultaneous exposure to PAHs and metals result in more complex impacts than those exerted by the single pollutant due to additive, synergistic or antagonistic effects. Indeed, metals can affect PAHs degradation by changing the surface properties of bacterial cells and interfering with enzymes on one side (Biswas et al., 2015); on the other, degradation of PAHs by the cytochrome P450 generate ROS reducing the tolerance to toxic metals (Kuang et al., 2013). In marine ecosystems, the impact of pollutants on benthic communities may also depend on the system attributes, such as hydrology, tidal energy, and climatic conditions (Nogales et al., 2011). In the last decade, the effects of co-contamination by PAHs and metals on benthic prokaryotic assemblages in marine sediments have been addressed in few studies (Iannelli et al., 2012; Sun et al., 2012; Chiellini et al., 2013; Misson et al., 2016) and even less have pursued this challenging goal by exploiting NGS techniques (Sun et al., 2013; Quero et al., 2015).

With these premises, the general objective of this work was to evaluate the impacts of organic and inorganic co-contamination on the prokaryotic communities in port sediments. In the framework of the ENPI CBCMED project MAPMED, sediments were collected from three touristic ports located along the Mediterranean Sea. The pollution status of the three harbors

has been recently determined by Chatzinikolaou et al. (2018) and Vitali et al. (2019). Moreover, their environmental properties have been defined based on the combined assessment of physical parameters, chemical variables (i.e., nutrients, pigments), and macrobenthic diversity (Chatzinikolaou et al., 2018). In this study, the structure and composition of the bacterial and archaeal communities were assessed in surface sediments from the three ports by targeted metagenomic analysis of the 16S rRNA gene, and the links between prokaryotic communities and both environmental and pollution variables were investigated.

MATERIALS AND METHODS

Study Sites and Sampling

A coordinated sampling campaign was performed in September 2012 at the end of the tourist season at three Mediterranean ports (**Figure 1**): Cagliari (Sardinia, Italy), El Kantaoui (Sousse, Tunisia), and Heraklion (Crete, Greece). Within each harbor, three to five sampling stations were located in sectors dominated by different port activities achieving an adequate spatial coverage of the whole port area (**Supplementary Table S1**). The collected samples were labeled as follows: the letter specifies the port (C: Cagliari; E: El Kantaoui; H: Heraklion), while the digit identifies the sampling station within each port sector (**Supplementary Table S1**).

The environmental parameters (Chatzinikolaou et al., 2018) included in the statistical analysis were salinity measured in surface seawater (S_W), temperature (T_S), redox potential (Eh), silt-clay ratio (SC), and organic carbon (OC). The sum of chlorophyll-a and phaeopigment concentrations (CPE, Danovaro et al., 1999) and the ratio of phaeopigments to the sum of chlorophyll-a and phaeopigments in sediments (PAP, Boon et al., 1998) were also calculated.

Concentrations of 31 individual aliphatic hydrocarbons (AHs) in the range C10-C40, Unresolved Complex Mixture (UCM), and 16 US EPA priority PAHs in superficial sediments were previously evaluated by gas chromatography-mass spectrometry in Chatzinikolaou et al. (2018) and Vitali et al. (2019). Abbreviations for the 16-EPA priority PAHs are as follows: 2-ring PAH – naphthalene (Naph); 3-ring PAHs – acenaphthylene (Aceph), acenaphthene (Ace), fluorene (Fl), phenanthrene (Phen), anthracene (Ant); 4-ring PAHs – fluoranthene (Flu), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chr); 5- ring PAHs – benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), indeno[1,2,3-c,d]pyrene (Inp), benzo[g,h,i]perylene (BgP), dibenzo[a,h]anthracene (DBA). The following hydrocarbon pollution descriptors were used: (i) the total level of AHs, calculated as the sum of individual compounds in the range C10-C40, (ii) the sum of four low-molecular-weight PAHs (Phen, Ant, Flu, Pyr, LPAHs) and the sum of eight high-molecular-weight (BaA, Chr, BbF, BkF, BaP, Inp, BgP, DBA, HPAHs) as descriptors of PAH pollution levels; (iii) the molar ratios of selected PAHs (ΣLPAH/ΣHPAH, Ant/Ant + Phen, Flu/Flu + Pyr, BaA/BaA + Chr, Inp/Inp + BgP), as descriptors of PAH sources (Vitali et al., 2019).

The concentrations of metals (Al, Cd, Cr, Cu, Fe, Ni, Pb, V, and Zn) and metalloids (As, Sb) were previously determined by Inductively Coupled Plasma optical emission spectrometry in Chatzinikolaou et al. (2018). A normalization

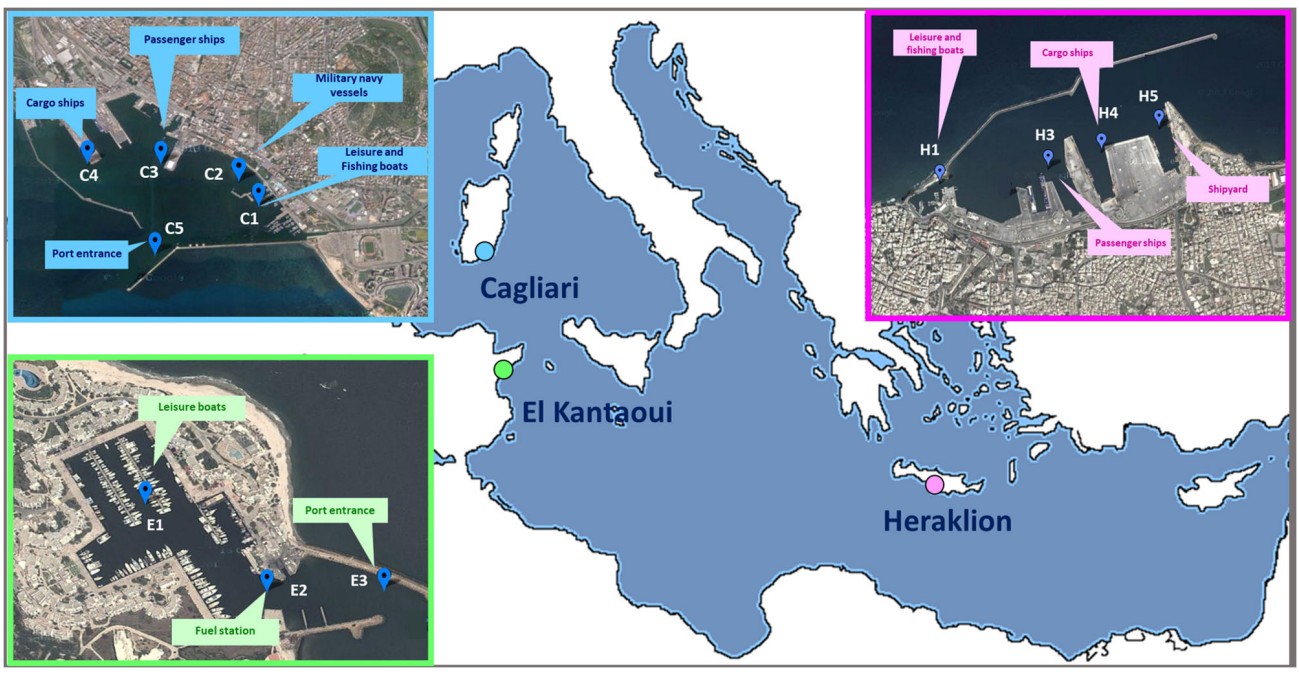

FIGURE 1 | Maps of the touristic ports of Cagliari (C), El Kantaoui (E), Heraklion (H). The position of the sampling stations and the main sector uses are indicated on the maps. Map image: Google Earth Pro, Maxar Technologies.

of metal data by Al as conservative metal was applied according to the geochemical approach implemented for port sediments by Ho et al. (2012). In literature, Al has been extensively used as normalizer since it complies with a number of prerequisites (Schropp and Windom, 1988; Huntingford and Turner, 2011; UNEP/Med, 2011). More specifically, the element is one of the most important constituents of the aluminosilicate minerals, which represent the main group of minerals in the fine sediment fractions and tightly bind naturally occurring metals within their structure. Aluminum is also stable and is not significantly subject to environmental influences, such as reduction/oxidation, adsorption/desorption, and other diagenetic processes. Finally, the aluminum content is not generally influenced by anthropogenic sources.

Analysis of Prokaryotic Communities by NGS of 16S rRNA Gene

Sample collection and DNA extraction were performed as previously described by Vitali et al. (2019). Briefly, three samples of surface sediments (0–1 cm) were collected using small plastic corers from each station (Chatzinikolaou et al., 2018). Extracted DNA (10 ng) was used as template in PCR reactions. The V4 region of the bacterial 16S rRNA gene was amplified by the bacterial-specific primer pair 563f/802r according to Claesson et al. (2010). For amplification of the archaeal 16S rRNA gene, a nested PCR approach was adopted. The first amplification was performed with archaeal-specific primers 21f/958r according to DeLong (1992). Then, the hypervariable V3 region of the 16S rRNA was amplified using the archaeal-specific primer pair 344f/519r (Yu et al., 2008). For each sample, three replicate reactions with each primer pair were combined to minimize stochastic PCR bias. The PCR products were purified from the agarose gel using the QIAquick Gel extraction kit (Qiagen). Sequencing was performed by the sequencing facility Source Bioscience (Nottingham, United Kingdom).

For data processing, raw sequences obtained by Illumina Miseq were demultiplexed by the sequencing facility. For pre-treatment, reads were quality checked with FastQC (Andrews, 2010), primers were removed with Cutadapt (Martin, 2011), and forward and reverse reads were merged using Pear (Zhang et al., 2014). The quality check with FastQC revealed a region of low quality at the end of the sequences. Thus, the sequences were subjected to a trimming and filtering step using Sickle (Joshi and Fass, 2011) and FastX-trimmer (Gordon and Hannon, 2012). A final quality control was carried out with MultiQC (Ewels et al., 2016) to evaluate the overall quality of the reads by aggregating the whole dataset. "Good Quality Reads" were subsequently imported into Quantitative Insights into Microbial Ecology (QIIME 2) version 2018-11 (Bolyen et al., 2019) and dereplicated. Illumina sequencing reads are available at the European Nucleotide Archive under accession study PRJEB36504.

For each distinct community (Bacteria, Archaea), the operational taxonomic units (OTUs) were assigned with a default identity of 97% using open reference OTU picking approach, then low abundance OTU $< 0.005\%$ (Bokulich et al., 2013), chimeras and singletons were identified and removed from the dataset, thus obtaining a filtered OTU-abundance table.

For each OTU, a representative sequence was used for taxonomy assignment against the Silva database release 132 (Quast et al., 2013). For the analysis of sulfate reducing bacteria (SRB), the OTUs assigned to the families Thermodesulfovibrionaceae, Desulfarculaceae, Dethiosulfovibrionaceae, Desulfobacteraceae, Syntrophaceae, and Syntrophobacteraceae were extracted from the normalized OTU-abundance table of Bacteria according to Robador et al. (2016). For community composition, the barplots and Venn diagrams were plotted using the ggplot2 package and the online tool Venny 2.1, respectively (Oliveros, 2015; Wickham, 2016).

Statistical Analyses

Statistical analyses were performed using R (R Core Team, 2013) in RStudio (RStudio Team, 2015). Linear correlations between abiotic variables were computed by using the *corr.test* function of the psych package (Revelle, 2018) (**Supplementary Figure S1**). As pre-treatment transformation, data for each variable were subjected to the z-score transformation by subtracting their mean to each value and then dividing by their standard deviation. For multivariate analysis, the Principal Component Analysis (PCA) was performed on normalized (z-score) variables. PCA was obtained using *prcomp* function, while the *fviz_pca* function in factoextra package was used for plotting (Kassambara and Mundt, 2017). According to Ho et al. (2012), metal concentrations were included without normalization to Al in correlation analysis and PCA, while normalization was applied in all the other statistical tests.

In distance-based methods (i.e., Permutational multivariate analysis of variance, BIOENV, Mantel and partial Mantel tests), matrices were calculated by means of Bray–Curtis dissimilarity coefficient between sampling stations based on biotic data (i.e. Bacteria, Archaea, and SRB) by using the *vegdist* function in vegan R package (Oksanen et al., 2019), while Euclidean distance was calculated using the *dist* function based on abiotic data and geographical coordinates of the sampling stations.

For the two distinct prokaryotic communities (Bacteria, Archaea) read count data were firstly normalized by Cumulative Sum Scaling (CSS) transformation, using metagenomeSeq package (Paulson et al., 2013; Paulson, 2014). The indices of diversity (richness as number of observed OTU, Shannon with an e log base) and evenness (Pielou's) were used to assess the alpha-diversity. All indices were calculated for all samples using the function *global* in the microbiome package (Lahti et al., 2017). Beta diversity was inspected by ordination analysis [principal coordinate analysis (PCoA)] based on Bray–Curtis dissimilarity using the function *ordinate* of the phyloseq package (McMurdie and Holmes, 2013). Permutational multivariate analysis of variance (PERMANOVA) was then used to evaluate the null hypothesis that there were no significant differences between ports. PERMANOVA was performed using the *adonis* function in the vegan package on the Bray–Curtis dissimilarity matrix with 9,999 permutations.

The relation between the structure of the prokaryotic communities (i.e., Bacteria, Archaea, SRB) and the measured

abiotic variables was investigated by the BIOENV test. In order to identify the best subsets of variables associated with each community structure (BestBIOENV), the Spearman rank correlation coefficient between the matrix for abiotic variables (calculated with Euclidean distance) and the matrix of each distinct community (calculated with Bray–Curtis) was determined by the *bioenv* function in the vegan package. A Mantel test was then performed to assess the significance of the biotic-abiotic relation using the *mantel* function (9,999 permutations) in the vegan package.

The Mantel test was also applied to correlate each Bray–Curtis distance matrix of community structure with the Euclidean distance matrix of the best subset of abiotic variables (BestBIOENV) selected for each distinct prokaryotic community. The partial Mantel test was subsequently performed according to Vitali et al. (2019) to evaluate the relationship between the community structures and the best subsets of variables (BestBIOENV) after the effects of spatial autocorrelation have been removed. Briefly, the *mantel.partial* function in vegan package was used to determine the correlation between each Bray–Curtis distance matrix of community structure and the Euclidean distance matrix of the best subsets of variables (BestBIOENV) while controlling the effect of spatial autocorrelation with the Euclidean distance matrix of geographical coordinates of sampling stations (GEO).

A redundancy analysis (RDA) was performed on the Hellinger transformed OTU-abundance table (Legendre and Gallagher, 2001) to investigate the effects of the best subset of abiotic variables selected for each distinct prokaryotic community. The *rda* function in the vegan R package was used to test the independent and combined effects of the variables in the best subsets (i.e., model was formalized in R with the * operator). The ANOVA test for constrained analysis was performed to assess the significance of the RDA model (i.e., overall and by terms) using the *anova.cca* function of vegan package.

To further investigate the relations between community structure and measured abiotic variables, Spearman correlation analysis was performed between all OTUs and between all OTUs and abiotic variables. Correlations were calculated with the *corr.test* function in the psych package, with false discovery rate correction for multiple testing (i.e., option "fdr" in *corr.test* function). For archaeal communities, few correlations were retained and therefore the dataset was not further analyzed. For bacterial communities, the strong and significant (i.e., spearman's rho > 0.8 and p-val < 0.05) correlations were selected, which were imported in Cytoscape (Shannon et al., 2003) to construct and visualize a correlation network. In the network, each node is a genus-level OTU or an abiotic variable, and edge connecting two nodes indicate the presence of a significant and strong correlation between two nodes (i.e., between two bacteria genus or between a bacterial genus and an abiotic variable). Clusters in the network were identified and calculated with clusterMaker2 (Morris et al., 2011) using the Community Clustering (GLay) algorithm. Network properties were calculated with Cytoscape "NetwrokAnalyzer" plugin (assuming an un-directed network on the unclustered network) and node degree was used to color nodes in the network clusters.

RESULTS

Environmental and Pollution Status

A PCA analysis was performed including all the abiotic variables in order to identify which of them contributed the most to the description of the environmental and contamination status of the three investigated ports (**Figures 2A,B**). The first three components accounted for 75.1% of the total variance. Sediments collected in Cagliari were separated from those collected in the other two ports on the first component (PC1) based on higher concentrations of the four metals Al, Pb, Zn and Fe, higher levels of CPE as well as lower values of temperature and salinity (see vectors in **Figures 2A,B**, and variable contribution in **Figures 2C–E**). On the second component (PC2), higher levels of the three metals Ni, Cr, and V, aliphatic hydrocarbons (AHs, UCM) and silt-clay ratio as well as lower values of Sb separated the majority of Heraklion samples from sediments collected in El Kantaoui. Cagliari exhibited intermediate levels of the variables included in PC2. Finally, sediments collected from the inner part of the El Kantaoui port (E1, E2), in the sectors hosting the leisure boat (C1) and military navy vessels (C2) in Cagliari, and near the shipyard in Heraklion (H5) were separated from the other samples on the PC3 based on lower redox potential and higher levels of Cu, OC and UCM.

Structure and Composition of Prokaryotic Communities

The richness displayed a low variation (coefficient of variation $< 5\%$) with average values of 1,880 OTUs and 597 OTUs for Bacteria and Archaea, respectively. The only exception was the sediments collected near the shipyard in Heraklion (H5), which differed from all the other stations with 654 OTUs for Bacteria and 361 OTUs for Archaea. In station H5, the lowest values of Shannon (H′) and Pielou's evenness (J') were also found for both prokaryotic communities (**Supplementary Table S2**).

The first two ordination axis of the PCoA analysis explained 68.7 and 49.9% of the variance in the bacterial and archaeal communities, respectively (**Figures 3A,B**). A clear segregation of the two prokaryotic communities in the ordination space was evident on the basis of the factor "port." Indeed, the PERMANOVA analysis demonstrated that the port was a factor significantly ($p < 0.01$) affecting the bacterial and archaeal communities. Most noticeably, sediments near the shipyard (H5) differed from all the other samples in community structure of both Bacteria and Archaea (**Figures 3A,B**).

The most abundant phyla of Bacteria was Proteobacteria ($41 \pm 3.5\%$) followed by Acidobacteria ($11 \pm 0.8\%$), Latescibacteria ($5.3 \pm 0.8\%$), Spirochaetes ($5.2 \pm 0.9\%$), Actinobacteria ($4.7 \pm 0.8\%$), Bacteroidetes ($3.5 \pm 0.5\%$), Chloroflexi ($3.3 \pm 0.9\%$), Planctomycetes ($3.2 \pm 0.4\%$). For archaeal communities, the most abundant phyla were Euryarchaeota ($58 \pm 4.9\%$), followed by Crenarchaeota ($21 \pm 3.9\%$) and Nanoarchaeaeota ($9.9 \pm 2.5\%$). The other less abundant phyla were all below the 3%, while the unassigned sequences accounted on average for 2.1 ± 1.0 and $3.5 \pm 2.5\%$ in the composition of bacterial and archaeal communities,

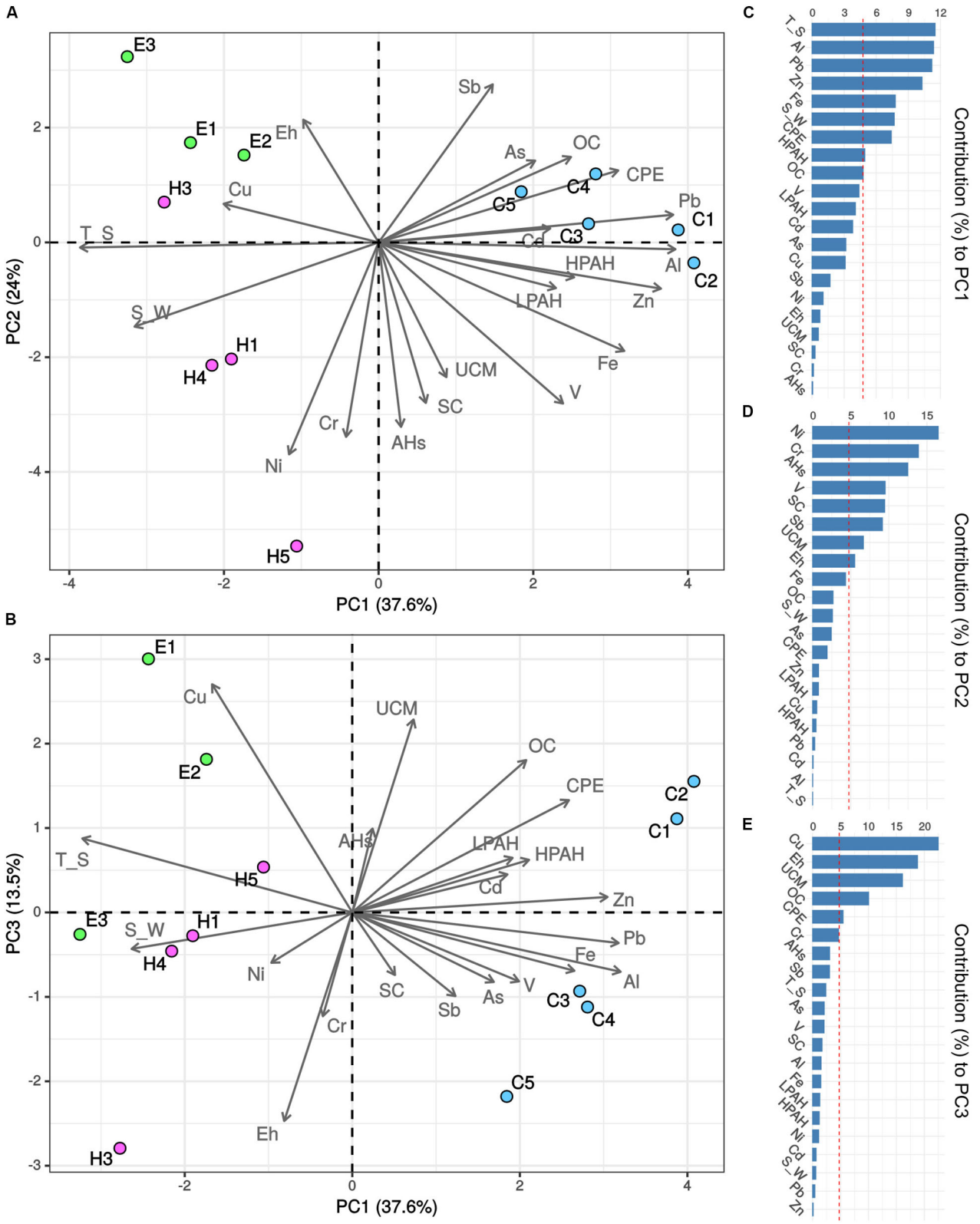

FIGURE 2 | Principal component analysis (PCA) of environmental and pollution variables recorded in the ports of Cagliari (C, blue), El Kantaoui (E, green), and Heraklion (H, pink). Biplots showing **(A)** PC1 vs. PC2, **(B)** PC1 vs. PC3. The percentage of the variation explained by each axis is indicated in parentheses after the axis label. Variable contribution to the first **(C)**, second **(D)**, and third **(E)** component is reported as percentage.

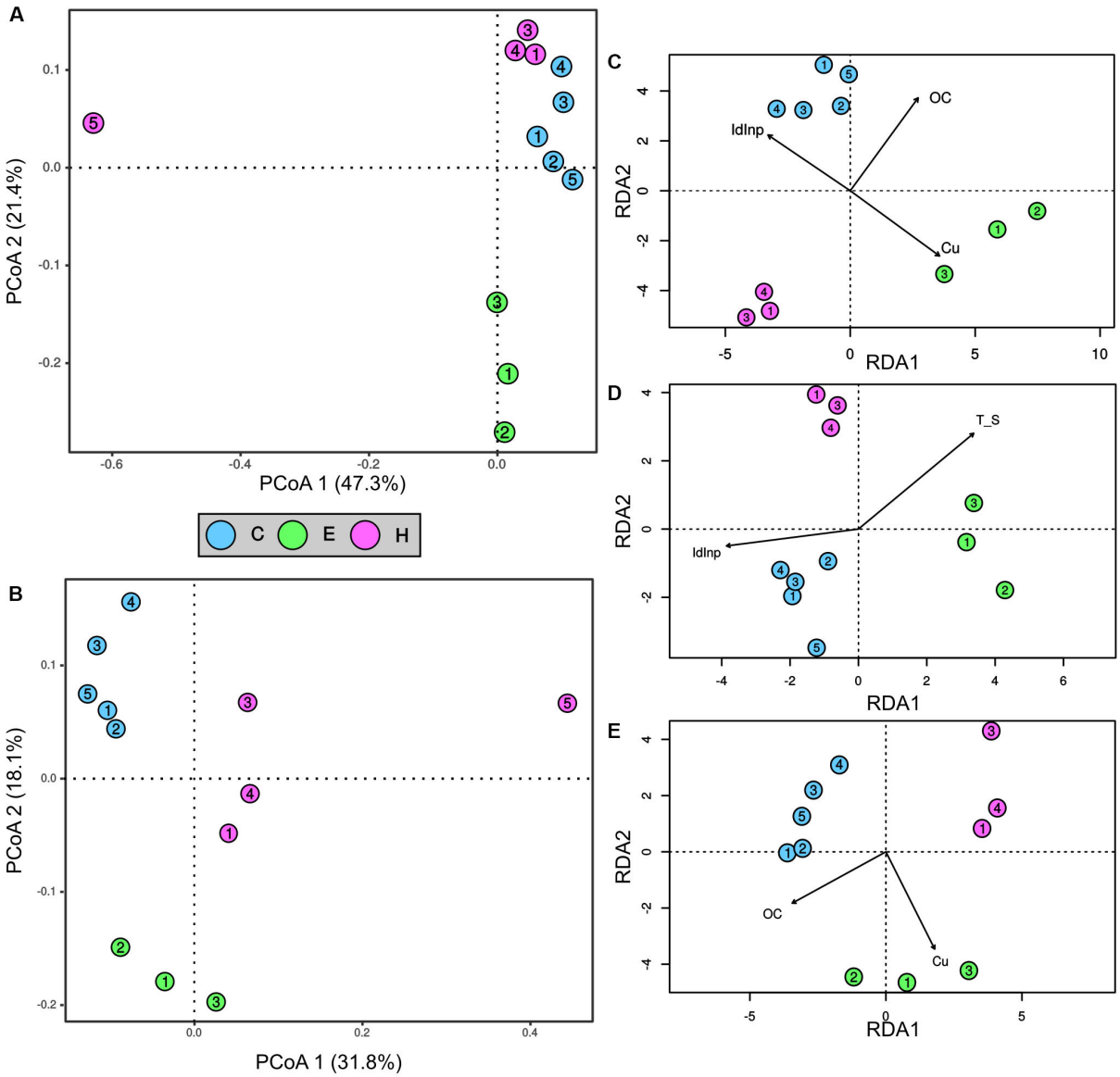

FIGURE 3 | Ordination and contribution of abiotic variables to spatial variation of prokaryotic community structures in surface sediments collected at the port of Cagliari (blue), El Kantaoui (green), Heraklion (pink). PCoA ordination of bacterial **(A)** and archaeal **(B)** communities. The percentage of the spatial variation in community structure explained by each axis is indicated in parentheses after the axis label. Redundancy analysis (RDA) ordination diagrams of the first two axes for bacterial **(C)**, SRB **(D)**, and archaeal **(E)** communities. The constrained sets of environmental variables analyzed are indicated as vectors. Only significant constrained variables are reported.

respectively. The community contribution of Bacteria and Archaea for each port is shown in **Figure 4**. Proteobacteria showed the highest values in Heraklion (44 ± 1.8%) and the lowest in El Kantaoui (36 ± 0.9%), with intermediate percentages in Cagliari (42 ± 1.7%). Sediments from El Kantaoui showed the highest percentages of Euryarchaeota (63%) and Heraklion the lowest one (51%), while Cagliari (58%) displayed intermediate values. A total of 2,482 bacterial OTUs and 909 archaeal OTUs were identified across all samples and, among them, 87 and 73% were shared among the three ports for Bacteria and Archaea, respectively (**Supplementary Figure S2**). Actually, sediments collected near the shipyard in Heraklion (H5) exhibited a peculiar community composition as compared to all the other samples (**Supplementary Figure S2**). More specifically, the highest percentages of Spirochaetes (12%), Actinobacteria (8.2%), and Firmicutes (7.8%) as well as the lowest percentages of Proteobacteria (26%), Acidobacteria (3.3%), Latescibacteria (3.3%), Chloroflexi (2.6%), and Planctomycetes (1.5%) were found in station H5, which also exhibited the highest percentages of Thaumarchaeota (11%) and Asgardaeota (3.0%). Because of its peculiar community compositions, sediments collected in station H5 were not included in all the average

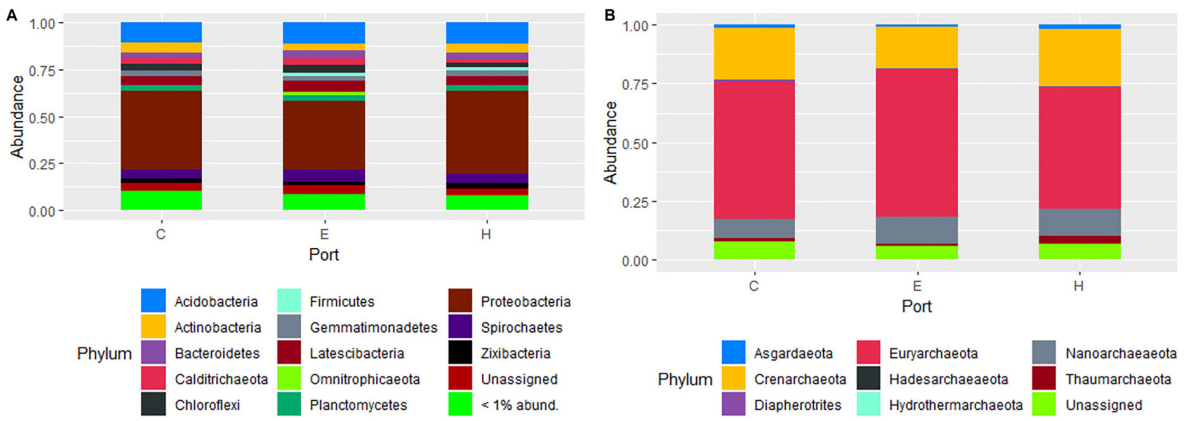

FIGURE 4 | Composition of prokaryotic communities in surface sediments of the study ports (C: Cagliari, E: El Kantaoui, and H: Heraklion). Bar plot showing the average contribution for each port of Bacteria **(A)** and Archaea **(B)** at phylum level. Taxa that on average comprised less than 1% of the libraries were grouped. The station H5 was excluded from calculation of average values for the Heraklion port.

calculations of community composition (**Figure 4**) as well as in subsequent analyses.

Relation Between Prokaryotic Communities and Environmental and Pollution Variables

The linking between the measured abiotic variables and the prokaryotic communities of Bacteria and Archaea was predicted using the BIOENV test. Moreover, the specific group of SRB was separately analyzed for its crucial role in ecosystem functioning in marine sediments. The best subsets of abiotic variables predicted by BIOENV test were: (i) concentrations of OC and Cu for Archaea ($\rho = 0.9025$, $p = 0.0001$), (ii) the concentrations of OC and Cu as well as a descriptor of PAH sources, namely the Inp/Inp + BgP ratio for Bacteria ($\rho = 0.8060$, $p = 0.0001$), (iii) temperature, OC, Cu, and the Inp/Inp + BgP ratio for SRB ($\rho = 0.8400$, $p = 0.0002$). The relation between each prokaryotic community and the best subset of abiotic variables predicted by BIOENV, was further explored by RDA ordination analysis (**Figures 3C–E**). The analysis confirmed significant relations between the community structure and each single abiotic variable predicted by BIOENV for Bacteria and Archaea (ANOVA, $p < 0.05$), but interactions between abiotic variables were never significant in ANOVA by term tests (ANOVA, $p > 0.05$). For SRB community, RDA showed a significant effect of the single variables temperature and the Inp/Inp + BgP ratio ($p < 0.05$), while the single variables OC and Cu, as well as the interactions among abiotic variables were not significant in ANOVA by term tests (ANOVA, $p > 0.05$).

Cogently the results of PERMANOVA analysis, Mantel test (**Table 1**) confirmed the presence of significant correlations ($p < 0.05$) between the geographic locations of the sampling stations and the community structures of Bacteria, Archaea, and SRB (spatial autocorrelation; row "GEO" in **Table 1**). Correlations were weak for SRB ($\rho = 0.3450$), moderate for Bacteria ($\rho = 0.4794$), while Archaea showed the highest correlation between community structure and geographic locations of the sampling stations ($\rho = 0.6805$). The effect of the best subsets of abiotic variables was thus untangled from the one owed to autocorrelation by partial Mantel test (**Table 1**, row "BestBIOENV – GEO"). The partial Mantel test demonstrated a significant and strong correlation ($\rho > 0.8$, $p < 0.001$) between each prokaryotic community and the best subset of selected abiotic variables (**Table 1**, row "BestBIOENV – GEO").

TABLE 1 | Spearman correlation coefficients (R) of Mantel and partial Mantel tests between the distance matrices of the best subsets (selected by the BIOENV test) of abiotic variables (Euclidean distance), geographical locations (Euclidean distance), and community structures (Bray–Curtis) of Bacteria, Archaea and SRB.

Test	Matrices	Bacteria	SRB	Archaea
Mantel	BestBIOENV	0.8463**	0.8438**	0.8929**
	GEO	0.4794*	0.3450^{+}	0.6805**
Partial Mantel	BestBIOENV – GEO	0.8038**	0.8378**	0.8294**

*BestBIOENV: organic carbon, Cu (Al normalized), Inp/Inp + BgP ratio for Bacteria; organic carbon, Cu (Al normalized) for Archaea; temperature, organic carbon, Cu (Al normalized), Inp/Inp + BgP ratio for SRB. GEO: geographical coordinates of the sampling stations. – GEO: the influence of distance matrix GEO is removed. +p < 0.05, *p < 0.01; **p < 0.001.*

The relationship between the bacterial communities and abiotic variables was further investigated by correlation analysis. **Figure 5** reports results of Spearman correlation analysis of the bacterial communities (cumulating all samples collected from the three ports and excluding the station H5) as a correlation network. Constructed network was composed of a total of 32 elements, 434 nodes (17 abiotic variables, 417 genus-level OTUs), and 1,131 edges (500 negative correlations, 631 positive correlations). The main element accounting for the majority of nodes and edges was used for further analysis. This main element was composed of 355 nodes (12 abiotic variables, 343 genus-level OTUs) and 1,077 edges (484 negative correlations, 593 positive correlations), while the average number of nodes connected to each node (i.e., the average node degree) was 6.07 ($SD = 5.95$). The network was divided into 11 clusters

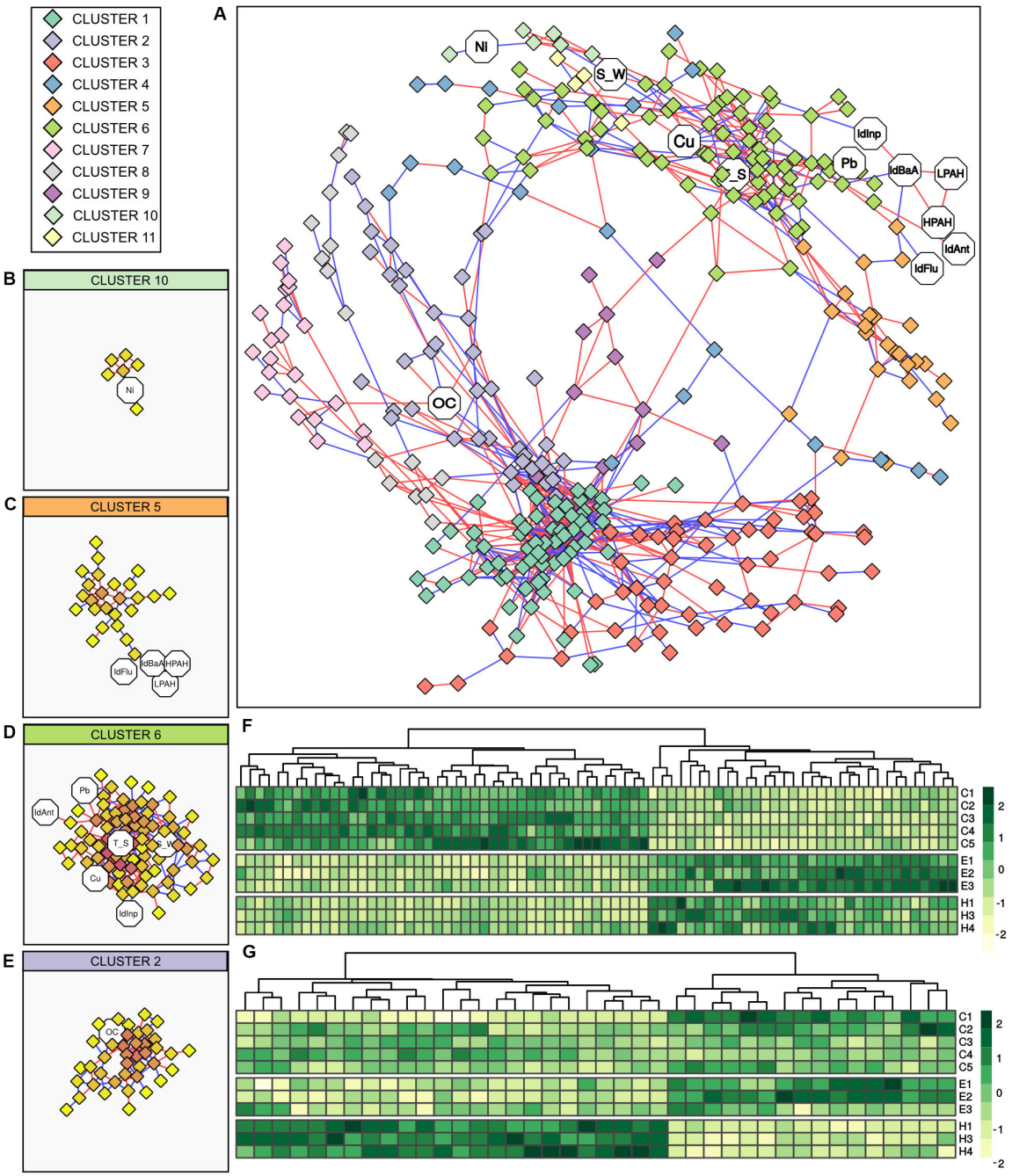

FIGURE 5 | Correlation analysis between bacterial genus-level OTUs and abiotic variables. **(A)** Network constructed with strong and significant Spearman correlations between OTUs, and between OTUs and abiotic variable. Node represents OTUs (rhombi) or abiotic variables (octagons). Node color is based on results of network clusterization analysis. Edges represent strong and significant Spearman correlation between nodes. Edge color is based on the direction of the correlation (blue for negative correlations, red for positive correlations). **(B–E)** Detailed view of the identified clusters in the network comprising abiotic variables. In those detailed views, node color is based on node degree (i.e., the number of nodes connected to a node). **(F)** Heatmap showing the distribution of OTUs in cluster 6 between samples. **(G)** Heatmap showing the distribution of OTUs in cluster 2 between samples. IdAnt: Ant/Ant + Phen; IdBaA: BaA/BaA + Chr; IdFlu: Flu/Flu + Pyr, IdInp: Inp/Inp + BgP; LPAHs: Phen, Ant, Flu, Pyr; HPAHs: BaA, Chr, BbF, BkF, BaP, Inp, BgP, DBA, S_W: salinity in surface water; T_S: temperature in surface sediments.

based on node connectivity (**Figure 5**). Overall, two distinct modules of genus-level OTUs can be observed in the network topology/morphology (**Figure 5A**). Those modules were not generated by groups of mutual excluding OTUs, as positive (which could be interpreted as co-occurrence) and negative (which could be interpreted as mutual exclusion) edges were

evenly distributed across the network. Moreover, inspection of the aforementioned modules showed that the OTUs distribution was not ascribable to differences in community composition among ports, a strong factor shaping the bacterial communities. The two modules were rather connected to different group of genera, having different relationships with abiotic variables. More specifically, the module on the lower part of the network was almost exclusively composed of genus-level OTUs and comprised only one abiotic variable, namely OC. The main clusters of this module were clusters 1, 2, 3, and 7. The clusters 1, 3, and 7 were exclusively composed of OTUs, cluster 1 showed the higher node degree values (data not shown), while cluster 2 comprised the abiotic variable OC (**Figure 5E**). Conversely, the module on the upper part of the network comprised all the connected abiotic variables. Main clusters of this module (**Figures 5B–D**) were cluster 6 (connected with the abiotic variables Ant/Ant + Phen, Cu, Inp/Inp + BgP, Pb, T_S, S_W), cluster 5 (connected with the abiotic variables BaA/BaA + Chr, Flu/Flu + Pyr, HPAHs, LPAHs) and cluster 10 (connected with the abiotic variable Ni).

The heatmaps showing the genus-level OTUs distribution among ports of cluster 6 (as the main abiotic connected cluster in the upper module) and cluster 2 (as the only abiotic connected cluster in the lower module) are shown in **Figure 5**. Even if the overall network did not reflect differences in genus-level OTUs distribution among ports, cluster 6 showed a marked similarity in OTUs composition and abundance distribution between El Kantaoui and Heraklion and a substantially different pattern in Cagliari. On the other hand, cluster 2 highlighted similarity between Cagliari and El Kantaoui, while Heraklion was substantially different. Mirroring abundance distribution, the PCoA ordinations of the OTUs included in cluster 2 and cluster 6 showed similar separations among the three ports with the first axis explaining 81.8 and 82.5% of the total variance, respectively (data not shown).

Upon detailed inspection of the OTUs included in cluster 2 (n = 40), four OTUs established a direct relationship with the abiotic variable OC. More specifically, an OTU assigned to the genus *Sedimenticola* exhibited a positive correlation with OC and a negative correlation with a second OTU belonging to the order Clostridiales, which in turn was negatively correlated to OC. Moreover, a positive correlation with OC was found for one OTU assigned to the uncultured lineage SJA-28 in the class Ignavibacteria and a negative correlation for an OTU attributed to the class Gammaproteobacteria. Among the OTUs included in cluster 6 (n = 78), the abiotic variables with the highest number of correlations were Cu (Node degree = 8) and sediment temperature (Node degree = 12), which established complex relationship with a total of 16 OTUs. Overall, those OTUs displayed a high connectivity level, with 5.5 median node degree (i.e., the median number of node connecter to each node of this network module) and values ranging from 1 to 12. Four OTUs established direct relationships with both Cu and temperature. Among them, two OTUs identified as belonging to the class Thermodesulfovibrionia and one to the phylum Schekmanbacteria were negatively correlated to Cu and temperature, while one OTU assigned to the class Anaerolineae was positively correlated to both variables. In addition to these four shared OTUs, four and eight OTUs were correlated singularly to Cu and temperature, respectively. More specifically, two OTUs were negatively correlated to Cu and assigned to the order Tistrellales in the class Alphaproteobacteria and to the uncultured clade BD7-8 in the class Gammaproteobacteria. On the other hand, Cu was positively correlated to one OTU identified as belonging to the genus *Sulfurovum* and one OTU assigned to the family Ruminococcaceae. As far as sediment temperature is concerned, negative correlations were found with one OTU assigned to Candidatus Moranbacteria order in the phylum Petescibacteria and one OTU assigned to the JS1 group in the phylum Atribacteria, while a positive correlation to temperature was found for six OTUs affiliated to the genus *Desulfosarcina*, the class Phycisphaerae, and the families Gemmatimonadetes, Spirochaetaceae, Oligoflexaceae, and Pedosphaeraceae.

Among the other abiotic variables included in cluster 6, one OTU identified as belonging to the Candidatus phylum Moranbacteria exhibited a negative correlation with salinity in surface water. A positive correlation with salinity was found for one OTU assigned to the family Terasakiellaceae and one OTU assigned to the class Gammaproteobacteria in the clade KI89A. The metal Pb correlated negatively to a single OTU identified as belonging to the genus Alkalispirochaeta and positively to two OTUs, one assigned to the family Nitrosococcaceae and one to the phylum Lentisphaerae. Finally, a single OTU affiliated to the family Desulfobacteraceae was negatively correlated to the Inp/Inp + BgP ratio and an unassigned OTU was positively correlated to the Ant/Ant + Phen ratio.

DISCUSSION

Prokaryotic communities play a fundamental role in ecosystem functioning in marine sediments regulating essential processes in global biogeochemical cycles, organic and inorganic contaminant transformation, and pollutant bioremediation. Recently, the impacts on benthic communities of organic and inorganic co-contamination as multiple stressors in harbors have been addressed by an increasing number of studies, such as extensive characterizations of complex commercial ports (Iannelli et al., 2012; Chiellini et al., 2013; Misson et al., 2016) as well as comparison between ports (as pollutant hot spots) and more natural (or less contaminated) coastal sediments (Sun et al., 2012, 2013). In this background, the present work represents the first assessment of the combined effects of multiple organic and inorganic pollutants on benthic prokaryotes in different ports at a large spatial scale (i.e., Mediterranean basin).

The sediment contaminations in the ports under study are markedly heterogeneous in compositions, levels and emission sources. Overall, the levels of PAHs vary over three orders of magnitude (25–49,000 ng/g), covering the range of concentrations previously reported for Mediterranean harbors (Vitali et al., 2019). The surface sediments in the artificial marina of El Kantaoui presents the highest levels of Cu (181 ± 10 mg/kg), a metal extensively used as antifouling agent in paints for ship hulls (Dahllöf and Andersen, 2009). Indeed, the copper level is

three orders of magnitude higher inside the marina than in the adjacent coastal sediments (Zakhama-Sraieb et al., 2016). On the other hand, sediments in the El Kantaoui port are contaminated by low/moderate levels of PAHs with fuel combustion as primary emission source (Vitali et al., 2019). The Heraklion port is characterized by a moderate level of PAHs emitted by different sources. Moreover, the co-occurrence of Ni (345 ± 11 mg/kg) and Cr (98 ± 43 mg/kg) was found, which could be reasonably ascribed to anthropogenic sources, such as nickel-chrome plating (Dahllöf and Andersen, 2009). The port of Cagliari shows the highest levels of PAHs, primarily originated by burning of coal and biomass (Vitali et al., 2019). As far as metals are concerned, sediments exhibit a 10-fold higher level of Pb (156 ± 38 mg/kg) than the other studied sites. Notably, the Cagliari port is located in the context of a peculiar mineralogical background near an important abandoned mining district (Cidu and Fanfani, 2002), even if anthropogenic inputs could not be ruled out inside the port area (Schintu et al., 2016). Finally, the three studied ports are markedly different in terms of environmental properties because of their different geographical positions in the Mediterranean Sea and local factors (Chatzinikolaou et al., 2018).

As expected for such a pronounced site-specificity in abiotic conditions, the archaeal and bacterial communities exhibited a strong spatial variation among the three investigated ports (**Figure 3**). On the contrary, we found an unforeseen overlap in composition of prokaryotic communities (**Figure 4**), down to the lowest taxonomic rank (i.e., OTU level, **Supplementary Figure S2**). On this core of shared taxa, the benthic communities in sediments collected near the shipyard in Heraklion clearly moved away from all the other stations for their structure and composition as well as the lowest richness and evenness. More specifically, Firmicutes and Spirochaetes were six- and two-fold more represented as compared to the other studied sediments, respectively. An increase in the relative abundance of Firmicutes has been found under anoxic conditions as compared to oxic ones (McKew et al., 2013). On the other hand, Spirochaetes are common and abundant in anoxic contaminated sites, where they have been suggested to drive necromass recycling (Dong et al., 2018). It is worth noting that the three most abundant OTUs were affiliated to the phylum Aegiribacteria and the family Ruminococcaceae. Members of the phylum Aegiribacteria have been found in an extreme meromictic system under anoxic conditions (Hamilton et al., 2016), while Ruminococcaceae are well-known anaerobic bacteria. The fourth most abundant OTU belonged to the phylum Acetothermia (previously candidate OP1 phylum), which has been involved in biogeochemical transformations in oil reservoirs (Hu et al., 2016). The peculiar prokaryotic assemblage in the Heraklion shipyard station is paralleled by the distinct abiotic status of its sediments (**Figure 2**), which exhibit the finest particle size, the most strict anoxic conditions, and the highest contamination by aliphatic hydrocarbons, petrogenic PAHs, and Ni throughout all stations, but also the highest amount of organic carbon among Heraklion stations (Chatzinikolaou et al., 2018; Vitali et al., 2019). The strict anoxic condition and pollution status in Heraklion shipyard sediments can be reasonably ascribed to the high organic carbon load and consequent increased respiration (Acosta-González et al., 2015) as well as to the high contaminant sorption and the slow oxygen diffusion in fine grain size sediments (Eggleton and Thomas, 2004). Consistently with our results, a negative impact on diversity of benthic prokaryotic assemblages with a reduction of species richness and changes in community structure has been extensively documented in chronic contaminations by petrogenic hydrocarbons (Orcutt et al., 2010; Acosta-González et al., 2015). Notably, a coordinated study of the three investigated Mediterranean ports also found the most heavily disturbed conditions in the sediments near the shipyard in Heraklion, as highlighted by the lowest species richness of the macrozoobenthic communities (Chatzinikolaou et al., 2018) and a "poor" ecological status ("unacceptable" under the WFD) based on benthic macrofaunal indices (Dimitriou et al., 2020).

The different statistical approaches implemented in this study demonstrated a strong link between the prokaryotic communities and organic matter in port sediments with numerous connections among bacterial genus-level OTUs (**Figure 5**). More specifically, a first module in the network gathers bacterial genera, which mainly take part to complex biotic interactions and are basically unrelated to abiotic variables except for a small proportion of taxa interconnected with OC (**Figure 3A**, lower module). A direct positive link with OC was found in the network for Ignavibacteria and *Sedimenticola*. The cultivable members of Ignavibacteria are facultatively anaerobic with an obligately organotrophic mode of life either by fermentation or respiration with several electron acceptors (Podosokorskaya et al., 2013). On the other hand, *Sedimenticola* have been demonstrated to grow autotrophically by sulfur oxidation coupled to denitrification under hypoxic or anaerobic conditions, but also organotrophically under aerobic and anaerobic conditions (Flood et al., 2015). In line with these results a role in decomposition of organic materials in the investigated surface sediments may be suggested for these recently described groups characterized by a pronounced metabolic versatility. Overall, opposite trends between Cagliari and El Kantaoui on one side, and Heraklion, on the other, were found in genus-level OTUs distribution in cluster 2 (connected with the abiotic variable OC, **Figure 5F**), reasonably related with the highest amount of organic matter in sediments (**Figure 2**, PC3). Cogently, RDA assigned the detected variations in bacterial community structure to changes in organic carbon levels with opposite trends for Heraklion and the other two ports (**Figure 3C**). We found neither an interaction of OC with Cu or descriptors of PAH sources in RDA nor a direct interconnection with other abiotic variables in the network. Our results should not be interpreted as a lack of reciprocal effects between pollutants and organic matter. It is well-known that the organic carbon fraction in sediments plays an important role in binding metals and hydrophobic contaminants, including PAHs. However, organic matter is present in sediments in different forms that may have very different sorption capacities for hydrophobic contaminants. Therefore, the nature of organic matter (e.g., coal, vegetable debris) determines the bioavailability, biodegradability and biological effects exerted by PAHs in sediments (Yunker et al., 2002; Ghosh et al., 2003). On the other hand, the ability to bind toxic metals in the colloidal fraction of the organic carbon

pool is important in the cycling of metals in aquatic systems (Ford and Ryan, 1995).

Network analysis identifies a second module composed of connections among bacterial taxa as well as strong relationships between them and abiotic parameters (**Figure 5A**, upper module). More specifically, there were numerous connections between OTUs affiliated to different lineages and temperature, and to a lesser extent with salinity. Both temperature and salinity have been found to be important drivers of bacterial communities over large spatial scale in coastal sediments (Sun et al., 2013; Bargiela et al., 2015). In the network, several OTUs ascribable to SRB (i.e., *Desulfosarcina* and Thermodesulfovibrionia) exhibited positive link with temperature. Accordingly, we also found a strong relation between this abiotic variable and the structure of SRB communities. These findings are consistent with previous results, which have demonstrated the prevailing ambient temperature exerts strong environmental selection on the composition of the SRB community in marine sediments from different climatic regions (Robador et al., 2016). Overall, temperature seems to play an important role in shaping SRB communities in sediments from the three investigated ports. In this context is important to mention that the Mediterranean Sea is characterized by well-known longitudinal gradients with a west to east increase in salinity and temperature (e.g., Coll et al., 2010), which may at least partially account for the correlation between the bacterial assemblages and the geographical locations of sampling stations. Moreover, the specific hydromorphological properties in ports (i.e., confinement) may reasonably contribute to local variation in salinity and temperature in the studied harbors, contributing to the site specificity of the benthic prokaryotic assemblages observed in this study. On the other hand, temperature has a crucial effect on the fate of PAHs and metals as it causes marked changes in the interactions (i.e., degradation, transformation, accumulation) between microorganisms and pollutants (Liu et al., 2017). Indeed, the solubility as well as the adsorption capacity and adsorption intensity on microbial cells and abiotic particles of PAHs and metals increase with increasing temperature (Liu et al., 2017). Even if descriptors of PAHs (i.e., Ant/Ant + Phen, BaA/BaA + Chr, Flu/Flu + Pyr, Inp/Inp + BgP, HPAHs) and metals (i.e., Cu, Pb, Ni) are connected with bacterial OTUs in the network, the biotic interconnections are more numerous (**Figure 5A**). Notably, the variable HPAHs interacts with a single OTU, while the variable LPAHs takes no direct connection with any genus-level OTU. This result is in line with that recently obtained for metals by Coclet et al. (2019) in a study on bacterioplankton in Toulon Bay; the authors have suggested that metals significantly influence the dynamics of few microbial groups, and could rather influence indirectly, via biotic interactions, the whole bacterial community. A clear separation of the Cagliari port from the other two ports was found based on the genus-level OTUs distribution in cluster 6. This result clearly mirrors differences in environmental and pollution status described by the abiotic variables included in this cluster (**Figure 2**, PC1).

Statistical analyses employed in this study cogently highlighted a strong link between descriptors of PAH sources and the structures of the whole bacterial communities as well as the group of SRB. These results support our previous study, which has recently provided a first evidence of the role of PAH emission sources in structuring the benthic communities of SRB as targeted by terminal restriction fragment length polymorphism of the *dsrAB* (dissimilatory sulfite reductase) gene (Vitali et al., 2019). Indeed, the combustion process by which PAHs are formed determines not only the composition of the contaminant mixture but also bioavailability of PAHs in sediments (Akkanen et al., 2012). In the network, we found a negative correlation between diagnostic ratio of PAH emission sources and Desulfobacteraceae. Bacteria belonging to Desulfobacteraceae have been previously found to be the dominant microorganisms in anaerobic enrichment cultures able to oxidize phenanthrene under sulfate reducing condition (Davidova et al., 2007; Himmelberg et al., 2018). Collectively, these findings might suggest the relative abundance of Desulfobacteraceae decrease in sediments where burning of biomass combustion was the dominant pollution source due to the low bioavailability of PAHs.

Among the investigated metals, both bacterial and archaeal communities showed a strong link with copper contamination in surface sediments. Our observations comply with similar studies on metal pollution in coastal areas, where copper has been suggested to be responsible for driving community changes in bacterioplankton from Toulon Bay (Coclet et al., 2019) and benthic bacteria from Australian coastal sediments (Sun et al., 2012). Moreover, a coordinated study on the three Mediterranean harbors investigated in the present work has previously demonstrated that copper affects macrobenthonic assemblages (Chatzinikolaou et al., 2018), a well-consolidated ecological tool for sediment quality assessment (McPherson et al., 2008; Gislason et al., 2017). The network analysis employed in this study allows us to identify potential copper sensitive- and tolerant- OTUs in the benthic bacterial community. More specifically, OTUs assigned to uncultured lineages of Alphaproteobacteria, Gammaproteobacteria, Schekmanbacteria, and Thermodesulfovibrionia seem to be negatively impacted by copper. In literature, similar results can be documented for Alphaproteobacteria and Gammaproteobacteria (Yin et al., 2015). Currently, Thermodesulfovibrionia are known for reduction of sulfate and other sulfur compounds but this class has not been previously linked to metals. The biological and geochemical importance of Candidatus phylum Schekmanbacteria is still unclear. On the other hand, the relative abundance of *Sulfurovum*, Anaerolineae and Ruminococcaceae increases with higher copper levels in the investigated sediments. Consistently with our results, an increase has been found in literature in metal contaminated environments including mangrove sediments and hydrothermal vents for Sulfurovum (Nakagawa et al., 2007; Fernández-Cadena et al., 2020), river sediments, soils, and coastal sediments for Anaerolineae (Sun et al., 2013; Yin et al., 2015; Meng et al., 2019), as well as barrier for mine tailings for Ruminococcaceae (Zhang et al., 2019).

CONCLUSION

Our study gives strong evidences supporting the notion that organic matter, metals and PAHs as well as temperature and salinity shape prokaryotic communities in port sediments. The "port" is the main factor affecting the structure of archaeal and bacterial communities in surface sediments, a result consistent with the pronounced differences in sediment pollution status, geological background, and geographical position among the investigated sites. Nevertheless, a marked overlap in the composition of prokaryotic communities was found among ports. In this contest, the targeted NGS analysis of the benthic bacterial community allows us to detect local variation in the community composition and loss of prokaryotic diversity in the heavily impacted sediments near the shipyard in Heraklion. On the other hand, multiple statistical tools recognize copper as strongly associated with the observed changes in structure and composition of the benthic bacterial community and allows us to identify the bacterial populations more directly linked to the pollutant. These findings would deserve further investigations under controlled experimental conditions to verify a direct causal relation between stressor and candidate indicators and a more in-depth analysis of bacterial genomes and functions. Overall, the results obtained under the umbrella of the ENPI CBCMED project MAPMED designate the benthic bacterial community as a good candidate tool for monitoring of the sediment status in port management, a crucial prerequisite to plan bioremediation intervention, and lay the foundation for the developing of a proper benthic microbiota-based index of sediment quality status. In a wider perspective, our results provide a significant contribution to the understanding of responses of benthic prokaryotic communities to anthropogenic perturbations in marine coastal areas. Admittedly, the main limitation of the employed DNA-based target metagenomic analysis resides in the substantial inability to directly evaluate functional and metabolic pathways in the active community, and how bacteria are affected by and affect anthropogenic pollutants, a goal that will be pursued by untargeted metagenomic analysis coupled with transcriptomics.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the https://www.ebi.ac.uk/ena/data/view/PRJEB36504.

AUTHOR CONTRIBUTIONS

ET, AC, and FV developed the concept for this study. ET and GC carried out the field work. ET, AC, GM, and EC designed the experiments and contributed reagents, materials, and analysis tools. FV and ET performed the microbiological experiments. GC performed the analysis of metals and metalloids. LD, FM, and ET analyzed abiotic data. FV and LD performed bioinformatic analyses. ET, LD, FV, and RL drafted the manuscript. All authors interpreted the results, edited and reviewed the final version of the manuscript and approved it before submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01234/full#supplementary-material

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MINI REVIEW
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Bacterial Calcium Carbonate Mineralization *in situ* Strategies for Conservation of Stone Artworks: From Cell Components to Microbial Community

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Calcareous stones have been widely used in artworks and buildings by almost all human cultures. Now, more than ever, the increased environmental pollution and global warming are threatening the stone cultural heritage. Weathering due to physical, chemical and biological factors results in monumental calcareous stone deterioration. These agents induce a progressive dissolution of the mineral matrix, increase porosity, and lead to structural weakening. Bacterial Calcium Carbonate Mineralization is a widespread naturally occurring process which in the last decades was proposed as an environmentally friendly tool to protect monumental and ornamental calcareous stones. The advantage of this treatment is that it mimics the natural process responsible for stone formation, producing a mineral product similar to the stone substrate. This mini review highlights the milestones of the biomineralization approaches with focus on *in situ* stone artworks protection. The strategies explored to date are based on three main approaches: (i) the use of allochthonous and (ii) autochthonous alive cells that, due to the bacterial metabolism, foster biomineralization; (iii) the cell-free approach which uses fractionated cellular components inducing biomineralization. We discuss the challenging aspects of all these techniques, focusing on *in situ* applications and suggesting perspectives based on recent advances.

Keywords: calcite biomineralization, biodeposition, bioremediation, stone conservation, stone microbiota, cultural heritage

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INTRODUCTION

Bacterial Calcium Carbonate Mineralization (BCCM) is a widespread natural process of many bacterial taxonomic groups in different environments, ranging from microscopic crystals to large geological formations (Boquet et al., 1973; Ehrlich, 2002; Zavarzin, 2002; Dupraz et al., 2009; Perito and Mastromei, 2011).

According to Hammes and Verstraete (2002), BCCM is regulated by four key factors: calcium concentration, concentration of dissolved inorganic carbon (DIC), pH, and the availability of nucleation sites. Bacteria can foster an alkaline environment and increase DIC through different autotrophic and heterotrophic metabolic pathways (Castanier et al., 1999; Dhami et al., 2014;

Zhu and Dittrich, 2016). If calcium ions and nucleation sites are available in the environment, BCCM then occurs.

Bacterial surfaces such as cell walls or esopolymeric substances (EPS), due to their metal binding properties, serve as nucleation sites and constitute particularly favorable templates for heterogeneous nucleation and crystal growth (Fortin et al., 1997; Douglas and Beveridge, 1998). The EPS act as matrix templates influencing $CaCO_3$ crystal morphology, polymorphism, spatial position and growth (Braissant et al., 2003; Tourney and Ngwenya, 2009; Ercole et al., 2012; Oppenheimer-Shaanan et al., 2016). $CaCO_3$ crystals usually grow on bacterial cell surfaces (Rivadeneyra et al., 1998; Castanier et al., 1999). The polymorph produced (mainly calcite, aragonite and vaterite) depends both on environmental conditions and bacterial strains (Ben Omar et al., 1997; Rivadeneyra et al., 1998; Brennan et al., 2004).

During the last decades, BCCM application was proposed as an environmentally friendly tool for conservation and reinforcement of monumental and ornamental calcareous stones (Orial et al., 1993). Weathering by physical, chemical and biological factors increases the porosity and dissolution of the mineral matrix thus progressively weakening the structure (Tiano et al., 1999). Organic products used to reduce monument deterioration present several drawbacks related to incompatibility with the stone, while inorganic consolidants show poor performance (De Muynck et al., 2010). The advantage of a BCCM-mediated treatment is that it mimics the natural process responsible for stone formation, producing a mineral product similar to the stone substrate. The aim is dual: to provide a coherent $CaCO_3$ layer on the surface of deteriorated stone, protecting against the intake of water or chemicals, and to consolidate the inner, weakened structure. In literature a number of comprehensive reviews are available about biodeposition of $CaCO_3$ on stone and building materials, highlighting mechanisms, limitations, challenges, and perspectives of this technology (De Muynck et al., 2010; Dhami et al., 2014; Anbu et al., 2016; Nazel, 2016; Zhu and Dittrich, 2016; Castro-Alonso et al., 2019). In this mini review, we fill a literature gap, by focusing on current BCCM technologies for *in situ* cultural stone conservation. We highlight the typology of interventions and recent improvements of *in situ* applications and provide viewpoints based on recent advances.

BCCM-Based Approaches for Cultural Stone Conservation

Living Cells, Single Selected Bacterial Strain

The application of BCCM for cultural heritage conservation was proposed by a pioneer French group that developed the so-called *Calcite Bioconcept* technology, covered by a now expired patent (Adolphe et al., 1990). This methodology was based on the application of cultures of selected bio-calcifying strains by spraying them on the stone surface and then feeding them by applications of a nutrient medium. The result was the formation of a new calcareous coating layer called *biocalcin*. This few μm thick layer was coherent to stone and made of encrusted bacterial bodies mixed with $CaCO_3$ (**Figures 1A,B**). A preliminary screening of bacteria isolated from natural carbonate environments allowed the selection of a *Bacillus cereus* strain exhibiting the highest precipitation performance via the ammonification of amino acids (**Table 1**; Castanier et al., 2000). After testing it on limestone specimens, the technology was transferred to *in situ* applications (Le Métayer-Levrel et al., 1999). The first application was made in 1993, testing an area of 50 m^2 of the tower of the Saint Médard Church in Thouars. Evaluation of the treatment was carried out 6 months and 1 year after the application (**Table 1**). The treatment had no influence on the color or other aesthetical features and the water absorption rate was up to five times less. Following this approach, a number of façades of French historic and private buildings were treated by the Calcite Bioconcept Company (Castanier et al., 2000; Anne et al., 2010; De Muynck et al., 2010). No scientific reports can be found about these treatments. At the same time, several groups have worked to improve this system by isolating and testing different microorganisms, exploring different metabolic pathways and application conditions mainly in laboratory settings, showing, in many cases, similar results (reviewed by Nazel, 2016).

Over the last 20 years, a Spanish group of Granada made efforts to further develop this technology. They promoted the use of *Mixococcus xanthus*, a Gram-negative, non-pathogenic soil bacterium, to overcome drawbacks of previous treatments: the thin layer of the new formed bio-cement, the possible formation of endospores, and uncontrolled biofilm by *Bacillus* clogging stone pores. In an *in vitro* model, sterilized calcarenite slabs were immersed in a liquid medium containing *M. xanthus* and nutrients activating the ammonification of amino acids (Rodriguez-Navarro et al., 2003). Newly formed coherent carbonate cement of calcite grains was deposited into the pores without plugging them to a depth $\geq$500 μm. No myxospore formation was found in the tested culture media.

Living Cells, Microbial Community of Stone

A further step in the development of this technology proposed by Jimenez-Lopez et al. (2007) was bio-precipitation fostered by the microbial community inhabiting the stone. The advantage was that it supported the autochthonous $CaCO_3$ producing-bacteria without introducing exogenous microorganisms. Initially, quarry porous limestone slabs were immersed in a M-3P nutritive buffered solution with/without *M. xanthus* (Jimenez-Lopez et al., 2008). Treated stones showed newly precipitated $CaCO_3$ overgrowth without pore plugging and, accordingly, weight increase, regardless of the presence or absence of *M. xanthus*. In comparison to sterilized slabs used as controls, the treated slabs maintained their original pore size distribution and were more resistant to mechanical stress. The M-3P medium, stimulating heterotrophic carbonatogenic bacteria via the ammonification of amino acids (**Table 1**), was patented (González-Muñoz et al., 2008).

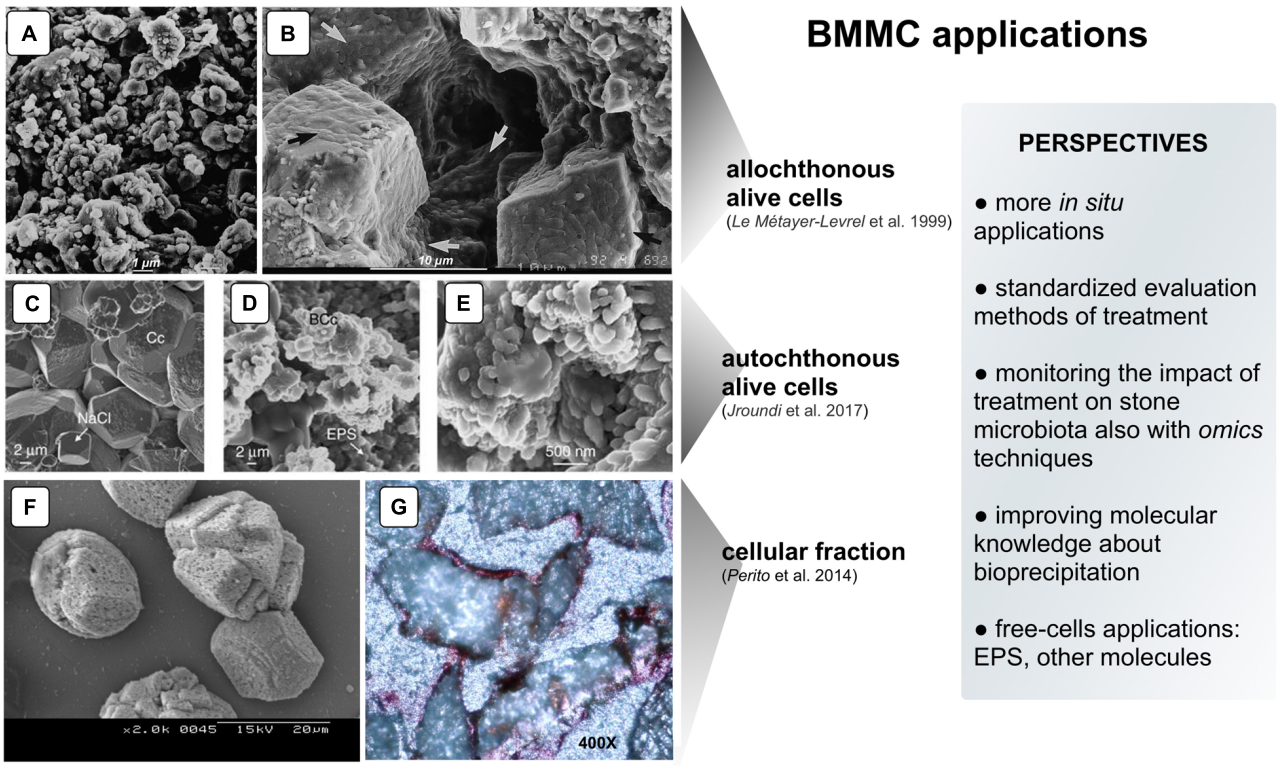

FIGURE 1 | Examples of $CaCO_3$ precipitation on limestone by different BCCM strategies. Panels **(A,B)** SEM micrographs of "biocalcin" formed by allochthonous alive cells on Saint Maxim (SM) limestone: **(A)** Untreated surface of SM limestone; **(B)** Pores filled with the superficial bacterial coating (arrows). Panels **(C–E)** SEM micrographs of calcite (determined by XRD) formed by autochthonous alive cells on calcarenite: **(C)** Untreated calcarenite. Chemically precipitated calcite crystals (Cc) in a control stone show dissolution pits and NaCl crystals; **(D)** In the treated stone, bacterial calcite (BCc) are organized in nanogranular structure surrounded by EPS; **(E)** Magnification of the nanogranular structure of calcite biocement. Panels **(F,G)** $CaCO_3$ induced by cellular fraction: **(F)** SEM micrograph of calcite crystals (determined by XRD) induced by BCF in $CaCl_2$ solution; **(G)** Representative thin section made from cores taken from stone slabs of the Angera Cathedral stained with Alizarine red (optical microscope, 400×). The metabolic pathway of allochthonous and autochthonous alive bacteria promoting BCCM (panels **A–E**) is the oxidative deamination of amino acids present in the nutrient medium. As a result, calcifying bacteria produce CO_2 and NH_3 creating an alkaline microenvironment and shifting the $HCO_3^- = CO_3^{2-} + H^+$ equilibrium toward the right. In the presence of Ca^{2+}, supplied in the nutrient medium, precipitation occurs via the reaction $Ca^{2+}\ CO_3^{2-} = CaCO_3$ preferentially on the bacterial cell surface in a microenvironment highly supersaturated with respect to $CaCO_3$ (e.g., bacterial biofilm; Jroundi et al., 2017) (With permission from: Le Métayer-Levrel et al., 1999 for Panels **A,B**.; Jroundi et al., 2017 for Panels **C–E**; and Perito et al., 2014 for Panels **F,G**).

The M-3P treatment was then tested *in situ*, with and without *M. xanthus*, on selected areas of decayed calcarenite stone of three historic buildings in Granada: San Jeronimo Monastery, Hospital Real and Royal Chapel (Jroundi et al., 2010; Rodriguez-Navarro et al., 2015). The evaluation included both the technical efficacy and, for the first time, the monitoring of the bacterial community of the decayed stone by culture-dependent and independent techniques (**Table 1**). Medium/long-term efficacy and detrimental side-effects were monitored up to 4 years after treatments (Rodriguez-Navarro et al., 2015). In all the three cases, the newly formed $CaCO_3$ (mostly calcite) created a cement that consolidated the deteriorated calcarenite with a significant surface strengthening neither plugging pores nor causing aesthetical changes. The efficacy of the treatment *in situ* was independent of the presence of *M. xanthus*. The carbonatogenic bacterial population initially increased after treatment applications, but over time reached values close to those observed before treatment.

In those cases where the stone microbiota was altered and/or suppressed (e.g., application of biocides), the same authors proposed a bioconsolidation treatment with carbonatogenic bacteria selected from calcareous stones as inoculants (Jroundi et al., 2012). Bacteria were isolated from altered calcarenite stone slabs by the application of M-3P medium, then precipitating bacteria belonging to Actinobacteria, Gamma-proteobacteria and Firmicutes were selected and single strains were tested for bio-consolidation capability *in vitro*, with and without *M. xanthus*. They found that *Acinetobacter* spp. strains were the most appropriate candidate bacteria.

To test the self-inoculation biotreatment *in situ*, an indigenous community was recovered by cultivation from salt damaged carbonate stone in a historic building (San Jeronimo Monastery), activated via M-3P, and applied back onto the same stone (Jroundi et al., 2017). Firmicutes was the dominant phylum in the inoculum (∼79%). Test evaluation methods are reported in **Table 1**. The effective consolidation was due to the formation of

TABLE 1 | Features of *in situ* stone treatments of the three BCCM-based approaches. Details of the methodology of treatment, as found in the cited literature, are reported in the notes.

BCCM-based approach	Bacterium/a (concentration) or medium	Mechanisms driving precipitation	Monumental Site-kind of limestone	Testing area (m^2)	Evaluation of treatment[1]				References
					Parameter	Method	Effect on stone	Monitoring Time	
Selected viable strain	[a] *Bacillus cereus* (Not found)	Deamination of amino acids and nucleation sites	Saint Médard Church- Tuffeau (Thouars, France)	50	Water absorption Colour	Water pipe Colorimetry	Biodeposition Safety	6 and 12 months after treatment	*Le Métayer-Levrel et al., 1999*; *Castanier et al., 2000*
Community inhabiting stone	[b]M 3-P Nutrient medium	Deamination of amino acids and nucleation sites	San Jeronimo Monastery-calcarenite, Royal Hospital-calcarenite, Royal Chapel- calcarenite (Granada, Spain)	From 0.15 to 2.0 if flat surfaces	New calcite microtexture	XRD SEM/EDS	Biodeposition		*Jroundi et al., 2010*; *Rodriguez-Navarro et al., 2015*
					Surface cohesion Colour	Peeling tape test Spectrophotometry	Reinforcement Safety	Before and up to 4 years after treatment	
					Stone microbiota	Culture-dependent (Total title, selected groups). Culture-independent (total DNA, 16S rDNA amplification, DGGE and clone libraries)	Safety		
Community inhabiting stone	[c] Self-inoculation, indigenous bacterial community (~8.2×10^8 CFU ml^{-1})	Deamination of amino acids and nucleation sites	San Jeronimo Monastery-calcarenite (Granada, Spain)	Not found	New calcite	SEM/XRD	Biodeposition		*Jroundi et al., 2017*
					Porosity and pore size distribution	Porosimetry (mercury intrusion)	Biodeposition		
					Surface cohesion Cohesion profiles Colour	Peeling tape DRMS[2] Spectrophotometry	Reinforcement Reinforcement Safety	Before and 5, 12, and 24 months after treatment	
					Stone microbiota	Cultivation (total number, selected groups)	Safety		
Cell Components	[d] BCF from *B. subtilis* (8.5 g l^{-1})	Nucleation sites	Angera Church-Angera (Angera, Italy)	0.29	Water absorption	Contact sponge	Biodeposition	Before and 4 months after treatment	*Perito et al., 2014*

(Continued)

TABLE 1 | Continued

BCCM-based approach	Bacterium/a (concentration) or medium	Mechanisms driving precipitation	Monumental Site-kind of limestone	Testing area (m^2)	Evaluation of treatment[1]				References
					Parameter	Method	Effect on stone	Monitoring Time	
					New $CaCO_3$ Cohesion profiles Colour	Alizarine red staining DRMS[2] Colorimetry	Biodeposition Reinforcement Safety		

[1] The parameter assesses the effect of the treatment on: biodeposition, safety (condition of being protected from aesthetical, structural, or microbiological changes), and reinforcement. [2] Drilling Resistance Measurement System. [a] The treatment is generically described in Le Métayer-Levrel et al. (1999). It consists of first spraying the entire surface to be protected with a suitable bacterial suspension culture. Afterward, the deposited culture is fed every 24–48 hours, usually for the next four days, with a suitable medium. [b] At the San Jeronimo Monastery and the Royal Hospital, the M-3P treatment was compared with the M. xanthus treatment. The treatments were applied by spray, twice a day, for 6 days (total volume applied 1–1.5 ml cm^{-2}) on flat surfaces with similar wideness of calcarenite stone blocks. Treatment with M. xanthus involved two initial applications with inoculated M-3P (~10^9cells/ml), followed by successive applications with sterile M-3P solution. At the Royal Chapel, only the M-3P treatment was performed on elements of the crest with carved surfaces. In all cases, the treated area remained covered with an aluminum/plastic (bubble wrap) foil up to two/ three days after treatment, to avoid the direct effect of sunlight and to minimize evaporation (Rodriguez-Navarro et al., 2015). [c] The self-inoculation treatment was compared with the M. xanthus and the sterile M-3P treatments. All three treatments were applied by spraying on adjacent stone blocks with similar exposure and decay levels. Approximately 0.125 ml cm^{-2} were used for each application (twice a day for 6 days). In the M. xanthus and the self-inoculation treatments, the stone was treated on the first day with the bacterial culture and then with only the sterile M-3P nutritive solution. The treated areas were covered with an aluminum/ plastic foil up to 3 days after treatment (Jroundi et al., 2017). [d] The field test was performed on the main façade of the Church of Angera. BCF in Super C solution) and REF (only Super C) were sprayed on the treated surface in selected areas of 0.29 m^2 and 0.28 m^2, respectively, for a total application of 1 l m^{-2} solution for each spray application. Spray application steps: the first day BCF solution at concentration of 8.5 g l^{-1} in Super C and REF were sprayed, the second day 0.032 g l^{-1} BCF in SuperC plus nanoparticles and REF plus nanoparticles were sprayed, while the third day only SuperC plus nanoparticles solution was sprayed on the selected areas (Perito et al., 2014).

an abundant and exceptionally strong hybrid cement consisting of nanostructured $CaCO_3$ and bacterial EPS covering the substrate (**Figures 1C–E**). After 5 months, the viable titer of culturable microbiota increased and then after 24 months dropped back to about pre-treatment values.

Cell Components

An Italian team of Florence investigated and assessed $CaCO_3$ mineralization on stone induced by a bacteria-mediated system in absence of viable cells (Perito et al., 2014). This investigation used the *Bacillus subtilis* strain 168 to identify bacterial structures or molecules inducing precipitation. The precipitation capability of bacterial dead cells was tested in a $CaCl_2$ solution as calcium source and with the sublimation of ammonium carbonate for alkalization. Dead cells were able to promote calcite formation, then cell fractions were tested and a bacterial cell fraction (BCF) containing the cell wall induced $CaCO_3$ formation (**Figure 1F**). Interestingly, the system was specific in generating crystal polymorphisms, since only calcite was found by X-ray diffraction.

Apparently, dead cells as well as BCF acted as crystallization nuclei in liquid medium. This hypothesis is supported by the capacity of cell walls to uptake cations such as Ca^{2+}, as previously demonstrated for isolated *B. subtilis* walls (Beveridge and Murray, 1980), and fostering heterogeneous nucleation (Fortin et al., 1997). According to Dupraz et al. (2009), this process can be referred to biologically influenced mineralization.

BCF was stored as easy-to use lyophilized preparations, maintained a long-lasting activity and showed heat resistance. BCF treatment was tested on slab stones and then *in situ* on selected areas of the main façade of the Angera Cathedral, a 6th century monumental site in Italy (Perito et al., 2014). Lyophilized BCF was dissolved in a $CaCl_2$ solution, then sprayed on stone surface with a supersaturated calcium bicarbonate $Ca(HCO_3)_2$ solution (Super C solution) for supplying calcium ions and CO_2. The solution was supplemented with calcite nanoparticles to maintain supersaturation in the pore and increase calcium ions. Field evaluation tests after treatment showed that BCF treated areas had negligible color changes (**Table 1**). New crystals formed inside stone pores (**Figure 1G**) and, accordingly, there was a significant decrease in water absorption (up to 6.8%). The cohesion profiles were significantly increased in the first 3 mm (if compared with the control area treated with Super C alone). These results show that this application has potential, even if the authors concluded that further testing was needed to fully assess the treatment conditions for *in situ* applications.

Perspectives: From Cell Components to the Microbial Community

BCCM biotechnology could be an ecological alternative to chemical treatments due to the low environmental impact and the production of a layer of $CaCO_3$ compatible with and coherent to the stone. A common point of improvement for the BCCM technologies is the consolidation performance, not yet comparable to that of synthetic polymers. The appropriate selection of stone types before application is important because pore structure affects penetration depth and treatment

performance (De Muynck et al., 2011). Nanomechanical properties of $CaCO_3$ polymorphs can also be improved by a better understanding of the bio-geo-chemical processes governing the formation of biominerals with high mechanical performance in natural environments (Dhami et al., 2018).

While the literature shows the potential in the laboratory of different bacterial applications to promote $CaCO_3$ mineralization, very few attempts have been made to test the technology *in situ* (**Table 1**). Based on these few studies, some companies have developed biomineralization products for cultural heritage by using cultures of selected strains (Amonit, France[1]) or media stimulating stone microbiota (KBYO Biological, Spain[2]). Nevertheless, the translation of other promising results obtained *in vitro* into practical bioremediation applications on heritage stone *in situ* remains the challenge for the immediate future (Webster and May, 2006); as examples, the well documented biocalcite production by bacterial urease or carbonic anhydrase (Castro-Alonso et al., 2019). Scaling up will be needed in order to develop this technology (**Figure 1**, box perspectives). *In situ* applications always have additional problems when compared with the *in vitro* conditions, especially concerning heterogeneity and conservation state of the stone, delivery systems, outdoor or indoor environmental conditions, type of feasible evaluation tests and the value of the artwork. For this reason, preliminary *in situ* small-scale testing should adopt treatment conditions mimicking those to the follow in larger-scale applications.

Another general comment concerns the heterogeneity of the treatment evaluation tests (**Table 1**). Although the choice of the monitoring methods sometimes depends on the experimental set-up, evaluating methods must be rapidly standardized for comparing results and for metadata analyses. Standard methods should consider the effectiveness of the treatment in terms of both consolidation and safety of stone (impact on structural and aesthetical features as well as on resident microbiota).

Living bacteria require the application of nutrient media on the stone. The possibility of undesirable side-effects on stone is controversial and it needs to be carefully evaluated (González-Muñoz, 2008; Nazel, 2016). The metabolic pathway activated *in situ* is the oxidative deamination of amino acids (**Table 1**), which increases the alkalinity by production of ammonia (Castanier et al., 1999; Lee and Park, 2019). The convenience of obtaining byproducts as ammonia and using spore-forming bacteria as *Bacillus* on stone has been recently discussed (Dhami et al., 2014; Zhu and Dittrich, 2016). More generally, promotion of undesired microbial growth can produce mineral changes or appearance of stained patches on stone, as found by Tiano et al. (1999). Such drawbacks may be encountered both in case of activating allochthonous or autochthonous strains. While aesthetical changes can be easily evaluated, growth of unwanted microorganisms and/or changes in the autochthonous community structure affecting the original ecological niche is harder to analyze. Microbes can strongly contribute to stone deterioration (Pinna, 2017) and the application of new biotechnologies by conservators requires knowledge about the risk factors, in particular on the long-term effects (Webster and May, 2006; De Muynck et al., 2010). In this respect, the work about the long-term monitoring of stone microbiota carried by Ettenauer et al. (2011) and Jroundi et al. (2017) is remarkable. However, knowledge about microbial communities inhabiting heritage stone mainly comes from cultivation studies (Scheerer et al., 2009). Microbial communities of stone were only recently investigated using Next Generation Sequencing and omics techniques (Perito and Cavalieri, 2018; Marvasi et al., 2019). The latest studies suggest that natural community structure detected by metagenomics is quite different from that of enriched communities cultivated from calcareous stone in precipitating media where Firmicutes are dominant (Dhami et al., 2018; Li et al., 2018).

Meta-omics techniques as a whole (metagenomics, metatranscriptomics and metabolomics) will promote a further step to improving BCCM technology, because they provide a wider view of the microbial community structure, fluctuations and metabolic potential (Marvasi et al., 2019). In regard to the cultivation bias (Hardoim et al., 2014), omics technologies will provide a better understanding of the stone microbial community structure to allow treatment monitoring as well as the identification of the community components with biomineralization potential. Chimienti et al. (2016) used metagenomics to identify the presence of microorganisms known as carbonatogenic (i.e., *Arthrobacter*) within the overall microbial community from stone slabs of a medieval church. Zanardini et al. (2019) reconstructed the carbon, nitrogen and sulfur cycles and their biodeterioration potential within the prokaryotic community of decayed sandstone of a medieval castle by 16S rRNA and functional gene analyses. Using a similar approach, the carbonatogenic potential of metabolic pathways linked to these biogeochemical cycles could be inferred. On the other hand, cultivation is more valuable than ever in the omics era (Gutleben et al., 2018) because it is needed to confirm the predicted carbonatogenic ability of stone populations as well as for other applications. But then again, meta-omics techniques can also provide useful information to improve cultivation strategies for the isolation of potential calcinogenic bacterial populations from calcareous environments.

The cell-free approach offers several advantages: the cellular components act as mineral nucleation and growth sites in the absence of nutrients, components smaller than cells penetrate more in depth into pores and microcracks, interventions on the chemical environment governing precipitation are easier (Hammes and Verstraete, 2002). Alkaline buffering or different supersaturated calcium solutions should be further developed and compared to that used by Perito et al. (2014). However, the preparation of the BCF product is more complex compared to alive cellular strategies but could have as target calcareous objects where minimum change in their chemistry is required (Perito et al., 2014). A cell-free approach has not been explored further.

Very little is still known about the molecular basis of the calcium biomineralization process (Perito and Mastromei, 2011). *B. subtilis* laboratory strain 168 was used to identify cellular fractions as well as genes and molecules with key roles in inducing

[1]http://www.amonit.fr/fr/calcite__1

[2]http://kbyobiological.com/en/

precipitation (Barabesi et al., 2007), as found for mollusks (Falini et al., 1996). Characterization of *B. subtilis* mutants impaired in $CaCO_3$ precipitation suggested a link between biomineralization, redox reactions of fatty acid metabolism, changes in phospholipids membrane composition and surface properties (Barabesi et al., 2007; Marvasi et al., 2010, 2016; Frandi et al., 2011; Perito et al., 2018a). In *Lysinibacillus*, $CaCO_3$ precipitation can modify membrane rigidity by upregulating the branched chain fatty acid synthesis (Lee and Park, 2019). We speculate that intervention on these metabolic switches could help in the search for bacterial molecules fostering precipitation and, at the same time, improving precipitation performance by bacteria.

On the other hand, it is well known that bacterial macromolecules, like the EPS, act as matrices which promote mineralization and are trapped in the growing calcite (Decho, 2010; Marvasi et al., 2012; Perito et al., 2018b). According to Jroundi et al. (2017), the hybrid cement due to the incorporation of organisms and EPS within the nanostructured $CaCO_3$ in the self-inoculation biotreatment was responsible for the high consolidation effectiveness. Further studies are needed in order to identify and test different EPS or to design bacteria-based biomimetic matrices promoting calcite growth on stone. This would represent a further advancement of the cell-free technology since it would reduce the complexity of organic matter to apply, increasing its penetration inside stone.

Concluding, in our opinion all the different approaches explored in this mini review are worth further development for *in situ* applications, even if two of them are already available on the market. Fascinating challenges for the future include advances in exploitation of bacterial pathways, cell components and single (macro)molecules.

AUTHOR CONTRIBUTIONS

BP provided the general concept. BP and MM wrote the manuscript. MM, GM, and BP revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Vibrio parahaemolyticus-specific *Halobacteriovorax* From Seawater of a Mussel Harvesting Area in the Adriatic Sea: Abundance, Diversity, Efficiency and Relationship With the Prey Natural Level

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This research aimed to study the abundance and molecular diversity of *Vibrio parahaemolyticus*-specific *Halobacteriovorax* strains isolated from seawater of the Adriatic Sea and the relationship between predator and prey abundances. Moreover, predator efficiency of the *Halobacteriovorax* isolates toward *V. parahaemolyticus* and *Vibrio cholerae* non-O1/O139 strains was tested. *V. parahaemolyticus* NCTC 10885 was used as primary host for the isolation of *Halobacteriovorax* from seawater by the plaque assay. Molecular identification was performed by PCR detection of a fragment of the 16S rRNA gene of the *Halobacteriovoraceae* family members. Moreover, 700 bp PCR products were sequenced and compared between them and to clones described for other sampling sites. *Vibrio* counts were performed on TCBS agar from 100 ml of filtered water samples and presumptive colonies were confirmed by standard methods. Predatory efficiency of *Halobacteriovorax* isolates was tested by monitoring abilities of 3-day enrichments to form clear lytic halos on a lawn of *Vibrio* preys, by the plaque assay. Out of 12 seawater samples monthly collected from June 2017 to May 2018, 10 were positive for *V. parahaemolyticus* specific *Halobacteriovorax* with counts ranging from 4 to 1.4 $\times$ 10^3 PFU per 7.5 ml. No significant relationship was found between *Halobacteriovorax* and *Vibrio* abundances. The 16SrRNA sequences of our *Halobacteriovorax* strains, one for each positive sample, were divided into three lineages. Within the lineages, some sequences had 100% similarity. Sequence similarity between lineages was always <94.5% suggesting that they may therefore well belong to three different species. All *Halobacteriovorax* isolates had the ability to prey all tested

Vibrio strains. Additional research is necessary to assess whether stable strains of *Halobacteriovorax* are present in the Adriatic Sea and to understand the mechanisms by which *Halobacteriovorax* may modulate the abundance of *V. parahaemolyticus* and other vibrios in a complex marine ecosystem.

Keywords: *V. parahaemolyticus*-specific *Halobacteriovorax*, *Vibrio* spp., *V. parahaemolyticus*, *V. cholerae* non-O1/O139, Adriatic Sea

INTRODUCTION

In many ecological communities, predation has a key role in regulating community structure or function. In the marine environment, predation has been extensively explored in animals, microbial eukaryotes and viruses (Baum and Worm, 2009) while predation by bacteria is less well understood (Williams et al., 2015). The genus *Halobacteriovorax*, in the class Deltaproteobacteria, family Bacteriovoracaceae, consists of small, Gram-negative, flagellated, marine predator bacteria that are members of a broader group of predatory bacteria known as *Bdellovibrio* and like organisms (BALOs), also including non-marine (terrestrial and freshwater) forms (Williams and Baer, 2005). BALOs enter into a susceptible Gram-negative prey bacterium and reside within the periplasmic space where they use the cytoplasmic nutrients of the prey to support growth and replication. The replicative form, known as bdelloplast, extends within the prey and divides into progeny cells that are released as soon as the host is lysed and are able to attack other preys (Williams and Baer, 2005). The genus *Halobacteriovorax*, according to the results of the analysis of the 16S rRNA gene sequence, includes two species *Halobacillus litoralis* and *Halobacteriovorax marinus* for which the similarity between the type strains is 92.61% (Koval et al., 2015). Recently, the new species *Halobacteriovorax vibrionivorans* has been also proposed (Ye et al., 2019). Furthermore, analysis of the 16S rRNA gene sequence from *Halobacteriovorax* saltwater strains identified multiple distinct phylogenetic clusters in different marine habitats, grouped based on a sequence identity with values >96.5% (Pineiro et al., 2007). Many of these *Halobacteriovorax* could represent potential new species. *Halobacteriovorax* favors predation on *Vibrio* species and other saltwater prey (Rice et al., 1998; Chen et al., 2012; Koval et al., 2015; Ye et al., 2019). Different lineages within this genus favor estuarine or marine waters and some isolates have been found in salt lakes (Koval et al., 2015). There are no known freshwater isolates (Koval et al., 2015). Recent studies reported that *Halobacteriovorax* were capable of containing *Vibrio parahaemolyticus* levels in seawater and oysters, at laboratory scale (Chen et al., 2011; Li et al., 2011; Richards et al., 2012, 2016; Williams et al., 2015; Ottaviani et al., 2018a). Furthermore, there is evidence that *Halobacteriovorax* contributes to *Vibrio vulnificus* mortality in a simulated natural seawater system with greater efficiency than other natural predators, such as bacteriophages (Chen et al., 2018). A previous study demonstrated that *Halobacteriovorax* was part of the coral microbiome (Welsh et al., 2016). In light of this evidence to understand if *Halobacteriovorax* has a role in modulating the natural levels of *V. parahaemolyticus* and other vibrios in the marine environment it is essential to know its natural abundance and understand in details how the predator infects and kills the prey. Quantitative PCR has been used to detect total *Halobacteriovorax* abundances in the marine environment, but it does not identify strains capable of targeting specific host bacteria (Zheng et al., 2008). On the other hand, Pineiro et al. (2007) isolated and characterized selected *Halobacteriovorax* phylotypes against *V. parahaemolyticus* in seawater, but results were not quantitative. To date, only two previous (plaque assay-based) studies have documented the abundance of *V. parahaemolyticus* specific *Halobacteriovorax* in seawater (Richards et al., 2013; Ottaviani et al., 2018a). However, as far as we know, there are no data on correlations between levels of *Halobacteriovorax*, *V. parahaemolyticus* and total vibrios in the marine environment. *V. parahaemolyticus* strains producing thermostable direct haemolysin (TDH) and/or TDH-related haemolysin (TRH) are recognized as a cause of diarrhoeal diseases worldwide, with bivalves, eaten raw or undercooked being the most frequent sources of infection (Potasman et al., 2002; Letchumanan et al., 2014). In Italy *Halobacteriovorax* and pathogenic vibrios, including *V. parahaemolyticus* and non-O1/O139 *Vibrio cholerae*, have been isolated in seawater and mussels coming from the Adriatic Sea (Masini et al., 2007; Ottaviani et al., 2018a). Moreover, in the last years, illness due to *V. parahaemolyticus* and non-O1/O139 *V. cholerae*, with mussels or seawater of Adriatic Sea as the source of infection, has been reported (Ottaviani et al., 2010, 2013, 2018b). In this work we studied the abundance and molecular diversity of *V. parahaemolyticus*-specific *Halobacteriovorax* strains isolated from seawater of a mussels growing area at the Conero Riviera of the Adriatic Sea. From the same samples *V. parahaemolyticus* and total vibrios were also counted and the relationship between these and *Halobacteriovorax* was studied. Finally, the predatory efficiency of *Halobacteriovorax* isolates toward *V. parahaemolyticus* and *V. cholerae* non-O1/O139 strains from clinical and environmental sources, the majority linked to the Adriatic sea of Italy, was tested.

MATERIALS AND METHODS

Sampling Site

The Conero Riviera, located halfway of the Italian Adriatic coast, is a coastal marine ecosystem with an extension of 20 km and a low anthropic impact. This riviera includes several important sea towns, such as Ancona, Portonovo, Sirolo, and Numana, and lies in a protected area representing the most important marine reserve of the Marches where there are also many natural banks

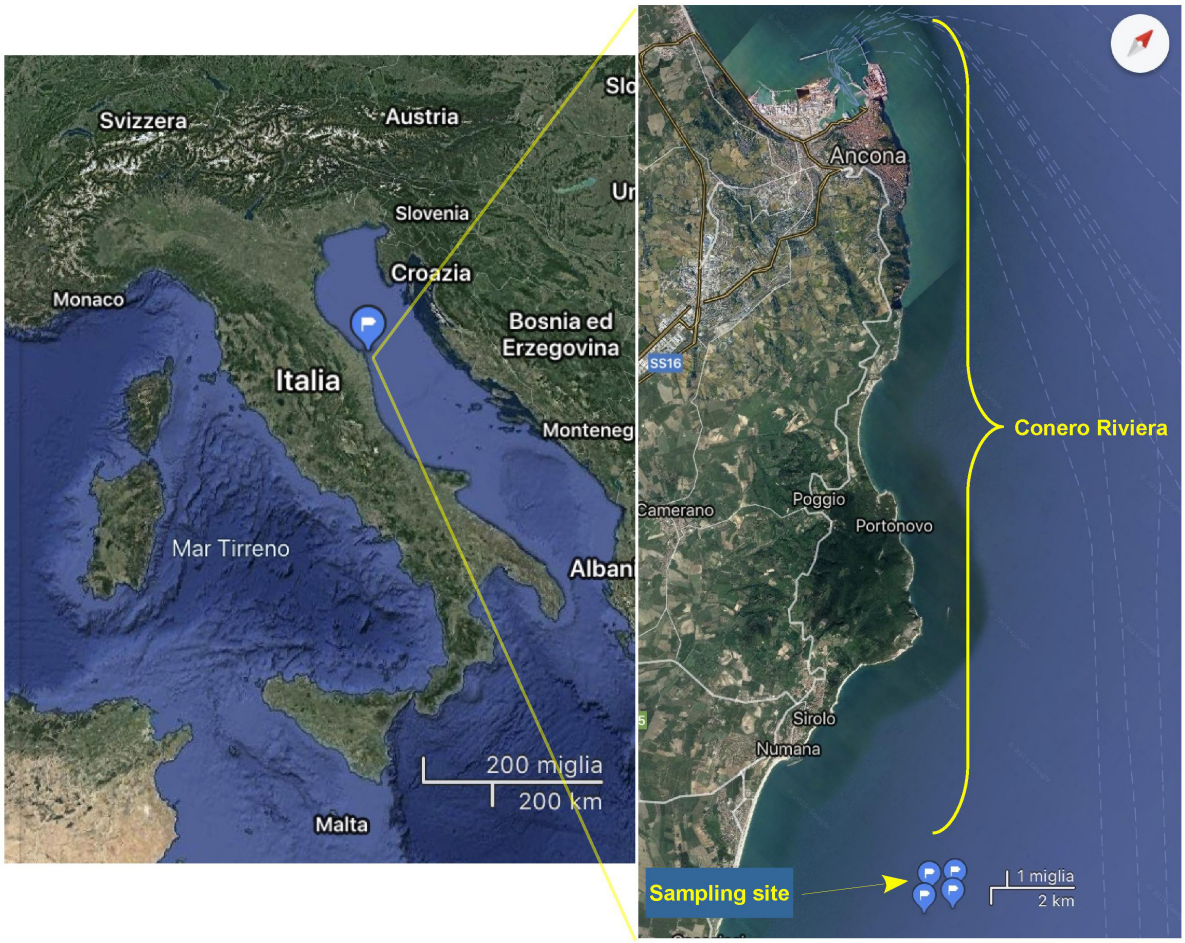

FIGURE 1 | Map of the Conero Riviera showing the location of the sampling site.

of mussels (*Mytilus galloprovincialis*) (**Figure 1**). Subsurface seawater samples were monthly collected from a site of approved mussels growing area in the Conero Riviera (43°30′12″N–13°41′12″E) from June 2017 to May 2018 (**Figure 1**). Two liters of seawater for each sample were collected using sterile polypropylene bottles. Water temperature and salinity were determined in situ with a multiparametric probe (Handy Gamma, Oxyguard, Denmark). All samples were transported to the laboratory on ice for analysis. Salinity remained steady between 38 and 39 ppt. Seawater was immediately transported to the laboratory in an insulated cooler and analyzed within 4 h. The same seawater samples that were used for *Halobacteriovorax* analyses were also used for *V. parahaemolyticus* and total *Vibrio* analyses.

Prey Strains

Vibrio parahaemolyticus NCTC 10885 strain was used as prey. Prey specificity and predatory efficiency of *Halobacteriovorax* isolates were tested on *V. parahaemolyticus* and *V. cholerae* strains of environmental and clinical origin, the majority directly or indirectly linked to the Adriatic sea of Italy (**Table 1**). For all *Vibrio* strains, fresh enrichments were prepared from a stock culture grown on 3% NaCl Luria-Bertani broth until prey reached an OD_{600} of 0.20 (∼1.8 × 10^8 CFU/ml).

Halobacteriovorax Enumeration

It was performed by a double layer agar plating technique (Richards et al., 2012). The prey was grown in LB–3% NaCl broth until it reached an OD_{600} of 0.20 (∼1.8 × 10^8 CFU/ml). For the analysis, 500 ml of test seawater was first filtered through a 0.45-μm, 500-ml filter to remove particulates and bacteria. Then, the filtered seawater was serially diluted 10-fold in sterilized artificial seawater (30 ppt). For each assay, 25 ml of bottom-layer Pp 20 agar (polypeptone peptone supplemented with Bacto agar) were dispensed into a 100-mm Petri dish and allowed to harden. Top agar was pipetted into sterile glass tubes (7.5 ml/tube) while the agar was hot and allowed to cool to 48°C in a water bath. The plaque assay was conducted by combining 1 ml of host *V. parahaemolyticus* culture, at an OD_{600} of 0.20 and 7.5 ml of undiluted and diluted test seawater to 7.5 ml of molten (48°C) Pp 20 agar in tubes. The tubes were inverted 3 times to mix and poured on top of the existing bottom layer. Counts of

TABLE 1 | *Vibrio* preys tested with *Halobacteriovorax* strains.

No.	Prey strains Toxin genes*	Source and year of isolation	Origin
1	*V. parahaemolyticus tdh*+**; *trh*−***; *toxRS*+ (pandemic strain)	Feces, 2007	Mussels of Adriatic sea, Italy as the most probable source of infection
2	*V. parahaemolyticus tdh*−/*trh*−; *toxRS*−	Feces, 2010	Mussels of Adriatic sea, Italy as the most probable source of infection
3	*V. parahaemolyticus tdh*−; *trh*−; *toxRS*−	Feces, 2010	Mussels of Adriatic sea, Italy as the most probable source of infection
4	*V. parahaemolyticus tdh*−/*trh*+; *toxRS*−	Marine water, 2011	Conero Riviera, Italy
5	*V. parahaemolyticus tdh*+/*trh*−; *toxRS*−	Mussels, 2014	Conero Riviera, Italy
6	*V. parahaemolyticus tdh*−; *trh*−; *toxRS*−	Mussels, 2015	Conero Riviera, Italy
7	*V. parahaemolyticus tdh*−; *trh*+; *toxRS*	Mussels, 2016	Conero Riviera, Italy
8	Non O1/O139 *V. cholerae hly*AClass−; *hly*ET+; *ctx*A−; *tcp*AClass+; *tcp*AET−; *stn/sto*−	Subcutaneous tissue, 2009	Seawater of Adriatic sea, Italy, as the most probable source of infection
9	Non O1/O139 *V. cholerae hly*AClass−; *hly*ET+; *ctx*A−; *tcp*AClass−; *tcp*AET−; *stn/sto*−	Subcutaneous tissue, 2012	Seawater of Adriatic sea, Croazia, as the most probable source of infection
10	Non O1/O139 *V. cholerae hly*AClass−; *hly*ET+; *ctx*A−; *tcp*AClass−; *tcp*AET−; *stn/sto*+	Marine water, 2011	Conero Riviera, Italy
11	Non O1/O139 *V. cholerae hly*AClass−; *hly*ET+; *ctx*A−; *tcp*AClass−; *tcp*AET−; *stn/sto*−	Feces, 2012	Bivalves as the most probable source of infection
12	Non O1/O139 *V. cholerae hly*AClass−; *hly*ET+; *ctx*A−; *tcp*AClass−; *tcp*AET−; *stn/sto*−	Mussels, 2014	Conero Riviera, Italy
13	*V. parahaemolyticus* NCTC 10903		
14	*V. parahaemolyticus* NCTC 10884		
15	*V. cholerae O1* NCTC 9459		

tdh: thermostable direct haemolysin gene; trh: TDH-related haemolysin gene; toxRS: pandemic genetic marker; ctxA: cholera toxin gene; stn/sto: heat stable enterotoxin gene; hlyAClass: hemolysin classical gene; hlyET: hemolysin El Tor gene; tcpAClass: toxin-coregulated pilus classical gene; tcpAET: toxin-coregulated pilus El Tor gene; **+: positive; *−: negative.*

Halobacteriovorax were performed after incubation at 26°C for 7 days. The number of viable *Halobacteriovorax* isolates was estimated as PFU per 7.5 ml seawater.

Halobacteriovorax Molecular Identification and Sequencing Analysis

For each presumptively positive sample, five plaques appearing on plates of the highest dilution were picked up for molecular identification. The templates used for PCR were individual plaques re-suspended in 100 ml of sterile double-distilled water and vortexed at a high speed. The liquid phase was transferred to a new tube and subjected to heating in boiling water for 3 min (Jurkevitch et al., 2000). 16S rRNA PCR analysis was performed on a fragment of the 16S rRNA gene of the *Halobacteriovoraceae* family members using the primers Bac676F and Bac1442R, as previously described (Davidov et al., 2006; Richards et al., 2013). Samples showing a band of 700 bp were considered *Halobacteriovorax*. For each positive sample, sequencing analysis was performed on PCR product of 700 bp from a unique plaque. PCR products were purified with the High Pure PCR Product Purification kit Roche Diagnostics (GmbH, Mannheim, Germany). Sequencing analysis was performed using the reverse primer BAC1442R and ABI Prism® BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems™, Life Technologies, United States), according to the manufacturer's instructions. Sequenced products were analyzed in an automated capillary sequencer ABI Prism® 310 Genetic Analyzer (Applied Biosystems™, United States). Nucleotide sequences were manually edited, aligned and analyzed using CLC genomics workbench V.12 (Qiagen Bioinformatics). Phylogenetic trees of the 16S rRNA gene sequences from the isolates generated in this study were reconstructed using the maximum likelihood (ML) method. These sequences were also compared to those of the following type strains of the *Halobacteriovorax* species: *H. marinus* SJ (GenBank accession number 102485), *H. litoralis* JS5 (GenBank accession number 028724) and *H. vibrionivorans* BL9 (GenBank accession number MH150810) Initially the 16SrRNA sequences were aligned with a progressive alignment tool (Feng and Doolittle, 1987) within the CLC genomics workbench 20 environment. The best substitution model fitting the alignment was the General Time Reversible (GTR), gamma distribution 0 and transition/transversion rate ratio 2. The tree topology was tested with 1000 bootstrap replicates.

Total *Vibrio* spp. and *V. parahaemolyticus* Enumeration

Each water sample was mixed and 100 ml of undiluted and 1:10, 1:100, 1:1000 diluted sample was filtered using a 0.45-mm-pore membrane filter (Millipore, Bedford, MA, United States); the filter was placed on thiosulfatecitrate-bile-salts-sucrose-agar (TCBS, Difco Laboratories, Detroit, MI, United States) and incubated at 37°C for 24 h. For each presumptively positive sample, five colonies appearing on plates of the highest dilution were selected and subcultured on trypticase soy agar with 2% NaCl (TSAs, Oxoid). Presumptive *Vibrio* spp. were biochemically

identified at genus level by a standardized protocol (Ottaviani et al., 2003). Presumptive *V. parahaemolyticus* strains were confirmed by a standard method (ISO/TS, 2017). The number of viable *V. parahaemolyticus* and total *Vibrio* isolates was estimated as CFU per 100 ml seawater.

Prey Specificity and Predatory Efficiency of *Halobacteriovorax* Isolates

Three-day enrichments of each *Halobacteriovorax* isolate (approximately 1×10^6 PFU ml^{-1}) were filtered through a 0.45- m-pore-size Millex HV syringe filter (Millipore Corp., Billerica, MA, United States) to remove the primary prey. Prey specificity and predator efficiency of *Halobacteriovorax* strains were determined by monitoring their abilities to form clear lytic halos on a lawn of the reference *V. parahaemolyticus* and *V. cholerae* preys listed in **Table 1**, using a double layer agar plating technique. Briefly, 1 μl of filtered predator undiluted enrichment, 1 ml of prey culture at an OD_{600} of 0.2 for *Vibrio* preys (approximately 10^8 PFU ml^{-1}), were added to 7.5 ml of sterilized artificial seawater (30 ppt salinity) and 7.5 ml of Pp 20 top agar (Richards et al., 2012, 2016). For each prey specificity assay a positive control, represented by *V. parahaemolyticus* NCTC 10885 and the *Halobacteriovorax* strain, and a negative control, which consisted of the prey alone, were performed.

Statistical Analysis

On each 2 l seawater sample the analyses were performed in six independent replicates for *Halobacteriovorax* and *Vibrio* ($n = 6$). Results of microbiological analyses were reported as mean values (log-transformed) ± standard deviation. Correlation coefficients were determined and associated p-values < 0.05 were considered significant.

RESULTS

Halobacteriovorax Enumeration

Out of 12 seawater samples collected, 10 (83.3%) were positive for presumptive *V. parahaemolyticus* specific *Halobacteriovorax,* with counts ranging from 4 PFU per 7.5 ml (November 2017) to 1.4×10^3 PFU per 7.5 ml (January 2018) (**Figure 2**). Plaques on primary prey became visible after 72 h. After that, sizes expanded over time, reaching the maximum diameter of 9 mm after 5 days of incubation at 26°C. A lack of plaques was observed on all replicates of February and May 2018 samples even when the incubation was prolonged up to 10 days. No significant relationship was found between *Halobacteriovorax* levels and seawater temperature (**Figure 2**).

Halobacteriovorax Molecular Identification and Sequencing Analysis

All plaques for each seawater sample were confirmed by molecular methods as *Halobacteriovorax.* The sequences obtained by analysis of the 10 16SrRNA 700pb PCR fragments, one for each positive sample, were named DOGA1-DOGA10. The partial 16SrRNA gene sequences were deposited in GenBank under accession numbers MN750616–MN750625 (**Table 2**). Sequences from DOGA1 to DOGA7 were obtained from the strains isolated in 2017 while sequences from DOGA8 to DOGA10 were from the strains isolated in 2018 (**Table 2**). Overall, the divergence between our sequences was always <10% (**Supplementary Figure 1**). A similarity >99% was between the sequences DOGA1-DOGA5, DOGA6-DOGA7 and DOGA8-DOGA10. Between the three groups, the sequence similarity ranged from 90.63% (between DOGA 1 and DOGA 8) to 94.51% (between DOGA 6 and DOGA 9–10) (**Supplementary Figure 1**). These three distinct lineages were named L1, L2, L3, respectively (**Table 2**). DOGA2, DOGA3, and DOGA5 within L1 showed 100% sequence identity (**Figure 3** and **Table 2**). Also DOGA9 and DOGA10 within L3 showed 100% sequence identity (**Figure 3** and **Table 2**). Sequences within L3 shared 98.54–99.19% similarity with *H. vibrionivorans* BL9, 94.83–95.32% with *H. litoralis* JS5 and 91.75–92.23%. with *H. marinus* SJ (**Figure 3** and **Supplementary Figure 1**). Sequences within L2 shared 94.18–94.51% similarity with *H. vibrionivorans* BL9, 95.64–95.96% with *H. litoralis* JS5 and 93.38–93.70%. with *H. marinus* SJ (**Figure 3** and **Supplementary Figure 1**). Sequences within L1 shared 91.44–92.57% similarity with *H. vibrionivorans* BL9, 91.11–92.25% with *H. litoralis* JS5 and 92.41–93.21%. with *H. marinus* SJ (**Figure 3** and **Supplementary Figure 1**).

Total *Vibrio* spp. and *V. parahaemolyticus* Enumeration

Six samples (50%) were positive for *V. parahaemolyticus* with counts ranging from 1.0×10^2 CFU per 100 ml (May 2018) to 1.7×10^4 CFU per 100 ml (August 2017). A lack of detection was reported from November to December 2017 and from January to April 2018. Nine samples (75%) were positive for total vibrios with counts ranging from 2.0×10^2 CFU per 100 ml (January 2018) to 1.3×10^5 CFU per 100 ml (August 2017). A lack of detection was reported from February to April 2018. No significant relationship was found between levels of *Halobacteriovorax* and those of total *Vibrio* and *V. parahaemolyticus* (**Figure 2**).

Prey Specificity and Predatory Efficiency of *Halobacteriovorax* Isolates

All *Halobacteriovorax* isolates were able to lyse all tested *V. parahaemolyticus* and *V. cholerae* reference strains. Plaques of lysis became visible after 72 h and then, their sizes expanded over time, reaching the maximum after 5 days of incubation at 26°C. At the end of the incubation, the diameter of lysis plaques on vibrios was similar to those on primary prey, ranging between 7 and 9 mm diameter.

DISCUSSION

Halobacteriovorax Enumeration

Indigenous *V. parahaemolyticus*-specific *Halobacteriovorax* is present in seawater of a mussel growing area of the Conero Riviera at levels higher than those reported for Atlantic and

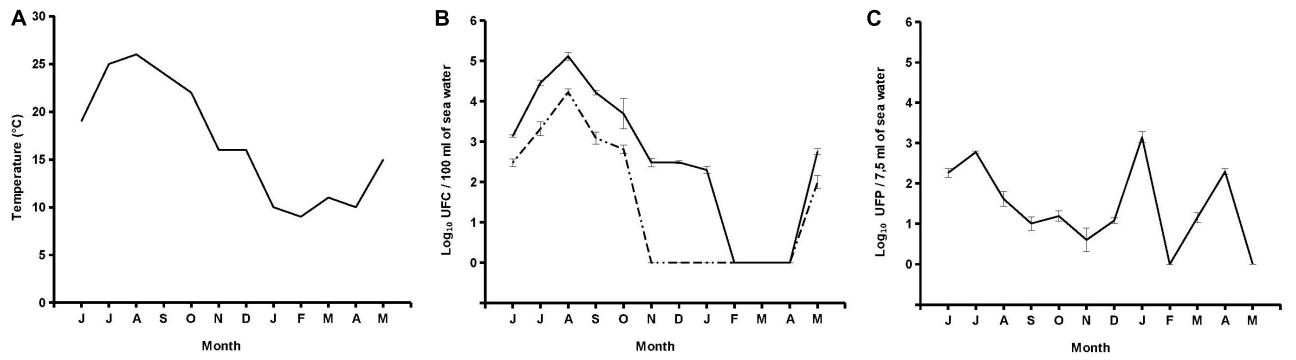

FIGURE 2 | Trend of temperature **(A)**, *V. parahaemolyticus* and total *vibrio* **(B)**, and *Halobacteriovorax* **(C)**.

TABLE 2 | *Halobacteriovorax* strains molecularly characterized in this study.

16S-rRNA sequences	GenBank accession number	Period of isolation	Seawater temperature	16S-rRNA lineages
DOGA1	MN750616	June 2017	20°C	L1
DOGA2	MN750617	July 2017	25°C	L1
DOGA3	MN750618	August 2017	26°C	L1
DOGA4	MN750619	September 2017	24°C	L1
DOGA5	MN750620	October 2017	22°C	L1
DOGA6	MN750621	November 2017	16°C	L2
DOGA7	MN750622	December 2017	16°C	L2
DOGA8	MN750623	January 2018	10°C	L3
DOGA9	MN750624	March 2018	11°C	L3
DOGA10	MN750625	April 2018	10°C	L3

Pacific Oceans which never exceeded values of 10^2 PFU per mL (Richards et al., 2013). Moreover, the *Halobacteriovorax* counts in this study should be considered minimum counts since some portion of the predators were undoubtedly removed by filtration (Richards et al., 2013). Surprisingly, we found the *Halobacteriovorax* higher abundances on January 2018 while *Halobacteriovorax* in Atlantic and Pacific Oceans were mainly concentrated in the warmer months of the year (Richards et al., 2013). It is known that *Halobacteriovorax* against *V. parahaemolyticus* is able to proliferate in the temperature range between 10 and 30°C (Williams et al., 2015). In the marine areas investigated by Richards et al. (2013) temperatures over a 12-month period ranged from lows of 5°C in winter and highs of 30°C in summer. *Halobacteriovorax* replication was therefore completely inhibited in the winter months. In the Conero Riviera temperatures over a 12-month period ranged from lows of 10°C in winter and highs of 25°C in summer. Therefore, throughout the year, the temperatures were suitable for a good replication of *Halobacteriovorax*.

Halobacteriovorax Molecular Identification and Sequencing Analysis

In BALOs, as in other bacteria, a 90% sequence similarity means grouping at the genus level while the species level is identified by a >98% similarity (Koval et al., 2015). In this study the sequence similarity of *Halobacteriovorax* strains inter-lineages was always <94.5% suggesting that they may therefore well belong to three different species. Moreover, sequences in L1 and L2 formed independent branches separated from the type strains of *Halobacteriovorax.* Instead the sequences in L3 shared >99% similarity with *H. vibrionivorans* BL9. For this reason these *Halobacteriovorax* strains would clustered with the proposed new species *H. vibrionivorans.* In this period there is a great interest from the scientific world in the taxonomic and phylogenetic study of this genus. Shortly we will perform the genome sequencing of our *Halobacteriovorax* strains in order to obtain more detailed information on their taxonomic position and understand if they could belong to new species within the genus. Our results show that different strains of *Halobacteriovorax* circulated in the Conero Riviera in the 2017–2018 period. In this study we have limited ourselves to the molecular characterization of the dominant population of *Halobacteriovorax*. For this reason, the plaques for each isolate were enumerated and treated as if they were a single clonal population. Moreover, in this study we have used a unique *V. parahaemolyticus* strain as prey to recover *Halobacteriovorax.* These working methods may have limited the *Halobacteriovorax* diversity detected in the marine area investigated. Previous studies associated different clusters of *Halobacteriovorax* to marine waters with different salinity (Pineiro et al., 2007, 2013; Crossman et al., 2013; Richards et al., 2013). In disagreement, our strains belonging to different lineages, were isolated from a marine area with a constant level of salinity equal to 38–39 ppt throughout the year. An association

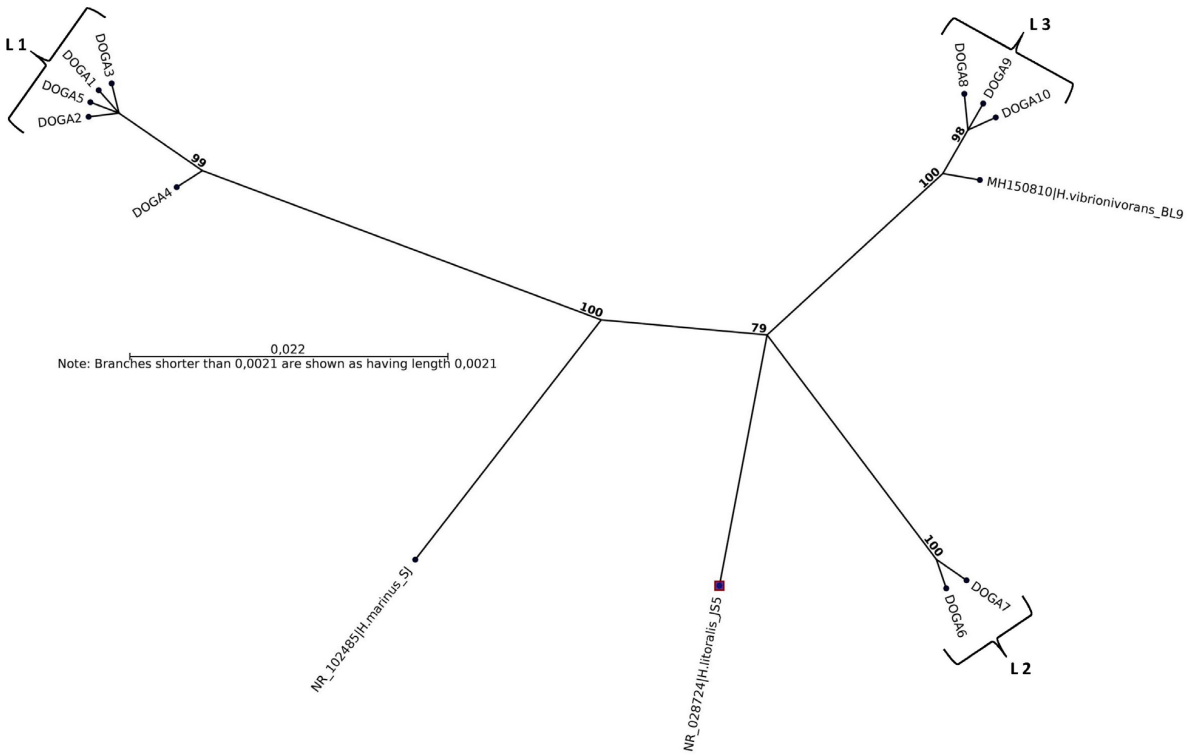

FIGURE 3 | Maximum-likelihood phylogenetic tree, based on 16S rRNA gene sequence comparisons, showing the position of strains DOGA1-10 and related type strains. Numbers at branch nodes are bootstrap values (per 1000 trials).

was found among the lineages of our *Halobacteriovorax* strains and the temperature of seawater. The strains in L1 were isolated in the period June–October 2017 when the temperature ranged between 20 and 25°C. The strains in L2 were isolated in the period November–December 2017 when the temperature was 16°C. Finally, the strains in L3 were isolated in the period January–April 2018 when the temperature ranged between 10 and 11°C. In a future investigation we will extend the period and marine area of the study, by using different *Vibrio parahemolyticus* prey strains and sequencing 16SrRNA fragments from more plaques for each sample. The scope will be to assess if, in the Adriatic Sea, diversities of *Halobacteriovorax* may exist in respect of seasonality and prey specificity and whether particular strains can be present here stably.

Total *Vibrio* spp. and *V. parahaemolyticus* Enumeration

No significant correlations between *Halobacteriovorax*, total *Vibrio* spp. and *V. parahaemolyticus* abundance was detected in the marine area investigated in this study. A previous study confined to microcosms in which *Halobacteriovorax*/*V. parahaemolyticus* at a ratio of 10^5 PFU/10^5 CFU per ml were added, produced a different effect, where *Halobacteriovorax* increased as *V. parahaemolyticus* decreased (Ottaviani et al., 2018a). The fact that no relationship between the abundance of *Halobacteriovorax* and *V. parahaemolyticus* was observed in the present study could be related to the very low abundance of both genera in seawater which was about 10–100 cells per ml. This may have reduced the chance for high predation and consequently a significant increase and decrease in the abundance of *Halobacteriovorax* and *V. parahaemolyticus*, respectively. Moreover, it is known that some *Halobacteriovorax* has broader host specificity than others and the mechanisms that drive *Halobacteriovorax* host specificity within a complex bacterial community are not known. In this regard, our *Halobacteriovorax* strains demonstrated predatory activity *in vitro* toward all *V. parahaemolyticus* and *V. cholerae* reference strains tested, many of them isolated from the same marine area. *Halobacteriovorax* may well target one microorganism over another within the same species and also between different species of marine bacteria. The extent to which this occurs remains unresolved. In the next future it would be essential to perform microcosm-studies to investigate the unwanted effect of *Halobacteriovorax* isolates toward the vital core-microbiome bacteria in marine seawater. Moreover, *Halobacteriovorax* and *Vibrio* abundances may be subject to control by bacteriophages, other predators, environmental conditions, nutrient levels, the effects of competing microbes, and the development of host resistance. Finally, the lack of correlation between predator and prey levels could be linked to the different speed of *Halobacteriovorax* and *Vibrio* replication, so that they cannot compete with maximum efficiency between them.

Prey Specificity and Predatory Efficiency of *Halobacteriovorax* Isolates

Our *Halobacteriovorax* isolates, although captured using a single *V. parahaemolyticus* strain as primary prey, were able to lyse a wide range of *V. parahaemolyticus* and *V. cholerae* strains, including those toxigenic and/or involved in human infections. Previous scientific information reported that *V. parahaemolyticus* is the most efficient prey known for *Halobacteriovorax* recovery, at least for the strains against *Vibrio* (Pineiro et al., 2007). The broad spectrum of action of our isolates would seem to confirm this hypothesis. Most of the vibrio prey came from the same marine area from which *Halobacteriovorax* strains were also isolated. Some had been isolated from mussels, others from seawater in the Conero Riviera. We believe that *Halobacteriovorax* similarly to other bacteria through the filtration from seawater passes inside the mollusk where continues to parasitize vibrios and other potential pathogens. A previous study investigated the *Halobacteriovorax* predation in coral-associated microbiome (Welsh et al., 2016). The authors speculated that coral microbiome could allow the predator to remain alive and vital while *Halobacteriovorax* could regulate and maintain the microbiome structure in balance and at the same time protect the host by consuming potential pathogens. Similar symbiotic relationships may also exist between *Halobacteriovorax* and the microbiome associated with other aquatic organisms, such as bivalves. Laboratory-scale studies are currently underway to evaluate the effects of *Halobacteriovorax* in the reduction of *V. parahaemolyticus* in bivalves during the purification phase and to understand the potential unwanted effects of the predator on the mussel microbiome.

CONCLUSION

In conclusion, different *V. parahaemolyticus*-specific *Halobacteriovorax* strains are present in seawater of a mussel growing area on the Conero Riviera where they, probably, play a physiological role as natural modulators versus *V. parahaemolyticus* and other vibrios populations. These predatory bacteria could have a primary role in regulating and structuring marine bacterial communities and the nutrient cycle but have not received the attention they deserve to date. Additional research is necessary to better understand the mechanisms by which *Halobacteriovorax* may modulate *V. parahaemolyticus* and other vibrios in a complex marine ecosystem, and the overall effect they exert on the structure of seawater microbial communities and marine host-associated microbiomes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank under the following accession numbers: SUB6636935 DOGA1 MN750616, SUB6636935 DOGA2 MN750617, SUB6636935 DOGA3 MN750618, SUB6636935 DOGA4 MN750619, SUB6636935 DOGA5 MN750620, SUB6636935 DOGA6 MN750621, SUB6636935 DOGA7 MN750622, SUB6636935 DOGA8 MN750623, SUB6636935 DOGA9 MN750624, and SUB6636935 DOGA10 MN750625.

AUTHOR CONTRIBUTIONS

GA, SP, and FM isolated bacteria and performed the laboratory measurements. DO, FL, ER, ML, and PT made substantial contributions to conception and design. AP aligned and compared 16SrRNA sequences. DO wrote and revised the manuscript. DO and GA drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01575/full#supplementary-material

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ORIGINAL RESEARCH
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Temporal Evolution of Bacterial Endophytes Associated to the Roots of *Phragmites australis* Exploited in Phytodepuration of Wastewater

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Improvement of industrial productions through more environment-friendly processes is a hot topic. In particular, land and marine environment pollution is a main concern, considering that recalcitrant compounds can be spread and persist for a long time. In this context, an efficient and cost-effective treatment of wastewater derived from industrial applications is crucial. Phytodepuration has been considered as a possible solution and it is based on the use of plants and their associated microorganisms to remove and/or transform pollutants. In this work we investigated the culturable microbiota of *Phragmites australis* roots, sampled from the constructed wetlands (CWs) pilot plant in the G.I.D.A. SpA wastewater treatment plant (WWTP) of Calice (Prato, Tuscany, Italy) before and after the CW activation in order to check how the influx of wastewater might affect the resident bacterial community. *P. australis* specimens were sampled and a panel of 294 culturable bacteria were isolated and characterized. This allowed to identify the dynamics of the microbiota composition triggered by the presence of wastewater. 27 out of 37 bacterial genera detected were exclusively associated to wastewater, and *Pseudomonas* was constantly the most represented genus. Moreover, isolates were assayed for their resistance against eight different antibiotics and synthetic wastewater (SWW). Data obtained revealed the presence of resistant phenotypes, including multi-drug resistant bacteria, and a general trend regarding the temporal evolution of resistance patterns: indeed, a direct correlation linking the appearance of antibiotic- and SWW-resistance with the time of exposure to wastewater was observed. In particular, nine isolates showed an interesting behavior since their growth was positively affected by the highest concentrations of SWW. Noteworthy, this study is among the few investigating the *P. australis* microbiota prior to the plant activation.

Keywords: *Phragmites australis*, phytodepuration, wastewater, endophytes, antibiotic resistance, metal resistance

INTRODUCTION

Plants and microorganisms have been living in association for a very long time. In fact, arbuscular mycorrhizal mutualism is believed to have had a key importance in the terrestrialization process and in the evolution and diversification of plant phototrophs (Selosse and Le Tacon, 1998; Heckman et al., 2001). Different microorganisms (bacteria and fungi) can establish (more or less) deep associations with plants; some of them exhibit an endophytic lifestyle, in that they colonize plant tissues internally, although a more specific definition of endophytes states that they are organisms which, at some moment of their life cycle, colonize the internal plant tissues without causing any type of harm to the host (Patriquin and Döbereiner, 1978). Potential endophytes often inhabit the surrounding soil, especially rhizosphere, from where they can enter plant tissues switching to an endophytic lifestyle. They may thus enter plant tissues through wounds, germinating radicles, emergence points of lateral roots or root elongation and differentiation zones (Reinhold-Hurek et al., 2006; Sturz et al., 2010). Once inside, bacteria adapt to different environmental conditions (e.g., pH, osmotic pressure, carbon source, and availability of oxygen) and overcome plant defense responses (Zeidler et al., 2004).

The plant host and the bacterial endophytes create a mutualistic interaction, with bacteria gaining nutrients and a niche to colonize (Sturz et al., 2010). Even though the exact role of endophytes within plant tissues has not been fully understood yet, it is well-established that in many cases endophytes are beneficial to plants (Schlaeppi and Bulgarelli, 2015; Wani et al., 2015). The most common functions observed for bacterial endophytes are (i) uptake of nutrients (e.g., N, P, S, Mg, Fe, and Ca; Duijff et al., 1999; Çakmakçi et al., 2006), (ii) biosynthesis of phytohormones promoting plant growth (Spaepen et al., 2007), (iii) 1-aminocyclopropane-1-carboxylate deaminase activity (ACC; Glick et al., 2007), (iv) nitrogen fixation (Doty et al., 2009), (v) prevention of pathogenic infections (Weller, 2007; Pérez-García et al., 2011), (vi) acceleration of seedling emergence (Hardoim et al., 2008), and (vii) tolerance to pollution and stresses (Ryan et al., 2008; Lugtenberg and Kamilova, 2014).

In the context of the present work, particularly important is the ability of plant-associated bacteria to increase tolerance to pollution and/or increase the ability of plants to detoxify polluted environments. Environmental pollution, especially water pollution, represents a concern of considerable prominence in the current society. In this regard, phytodepuration is the over-arching term for a group of technologies that utilizes plants and the associated rhizospheric microorganisms to remove and/or transform contaminants leached from soils/sediments and from used water streams (He et al., 2017; Saxena et al., 2020). It represents an environmental-friendly and a valuable solution for environmental cleanup, in particular for wastewater treatment, and it is popular because of its cost effectiveness, aesthetic advantages, and long-term applicability (Puvanakrishnan et al., 2019). In the present manuscript the term "phytodepuration" has been used to indicate specifically the remediation process regarding water and wastewater, rather than "phytoremediation," which has a more general meaning, encompassing applications regarding, for example, soil remediation.

TABLE 1 | Antibiotics used in this work.

Antibiotic	Class	Target
Ampicillin	Penicillins	Cell wall synthesis: inhibitor of D-Ala-D-Ala carboxypeptidase
Chloramphenicol	Phenicols	Ribosome: inhibitor of peptidyl transferase activity of 23S rRNA
Ciprofloxacin	Fluoroquinolones	Topoisomerases
Kanamycin	Aminoglycosides	Ribosome: inhibitor of 30S ribosomal subunit
Rifampicin	Ansamycins	DNA-dependent RNA polymerase
Streptomycin	Aminoglycosides	Ribosome: inhibitor of 30S ribosomal subunit
Tetracycline	Tetracyclines	Ribosome: it blocks the binding of aminoacyl-tRNAs
Trimethoprim	Diaminopyrimidines	DNA replication: inhibitor of dihydrofolate reductase

TABLE 2 | Composition of synthetic wastewaters (SWWs).

Compound	1X SWW	2X SWW	3X SWW
H_3BO_3	20	40	60
$FeCl_2 \cdot 4H_2O$	15	30	45
Na_2SeO_3	0.03	0.06	0.09
NaCl	5,000	10,000	15,000

Concentrations are expressed as mg/L.

The constructed wetlands (CW) are engineered systems designed to mimic the self-purification processes of natural wetlands. For decades, CW have been successfully used for treating wastewater of different origins and have been identified as a sustainable wastewater management option worldwide (Wang et al., 2017), demonstrating their ability to eliminate diffuse pollutants from urban, rural, and industrial emissions. In literature, the effectiveness of the use of CW in the treatment of sewage containing heavy metals and high salinity is reported (Vymazal, 2011). This process is due to the interaction between plants, microorganisms, soil, and polluting substances (Zhou et al., 2013).

In CW, the rhizosphere is the mainly involved plant compartment, where multiple different physiochemical and biological processes occur (Stottmeister et al., 2003). The common reed *Phragmites australis* is one of the most employed plant species, because of its ability to flourish in marshy areas and swamps and the high detoxification and phytodepuration potential. Moreover, it is widely used to treat industrial wastewater containing heavy metals (Zhang et al., 2017). One peculiar characteristic of *P. australis* is that its internal environment is characterized by a relatively constant osmotic gradient determined by the downward transportation of Na^+ from stems to roots (Vasquez et al., 2006). For this reason, *P. australis* is also well-adapted to salty ecosystems. In CW, vegetation is responsible for only a small amount of pollutant removal (0.02%; Zhang et al., 2017), while its main function is

TABLE 3 | Bacterial counts in roots of *Phragmites australis* collected during the five samplings and meteorological conditions registered monthly.

	Samplings				
	1st (March 2017)	**2nd (July 2017)**	**3rd (November 2017)**	**4th (June 2018)**	**5th (December 2018)**
SFS-v (CFU/g)	4×10^6	1×10^7	1×10^6	3×10^6	6×10^5
SFS-h (CFU/g)	5×10^6	1×10^7	5×10^6	2×10^7	1×10^6
Average air temperature (°C)	13.8	26.4	10.6	23.5	7.4
Total precipitations (mm)	58.8	2.0	108.2	48.6	42.6

Bacterial counts are expressed as colony forming units per gram (CFU/g) of roots, air temperature in Celsius degrees, total precipitations in millimeters.

to provide additional oxygen and organic matter for microbial growth (Zhou et al., 2013). Indeed, microorganisms have been described as the main actors of pollutant removal in CW (Zhang et al., 2017). Phytodepuration has proved to effectively remove or neutralize hazardous environmental contaminants and it is predicted to have a growing application in the next years. However, this process presents some limitations, such as the toxic effects of pollutants on the growth and health of the plants (Glick, 2003). In fact, plant biomass is critical for phytodepuration (Germaine et al., 2010) and even hyperaccumulator plants, which can accumulate concentrations of toxic elements up to 100-fold higher than other plant species, usually exhibit a reduced growth. Also, phytodepuration may determine the accumulation of contaminants in plant tissues, which, in turn, is responsible for ecological and airborne exposure issues (Ho et al., 2012). In this scenario, rhizobacteria and endophytic bacteria can aid plants by supporting their growth (Tesar et al., 2002; Shaw and Burns, 2004; Chaudhry et al., 2005), reducing phytotoxicity effects, increasing pollutant uptake and removal (Glick and Stearns, 2011), reducing the release of toxic compounds into the atmosphere (Barac et al., 2004), removing contaminants and/or accumulating heavy metals (Germaine et al., 2010; Ho et al., 2012).

The experimental plant of Calice (Prato, Italy), managed by G.I.D.A. SpA, has therefore set itself as a goal to verify the action of this association in tertiary treatment of landfill leachate (LFL; Coppini et al., 2019).

The aim of this work was to characterize the cultivable bacterial communities associated to the roots of *P. australis* plants in Calice CW and to analyze their temporal dynamics before and after the activation of the plant for 22 months. This allowed the assessment of wastewater influx effect in shaping the composition of pre-existing bacterial communities. Moreover, bacteria isolated from roots were tested for their ability to grow in the presence of synthetic wastewater (SWW), along with their resistance against a panel of antibiotics commonly used to treat infections in humans. To the best of our knowledge, this work is among the few taking in consideration the bacterial composition of endophytes before the activation of CW, and likely the first regarding this issue in *P. australis*.

MATERIALS AND METHODS

Site Description

P. australis plants were obtained from the CWs pilot plant managed by G.I.D.A. SpA and located at Calice Wastewater Treatment Plant (WWTP) in Prato, Italy. The CW of Calice was designed for the tertiary treatment of LFL. This CW is located downstream of a membrane bio-reactor (MBR) designed to pretreat a mixture of LFLs before their discharging in the main line of a full-scale WWTP, which treats both urban and industrial wastewater.

Constructed wetlands medium, used as substrate for the growth of *P. australis*, consists of four layers of gravel and sand; proceeding from the top to the bottom they are (thickness of layers and diameter range of particles are reported in brackets, respectively): gravel (20 cm; 5–10 mm) – sand (60 cm; 0.1–0.4 mm and 0.02–0.1 mm) – gravel (10 cm; 5–10 mm) – gravel (10 cm; 40–70 mm). CW implant was designed with two parallel lines, named "Line A" and "Line B," respectively, with a total surface area of 1,620 m^2. Each line is a two-stage subsurface flow system (SFS), consisting of a vertical system (SFS-v) followed by a horizontal one (SFS-h). The SFS-v of Line A is subdivided into four parallel separated tanks (SFS-v1, SFS-v2, SFS-v3 e SFS-v4), while the SFS-v of Line B is composed by two tanks (SFS-v5 e SFS-v6). Furthermore, both SFS-h lines are composed by three tanks, each one receiving the same hydraulic load. The maximum hydraulic load supplied to the entire system was 95 m^3/day corresponding to a 1.9-day Hydraulic Retention Time for the horizontal stage (Coppini et al., 2019).

Sampling and Isolation of Bacteria

Samples from the roots of *P. australis* were collected using sterile plastic bags and immediately transported to the laboratory for the subsequent processing. All procedures described hereinafter were carried out under sterile conditions to avoid external contaminations. Samples of three different specimens of *P. australis* growing in three different tanks were grouped and pooled before starting any procedure. Two pools were obtained from both SFS-v and SFS-h for each sampling campaign. 1 g of fresh tissue from each pool was surface-sterilized with 1% v/v HClO solution at room temperature to remove epiphytic bacteria and then washed three times with sterile water. Aliquots of 100 μL of water from the last wash were plated in triplicate as sterility controls. Subsequently, samples were homogenously pottered in a sterile mortar with the addition of 2 mL of 0.9% w/v NaCl sterile solution. Serial dilutions of tissue extracts were plated in triplicate on trypticase soy agar (TSA) medium (Biolife) and incubated at 30°C for 48 h. The total number of aerobic heterotrophic fast-growing bacteria of each sample was expressed as colony forming units per gram of roots (CFU/g), and it was determined as an average of three replicates. Isolated bacteria

TABLE 4 | Number of bacterial isolates grouped for genus and sampling.

	Samplings						
	1st	2nd	3rd	4th	5th	Total	%
Achromobacter	–	1	3	4	4	12	4.08
Acinetobacter	–	4	–	–	–	4	1.36
Aeromonas	–	–	–	1	–	1	0.34
Agrobacterium	–	2	1	9	1	13	4.42
Arthrobacter	–	–	–	1	–	1	0.34
Bacillus	9	2	9	–	4	24	8.16
Buttiauxella	1	–	–	–	–	1	0.34
Chryseobacterium	–	–	–	–	3	3	1.02
Comamonas	–	1	–	–	–	1	0.34
Devosia	–	–	–	1	–	1	0.34
Enterobacter	–	–	2	–	–	2	0.68
Flavobacterium	1	–	–	2	2	5	1.70
Halomonas	–	1	6	–	–	7	2.38
Idiomarina	–	1	–	–	–	1	0.34
Isoptericola	–	–	1	–	–	1	0.34
Janthinobacterium	1	–	–	–	–	1	0.34
Lelliottia	2	–	–	–	–	2	0.68
Lysobacter	–	–	–	1	–	1	0.34
Microbacterium	–	2	1	1	–	4	1.36
Micrococcus	–	–	–	1	–	1	0.34
Ochrobactrum	–	2	–	–	1	3	1.02
Paenibacillus	–	–	–	–	5	5	1.70
Pannonibacter	–	1	2	3	–	6	2.04
Pantoea	7	–	–	–	2	9	3.06
Paracoccus	–	1	–	–	–	1	0.34
Pectobacterium	1	–	–	–	–	1	0.34
Planococcus	–	–	–	–	1	1	0.34
Pseudomonas	43	15	12	21	19	110	37.41
Pseudoxanthomonas	–	2	1	–	–	3	1.02
Rheinheimera	–	7	2	1	–	10	3.40
Rhizobium	–	–	2	13	1	16	5.44
Shinella	–	–	–	2	–	2	0.68
Sphingobium	–	–	–	1	–	1	0.34
Staphylococcus	1	2	–	–	–	3	1.02
Stenotrophomonas	1	–	8	1	10	20	6.80
Thalassospira	–	4	7	5	–	16	5.44
Vibrio	–	1	–	–	–	1	0.34
Total number of isolates	67	49	57	68	53	294	
Total number of genera	10	17	14	17	12	37	

–: absence of isolates.

were name-coded according to the portion of the CW from whom they were isolated (V for the SFS-v and H for the SFS-h, respectively) and the pool of origin collected during the five samplings (1–2, 3–4, 5–6, 7–8, and 9–10 for the first, second, third, fourth, and fifth sampling, respectively).

Amplification and Sequencing of 16S rRNA Gene

Polymerase chain reaction (PCR) were performed to amplify the 16S rRNA coding gene. 2 μL of colony thermal lysate were used as template for a PCR in 1X DreamTaq Buffer (Thermo Scientific) containing 200 μM of each dNTPs, 0.2 μM of primers P0 (5′-GAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′; Di Cello and Fani, 1996), 2 U of DreamTaq DNA Polymerase (Thermo Scientific) in a final volume of 25 μl. The PCR cycling for 16S rRNA gene amplification was 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, then a final extension at 72°C for 10 min. A Bio-Rad T100 thermal cycler was used. Sequencing of 16S rRNA gene was performed by IGA Technology Services Srl (Udine, Italy).

Taxonomic and Phylogenetic Analyses

Taxonomic affiliation of isolates was determined through the alignment of sequences to those of type strains downloaded from the ribosomal database project (RDP; Cole et al., 2014) using BioEdit (Hall, 1999). The obtained alignment was then used to build a phylogenetic tree through MEGA7 (Kumar et al., 2016), applying the Neighbor-Joining algorithm with a 1000-bootstrap resampling.

Antibiotic Resistance Assay

Isolates were tested for their resistance against eight antibiotics (i.e., rifampicin, ampicillin, kanamycin, tetracycline, chloramphenicol, streptomycin, trimethoprim and ciprofloxacin) at six different concentrations (i.e., 1 – 10 – 25 – 50 – 100 – 150 μg/mL; **Table 1**). Bacteria were firstly grown overnight on TSA (Biolife) at 30°C, then a single colony was resuspended in 100 μL of 0.9 w/v NaCl sterile solution. The obtained suspensions were streaked on Mueller–Hinton II Agar (Biolife) plates supplemented with the tested antibiotics. Bacteria were also cultivated on the same medium in the absence of antibiotics, using these cultures as control to evaluate the presence of growth inhibition in presence of antibiotics. All plates were incubated at 30°C and growth performances were evaluated after 48 h. The minimal inhibitory concentration (MIC) value for each antibiotic was considered as the lowest concentration of the compound that inhibited visible growth of the tested isolate.

Growth in Presence of Synthetic Wastewater

Growth of strains isolated from roots of *P. australis* in presence of SWW was assayed through the broth microdilution methods (Wiegand et al., 2008) using trypticase soy broth (TSB) medium (Biolife). The bacterial inoculum for the experiment was prepared by dissolving an isolated bacterial colony in 10 ml of TSB medium after 24 h-growth at 30°C on TSA. The inoculum was incubated overnight at 30°C under shaking. Upon incubation, absorbance at 600 nm was measured and adjusted to 0.1. The experiment was performed using 96-well plates. Each well contained 10 μL of bacterial inoculum, 80 μL of TSB medium and 10 μL of 10X, 20X, and 30X SWW, to reach the final concentration of 1X, 2X, and 3X, respectively. The composition of SWWs used for this assay is shown in **Table 2**. Growth performances in presence of SWW were evaluated calculating the ratio between the OD_{600} of cultures in presence of SWW (herein after indicated

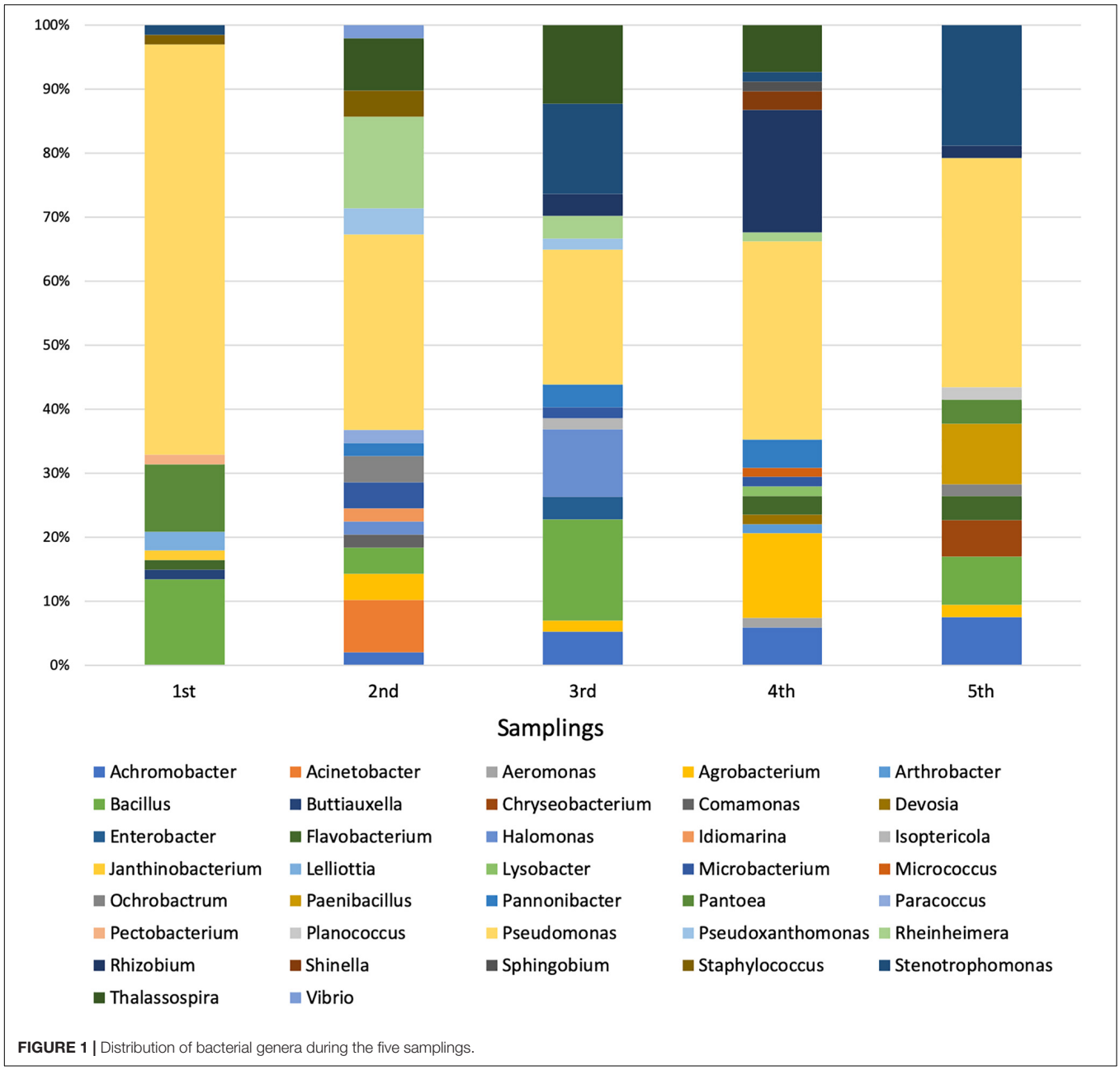

FIGURE 1 | Distribution of bacterial genera during the five samplings.

as OD_{600SWW}) and OD_{600} of controls (i.e., bacteria grown in TSB lacking SWW). Bacterial isolates were considered sensitive to SWW when this parameter assumed values <0.7, while they were evaluated as resistant when it was >1.3.

RESULTS

Bacterial Counts

P. australis plants were sampled from the CW in Calice during a period of 22 months, spanning from March 2017 to December 2018; 5 samplings were conducted, with the first one (i.e., March 2017) performed before the activation of the CW (**Table 3**). This experimental strategy allowed us to compare the composition of the cultivable bacterial community associated to the *P. australis* roots before and after the beginning of wastewater influx. The titer of viable bacteria associated to roots was determined as described in section "Materials and Methods". Data obtained revealed that there were no great differences between the CFU counts in SFS-v and SFS-h, exception for the third and fourth samplings in which CFU values were higher in SFS-h (**Table 3**).

In general, bacterial load was quite constant during the experiment and fluctuations might be related to different factors, such as wastewater composition, frequency of raining, and/or seasonal variations. Indeed, it is likely that the weather exerted a main effect on bacterial growth since the highest bacterial loads

TABLE 5 | Frequencies of minimal inhibitory concentration (MIC) values among bacterial isolates.

	MIC (μg/mL)	Samplings				
		1	**2**	**3**	**4**	**5**
Rifampicin	1	12	18	26	9	8
	10	26	28	9	21	20
	25	23	–	9	22	13
	50	–	–	11	1	5
	100	–	–	–	3	4
	150	–	–	–	–	2
	>150	–	–	–	–	–
Ampicillin	1	3	7	18	5	2
	10	1	8	1	1	3
	25	4	3	4	–	2
	50	2	7	6	5	5
	100	10	6	1	9	5
	150	1	4	2	3	5
	>150	39	8	23	33	30
Kanamycin	1	1	–	–	2	3
	10	50	25	31	16	27
	25	2	7	5	7	4
	50	3	3	2	14	1
	100	1	1	1	5	–
	150	1	2	1	–	–
	>150	1	6	15	12	17
Tetracycline	1	30	43	37	33	12
	10	28	4	8	19	31
	25	–	–	8	3	1
	50	–	–	1	–	–
	100	–	–	–	–	8
	150	–	–	–	–	–
	>150	–	–	–	1	–
Chloramphenicol	1	20	14	9	15	4
	10	8	10	17	5	9
	25	4	7	14	13	14
	50	4	7	8	6	14
	100	16	6	–	–	6
	150	2	2	2	–	1
	>150	7	–	4	17	4
Streptomycin	1	3	12	2	–	2
	10	32	13	24	16	10
	25	10	9	9	4	12
	50	10	3	1	5	–
	100	4	–	4	2	8
	150	1	–	–	9	–
	>150	3	7	14	20	20
Trimethoprim	1	12	15	8	–	5
	10	–	5	9	7	7
	25	1	3	2	2	7
	50	1	6	3	5	2
	100	2	7	15	4	5
	150	7	5	5	3	6
	>150	42	5	12	35	20
Ciprofloxacin	1	65	43	40	48	39
	10	–	3	14	8	5
	25	–	–	–	–	8
	50	–	–	–	–	–
	100	–	–	–	–	–
	150	–	–	–	–	–
	>150	–	–	–	–	–

–: absence of isolates showing a specific MIC value.

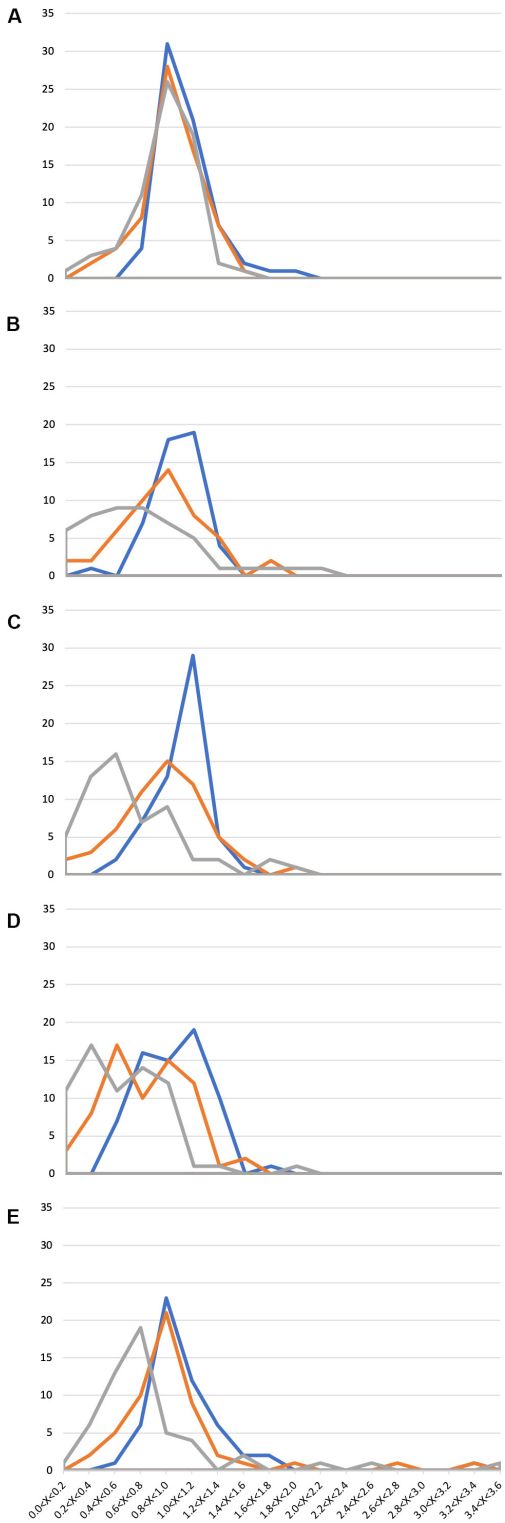

FIGURE 2 | Growth of 294 *Phragmites australis* root-associated bacteria in the presence of synthetic wastewater (SWW). Ranges of OD_{600SWW}/OD_{600} values are reported in the *x* axis, while the number of isolates is reported in the *y* axis. **(A)** 1st sampling; **(B)** 2nd sampling; **(C)** 3rd sampling; **(D)** 4th sampling; and **(E)** 5th sampling. Blue: 1X SWW; Orange: 2X SWW; Gray: 3X SWW.

were observed during summer (i.e., second and fourth samplings, respectively), when the higher temperatures probably facilitated bacterial growth and the poor precipitations probably caused a higher concentration of wastewater.

Taxonomic Affiliation of Cultivable Bacteria

A total of 294 isolates (67, 49, 57, 68, and 53 from the first, second, third, fourth, and fifth sampling, respectively) were isolated from the *P. australis* roots. The attention was focused on bacteria isolated from this plant compartment because it has been reported that it is primarily involved in the depuration process (Riva et al., 2020). Each of the 294 isolates underwent a taxonomic characterization; to this purpose the amplification, sequencing, and analysis of 16S rRNA coding gene(s) were performed as described in section "Materials and Methods."

Each sequence was submitted to Genbank and was assigned the accession number reported in **Supplementary Table S1**. The comparative analysis of each sequence with those available in databases allowed to split the 294 isolates into 37 different genera (**Supplementary Figures S1–S30**). The analysis revealed that 254 isolates were Gram-negative while 40 were Gram-positive bacteria. Moreover, a total of four different phyla were represented, with 246 belonging to *Proteobacteria* (59 *Alphaproteobacteria*, 14 *Betaproteobacteria,* and 173 *Gammaproteobacteria*), 8 to *Bacteroidetes* (all belonging to *Flavobacteriia* class), 33 to *Firmicutes* (all belonging to *Bacilli* class), and 7 to *Actinobacteria* (all belonging to *Actinobacteria* class). The most represented genus was *Pseudomonas*, whose members accounted for 37% of all isolates, as shown in **Table 4**. The abundance of *Pseudomonas* was not directly related to the activation of the CW, because it was the most represented genus even before the influx of wastewater (**Figure 1**).

Among the 37 genera, only 4 were exclusively present during the first sampling (i.e., *Buttiauxella*, *Janthinobacterium*, *Lelliottia,* and *Pectobacterium*), while 27 started being present from the second one on (i.e., *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Agrobacterium*, *Arthrobacter*, *Chryseobacterium*, *Comamonas*, *Devosia*, *Enterobacter*, *Halomonas*, *Idiomarina*, *Isoptericola*, *Lysobacter*, *Microbacterium*, *Micrococcus*, *Ochrobactrum*, *Paenibacillus*, *Pannonibacter*, *Paracoccus*, *Planococcus*, *Pseudoxanthomonas*, *Rheinheimera*, *Rhizobium*, *Shinella*, *Sphingobium*, *Thalassospira,* and *Vibrio*). Hence, it is possible that the bacteria belonging to these 27 genera might derive from the wastewater, although it cannot be established whether they were present in wastewater with either urban or industrial origin. In addition to this, we cannot *a priori* exclude the possibility that they were already present in the pre-existing community (even though in low percentage) and that the presence of the wastewater might have exerted a selective pressure favoring their reproduction. In most cases, the phylogenetic trees showed a narrow taxonomic distribution of isolates, which clustered together in the same branch. For example, all *Acinetobacter* strains were phylogenetically close to *A. haemolyticus* (**Supplementary Figure S1**), all *Achromobacter* isolates formed a distinct cluster and were close to *A. spanius* (**Supplementary Figure S5**), all *Chryseobacterium* were related to *C. indoltheticum* (**Supplementary Figure S7**), all *Paenibacillus* belonged to the same cluster and were close to *P. tundrae* (**Supplementary Figure S19**), all *Pannonibacter* were affiliated to *P. phragmitetus* (**Supplementary Figure S24**), and all *Thalassospira* isolates formed a separate branch in the phylogenetic tree (**Supplementary Figure S29**). Moreover, *Rheinhemera* isolates distributed in close branches which included *R. aquimaris*, *R. pacifica*, and *R. nanhaiensis* (**Supplementary Figure S22**). On the contrary, a higher phylogenetic diversity was observed in the case of *Bacillus* (**Supplementary Figure S4**), *Enterobacteriales* (**Supplementary Figure S10**), *Pseudomonas* (**Supplementary Figure S20**), *Rhizobiales* (**Supplementary Figure S23**), and *Stenotrophomonas* (**Supplementary Figure S28**). However, with the exception of the bacteria belonging to the *Enterobacteriales* order, in the case of these genera the formation of distinct clusters was observed.

Resistance Against Antibiotics

All 294 isolates were tested for their resistance against a panel of eight antibiotics used to treat human infections as described in section "Materials and Methods." These compounds were chosen because they are representatives of diverse antibiotic classes and they are directed toward different cellular targets (**Table 1**). Data obtained are shown in **Table 5** and **Supplementary Table S2** and revealed that, overall, the most effective antibiotics were rifampicin, tetracycline and, above all, ciprofloxacin. On the contrary, the more tolerated antibiotic was ampicillin, especially in the case of *Pseudomonas* and *Stenotrophomonas* (**Supplementary Table S2**). Although resistant bacteria were isolated since the first sampling, a correlation between the time of exposure to the wastewater (i.e., earlier vs later samplings) and the increase of MIC values was observed for almost all antibiotics (**Table 5** and **Supplementary Table S2**).

On one hand, tests with rifampicin, ciprofloxacin and tetracycline showed a progressive increase in the number of isolates having the highest MIC values going from the first to the fifth sampling. On the other one, although MIC values were not determined in the assayed conditions because isolates were able to grow even in presence of the highest concentration of antibiotic, in the case of kanamycin and streptomycin the number of isolates with MIC > 150 μg/mL increased during time. Finally, clear trends were not detected using ampicillin, chloramphenicol and trimethoprim: indeed, there were bacteria able to survive in the presence of the highest concentration since the first sampling and, also, the frequency of resistant isolates was not subjected to temporal variations.

According to the MIC breakpoints provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST – Breakpoint tables for interpretation of MICs and zone diameters; Version 10.0, 2020[1]), relatively to the antibiotics assayed in this work and limiting to the species reported by EUCAST, six isolates could be defined as multi-drug resistant strains, since they were resistant against at least three different antibiotics (**Supplementary Table S2**). In detail, two *Lelliottia* (V2R14 and

[1]http://www.eucast.org

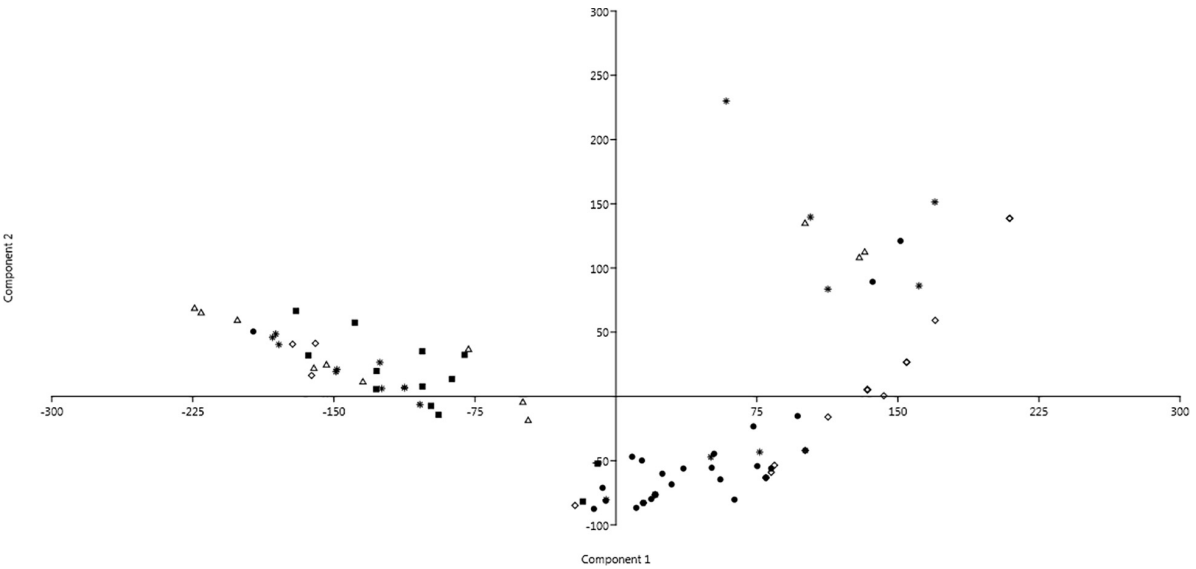

FIGURE 3 | Principal component analysis (PCA) showing profiles of resistance against antibiotics and SWW of isolates belonging to the genus *Pseudomonas*. Dot: 1st sampling; Filled square: 2nd sampling; Triangle: 3rd sampling; Diamond: 4th sampling; and Star: 5th sampling.

H1R21) and two *Enterobacter* (H5R6 and H5R7) isolates were resistant to ampicillin, chloramphenicol, and ciprofloxacin; lastly, the two *Pantoea* isolates H9R2 and H9R15 that were resistant to ampicillin, chloramphenicol, trimethoprim, and ciprofloxacin.

Growth in the Presence of SWW

The 294 isolated *P. australis* root-associated endophytes were also tested for their ability to grow in the presence of SWW containing B, Fe, and Se since these elements are critical for the WWTP studied in this work. Selection of bacteria able to grow efficiently in the presence of these compounds is of relevant interest because CW might be enriched with these more tolerant microorganisms, which, in turn, might increase the pollutant removal efficiency in wastewater. All bacterial isolates were assayed for their growth in TSB medium supplemented with three different concentration of SWW: 1X (i.e., a mix of H_3BO_3, $FeCl_2 \cdot 4H_2O$, Na_2SeO_3, and NaCl at the maximum concentrations allowed by law for sewer emission), and 2X and 3X in which TSB medium was supplemented with two- and threefold higher concentrations of 1X SWW, respectively.

In general, the presence of 1X SWW did not alter the growth of isolates, indicating that these endophytes can tolerate the presence of the tested compounds (**Figure 2** and **Supplementary Table S3**). The analysis of data shown in **Supplementary Table S3** and **Figure 2** also revealed that 211 bacterial isolates were able to grow efficiently also in the presence of either 2X SWW or 3X SWW. Interestingly, eight isolates (i.e., V2R8, H3R17, H4R18, V6R1, V5R1, V8R24, H9R1, and H10R8), belonging to the genera *Bacillus*, *Planococcus*, *Pseudomonas*, and *Rheinheimera*, showed a positive correlation between growth and concentration of SWW: indeed, the higher the SWW concentration, the higher the growth of these bacteria. This finding suggests that these bacteria could represent good candidates for future applications and for improvements of phytodepuration efficiency and pollutant removal. Moreover, the isolate H9R16 deserves further investigations, since it showed the highest growth increase (about 350%) in presence of SWW. The analysis of the 16S rDNA phylogenetic tree revealed that it joined bacteria belonging to the *Bacillus gibsonii* species (**Supplementary Figure S4**), alkaliphilic bacteria exploited for production of alkaline proteases (Martinez et al., 2013; Deng et al., 2014).

Similarly to the case of antibiotic resistance, also in this assay a correlation between the time of exposure to wastewater in the CW and the appearance of more resistant isolates was highlighted. Indeed, a progressive increase of the number of isolates showing an augmented growth (measured as OD_{600SWW}/OD_{600} ratio as described in section "Materials and Methods") was observed from the first to the last sampling. For instance, several isolates with an $OD_{600SWW}/OD_{600} > 2.4$ (**Figure 2E**) were detected only during the last sampling.

Changings of resistance profiles along time, considering those against either antibiotics or SWW, were particularly clear in the case of isolates belonging to the genus *Pseudomonas*. As shown in **Figure 3** by the mean of principal component analysis (PCA) performed with the PAST4 software (Hammer et al., 2001), the formation of two different main clusters was observed with the main part of isolates from the first sampling clustering independently from those isolated from all the other four samplings.

DISCUSSION

The aim of this work was the analysis of the composition, the phenotypic characterization, and the temporal dynamics of the cultivable microbiota associated to the roots of *P. australis*

grown in the CW of Calice (Prato, Italy) before and after the activation of the CW.

The composition of root cultivable microbiota was determined through five samplings spanning from March 2017 to December 2018. We focused on cultivable bacterial communities because their isolation and characterization might permit the identification of strains particularly resistant to the antibiotics and/or to the compounds present in the wastewater. Hence, these strains could be used to construct a "synthetic" consortium that, in turn, might be exploited in pilot-experiments with the goal of increasing the phytodepuration efficiency of the plant. The taxonomic analysis was performed on 294 cultivable bacteria through the analysis of the 16S rRNA genes. Even though we are completely aware that the number of isolates could not be representative of the entire community, the analysis performed gave useful hints on the effect of the activation of CW on bacterial community composition. Data obtained revealed that the wastewater income exerted a shaping effect on the bacterial composition. Overall, 37 bacterial genera were disclosed and six of them were detected both before and after the activation of CW. Moreover, bacteria belonging to 27 different genera were detected only after the activation of the CW, while, on the contrary, 4 genera that were present at the beginning were not found in the following samplings. The most represented genus in all five samplings was *Pseudomonas*, which accounted for the 37% of all isolates.

As it might be expected, the effect of the wastewater income was exerted not only at the taxonomic level, but also at the phenotypic one. Each of the 294 bacterial isolates was assayed for its resistance against a panel of eight antibiotics belonging to different chemical classes and acting toward different cellular targets. This analysis was performed since it is known that WWTP are reservoirs of antibiotic resistance genes and/or resistant bacteria. Data obtained revealed the presence of several resistant (or multi-resistant) strains and, mostly important, that the number of antibiotic resistant isolates and the degree of antibiotic resistance increased over time, from the first to the last sampling. This strongly suggests that the wastewater income might generate a selective pressure favoring the growth of those isolates intrinsically resistant to antibiotics, even though it cannot be *a priori* excluded the possibility of horizontal gene transfer (HGT) events and/or the acquisition of resistance through mutations in chromosomal genes.

All the 294 isolates were assayed also for their ability to grow in SWW containing three different concentrations of B, Se, Fe, and NaCl. Analogously to the antibiotic resistance pattern, we detected a similar correlation between SWW exposure and the ability to grow in its presence. Interestingly, among isolates able to grow in the presence of these compounds, nine of them showed an increasing growth at the highest concentrations of SWW: these isolates will deserve a specific focus to identify the molecular mechanisms behind this intriguing behavior. Moreover, a PCA carried out on *Pseudomonas* strains, isolated from all the five samplings, furtherly suggested that the main event changing the resistance patterns against antibiotics and SWW was the activation of the plant (i.e., when conveyance of the permeate into the tanks occurred). The characterization of heavy metal resistant strains may be crucial to better understand the diffusion of antibiotic resistance genes in the environment. As a matter of fact, it has been reported that the occurrence of multiple heavy metal resistance markers is associated with the onset of antibiotic resistance (Wales and Davies, 2015; Wu et al., 2018; Zhu et al., 2019). This might be due to the co-localization of resistance genes against antibiotics and heavy metals in the same mobile genetic element(s) and, as a consequence, the accumulation of heavy metals in the environment can cause the selection of antibiotic resistant species. So, the dissemination of these heavy metal resistance genes represents an issue that should not be underestimated. Moreover, monitoring the presence of bacteria resistant to antibiotics and/or heavy metals specifically in WWTPs should be considered as a priority to contrast the spreading of multi-drug resistant (MDR) pathogens. Indeed, WWTPs represent hotspots for HGT events, because of the mixing of bacteria from diverse sources (e.g., households, hospitals, industries, etc.), the high bacterial densities, stressful conditions triggering SOS responses and presence of antibiotics at sublethal concentrations (Karkman et al., 2018). It must be also considered that although HGT occurring in WWTPs might not directly regard human pathogens, these could acquire resistance markers from harmless bacteria which act as vectors as soon as effluent is released in the environment (Manaia, 2017).

To deeply characterize this phenomenon, future work could take advantage of emulsion, paired isolation and concatenation PCR (epicPCR) as previously reported (Spencer et al., 2016; Hultman et al., 2018). Indeed, this kind of analysis could allow the "tagging" of resistance genes with phylogenetic markers, such as 16S rRNA gene, helping to compare these pairs in wastewater entering the WWTP and in effluents. However, this would be limited to target resistance markers with known sequence and for whom it is thereof possible to design specific primers.

CONCLUSION

The experimental approach used in this work revealed that the cultivable bacterial community existing prior to the plant activation underwent fluctuations in terms of both taxonomy and resistance to antibiotics and SWW compounds. As it might be expected, the influx of wastewater exerted a selective pressure on the resident bacterial community, selecting and/or bringing bacterial strains progressively more resistant to SWW and/or antibiotics. We are completely aware that the analysis of the entire community (both cultivable and uncultivable) might give more detailed insights into the composition of the total community. In spite of this, only the selection of particular cultivable strains, i.e., more resistant to SWW and antibiotics, can permit the formulation of a synthetic bacterial community to improve the phytodepuration properties of *P. australis*.

DATA AVAILABILITY STATEMENT

The datasets generated in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, MK110895, MK110946, MK110896, MK110920, MK110921, MK110947, MK110948, MK110922, MK110897, MK110949, MK110898, MK110950, MK110899, MK110959, MK110923, MK110925, MK110924, MK110960, MK110926, MK110945, MK110957, MK110927, MK110928, MK110929, MK110930, MK110931, MK110932, MK110900, MK110901, MK110902, MK110933, MK110934, MK110935, MK110958, MK110936, MK110937, MK110938, MK110939, MK110940, MK110941, MK110942, MK110943, MK110903, MK110904, MK110905, MK110906, MK110907, MK110908, MK110909, MK110910, MK110911, MK110912, MK110951, MK110913, MK110914, MK110915, MK110952, MK110916, MK110953, MK110917, MK110954, MK110918, MK110944, MK110961, MK110919, MK110955, MK110956, MK134509, MK134489, MK134488, MK134487, MK134486, MK134554, MK134508, MK134547, MK134496, MK138850, MK134502, MK134511, MK134551, MK134555, MK134559, MK134558, MK134549, MK138851, MK134553, MK134542, MK134557, MK134546, MK134544, MK134543, MK134541, MK134540, MK134510, MK134497, MK134490, MK134495, MK134494, MK134493, MK134499, MK134505, MK134552, MK134556, MK134545, MK134548, MK134500, MT165525, MK134507, MK134504, MK134503, MK134550, MK134501, MK134498, MK134506, MK134492, MK134491, MK134518, MK130934, MK130935, MK134524, MK134539, MK134538, MK134515, MK138852, MK134526, MK130907, MK130906, MK130937, MK134534, MK134533, MK138853, MK130915, MK130913, MK130910, MK130921, MK130917, MK134514, MK130914, MK134516, MK130911, MK134532, MK134531, MK134530, MK134528, MK134485, MK138854, MK130912, MK130908, MK130920, MK130932, MK130933, MK130936, MK134513, MK130922, MK130919, MK134521, MK134512, MK134537, MK134536, MK134535, MK134522, MK134519, MK134517, MK134529, MK134525, MK134523, MK134520, MK134527, MK130916, MK130931, MK130909, MK130923, MK130918, MK130945, MK130957, MK130901, MK130905, MK133358, MK138868, MK138869, MK138870, MK138872, MK138874, MK138875, MK130924, MK130928, MK138881, MK138862, MK130903, MK138867, MK138878, MK138861, MK130939, MK138863, MK130949, MK130953, MK130904, MK138876, MK138879, MK130926, MK130927, MK130929, MK138880, MK130930, MK138882, MK138883, MK138884, MK138885, MK138886, MK138887, MK130940, MK130941, MK130943, MK138889, MK130944, MK130902, MK130900, MK130946, MK138877, MK138855, MK138856, MK138857, MK138858, MK138859, MK138860, MK138864, MK138865, MK138866, MK138871, MK138873, MK130925, MK130942, MK130948, MK130950, MK130955, MK138888, MK130951, MK130952, MK130954, MK130956, MK130947, MT165526, MT165527, MT165547, MT165551, MT165553, MT165552, MT165528, MT165529, MT165530, MT165531, MT165532, MT165533, MT165534, MT165557, MT165554, MT165535, MT165536, MT165555, MT165558, MT165559, MT165561, MT165548, MT165562, MT165560, MT165549, MT165563, MT165564, MT165537, MT165538, MT165565, MT165539, MT165540, MT165566, MT165570, MT165569, MT165567, MT165571, MT165568, MT165578, MT165550, MT165541, MT165542, MT165543, MT165572, MT165544, MT165545, MT165573, MT165546, MT165574, MT165556, MT165575, MT165576, and MT165577.

AUTHOR CONTRIBUTIONS

RF, EC, and DF conceived the project. AV, EM, CF, and RF designed the experiments. AV, EM, CF, SV, SD, LC, and SC performed the experiments. RF supervised the experiments. AV, EM, and RF analyzed the results. AV wrote the original draft of the manuscript. AV, SD, LC, SC, CF, EM, EC, and RF reviewed and edited the manuscript. All authors read and approved the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01652/full#supplementary-material

FIGURE S1–S30 | Phylogenetic trees.

TABLE S1 | List of bacteria isolated from roots of *Phragmites australis* and used in this work. Accession numbers of 16S rRNA gene partial sequences are reported.

TABLE S2 | MIC (μg/ml) values of bacterial isolates described in this work.

TABLE S3 | Growth of 294 bacterial isolates associated to *Phragmites australis* roots in TSB in presence of three different concentrations of synthetic wastewater (SWW).

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Exploring the Animal Waste Resistome: The Spread of Antimicrobial Resistance Genes Through the Use of Livestock Manure

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Antibiotic resistance is a public health problem of growing concern. Animal manure application to soil is considered to be a main cause of the propagation and dissemination of antibiotic residues, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in the soil-water system. In recent decades, studies on the impact of antibiotic-contaminated manure on soil microbiomes have increased exponentially, in particular for taxonomical diversity and ARGs' diffusion. Antibiotic resistance genes are often located on mobile genetic elements (MGEs). Horizontal transfer of MGEs toward a broad range of bacteria (pathogens and human commensals included) has been identified as the main cause for their persistence and dissemination. Chemical and bio-sanitizing treatments reduce the antibiotic load and ARB. Nevertheless, effects of these treatments on the persistence of resistance genes must be carefully considered. This review analyzed the most recent research on antibiotic and ARG environmental dissemination conveyed by livestock waste. Strategies to control ARG dissemination and antibiotic persistence were reviewed with the aim to identify methods for monitoring DNA transferability and environmental conditions promoting such diffusion.

Keywords: veterinary antibiotics, animal manure, antibiotic resistance genes, crop soils, antimicrobial resistance

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INTRODUCTION

In recent decades, the overuse and misuse of antibiotics in human and veterinary medicine has become a serious public health issue (World Health Organization, 2014; Aidara-Kane et al., 2018). The increased number of resistant pathogens and commensal bacteria has been associated with the environmental spread of antibiotics and the propagation of antimicrobial resistant genes (ARGs; Levy, 1998; Witte, 1998; He et al., 2020). Furthermore, the environmental diffusion of antibiotics may lead to the change (Han et al., 2018) and loss (Chen et al., 2019) of microbial community diversity in soil (Kemper, 2008).

Antibiotics are used worldwide in livestock production, thus increasing the risk of antimicrobial resistance (AMR) spread. When administered for prophylactic treatments, antibiotics can directly increase selective pressure, thus favoring the generation of antibiotic-resistant bacteria (ARB;

Pruden et al., 2013; Troiano et al., 2018; Blau et al., 2019). For these reasons, improved livestock and waste management strategies (i.e., diets, proximity between animals, waste treatment, use of additives, and operating conditions) should be adopted to limit the use of antibiotics in animal husbandry.

Antimicrobial resistant genes can enter and persist in ecosystem through multiple pathways. They spread across soil (Binh et al., 2007), crops (Su et al., 2015), and gut microbial communities of wild and livestock animals and of humans (Yadav and Kapley, 2019). Antimicrobial resistant genes' spread occurs through horizontal gene transfer (HGT) of mobile genetic elements (MGEs), as phages, plasmids (Fondi and Fani, 2010), transposons, or integron gene cassettes (**Figure 1**). The acquisition of AMR by bacteria may be due to spontaneous mutations (Woodford and Ellington, 2007) or, more frequently, by gaining specific ARGs from other bacteria through HGT. High density of microbial cells in the presence of antimicrobial compounds and nutrients, as observable in manure (Blau et al., 2018), triggers HGT events among bacteria, thus conferring selective advantage to the hosts (Thomas and Nielsen, 2005). Mutations are essential for the continuous evolution of ARGs, producing hundreds of variants which are hardly identifiable and increasingly dangerous for the environment (Woodford and Ellington, 2007).

In this review, the effect of antibiotic occurrence in animal manure on the dissemination of AMR and ARGs in agricultural fields are discussed in a critical way. The main strategies to mitigate ARGs' dissemination and to control antibiotic persistence are also reported. Methods monitoring changes in microbial communities and transferability and environmental diffusion of DNA were addressed as well.

THE DISSEMINATION MECHANISMS OF ENVIRONMENTAL RESISTOME

The "resistome," i.e., the total amount of resistance genes associated with an ecosystem (Finley et al., 2013), is generally mediated by conjugative plasmids. The resistome confers resistance of antibiotics and heavy metals to microorganisms, thus enhancing their survival in hostile environments (Bennett, 2008; Song et al., 2017). IncP-1, a common environmental plasmid group, is largely known for its efficient conjugative transferability potential and stable replication in a wide range of Gram-negative bacteria (Heuer et al., 2012). Conversely, plasmids IncF (Villa et al., 2010), IncI (Blau et al., 2018), and IncQ (Rawlings and Tietze, 2001) show a narrower host range. These plasmids are assumed to be important for the dissemination of ARG in *Escherichia coli* and other Enterobacteriaceae (Johnson and Nolan, 2009; Suzuki et al., 2010; Heuer et al., 2012; Van Houdt et al., 2013). As evidence, the study of the mechanisms of diffusion of these plasmids (Teuber, 2001) and compatibility evolution with broad or narrow host ranges should allow for ARG diffusion prediction.

Integrons play a key role in the fast spread of resistance determinants toward antibiotics. They are genetic elements composed of a gene encoding an integrase and an integration site for exogenous gene cassettes, which can be acquired and converted in functional and expressed genes (Mazel, 2006). Integrons can move horizontally in bacterial populations by frequent integration in plasmids or in transposons (Rowe-Magnus and Mazel, 2002). According to their aminoacidic sequence, integrases are divided into several classes. Classes 1, 2, and 3 (Inti1, Inti2, Inti3) were the first to be identified as associated with MGEs, while class 4 (Inti4) was associated with chromosomal integration (Deng et al., 2015). Among elements which facilitate DNA transfer, class 1 integron (*int1*) is the most frequently identified as responsible for spreading antibiotic resistance determinants amongst commensals and pathogens of humans and domesticated animals. Moreover, *int1* cassette was found in different environments, such as fresh water, sediments, and sludge (Collis and Hall, 1995; Hall and Collis, 1998; Nardelli et al., 2012; Borruso et al., 2016), where it showed significantly positive correlations with the relative ARG abundance (Zhao et al., 2019).

Antibiotic residues, once entered into soil through manure application, can enhance persistence and HGT of ARGs (Binh et al., 2007; Zhao et al., 2019) through plasmids and integrons (Gotz and Smalla, 1997; Smalla et al., 2000; Sengeløv et al., 2003), promoting the spread of ARB in the environment and affecting the microbial community composition (Chen et al., 2019). Although manure-derived bacteria cannot always adapt to new environments, the antimicrobials can favor the enrichment of specific bacterial taxa in soils (through positive selection) and suppress others (Ding et al., 2014). In addition, the concentration of antibiotics in manure, usually at a sub-inhibitory level, can affect the interactions among strains and impact on gene expression and regulation (Gillings, 2013; Jechalke et al., 2014; Brüssow, 2015).

When manure is used as a fertilizer for crop production, both the increased ARB load and the antibiotic residues contained within may have negative effects on plant development and food product quality (Verraes et al., 2013; Mirza et al., 2020; Muhammad et al., 2020). In addition, antibiotic residues can persist and accumulate in the environment (Jechalke et al., 2014) by adsorption on soil solid phases (Du and Liu, 2012).

ARGs IN THE ENVIRONMENT

The majority of antibiotics are naturally produced by microbes as a self-protection mechanism against other microorganisms. ARGs have been always present in the environment. ARGs encoding resistance for a large set of antibiotics have been found in 30,000-year-old Beringian permafrost and in bacteria isolated from prehistoric caves (D'Costa et al., 2011; Berglund, 2015). When present in the environment at a sub-inhibitory concentration, antibiotics frequently play a role in transcription regulation and in the exchange of signals among cells (i.e., quorum sensing mechanism and conjugation) (Reygaert, 2018).

Antibiotic resistance consists of a large variety of mechanisms, such as inactivation by specific cleaving enzymes, exclusion from cells via efflux pumps, interference with protein synthesis, limitation of drug uptake, and modification of antibiotic target.

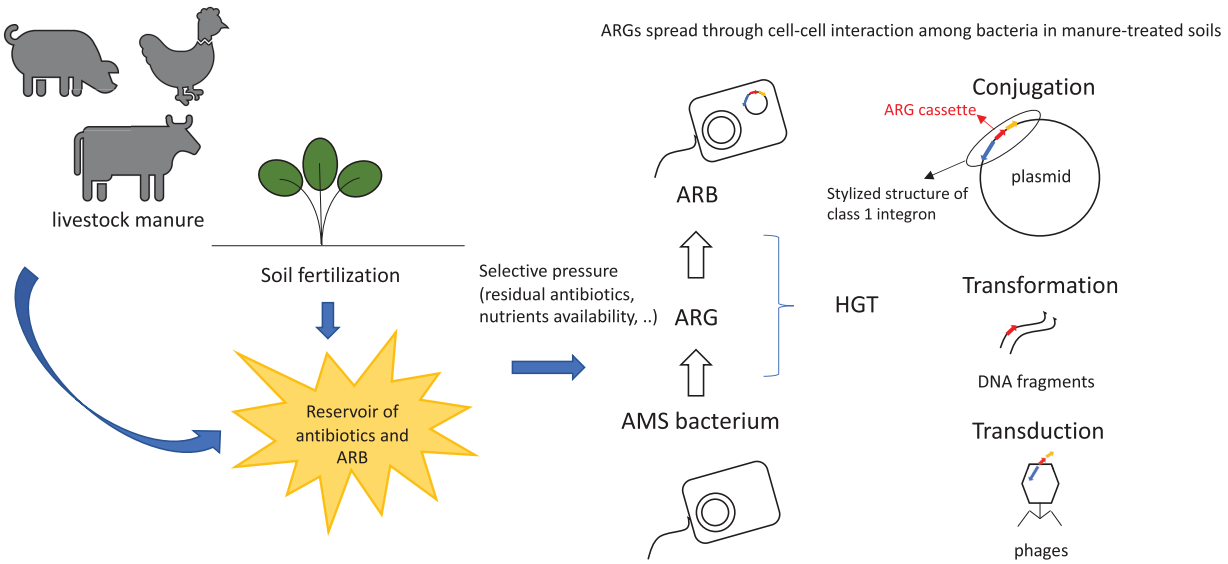

FIGURE 1 | Spread of ARGs and ARB in farm-related environments. ARB, antimicrobial resistant bacteria; ARG, antibiotic resistance gene; AMS, antimicrobial sensitive; HGT, horizontal gene transfer.

Resistance acquired through MGEs and plasmids is responsible for the last two mechanisms in which the resistance extent depends on bacterial species and acquired ARGs (Reygaert, 2018; Kraemer et al., 2019). The antibiotic selective pressure driving the acquired resistance determines accurate ARGs' specialization, thus making the environment a potential reservoir.

Anthropogenic activities affect antibiotic and ARGs' spread with somewhat predictable effects (Vikesland et al., 2017). In livestock farming, the use of antibiotics varies depending on the farming type and location, having a considerable effect on ARGs' concentration. Among the ARGs most frequently detected in livestock production, those related to sulfonamide resistance (*sul*) (**Table 1**) are particularly diffused in aquatic systems (Chen et al., 2015; Makowska et al., 2016). In surface and fresh waters, *sul* genes were found in IncQ plasmid group (Sköld, 2001; Berglund, 2015). Similarly, diaminopyrimidine genes (*dfr*), which confer resistance to antimicrobial trimethoprim, have been identified in both class 1 and class 2 integrons (Deng et al., 2015). Similarly, quinolone resistance *qnr* genes have been frequently associated with different plasmid groups. Both *dfr* and *qnr* genes easily disseminate in the environment, being found in surface waters (Berglund, 2015), wastewaters, and related irrigated soils (Dalkmann et al., 2012). Tetracycline resistance genes (*tet*) are widely diffused in different pathogenic and environmental bacteria (Roberts, 2005) and are often detected in sewage treatment plants, soil, and surface and ground water (Chee-Sanford et al., 2001; Berglund, 2015). In the same environments, *erm* genes, which are the most widespread macrolides resistance gene, were isolated.

Essentially, ARGs' diffusion is associated with a stress response activated by exposure to antibiotics as well as with the mobilization of several integrative and conjugative elements. ARGs' maintenance depends on their considerably low fitness cost. In fact, once a specific ARG has been acquired by a bacterial cell, it must evolve to produce more benefits than costs in order for multiple copies of the same gene to be kept and to maintain the expression control of genes in MGEs (Bengtsson-Palme et al., 2017). Furthermore, as already mentioned, nutrient rich environments can positively influence the ARGs' spread and facilitate cell–cell interactions (Manaia et al., 2018) (**Figure 1**).

THE USE OF VETERINARY ANTIBIOTICS

In veterinary medicine, antimicrobials can be used as therapeutics and/or growth promoters. Antibiotic growth promoters (AGPs) are antimicrobial substances administered at a sub-therapeutic dose for a prolonged time with the main purpose being to improve the feed conversion rate, especially in young animals, raising the economical profit of farmers. Since 2006, both the European Union and Australia have forbidden the use of AGPs. Nevertheless, in most other countries the use of AGPs is still permitted (Guardabassi et al., 2009).

Among breeding farms, poultry and pig livestock have received the majority of antibiotics for therapeutic or prophylactic use (Ungemach, 2000; Kim et al., 2011), resulting in an abundance of ARGs greater than three orders of magnitude compared to other farming systems, such as fish and cattle farming. Several studies confirmed swine farms as a hot-spot for ARB and ARGs (Rosen, 1995; Cromwell, 2002; de Greeff et al., 2019; Petrin et al., 2019). Recently, the scientific community investigated prevalence, abundance, and possible mobilization of ARGs in pig farms and surrounding environments (Hölzel et al., 2010; Marti Serrano, 2014; Petrin et al., 2019; Van den Meersche et al., 2019; Wu et al., 2019).

TABLE 1 | The most commonly used antibiotics and the relative ARGs in livestock production (DHPS, dihydropteroate synthase; DHPR, dihydropyridine-resistant).

Antibiotic family	Most used	Animal Farming	Use	Contrasted bacteria and recognized main targets	Resistance mechanism	Main ARGs
Macrolides	Tylosin	Cattle	Gastrointestinal and respiratory infections	Gram-positive bacteria.	Interference with protein synthesis (sequestration of mRNA ribosome-binding site)	*erm*, *msr*, *mef* genes
	Erytromycin	Pig		Main target: *Lawsonia intracellularis*		
	Clarithromycin	Poultry		*Staphylococcus aureus*		
Sulfonamides	Sulfamethazine	Cattle	Urinary tract infections	Gram-positive and Gram-negative bacteria. Main target: *Enterobacteriaceae, Pasteurellaceae*	Interference with folic acid synthesis competing for the enzyme DHPS	*sull, sulII* genes
		Pig	Respiratory infections			
		Poultry				
Tetracyclines	Chlortetracycline	Cattle	Systemic and local infections	Gram-positive and Gram-negative bacteria	Interference with efflux pump systems	*tet* genes
	Oxytetracyclines	Pig	Gastrointestinal and respiratory infections			
	Doxycycline	Poultry				
Quinolones	Fluoroquinolones (Enrofloxacin, Danofloxacin, Marbofloxacine)	Pig	Intestinal infections	Gram-positive and Gram-negative bacteria, including mycobacteria, and anaerobes	Mutations in the genes encoding quinolone target DNA gyrase and topoisomerase IV, interference with efflux pump systems	*qnr* genes
		Cattle				
β-lactams	Penicillins (Amoxycilline, Ampicillines) Cephalosporins, Carbapenems	Pig	Respiratory diseases	Gram-positive and Gram-negative bacteria	Interference with cell wall synthesis and permeability, inactivation through β-Lactamase enzyme	*bla, amp, pen* genes,
		Cattle	Necrotic enteritis			
		Poultry				
		Dog				
		Cat				
Aminoglycosides	Streptomycin, Spectinomycin, Neomycin, Aspramycin, Gentamycin, Lincomycin	Pig	Intestinal infections	Gram-positive, and Gram-negative bacteria, if aerobic	Inhibition of protein synthesis (rhibosome interference)	*aac, aad*, *aad aph* genes
		Poultry				
Phenicols	Chloramphenicol	Pig	Respiratory disease, foot rot	Broad spectrum. Main target: *Photobacterium, Salmonella, E. coli*	Enzymatic modification of antibiotic molecules	*cat, pp-flo, flo* genes
	Thiamphenicols (thiamphenicol, florfenicol)					
Diaminopyrimidines	Trimethoprim	Horse	Post-weaning scours	Gram-positive and many Gram-negative bacteria. Main target: *Enterobacteriaceae*	Interference with folic acid synthesis by binding the enzyme DHFR	*dfr* genes
		Pig				

(Continued)

TABLE 1 | Continued

Antibiotic family	Most used	Animal Farming	Use	Contrasted bacteria and recognized main targets	Resistance mechanism	Main ARGs
Polypeptides	Bacitracin, Colistin	Pig	Intestinal diseases	Gram-positive (Bacitracin) or Gram negative (Colistin) bacteria. Main Gram negative target: *E. coli Salmonela* spp. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, or *Acinetobacter*.	LPS modification, efflux pump systems regulation	*pmr,pho*, *mcr*, *kpn* genes
		Poultry		Main Gram positive target: *Campylobacter*		
Lincosamides	Lincomycin	Pig	Respiratory and Intestinal infections	Gram positive bacteria, most anaerobic and some mycolpasma species. Main target: *Staphylococcus aureus*	Alteration of the antibiotic target site	*lnu*, *lin*, *erm* genes
		Poultry				
		Cattle				
Pleuromutilins	Tiamulin	Pig	Respiratory and Intestinal infections	*Pasteurellaceae*, *Brachyspira*, *Micoplasma*	Alteration/protection of the antibiotic target site	*vga*, *sal*, *lsa* genes
	Valnemulin	Poultry				

References: (Schwarz et al., 2001; Petinaki et al., 2008; Guardabassi et al., 2009; Abbas et al., 2011; Van Hoek et al., 2011; Li et al., 2013; Shang et al., 2013; Tasho and Cho, 2016; Deng et al., 2017; Aghapour et al., 2019; https://www.msdvetmanual.com/pharmacology/antibacterial-agents).

Table 1 summarizes the main antibiotic families and the most used antimicrobics in livestock animals for therapeutic use. Nowadays, more than 150 antimicrobial compounds in livestock production are used. The residues inevitably end up in the environment because of manure application on agricultural lands (Baguer et al., 2000). In 2010, more than 63,000 tons of antimicrobials were consumed by livestock across the globe. The predicted growth of the world's population allows for an estimated increase in antibiotic consumption of up to 105,000 tons by 2030 (Tasho and Cho, 2016). For this reason, specific action plans have been defined to reduce the use of antibiotics as therapeutics for livestock in several countries (i.e., the European One Health Action Plan against Antimicrobial Resistance, 2017; the National Strategy to Combat Antibiotic-Resistant Bacteria, proposed by the White House, 2014; the National Action Plan to Contain Antimicrobial Resistance issued by the Chinese National Health and Family Planning Commission, 2016–2020).

MANURE TREATMENTS

Besides direct collection into aerobic or anaerobic lagoons, animal manure can undergo drying and liquid-solid phase separation. Manure solid phase, as well as whole manure if shovellable, is traditionally composted to produce biofertilizer. Currently, anaerobic digestion and biological treatments of animal manure are often adopted on intensive animal farms (Van Epps and Blaney, 2016).

Composting can substantially reduce the antibiotic load, especially during the thermophilic phase (Zhang et al., 2019), but recalcitrant antibiotics accumulate in compost products and in amended soil (Bohrer et al., 2019; Zang et al., 2019). A general ARG abatement (0.7–2.0 log decrease) is obtained through thermophilic composting of swine, cattle, and poultry manure, depending on manure type and operational conditions (He et al., 2020).

Biological treatments of animal manure and wastewater, which are adopted to reduce the environmental input of nitrates, slightly decreases the levels of antibiotic residues and pathogenic bacteria (Van den Meersche et al., 2019). Antimicrobial resistant gene reduction of 0.1–3.3 log is observed in swine manure after treatment (He et al., 2020).

Anerobic digestion (AD) is adopted to stabilize manure with a final production of methane (Fubin et al., 2016, 2017). A 0.3–52 log decrease of ARGs was observed in digestate from swine wastewater (He et al., 2020). Interestingly, the higher the content of volatile solids in manure and the mixing rate, the higher the ARGs number in the digestate (Turker et al., 2018). The combined pasteurization and AD of swine manure reduced sole archaeal communities, whereas simple AD affected bacteria and archea (Fubin et al., 2020). Manure pretreatment with bacterial strains is effective in degrading antibiotics (Liu et al., 2019) and enhancing biogas production, but the overall effect on ARB and ARGs was not addressed.

Constructed wetlands are vegetated aquatic systems that can be adopted for the treatment of wastewater and agricultural drainage water (Lavrnic et al., 2018). Their ability to reduce

ARGs in swine wastewater resulted in a 0.18–3 log decrease (He et al., 2020).

Oxidizing post-treatments, as ozonation or Fenton conditions, can be used on animal or treated wastewaters to degrade antibiotics and bacteria thanks to the activity of reactive oxygen species (Balcıoğlu and Ötker, 2003; Ikehata et al., 2006; Uslu and Balcıoğlu, 2009). Among advanced oxidation processes, highly costly ionizing radiations are known for their ability to destroy microbial DNA. Therefore, affordable combinations of ionizing radiation and oxidation allows for the degradation of antibiotics and ARGs in organic matrices, although with a high biological and environmental risk (Chu et al., 2019, 2020).

DIFFERENT APPROACHES TO RESISTOME PROFILING STUDY

Even though AMRs introduced in the environment with animal manure have been largely explored (Dolliver et al., 2008; Selvam et al., 2012b), contradictory information exists regarding the fate of ARGs (Selvam et al., 2012a; Wang et al., 2015; Xie et al., 2016). The growing need for the control of ARGs' spread prompted the scientific community to set up and to validate refined molecular methods for the study of ARGs' dissemination dynamics among environmental microbial communities.

Both 16S rRNA amplicon and untargeted sequencing can be considered exhaustive methods for the exploration of microbial community structure in manure-fertilized soil and farm waste. Several studies on resistome diffusion in wastewater treatment plants (Yadav and Kapley, 2019), sewage sludge composting units (Su et al., 2015), and urban sewage support the metagenomic approach (Hendriksen et al., 2019) in monitoring ARGs' level during treatments and seasonal changes. A recent work (Han et al., 2018) showed that the shift in soil bacterial communities caused by manure application leads to changes in the soil bacteria resistome.

Recently, studies on the detection of genetic markers associated with AMR (transposases and class 1 integron-integrase genes) and ARGs have been markedly increasing. The quantification of ARGs in soils amended with livestock and swine manure (Brooks et al., 2014; Tao et al., 2014) was performed with high-throughput qPCR assay (Rocha et al., 2018; Blau et al., 2019). In a recent study, both intracellular and extracellular DNA containing ARGs were quantified in sludge at about 10^{10} and 10^{12} copies per gram, respectively (Dong et al., 2019). Here, the intracellular ARGs were assessed through conjugation with cell-cell contact, whereas the extracellular ARGs were assessed through natural transformation. Several works on different manure types focused on the quantification of targeted genes *intI1* and *intI2* for class 1 and 2 integron-integrase genes and *korB* gene, specific for IncP-1 plasmids, together with ARGs (Hu et al., 2016; Blau et al., 2018, 2019).

As already reported, plasmid-mediated ARGs' diffusion is frequently used, especially for the role of plasmids in the rapid bacterial adaptation and fitness improvement (Smalla et al., 2000). Exogenous plasmid isolation techniques (Bale et al., 1988) clarified how plasmids diffuse in different environments. Recently, plasmids from municipal sewage sludge and recipient bacteria were analyzed for their transferability by exogenous isolation (Blau et al., 2018; Wolters et al., 2018). Referring to pig manure samples, four IncQ-like plasmids were isolated in recipient strains: *Pseudomonas putida* UWC1, *Acinetobacter* sp., *Ralstonia eutropha*, *Agrobacterium tumefaciens,* and *E. coli.* The plasmid transferability in *E. coli* strains was not efficient, underlying a broad but highly specific host range (Smalla et al., 2000).

Recently, simplified mathematical models have been applied to predict and quantify ARGs' spread in livestock animal gut microbiomes (Andersen et al., 2020) and in agricultural waste (Baker et al., 2016). In such environments, the variables involved in the ARGs' spread are countless and depend on a wide range of intrinsic and extrinsic factors, such as genetic mechanisms of ARB replication, HGT dynamics, environmental and stressor conditions, and microbiota composition. Therefore, future research should focus on the improvement of predictive models of ARGs' dissemination mechanism, exploitable for targeted operations in livestock waste management.

CONCLUSION

Although a decrease in the use of antibiotics in livestock production is highly recommended, antibiotics' overuse remains an important issue to solve. The uncontrolled spread of ARB and ARGs in the environment due to soil manuring is of serious concern. Many studies highlight ARGs' presence in microbial communities of livestock manure and manured agricultural fields, despite the improved livestock and waste management strategies to contain in-farm ARGs' spread. In the last thirty years, knowledge on pathways of ARGs' diffusion from animal waste to the environment was enriched by multidisciplinary research approaches.

In light of the current knowledge, the study of the dynamics of AMR and ARGs' spread in manure and environments surrounding livestock farms should combine molecular and functional genetics strategies with prediction models of the diffusion of MGEs (integrons and plasmids) and metagenomic data.

AUTHOR CONTRIBUTIONS

AC: original draft preparation, figure and table conceptualization, review, and editing. PT, MM, and SB: review. DL: original draft preparation and table preparation. IB and PM: original draft preparation and review. All authors contributed to critically revising the manuscript and gave final approval for publication.

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frontiers in Microbiology

MINI REVIEW
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Streptomycetes: Attractive Hosts for Recombinant Protein Production

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Enzymes are increasingly applied as biocatalysts for fulfilling industrial needs in a variety of applications and there is a bursting of interest for novel therapeutic proteins. Consequently, developing appropriate expression platforms for efficiently producing such recombinant proteins represents a crucial challenge. It is nowadays widely accepted that an ideal 'universal microbial host' for heterologous protein expression does not exist. Indeed, the first-choice microbes, as *Escherichia coli* or yeasts, possess known intrinsic limitations that inevitably restrict their applications. In this scenario, bacteria belonging to the *Streptomyces* genus need to be considered with more attention as promising, alternative, and versatile platforms for recombinant protein production. This is due to their peculiar features, first-of-all their natural attitude to secrete proteins in the extracellular milieu. Additionally, streptomycetes are considered robust and scalable industrial strains and a wide range of tools for their genetic manipulation is nowadays available. This mini-review includes an overview of recombinant protein production in streptomycetes, covering nearly 100 cases of heterologous proteins expressed in these Gram-positives from the 1980s to December 2019. We investigated homologous sources, heterologous hosts, and molecular tools (promoters/vectors/signal peptides) used for the expression of these recombinant proteins. We reported on their final cellular localization and yield. Thus, this analysis might represent a useful source of information, showing pros and cons of using streptomycetes as platform for recombinant protein production and paving the way for their more extensive use in future as alternative heterologous hosts.

Keywords: streptomycetes, recombinant proteins, heterologous expression, industrial enzymes, therapeutic proteins

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INTRODUCTION

Nowadays, we witness the increasing application of enzymes in industrial sectors, including food, detergent, and textile manufactures (Trono, 2019) and the bursting of interest in proteins for therapeutic and diagnostic purposes (Tripathi and Shrivastava, 2019). Developing efficient bioprocessing strategies for protein production is consequently of utmost importance. Most of valuable industrial enzymes and therapeutic proteins are recombinant versions, produced by heterologous platforms (Adrio and Demain, 2014). However, an ideal 'universal host' for protein heterologous expression does not exist. Those microbes (as *Escherichia coli* or yeasts) that are still considered the first-choices to this purpose possess intrinsic limitations inevitably restricting their use. Production of heterologous proteins in *E. coli* is limited by self-cytotoxicity, incorrect folding,

aggregation into inclusion bodies, and/or lack of secretion (Adrio and Demain, 2014). In yeasts, recombinant protein production is often associated with hyper-glycosylation and product retention within the periplasmic space (Vieira Gomes et al., 2018).

In this scenario, bacteria belonging to the *Streptomyces* genus might represent a promising alternative platform for recombinant protein production. Streptomycetes are Gram-positive, aerobic bacteria, characterized by a mycelial life style and commonly found in soils, where they secrete multiple hydrolytic enzymes to degrade complex organic substrates. This natural secretion capacity represents their most attractive feature for recombinant protein production. Secretion may prevent local accumulation of the overexpressed recombinant proteins, reducing toxicity to host cells and promoting correct folding (Anné et al., 2012). It facilitates downstream recovery decreasing production costs (Hamed et al., 2018). In addition, streptomycetes are characterized by low endogenous proteolytic activity; they grow relatively fast and in inexpensive media; they do not produce pyrogenic lipopolysaccharides and endotoxins; they are not pathogenic; and they might express G + C-rich genes without codon usage optimization (Anné et al., 2012; Sevillano et al., 2013). Thanks to the extensive fermentation knowhow deriving from their use as antibiotic producers (Ndlovu et al., 2015), streptomycetes are robust and scalable industrial strains, and a wide range of tools for their genetic manipulation have recently become available (Kieser et al., 2000). Notwithstanding these potential advantages, nowadays their use is not so common as it could be expected. To investigate this aspect, in this mini-review we cover – to the best of our knowledge – all studies published from 1980s to December 2019, in which streptomycetes were used as heterologous hosts for recombinant protein production. **Table 1** reports these 94 cases of proteins expressed in streptomycetes. **Figure 1** highlights the main results emerging from the analysis of **Table 1** in terms of protein class, homologous source, heterologous host, and molecular tools.

WHAT ARE THE RECOMBINANT PROTEINS PRODUCED IN STREPTOMYCETES?

50 (out of 94) proteins listed in **Table 1** are enzymes with potential industrial/environmental applications (**Figure 1A**). The most represented class is that of glycosyl hydrolases (23 proteins), including: (i) (hemi)cellulases, for lignocellulose saccharification and biofuel production; (ii) chitinases, for generating value-added chitin-derivatives as chitosan or biopesticides (Berini et al., 2018a); and (iii) amylases for starch processing. The lipase/esterase group (8 proteins) with applications in detergent, food, and biofuel industries, and the oxidoreductase class (7), including laccases and peroxidases for bioremediation (Berini et al., 2018b), follow. Interesting examples are the phospholipase D from *Streptomyces racemochromogenes,* for producing phosphatidyl derivatives from lecithin with emulsifying properties for food and cosmetics (Nakazawa et al., 2011), and the cutinase from *Thermobifida* sp. with polyester-degrading activity in bioplastic recycle (Sinsereekul et al., 2010). Dubé et al. (2008) produced in *Streptomyces lividans* up to 350 mg/L of *Streptomyces coelicolor* small laccase, a thermostable enzyme decolorizing synthetic dyes that is considered promising for pollutant degradation in urban or industrial wastewaters. Finally, **Table 1** and **Figure 1A** include transferases (6 proteins) for food processing, proteases/peptidases (5) for feed and detergent industries, and phosphatases (2), including a phytase used as supplement for animal nutrition (Carrillo Rincón et al., 2018). Additionally, Torres-Bacete et al. (2015) expressed a novel Penicillin V acylase for producing semisynthetic penicillins, whereas Rose et al. (2005) a latex clearing protein for bioconversion of rubber wastes. Unfortunately, only few of these studies reported a comparison of protein expression yield between streptomycetes and other microbial hosts. Hamed et al. (2017) succeed in producing 90 mg/L of a thermostable cellulase from the bacteroidetes *Rhodotermus marinus* using *S. lividans* TK24 as host; the same protein could not be produced in *E. coli*. Very recently, Šnajder et al. (2019) reported the first and so far the only case of expression of an archaeal thermozyme (pernisine) in *Streptomyces rimosus*. The homologous host – the hyperthermophilic *Aeropyrum pernix* – was uncultivable in industrial fermentation facilities. The protein productivity (10 mg/L) in this case was comparable to that achieved in *E. coli*, but with the advantage of simplified downstream processes due to protein secretion in the streptomycete (Šnajder et al., 2019). Similarly, the *Streptomyces halstedii* phospholipase expression was approximately 60 and 30 times higher in *S. lividans* TK24 than in *E. coli* and *Pichia pastoris,* respectively (Tao et al., 2019). Sianidis et al. (2006) and Sinsereekul et al. (2010) reported that their attempts to express a xyloglucanase from *Jonesia* sp. and a cutinase from *Thermobifida* sp. failed, respectively, in *E. coli* and *B. subtilis*, and *E. coli* and *P. pastoris*. Finally, Díaz et al. (2004) produced in *S. lividans* JI66 a xylanase from *Aspergillus nidulans* with a yield 3- and 19-fold higher than in lactic bacteria and *Saccharomyces cerevisiae,* respectively. Despite these successes at laboratory level, we are indeed unaware of any further scaling up at industrial level of recombinant enzyme production from streptomycetes. We can suppose that this is probably due to an overall limited protein productivity in streptomycetes that rarely reaches the g/L production level usually required for industrial application. As reported in **Table 1**, only in the case of a chitinase (Nguyen-Thi and Doucet, 2016), the protein productivity was more than 1 g/L. These results point out the crucial need to overcome intrinsic bottlenecks in protein productivity in streptomycetes, by redesigning their regulatory networks and secretion pathways by system biology, as recently proposed by Kim et al. (2020).

In **Table 1**, 21 are the recombinant proteins curing human diseases (**Figure 1A**), including those for treating cancer (interleukin, interferon, Tumor Necrosis Factor Alpha-TNF-α), cardiovascular pathologies (streptokinase, hirudin), and metabolic or auto-immune disorders (glucagon, phenylalanine ammonia-lyase, tendamistat). Recently, *S. lividans* TK24 was used for producing an *Actinoallomurus* A8-sourced glutenase, a promising candidate for oral enzymatic management of gluten toxicity (Cavaletti et al., 2019). Streptomycetes were also used to express 8 'target' proteins, as antigens from *Mycobacterium*

TABLE 1 | List of the heterologous proteins produced by streptomycetes (in chronological order).

References	Protein	Source	Heterologous host	Plasmid	Promoter	Signal peptide	Productivity (up to)	Localization
Berini et al., 2019	Chitinase	Metagenomics	*S. coelicolor* A3(2), *S. venezuelae* ATCC 10595, *S. lividans* TK24	pIJ86	*ermE*p*	Absent	45 mg/L	Extracellular
Cavaletti et al., 2019	Glutenase	*Actinoallomurus* sp. (Gram +)	*S. lividans* TK24	pIJ86	*ermE*p*	Native	1.4×10^6 U/L	Extracellular
Šnajder et al., 2019	Pernisine	*Aeropyrum pernix* (archaeon)	*S. rimosus* M4018	pVF	*tcp830*p	*srT*-SP	10 mg/L (codon usage optimization, pro-region removal)	Extracellular
Tao et al., 2019	Phospholipase D	*S. halstedii* (Gram +)	*S. lividans* TK24	pIJ12739	Dual promoter (*tipA*p/*ermE*p*)	Native	7.1×10^4 U/L	Extracellular
Carrillo Rincón et al., 2018	Phytase	*Escherichia coli* (Gram −)	*S. rimosus* M4018	pVF, pAB04	*ermE*p*, *nitA/nitR*p, *tcp830*p	*aml*-SP$_{Sv}$, *srT*-SP, *lip*-SP	5×10^3 U/L in extracellular fraction, $< 1 \times 10^3$ U/L in cytoplasm (codon usage optimization)	Extracellular + cytoplasm
Daniels et al., 2018	Cellulase	*Rhodothermus marinus* (Gram −)	*S. lividans* TK24	pIJ486	*vsi*p	*vsi*-SP	7.5 mg/L	Extracellular
Noguchi et al., 2018	Chitobiase	*S. avermitilis* (Gram +)	*S. lividans* 1326 and derivative (expressing a repressor to avoid protein production without inducer)	pIJ350	*xylA*p$_{Sa}$	Native	1.5×10^6 U/L	Extracellular
Hamed et al., 2017	Cellulase	*Rhodothermus marinus* (Gram −)	*S. lividans* TK24	pIJ486	*vsi*p	*vsi*-SP	90 mg/L (120 mg/g dry cell weight)	Extracellular
Sevillano et al., 2017	α-Amylase	*S. griseus* (Gram +)	*S. lividans* Δ*TA-Tox* (pGM160-YefMsl^{ts}, pALCre^{ts})	pNRoxAnti	*pstS*p	NA	1.1×10^6 U/L	Extracellular
	Xylanase	*S. halstedii* (Gram +)				NA	1.7×10^5 U/L	Extracellular
Gabarró et al., 2016	Agarase	*S. coelicolor* (Gram +)	*S. lividans* TK21, *S. lividans* Δ*sipY* (derivative deficient in the major signal peptidase SipY)	pIJ486	Native	NA	2.4×10^6 U/L	Extracellular
	Laccase	*S. lividans* (Gram +)	*S. lividans* Δ*sipY* (derivative deficient in the major signal peptidase SipY)	pFD666	*dagp*	NA	5.8 U/L	Extracellular
Liu et al., 2016	Transglutaminase	*S. hygroscopicus* (Gram +)	*S. lividans* TK24	pIJ86	Native (optimized by removal of negative element)	Native	5.7×10^3 U/L (codon usage optimization)	Extracellular
Nguyen-Thi and Doucet, 2016	Chitinase	*S. coelicolor* (Gram +)	*S. lividans* 10-164	pC109	NA	NA	1.1×10^3 mg/L	Extracellular

(Continued)

TABLE 1 | Continued

References	Protein	Source	Heterologous host	Plasmid	Promoter	Signal peptide	Productivity (up to)	Localization
Sevillano et al., 2016	Xylanase	*S. halstedii* (Gram +)	*S. lividans* 1326, *S. lividans* GSAL1 (derivative overexpressing the morphogene *ssgA*)	Derivative of pN702GEM3	Native, *vsi*p, *ermE*p*, *xysA*p, *pstS*p, *xylA*p_{Sc}, *glpQ*p	Native, *amy*-SP (as-it-is, or optimized)	2.5×10^5 U/L	Extracellular
	α-Amylase	*S. griseus* (Gram +)			*xysA*p, *pstS*p	Native	1.6×10^5 U/L	Extracellular
	Laccase	*S. coelicolor* (Gram +)	*S. lividans* 1326, *S. lividans* Δ*xlnR*, *S. lividans* Δ*bxlR* (derivatives knocked-out in putative *xysA*p repressor genes)	pHJL401	*xysA*p	Native	160 U/g dry weight	Extracellular
Guan et al., 2015	Transglutaminase	*S. hygroscopicus* (Gram +)	*S. lividans* TK24, *S. griseus*, *S. lividans* 1326, *S. hygroscopicus* FR008	pIJ86	Native, *ermE*p	Native (as-it-is, or optimized)	687 mg/L (9.6×10^3 U/L)	Extracellular
	Aminopeptidase	*Bacillus subtilis* (Gram +)			*tg*p	*tg*-SP (optimized)	2.8×10^3 U/L	Extracellular
	Phenylalanine ammonia-lyase	*Rhodotorula glutinis* (yeast)					2.1×10^4 U/L	Extracellular
Gullón et al., 2015	Agarase	*S. coelicolor* (Gram +)	*S. lividans* TK21, *S. lividans* Δ*secG*, *S. lividans* Δ*tatC* (derivatives knocked-out for components of the Sec- or Tat-route respectively)	pAGAs1	Native	Native, *aml*-SP_{Sl}	60 U/mg dry weight	Extracellular
Torres-Bacete et al., 2015	Penicillin V acylase	*S. lavendulae* (Gram +)	*S. lividans* 1326	pEM4	*ermE*p*	Native	11 mg/L (959 U/L)	Extracellular
Binda et al., 2013	D,D-peptidase/D,D-carboxypeptidase	*Nonomuraea gerenzanensis* (Gram +)	*S. venezuelae* ATCC 10595, *S. coelicolor* A3(2), *S. lividans* TK24	pIJ86	*ermE*p*	Native	12 mg/L	Cell wall fraction
Li et al., 2013	Endoglucanase	*Thermobifida fusca* (Gram +)	*S. lividans* 1326	pZRJ362	*xylA*p_{Am}	Native	173 mg/L (5.6×10^3 U/L)	Extracellular
Sevillano et al., 2013	α-Amylase	*S. griseus* (Gram +)	*S. lividans* pKC796, *S. lividans* Δ*TA-pKC796* (pGM160-YefMsl^{ts}), *S. lividans* Δ*TA-pKC796-Tox* (pGM160-YefMsl^{ts})	pN702Gem3-Anti	*pstS*p	NA	NA	Extracellular
	Xylanase	*S. halstedii* (Gram +)				NA	NA	Extracellular
Lule et al., 2012	Tumor Necrosis Factor α	Human	*S. lividans* TK24 and derivative (overexpressing phosphoenolpyruvate carboxykinase)	pIJ486	*vsi*p	*vsi*-SP	0.9 mg/g dry biomass	Extracellular
Dubeau et al., 2011	Chitosanase	*Kitasatospora* sp. N106 (Gram +)	*S. lividans* TK24, *S. lividans* Δ*csnR* (knocked-out for a negative transcriptional regulator)	Derivative of pHM8a, pFDES	Native (as-it-is or modified), *S. ghanaensis* phage I19 promoter	NA	2.4×10^4 U/L	Extracellular

(Continued)

TABLE 1 | Continued

References	Protein	Source	Heterologous host	Plasmid	Promoter	Signal peptide	Productivity (up to)	Localization
Nakazawa et al., 2011	Phospholipase D	*S. racemochromo genes* (Gram +)	*S. lividans* TK23	pES	Native	NA	3.0×10^4 U/L	Extracellular
Zhu et al., 2011	Interleukin A	Human	*S. lividans* TK24	Derivative of pSGL1	*ermE*p*	*melC1*-SP, *gpp*-SP (as-it-is, or optimized)	0.6 mg/L	Extracellular
Côté and Shareck, 2010	Lipase	Metagenomics	*S. lividans* 10-164	pIAFC109	NA	Native	NA	Extracellular
Noda et al., 2010	Transglutaminase	*Stv. cinnamoneum* (Gram +)	*S. lividans* 1326	pIJ702	*plD*p	*plD*-SP	230 mg/L	Extracellular
	β-1,4-Endoglucanase	*Thermobifida fusca* (Gram +)					64 mg/L	Extracellular
	β-Glucosidase						114 mg/L	Extracellular
Sinsereekul et al., 2010	Cutinase	*Thermobifida* sp. (Gram +)	*S. rimosus* R7	pIJ8600	*tipA*p	Native	58 μg/L	Extracellular
Meilleur et al., 2009	Lipase	Metagenomics	*S. lividans* IAF10-164	pIAFD95A	*D95A*p	Native	11.3 mg/L	Extracellular
Díaz et al., 2008	Alkaline phosphatase	*Thermus thermophiles* (Gram −)	*S. lividans* JI66	pIJ702	*xysA*p	Native	2.7×10^5 U/L	Extracellular
	β-Glycosidase					Absent	2.6×10^5 U/L in cytoplasm, 5.5×10^4 U/L in extracellular fraction	Extracellular + cytoplasm
Dubé et al., 2008	Laccase	*S. coelicolor* (Gram +)	*S. lividans* IAF10-164	pIAFD95A	*D95A*p	NA	350 mg/L	Extracellular
Hatanaka et al., 2008	Leucine aminopeptidase	*S. griseus* (Gram +)	*S. lividans* 1326	pTONA5	*ssmp, ermE*p*, kibilysin gene promoter	NA	1.5×10^5 U/L	Extracellular
	Proline aminopeptidase	*Streptomyces* sp. (Gram +)				Absent	$5.2\ 10^5$ U/L in extracellular fraction, 5.0×10^4 U/L in cytoplasm	Extracellular + cytoplasm
	Aminopeptidase P					Absent	3.5×10^4 U/L in extracellular fraction, up to 1.8×10^4 U/L in cytoplasm	Extracellular + cytoplasm
Lin et al., 2006, 2008	Tranglutaminase	*S. platensis* (Gram +)	*S. lividans* JT46	pIJ702	*melC1*p	Native	5.8×10^3 U/L	Extracellular
Qi et al., 2008	Glucagon (co-expressed with rat α-amidase gene)	Human	*S. lividans* TK24	Derivative of pIJ680	*aph*p	*melC1*-SP	24 mg/L	Extracellular

(Continued)

TABLE 1 | Continued

References	Protein	Source	Heterologous host	Plasmid	Promoter	Signal peptide	Productivity (up to)	Localization
Ayadi et al., 2007	α-Integrin A-domain	Rat	*S. lividans* 1326	pIJ699	*ermE*p	Long synthetic SP	8 mg/L	Extracellular
Merkens et al., 2007	Quercetinase	*Streptomyces* sp. (Gram +)	*S. lividans* TK23	pIJ702	Native	Absent	5.1 U/mg total protein	Cytoplasm
Pimienta et al., 2007	Streptokinase	*Streptococcus equisimilis* (Gram +)	*S. lividans* TK24	pUWL-218	*vsi*p	*vsi*-SP, *xlnC*-SP	15 mg/L	Extracellular
Vrancken et al., 2007	Tumor Necrosis Factor α	Human	*S. lividans* TK24 and derivative (over-expressing the phage-shock protein A homolog)	pSSV05	*vsi*p	*vsi*-SP	1.1 μg/mg dry weight	Extracellular
	Enhanced green fluorescent protein	*Aequorea victoria* (jellyfish)				*xlnC*-SP	20 mg/L (15.9 U/mg dry weight)	Extracellular
Côté et al., 2006	β-Glucosaminidase	*Amycolatopsis orientalis* (Gram +)	*S. lividans* TK24	pFD666	NA	Native	573 U/L	Extracellular
	β-Glucosaminidase	*S. avermitilis* (Gram +)			NA	NA	NA	Extracellular
Sianidis et al., 2006	Xyloglucanase	*Jonesia* sp. (Gram +)	*S. lividans* TK24	pIJ486	*vsi*p	Native, *vsi*-SP	150 mg/L	Extracellular
Vallin et al., 2006	Glycoprotein (antigen)	*Mycobacterium tuberculosis* (Gram +)	*S. lividans* 1326	pUWL-219	*dag*p	*dag*-SP	80 mg/L	Extracellular
Fukatsu et al., 2005	N-substituted formamide deformylase	*Arthrobacter pascens* (Gram +)	*S. lividans* TK24, *S. coelicolor* A3(2) M145, *S. avermitilis* K139	pSH19	*nitA/nitR*p	NA	8.5 U/mg total protein	Extracellular
Rose et al., 2005	Latex clearing protein	*Streptomyces* sp. (Gram +)	*S. lividans* TK23	pIJ702	Native	Native	NA	Extracellular
Díaz et al., 2004	Xylanase	*Aspergillus nidulans* (fungus)	*S. lividans* JI66	pIJ702	*xysA*p	Native, *xys1*-SP	1.9×10^4 U/L	Extracellular
Lara et al., 2004	Glycoprotein (antigen)	*Mycobacterium tuberculosis* (Gram +)	*S. lividans* 1326	pIJ486, pIJ6021	Native, *tipA*p	Native	5 mg/L	Extracellular
Lin et al., 2004	Transglutaminase	*Stv. ladakanum* (Gram +)	*S. lividans* JT46	pIJ702	Native	Native	1.5×10^3 U/L	Extracellular
Ogino et al., 2004	Phospholipase D	*Stv cinnamoneum* (Gram +)	*S. lividans* 1326	pUC702	Native	Native	118 mg/L (5.5×10^4 U/L)	Extracellular
Schaerlaekens et al., 2004	Tumor Necrosis Factor α	Human	*S. lividans* TK24, *S. lividans* Δ*tatB*, *S. lividans* Δ*tatC* (derivatives knocked-out for components of the Tat pathway)	pIJ486	*vsi*p	*xlnC*-SP, *melC1*-SP, *vsi*-SP	23 mg/L	Extracellular
	Interleukin-10						166 μg/L	Extracellular
Zhang et al., 2004	Interleukin-4 receptor	Human	*S. lividans* TK24	pSGLgpp	NA	*gpp*-SP	10 mg/L	Extracellular

(Continued)

TABLE 1 | Continued

References	Protein	Source	Heterologous host	Plasmid	Promoter	Signal peptide	Productivity (up to)	Localization
Béki et al., 2003	β-D-Mannosidase	*Thermobifida fusca* (Gram +)	*S. lividans* TK24	pIJ699	Native	Absent	0.015 U/mg total protein	Cytoplasm
Geueke and Hummel, 2003	L-Amino acid oxidase	*Rhodococcus opacus* (Gram +)	*S. lividans* 1326	pIJ6021, pUWL201	*tipA*p, *ermE*p*	Native	18 U/L	Cytoplasm
Hong et al., 2003	Calcitonin (co-expressed with rat α-amidase gene)	Salmon	*S. lividans* TK54	pIJ680	*aph*p	*melC1*-SP	30 mg/L	Extracellular
Tremblay et al., 2002	19 kDa major lipoprotein antigens	*Mycobacterium tuberculosis* (Gram +)	*S. lividans* IA F10-164	pIJ702	*xlnA*p	*celA*-SP (long)	200 mg/L	Extracellular
	38 kDa major lipoprotein antigens						80 mg/L	Extracellular
Lammertyn et al., 1997; Pozidis et al., 2001	Tumor Necrosis Factor α	*Mus musculus* (Mouse)	*S. lividans* TK24	pIJ486	*vsi*p	*vsi*-SP (as-it-is or modified)	300 mg/L	Extracellular
Isiegas et al., 1999	β-Lactamase	*Escherichia coli* (Gram −)	*S. lividans* TK21	pIJ487	*dag*p	*dag*-SP	60 U/L	Extracellular
Smith et al., 1999	Alkene monooxygenase	*Rhodococcus rhodochrous* (Gram +)	*S. lividans* TK24	pIJ6021	*tipA*p	NA	2.2 U/mg total protein	Cytoplasm
Lammertyn et al., 1998	Tumor Necrosis Factor α	*Mus musculus* (Mouse)	*S. lividans*	pIJ486	*vsi*p	*aml*-SP_{Sv}	50 mg/L	Extracellular
Park and Lee, 1998	β-Lactamase-inhibitory protein	*S. exfoliatus* (Gram +)	*S. lividans* TK24	pIJ702	*melC1*p	Native	3.0×10^4 U/L	Extracellular
Binnie et al., 1997	Extracellular domain of erythropoietin receptor	Human	*S. lividans* 66	pCAN46	*aph*p	*sprtB*-SP (modified)	15 mg/L	Extracellular
Motamedi et al., 1996	31-*O*-Demethyl-FK506 methyltransferase	*S. hygroscopicus* (Gram +)	*S. lividans*	pIJ943	NA	Native	NA	Cytoplasm
Taguchi et al., 1995	Transforming Growth Factor α (fused with *S. albogriseolus* subtilisin inhibitor)	Human	*S. lividans* 66	pIJ702	*ssi*p + *melC1*p	*ssi*-SP	10 mg/L	Extracellular
Paradkar et al., 1994	β-Lactamase inhibitor protein	*S. clavuligerus* (Gram +)	*S. lividans* TK24	pIJ486	Native	Native	NA	Extracellular

(Continued)

TABLE 1 | Continued

References	Protein	Source	Heterologous host	Plasmid	Promoter	Signal peptide	Productivity (up to)	Localization
Washizu et al., 1994	Transglutaminase	*Stv. mobaraense* (Gram +)	*S. lividans* 3131	pIJ702	*S. antibioticus* tyrosinase promoter	Native	0.1 mg/L	Extracellular
Fornwald et al., 1993	T cell receptor CD4 (as-it-is and derivatives)	Human	*S. lividans* 1326	pLTI450	*STI-II*p, βgalp	*STI-II*-SP	300 mg/L	Extracellular
Jung et al., 1993	Endoglucanase	*Thermobifida fusca* (Gram +)	*S. lividans* TK24	Derivatives of pIJ702	Native	Native	36 mg/L (1.9×10^3 U/L)	Extracellular
	Exoglucanase				Native	Native	17 mg/L (23 U/L)	Extracellular
Ueda et al., 1993	Fv domain of monoclonal antibody against hen egg-white lysozyme	Human	*S. lividans* 66	pIJ702	*ssi*p	*ssi*-SP	1 mg/L	Extracellular
Wolfframm et al., 1993	Chloroperoxidase	*Pseudomonas pyrrocinia* (Gram −)	*S. lividans* TK64	pIJ486	Native	NA	11.2 U/g wet weight	Cytoplasm
Hale et al., 1992	Esterase	*S. scabiae* (Gram +)	*S. lividans* 1326	pIJ486, pIJ702	NA	Native	100 mg/L	Extracellular
Taguchi et al., 1992	Apidaecin 1b (fused with *S. albogriseolus* subtilisin inhibitor)	*Apis mellifera* (Honeybee)	*S. lividans* 66	pIJ702	*ssi*p + *melC1*p	*ssi*-SP	>200 mg/L	Extracellular
Jørgensen et al., 1991	Lipase (co-expressed with a lipase modulator)	*Pseudomonas cepacia* (Gram −)	*S. lividans* TK24	pIJ702	*dag*p	*dag*-SP	Na	NA
Bender et al., 1990a	Hirudin	*Hirudo medicinalis* (Leech)	*S. lividans* TK24	pIJ702	*melC1*p	*AI*-SP	500 μg/L	Extracellular
Bender et al., 1990b	Interleukin-2	Human	*S. lividans* TK24	pIJ680	NA	*AI*-SP	7.1×10^5 U/L in extracellular fraction, 4.7×10^4 U/L in cytoplasm	Extracellular + cytoplasm
Koller and Riess, 1989	Human α-amylase inhibitor (tendamistat)	*S. tendae* (Gram +)	*S. lividans* TK24	pIJ61, pIJ350, pIJ486, pIJ702	Native, *melC1*p (or both in tandem)	Native	700 mg/L	Extracellular
Swan et al., 1989	Calcium-binding protein	*Sac. erythraea* (Gram +)	*S. lividans* TK24	pIJ702	Native	NA	NA	Extracellular

(Continued)

TABLE 1 | Continued

References	Protein	Source	Heterologous host	Plasmid	Promoter	Signal peptide	Productivity (up to)	Localization
Lamb et al., 1988	65-kilodalton antigen	*Mycobacterium leprae* (Gram +)	*S. lividans*	pIJ697	Native	NA	NA	Cytoplasm
Lichenstein et al., 1988	Interleukin-1β	Human	*S. lividans* 1326	pIJ350	β*gal*p	β*gal*-SP	3.8×10^6 U/L in extracellular fraction, 6.3×10^4 U/L in cytoplasm	Extracellular + cytoplasm
	Galaktokinase	*Escherichia coli* (Gram −)	*S. lividans* 1326, *S. lividans galK*$^-$ (galactokinase-deficient mutant)				345 U/L in extracellular fraction, 120 U/L in cytoplasm	Extracellular + cytoplasm
Noack et al., 1988	Interferon α1	Human	*S. lividans* TK24	pIJ487	*saK*p	*saK*-SP	2.0×10^8 U/L	Extracellular
Horinouchi et al., 1987	Streptothricin acetyltransferase	*S. lavendulae* (Gram +)	*S. lividans* TK21	pIJ41, pIJ702, pIJ487	*aph*p, *melC1*p, *Bacillus subtilis* cellulose promoter	NA	NA	Cytoplasm

The list was created by searching Pubmed database (accession on 18 December, 2019) with the following query: ((((heterologous[Title/Abstract]) AND expression[Title/Abstract]) AND protein[Title/Abstract]) AND streptomyces[Title/Abstract]), then manually checked and integrated. Gram + , Gram-positive; Gram −, Gram-negative; NA, data not available; SP, signal peptide; S., Streptomyces; Sac., Saccharopolyspora; Stv., Streptoverticillium. Promoters (CO, constitutive; IN, inducible): aphp from S. fradiae aminoglycoside 3′-phosphotransferase (CO); βgalp from S. lividans β-galactosidase (CO); D95Ap from S. coelicolor groEL2 heat-shock gene (NA); dagp from S. coelicolor agarase (CO); ermEp from Sac. erythraea erythromycin resistance gene (CO); glpQp from S. coelicolor glycerophosphoryl diester phosphodiesterase (IN by glycerol-3-phosphate); melC1p from S. antibioticus melanin operon (CO); nitA/nitRp from Rhodococcus rhodochrous nitrilase (IN by ε-caprolactam); plDp from Stv. cinnamoneum phospholipase D (CO); pstSp from S. lividans phosphate-binding protein (IN by phosphate starvation and carbon sources as fructose, xylose, or galactose); saKp from Staphylococcus aureus phage 42D staphylokinase (NA); STI-IIp from S. longisporus protease inhibitor (NA); ssip from S. albogriseolus subtilisin inhibitor (CO); ssmp from S. cinnamoneus metalloendopeptidase (CO in the presence of a rich inorganic phosphate source and glucose); tcp830p synthetic promoter from S. coelicolor (IN by tetracycline); tgp from S. hygroscopicus transglutaminase (CO); tipAp from S. lividans (IN by thiostrepton); vsip from S. venezuelae subtilisin inhibitor (CO); xlnAp from S. lividans xylanase A (NA); xylAp from S. avermitilis ($xylAp_{Sa}$), S. coelicolor ($xylAp_{Sc}$), or Actinoplanes missouriensis ($xylAp_{Am}$) xylose isomerase (IN by xylose); xysAp from S. halstedii xylanase (IN by carbon sources as xylose, xylan, or fructose). Plasmids (HN, high copy number; MN, moderate copy number; LN, low copy number; SN, single copy number; int, integrative; rep, replicative): pAB04 (LN, int); pAGAs1 (HN, rep); pC109 (HN, rep); pCAN46 (HN, rep); pEM4 (HN, rep); pES (HN, rep); pFD666 (HN, rep); pFDES (HN, rep); pHJL401 (MN, rep); pHM8 (SN, int); pIAFC109 (HN, rep); pIJ12739 (MN, rep); pIJ350 (HN, rep); pIJ41 (LN, rep); pIJ486 (HN, rep); pIJ487 (HN, rep); pIJ6021 (HN, rep); pIJ61 (LN, rep); pIJ680 (HN, rep); pIJ699 (HN, rep); pIJ702 (HN, rep); pIJ86 (HN, rep); pIJ8600 (SN, int); pIJ943 (LN, rep); pLTI450 (HN, rep); pN702GEM3 (HN, rep); pN702Gem3-Anti (HN, rep); pNRoxAnti (HN, rep); pSGL1 (HN, rep); pSGLgpp (HN, rep); pSH19 (HN, rep); pSSV05 (HN, rep); pTONA5 (HN, rep); pUC702 (HN, rep); pUWL201 (HN, rep); pUWL-218 (HN, rep); pUWL-219 (HN, rep); pVF (HN, rep); pZRJ362 (HN, rep). Signal peptide: AI-SP from S. tendae tendamistat (α-amylase inhibitor); aml-SP from S. venezuelae (aml-SP_{Sv}) or S. lividans (aml-SP_{Sl}) α-amylase; amy-SP from S. griseus α-amylase; βgal-SP from S. lividans (β-galactosidase); celA-SP from S. lividans cellulase; dag-SP from S. coelicolor agarase; gpp-SP from S. globisporus apoprotein C-1027; lip-SP from S. rimosus lipase; melC1-SP from S. antibioticus melanin operon gene; plD-SP from Stv. cinnamoneum phospholipase D; saK-SP from Staphylococcus aureus phage 42D staphylokinase; sprtB-SP from S. griseus protease B; srT-SP from S. rimosus trypsin-like protease; ssi-SP from S. albogriseolus subtilisin inhibitor; STI-II-SP from S. longisporus protease inhibitor; tg-SP from S. hygroscopicus transglutaminase; vsi-SP from S. venezuelae subtilisin inhibitor; xlnC-SP from S. lividans xylanase C; xys1-SP from S. halstedii xylanase.*

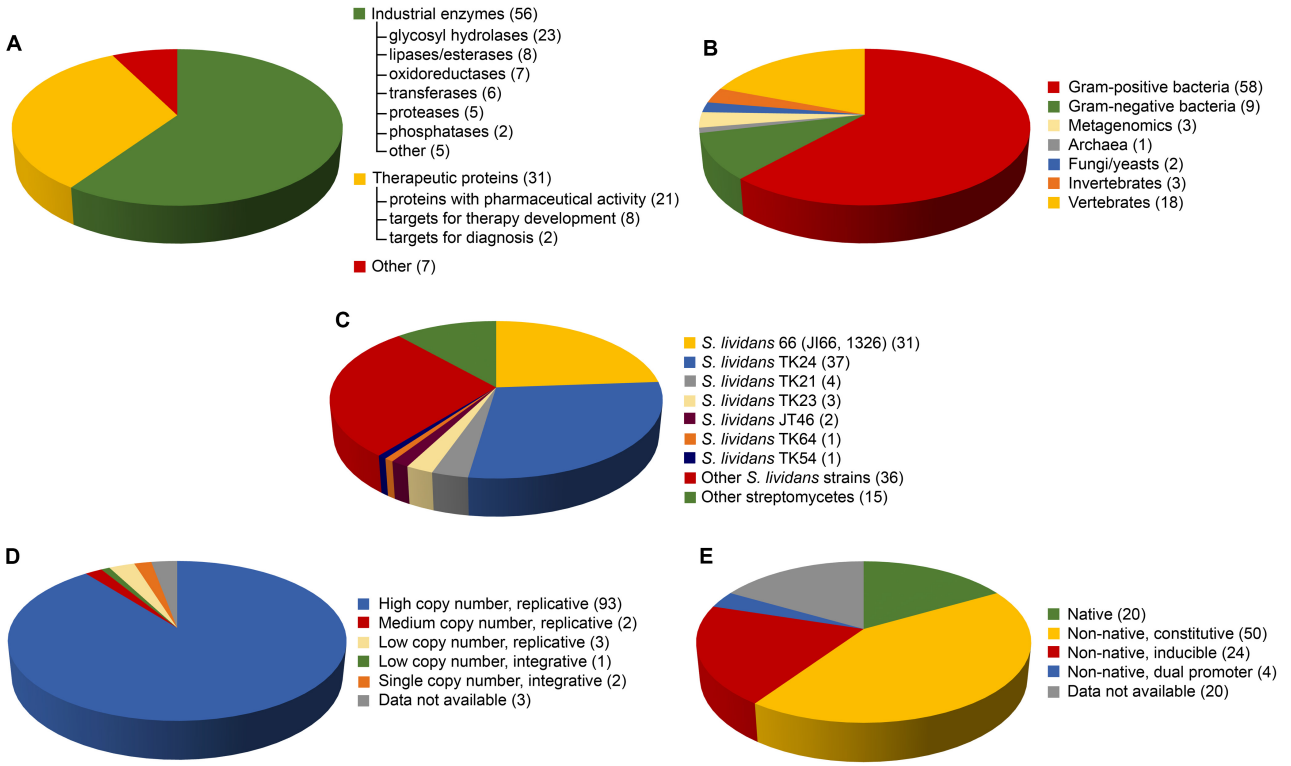

FIGURE 1 | Distribution of the 94 recombinant proteins heterologously produced by streptomycetes and listed in **Table 1**, according to: use/family of the recombinant protein **(A)**, homologous source **(B)**, heterologous host **(C)**, plasmid **(D)**, and promoter **(E)** used for heterologous production. **(C–E)** When for the same protein, multiple hosts and/or plasmids, and/or promoters were used, these different conditions were counted separately.

tuberculosis (Vallin et al., 2006) or the α-integrin A-domain for screening ligands for treating inflammatory disorders (Ayadi et al., 2007), and few diagnostic proteins (2) as the T Cell receptor CD4 for diagnosis of HIV infection (Fornwald et al., 1993). Biopharmaceutical production of proteins in streptomycetes is generally acceptable to the Food and Drug Administration and European Medicine Agency since these bacteria have been used for decades in industrial manufacturing of antibiotics, immunomodulating and antitumor drugs, and nutraceuticals (Marinelli and Marcone, 2011). Additionally, these naturally soil-inhabiting bacteria are recognized as useful components of natural ecosystem and they are considered safer than other microorganisms for agricultural use (Berini et al., 2019). Interestingly, besides the proteins listed in **Table 1**, *S. lividans* was employed by Cangene Corporation (now part of Emergent BioSolutions) for the recombinant production of the macrophage-colony stimulating factor Leucotropin™, a therapeutic agent that successfully completed Phase III trials for treating Hodgkin's and non-Hodgkin's lymphoma (Vrancken and Anné, 2009). To our best knowledge, this is the only reported case of a therapeutic protein production in streptomycetes that reached the clinical phases.

Finally, **Table 1** includes 7 proteins without any direct industrial/therapeutic application: they were produced in streptomycetes for studying biochemical/functional properties and/or mode of action, as in case of the novel N-substitute formamide deformylase from *Arthrobacter pascens* involved in the metabolism of isonitriles (Fukatsu et al., 2005). Another example is VanYn, a D,D-dipeptidase/D,D-carboxypeptidase identified as the sole resistant determinant in the glycopeptide producer *Nonomuraea gerenzanensis* (Binda et al., 2013; Dalmastri et al., 2016). VanYn expression in *Streptomyces venezuelae* allowed a higher production than in *E. coli* (Binda et al., 2012), and contributed to elucidating cell wall turnover during antibiotic production (Marcone et al., 2010a, 2014).

WHERE DO RECOMBINANT PROTEINS EXPRESSED IN STREPTOMYCETES COME FROM?

71 of the proteins listed in **Table 1** derive from prokaryotes and 23 from eukaryotes (**Figure 1B**). Most of prokaryote-sourced proteins come from Gram-positive bacteria: 49 are from *Streptomyces* or *Streptoverticillium* spp., or other actinomycetes as *Nonomuraea, Kitasatospora,* or *Thermobifida* spp. This is not surprising, as heterologous expression is facilitated when the host is phylogenetically related to the homologous producer, due to the similar metabolic and genetic background (Binda et al., 2013). Streptomycetes (DNA G + C > 60%) offer an optimized codon usage for high G + C content genes and they represent

a complementary tool versus *E. coli* (DNA G + C *ca.* 51%). For instance, chitinases, usually produced by soil-inhabitant actinomycetes, were successfully produced in streptomycetes (Berini et al., 2019). Cloning a *S. coelicolor* chitinase in *S. lividans* 10–164 resulted in 486-fold production improvement compared to *E. coli*, allowing gram-scale production for converting crystalline chitin in *N*-acetylglucosamine (Nguyen-Thi and Doucet, 2016). 9 additional recombinant proteins derive from the firmicutes *Bacillus subtilis* and *Streptococcus equisimilis*, and other 9 from the Gram-negative *Escherichia*, *Thermus,* and *Pseudomonas* spp. (**Figure 1B**). The thermostable cellulase from the bacteroidetes *Rhodothermus marinus* (Hamed et al., 2017) and the archaeal thermozyme (pernisine) (Šnajder et al., 2019), described above, complete the list of the prokaryote proteins.

Streptomycetes were successfully used for expressing metagenome-sourced bacterial enzymes (Berini et al., 2017). 2 lipases from enriched fed-batch bioreactors (Meilleur et al., 2009; Côté and Shareck, 2010) and 1 chitinase (named 53D1) from agricultural soil (Berini et al., 2019) were produced in different *Streptomyces* strains. In case of 53D1, the protein was secreted (45 mg/L) into the culture broth by *S. coelicolor* A3(2), with a clear improvement over its expression in *E. coli,* where the protein was mostly accumulated as inactive into inclusion bodies (Cretoiu et al., 2015). Enough 53D1 was produced in the streptomycete to test its activity as biopesticide (Berini et al., 2019).

The heterogeneity of eukaryote sources of the recombinant proteins expressed in streptomycetes confirms their versatility (**Table 1** and **Figure 1B**). The homologous producers of the eukaryote proteins listed in **Table 1** span from filamentous fungi or yeasts (2), to invertebrates (insect, leech, and jellyfish; 3) or vertebrates (fish and mammals; 18). Notably, 14 human proteins were produced in these hosts. A chronological analysis indicates that eukaryote protein expression in streptomycetes was more frequent in the 1990s, becoming after that rarer. The last example of eukaryote protein produced in *S. lividans* TK24 dated back to 2012 (Lule et al., 2012). This is probably due to recent developments in using engineered yeasts, and mammalian and insect cell lines for manufacturing high-value eukaryote proteins, especially those requiring post-translational modifications (Hunter et al., 2019).

WHICH IS THE BEST PROMOTER/VECTOR/HOST SYSTEM FOR RECOMBINANT PROTEIN PRODUCTION IN STREPTOMYCETES?

S. lividans strains are by far the most frequently used heterologous hosts, employed for producing 91 proteins listed in **Table 1**. 31 proteins were expressed in the parental *S. lividans* 66 (also named JI66 or 1326), whereas 37 in its derivative TK24, which is a two-plasmid-free mutant carrying streptomycin resistance mutation (*str-6*, SLP2$^-$, SLP3$^-$) (Kieser et al., 2000) (**Figure 1C**). 1 additional protein was produced in TK64, carrying the same mutations as TK24 plus the *pro-2* mutation, and 1 in TK54, characterized by *his-2, leu-2,* and *spc-1* mutations. The use of *S. lividans* TK24 has the following advantages: (i) low level of extracellular protease activity, (ii) poorly active restriction-modification system of exogenous DNA, (iii) known biochemistry/genetic background due to its high similarity to the model organism *S. coelicolor* A3(2) (Daniels et al., 2018). Other *S. lividans* used as hosts were the plasmid-free mutants *S. lividans* TK23 (for 3 proteins), carrying spectinomycin resistance mutation (*spc-1,* SLP2$^-$, SLP3$^-$), and its derivative JT46 (2 proteins) mutated in *rec-46* gene to reduce inter-plasmid recombination (Kieser et al., 2000). 4 proteins were produced in *S. lividans* TK21, which lacks only SLP2 plasmid. *Ad hoc* constructed *S. lividans* hosts were derivatives of *S. lividans* 66 or TK24, as the pleiotropic mutant *S. lividans* 10–164 (Hurtubise et al., 1995) defective in cellobiose and xylobiose uptake and used for producing a metagenome-sourced lipase (Meilleur et al., 2009; Côté and Shareck, 2010), and *S. lividans galK*$^-$ (galactokinase-deficient mutant) for the production of *E. coli* galactokinase (Lichenstein et al., 1988). *S. lividans* GSAL1, used for the production of a xylanase and a α-amylase, overexpresses the morphogene *ssgA*, which pleiotropically controls growth and cell division. *ssgA* overexpression markedly enhances septation in vegetative hyphae, leading to fragmented growth and to wider hyphae, a phenotype that apparently favors protein production and secretion (Sevillano et al., 2016). Other streptomycetes employed as hosts were *S. coelicolor* A3(2) and its derivative M145 (3 proteins), *Streptomyces griseus* (3), *S. rimosus* (3), *Streptomyces hygroscopicus* (3), *S. venezuelae* (2), and *Streptomyces avermitilis* (1) (**Table 1** and **Figure 1C**). Although less frequently used than *S. lividans,* in certain cases these alternative streptomycetes permitted the production of proteins poorly or not at all expressed in *S. lividans* (Binda et al., 2013; Berini et al., 2019), thus indicating that expanding the range of streptomycete hosts might be promising.

As regards to vectors, the mostly used are high copy number replicative ones (in 93 cases) (**Table 1** and **Figure 1D**) as for examples pIJ702 (25 proteins), pIJ486 (14), and pIJ86 (7 proteins). pIJ702 vector, which carries thiostrepton resistance (*tsrR*) and tyrosinase production (*mel*$^+$) markers, is the derivative of pIJ350, a non-conjugative broad host range vector (Kieser et al., 2000). pIJ486 (*tsrR*) derived from pIJ101, which contains the promoterless *neo* gene (kanamycin resistance) and lacks both the transfer function and the *sti* locus that usually confers 'strong incompatibility'. Removing the *sti* locus increases the chance that different plasmids can be retained at similar copy numbers (Deng et al., 1988; Kieser et al., 2000). The more recent pIJ86 carries apramycin resistance marker (*aprR*) and it is a conjugative vector used for the strong constitutive expression of proteins under erythromycin promoter (*ermE** promoter) from *Saccharopolyspora erythraea*. Recent works (Sevillano et al., 2013, 2017) described new replicative high copy number 'marker-free' systems, which allowed the production of high levels of proteins without using antibiotics as selection markers. One example is based on the presence of a toxin gene localized in the genome and of an anti-toxin gene located on the expression plasmid of the *yefM/yoeBsl* operon from *S. lividans* (Sevillano et al., 2013). Only for 5 proteins, replicative moderate or low copy number vectors

were used. For instance, the moderate copy number pIJ12739 was constructed for the expression of the phospholipase D from *S. halstedii* in *S. lividans* TK24, following the same approach previously described by Fernández-Martínez and Bibb (2014) to produce a dual-promoter expression vector (Tao et al., 2019). The low copy number pIJ943 was used for producing the 31-*O*-demethyl-FK506 methyltransferase in *S. lividans* (Motamedi et al., 1996). For only 3 proteins, integrative vectors were employed such as pAB04 – low copy number plasmid used for producing a phytase (Carrillo Rincón et al., 2018), or pIJ8600 – single copy number vector employed for the expression of the cutinase from the Gram-positive *Thermobifida* sp. in *S. rimosus* R7 (Sinsereekul et al., 2010). Although less explored, integrative vectors might present some advantages. When the integrative single copy number pHM8a plasmid was used for expressing a chitosanase, productivity was comparable to that achieved with replicative multicopy pFDES plasmid, but with the advantage of not requiring antibiotic addition for selection (Dubeau et al., 2011). Interestingly, this last work is the only one, among those cited in this mini-review, which allowed a direct comparison on the effect of different vectors on protein yield. Most of the studies were driven by an empirical case-by-case approach to optimize the tools for a specific protein production, making difficult to draw final conclusions on which is the preferable vector system to be used.

In 20 cases (out of 94), the heterologous protein genes were cloned under the control of their native promoters, but more frequently streptomycete (or other actinomycete) heterologous promoters were used (**Table 1** and **Figure 1C**). The heterologous promoters can be constitutive (e.g., *vsi*p from *S. venezuelae*; *dag*p from *S. coelicolor; ermE**p from *Sac. erythraea; ssi*p from *Streptomyces albogriseolus; aph*p from *Streptomyces fradiae*) or inducible (e.g., *xysA*p from *S. halstedii,* induced by xylane; *pstS*p from *S. lividans,* by phosphate starvation and different carbon sources; *tcp830*p from *S. coelicolor,* by tetracycline*; tipA*p from *S. lividans,* by thiostrepton). Constitutive promoters were more frequently used than inducible ones (50 vs. 24 cases, respectively). If in *E. coli*, a balance between the vector copy number and the promoter strength is needed for controlling protein production and slowing down inclusion body formation (Adrio and Demain, 2014), in streptomycetes this problem is overcome by protein secretion. On the other hand, in streptomycetes, constitutive expression may cause a growth rate reduction negatively impacting on protein productivity: in these cases, inducible expression could be advantageous, although weak points of an inducible system remain as low level of expression, a narrow host range, and the need of an expensive inducer (Herai et al., 2004). As in the case of vectors, only very few studies systemically compared the effect of different promoters on protein yield. Sevillano et al. (2016) investigated the expression of a xylanase from *S. halstedii* cloning the gene under the control of six strong promoters, including two commonly used (*vsi*p and *ermE**p) and four recently identified. Two belonging to the last group (*xysA*p and *pstS*p) performed better than those considered the golden standards, confirming that there is room for developing new tools for improving protein expression in streptomycetes.

In 30 out of the 94 proteins, the presence of native signal peptides (SP) guaranteed secretion in the heterologous hosts, while in 2 cases proteins expressed with their native SP accumulated into the cytoplasm and in 1 case the enzyme was recovered from the cell wall fraction (**Table 1**). In streptomycetes, the Sec pathway constitutes the main secretion system (Anné et al., 2012). Accordingly, proteins to be secreted have N-terminal hydrophobic SP, followed by a longer hydrophobic H-domain and a C-terminal part containing at the end three amino acids which form the signal peptidase recognition site. Other minor secretion systems were reported, including the twin-arginine dependent translocation (Tat) pathway (Anné et al., 2012). The Tat machinery exports fully folded proteins across the cytoplasmic membrane: SPs that target proteins to this pathway resemble Sec SPs, but contain a conserved twin-arginine motif in the N-region (Valverde et al., 2018). A comparison between the efficiency of these two pathways for recombinant protein production showed that replacing Sec-dependent SP with Tat-dependent SP drastically reduced protein expression (Schaerlaekens et al., 2004). When native SPs were absent or not functional, heterologous genes were fused to SP encoding sequences from genes for highly expressed/secreted endogenous *Streptomyces* proteins (Anné et al., 2016), such as the one from the subtilisin inhibitor (*vsi*) of *S. venezuelae* CBS762.70 (Van Mellaert et al., 1998). Other SP sequences, frequently used in *Streptomyces* expression-systems are also listed in **Table 1**. They derived from the genes for the trypsin-like protease (*srT*) from *S. rimosus*, for the α-amylase from *Streptomyces tendae*, *S. griseus, S. lividans,* or *Streptomyces limoseus,* for the melanin operon gene (*melC1*) from *Streptomyces antibioticus,* for the subtilisin inhibitor (*ssi*) from *S. albogriseolus*. The final result is that in 77 out of the 94 proteins listed in **Table 1**, the recombinant proteins were completely secreted with productivities up to 100s of mg/L (Guan et al., 2015). In the few cases (8) where proteins were accumulated into cytoplasm, their productivity was generally low. 7 proteins were found produced either inside or outside the cells, whereas VanYn was localized in the cell wall fraction where it plays its physiological role in antibiotic resistance (Marcone et al., 2010a; Binda et al., 2012, 2013).

CONCLUSION

From the analysis of the literature in the last four decades, it emerges that, although promising, streptomycetes have been used for heterologous protein production less than their potential indicates to do. Notwithstanding their efficient protein secretion machine – which definitively facilitates downstream operations and protein purification – the mycelial lifestyle of these bacteria has probably discouraged scientists to use them more frequently. In liquid media, streptomycetes grow as mycelial pellets consisting of cells in different physiological states, and cultures are not homogenous and might become very viscous. In this regard, combining different specific mutations as *ssgA* for improving disperse growth (Sevillano et al., 2016), and *galK* for generating auxotrophic mutants not requiring antibiotic-dependent selection (Lichenstein et al., 1988) might facilitate upstream processes. Additionally, formulation

of novel cultivation media – replacing those used for antibiotic biosynthesis – could facilitate protein downstream (Binda et al., 2013; Berini et al., 2019). Another aspect probably limiting their application is that streptomycetes cannot be genetically manipulated by the methods commonly used for *E. coli* and *S. cerevisiae*. They need *ad hoc* protocols based on intergeneric conjugation or protoplast transformation (Kieser et al., 2000; Marcone et al., 2010b,c). With time, these protocols have become available and, as reported in this review, nowadays we can count on a large variety of vectors, promoters, and SP sequences. What is still missing is the systematic and critical comparison of the available toolkits. Optimization of protein production is still conducted following a case-by-case – and somehow random – approach. Finally, an important issue is the intrinsic low protein productivity of streptomycetes in comparison with the mostly used *E. coli* and yeasts. Further improvements, in this sense, are urgently needed and may derive from system and synthetic biology approaches, still poorly applied to streptomycetes. Indeed, progresses in system biology and -omics technologies may shed light on the interplay of elements involved in protein expression, thus helping in the rational improvement of both expression platforms and fermentation conditions, finalized at reducing the metabolic burden due to heterologous protein production. A demonstration is present in the pioneering work conducted by Muhamadali et al. (2015) on a *S. lividans* strain producing the murine TNF-α, where heterologous protein expression determined profound changes in the metabolomics of the streptomycete causing an overflow of organic acids and sugars. In post-genomic era, a further ambitious goal is applying synthetic biology approaches for building a *Streptomyces* 'super host' with metabolic networks rewired to facilitate heterologous protein expression. Synergic application of genome minimization strategies (i.e., systematic removal of those elements – as secondary metabolites or proteases – that can hamper protein production) and engineering of translation and transcription machineries, might help reaching this goal (Kim et al., 2020). To this end, it is encouraging considering that performing *Streptomyces* 'super hosts' have been already constructed for the heterologous production of antibiotics (Gomez-Escribano and Bibb, 2011; Myronovskyi et al., 2018). We believe that integrating these tools could help in improving streptomycetes as robust producers of recombinant proteins, increasing their competitiveness to other platforms and stimulating their large-scale application as cell factories.

AUTHOR CONTRIBUTIONS

FB and EB collected the data and analyzed them. FB, FM, and EB co-wrote the review. FB prepared the figure and the table. EB coordinated the work. All authors contributed to the article and approved the submitted version.

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An Environmental *Escherichia coli* Strain Is Naturally Competent to Acquire Exogenous DNA

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The diffusion of antibiotic resistance determinants in different environments, e.g., soil and water, has become a public concern for global health and food safety and many efforts are currently devoted to clarify this complex ecological and evolutionary issue. Horizontal gene transfer (HGT) has an important role in the spread of antibiotic resistance genes (ARGs). However, among the different HGT mechanisms, the capacity of environmental bacteria to acquire naked exogenous DNA by natural competence is still poorly investigated. This study aimed to characterize the ability of the environmental *Escherichia coli* strain ED1, isolated from the crustacean *Daphnia* sp., to acquire exogenous DNA by natural competence. Transformation experiments were carried out varying different parameters, i.e., cell growth phase, amount of exogenous DNA and exposition to artificial lake water (ALW) and treated wastewater to mimic environmental-like conditions that may be encountered in the agri-food system. Results were compared with those showed by the laboratory *E. coli* strain DH5α. Our experimental data, supported by genomic sequencing, showed that, when exposed to pure water, ED1 strain was able to acquire exogenous DNA with frequencies (10^{-8}–10^{-9}) statistically higher than the ones observed for DH5α strain (10^{-10}). Interestingly, higher values were retrieved for ED1 than DH5α strains exposed to ALW (10^{-7} vs. 10^{-9}, respectively) or treated wastewater (10^{-8} vs. 10^{-10}, respectively). We tested, therefore, ED1 strain ability to colonize the rhizosphere of lettuce, a model plant representative of raw-consumed vegetables of high economic importance in the ready-to-eat food industry. Results showed that ED1 strain was able to efficiently colonize lettuce rhizosphere, revealing a stable colonization for 14 days-long period. In conclusion, ED1 strain ability to acquire exogenous DNA in environmental-like conditions by natural competence, combined with its ability to efficiently and stably colonize plant rhizosphere, poses the attention to food and human safety showing a possible route of diffusion of antibiotic resistance in the agri-food system, sustaining the "One Health" warnings related to the antibiotic spread.

Keywords: antibiotic resistance, horizontal gene transfer, treated wastewater, rhizosphere, root colonization, *E. coli* genomes, One Health

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INTRODUCTION

Antibiotic Resistance (AR) is a public concern for global health. About 700,000 people die every year from antibiotic resistant bacteria-infections and 10 million annual deaths caused by antibiotic resistant pathogens are estimated by 2050 (Lim et al., 2019). In the last century, antibiotics have been widely used in medicine, plant production and livestock industries, imposing a strong selective pressure on the environmental microbial communities (Van Hoek et al., 2011). The exposition of bacteria to a sub-lethal concentration of antibiotics has led to the generation and diffusion of antibiotic resistant bacteria (ARB), through mutations and horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs) (Smalla et al., 2018). This can be particularly enhanced in specific hot spots of natural and engineered ecosystems, such as mycosphere, residuesphere, rhizosphere and wastewater treatment plants (WWTPs) (Eckert et al., 2018; Riva et al., 2020). The spread of ARGs in different environments linked to anthropogenic activities has been largely demonstrated: for example, long-term applications of sewage sludge and chicken manure can improve the abundance and the diversity of ARGs and ARB in soil (Chen et al., 2016), while WWTPs can be considered as one of the main ARGs' contaminated aquatic systems for both ARB and free DNA (Czekalski et al., 2014; Amos et al., 2015). Despite several studies have described the presence and spread of ARGs and ARB in the environment, some gaps of knowledge about the selection, evolution, persistence and HGT of ARGs remain to be unveiled (Larsson et al., 2018; Smalla et al., 2018).

HGT is crucial for bacterial adaptation to new environments and, consequently, for bacterial evolution. DNA transfer is generally accomplished by three "classical" mechanisms, namely transduction, conjugation and transformation (Sun, 2018). While in transduction and conjugation specific apparatuses are required to transfer DNA from donor to recipient cells, i.e., phage virions and conjugative pili, respectively, in transformation the acquisition of DNA is usually transient and linked to the capability of the bacterial cells to express competence at a specific physiological phase. Concerning the environmental ARG diffusion through HGT mechanisms, researchers have highlighted that many aspects have yet to be clarified, e.g., the contribution of the different mechanisms to ARG spread or the drivers of gene transfer (Smalla et al., 2018). For instance, since conjugation-based experiments are more feasible in laboratory and field conditions than those based on the other HGT mechanisms, this might have underestimated the importance of transformation or transduction (Smalla et al., 2018).

Natural competence for transformation is a specific physiological state in which bacteria are able to acquire genetic material from their surroundings. The acquired DNA can be then integrated into the bacterial genome or be maintained as a plasmid in the cell (Blokesch, 2016). There are more than 80 prokaryotic species described to be naturally transformable and different species and strains can show peculiar traits: for instance, *Vibrio cholerae* has been described to acquire DNA in presence of chitin (Meibom et al., 2005), while *Acinetobacter baylyi* is constitutively competent for transformation with frequency rates depending on the bacterial growth phase (Blokesch, 2016; Domingues et al., 2019). For a long time, *Escherichia coli* has not been considered a naturally transformable bacterium. *E. coli* is routinely forced to acquire exogenous DNA by artificial laboratory treatments, i.e., following the exposure to (i) solutions with high concentrations of divalent metal ions followed by heat shock, (ii) polyethylene glycol solutions, or (iii) electrical shock pulses (Hasegawa et al., 2018). Nonetheless, in some specific conditions, not related to the artificial transformation, *E. coli* has been demonstrated capable to acquire exogenous DNA, e.g., in contact with environmental waters (Baur et al., 1996; Woegerbauer et al., 2002; Ishimoto et al., 2008), in food extracts (Maeda et al., 2003) or after freeze-thaw processes. Besides the "classical" exogenous DNA uptake machinery of natural transformation, based on conserved proteins for the transport of single-stranded DNA (ssDNA) into the cell cytoplasm, two new routes of DNA acquisition by transformation have been recently identified in this species. In the first way, double-stranded DNA (dsDNA) is internalized into *E. coli* cells on agar plates, while the second DNA uptake mechanism depends on a cell-to-cell contact, not related to conjugation, and occurs in a colony on agar plates (Sun et al., 2006, 2009; Etchuuya et al., 2011; Sun, 2018). While the latter mechanism has been recently reported to be induced by a P1*vir* bacteriophage (Sugiura et al., 2017), the former foresees the participation of several proteins, among which researchers have so far identified *ydcS* and *ydcV* genes, encoding for a putative periplasmic protein and a putative inner membrane protein, respectively (both located on the putative ABC transporter *ydcSTUV* operon for putrescine transport; Sun, 2016) and the general stress response regulator factor RpoS (Zhang et al., 2012; Sun, 2016).

One of the main recognized routes that could allow AR spread in environments related to the agri-food system is the use of reclaimed water for irrigation purposes. Nowadays the water reuse represents a common practice in several countries and is considered a priority also by the European water management policy to combat the water crisis exacerbated by global warming (Riva et al., 2020). Indeed, at least 20 million hectares of croplands worldwide are irrigated with urban treated wastewater (Bouaroudj et al., 2019). WWTPs have been indicated as one of the main contributors of both cell bound and free ARGs for the aquatic systems (Czekalski et al., 2014; Amos et al., 2015; Li et al., 2018; Zhang et al., 2018); the reuse of treated wastewater for irrigation purposes would enter the food production and could contribute to the diffusion of ARGs that finally could potentially be acquired by pathogenic strains. Indeed, it has been found that WWTPs can promote, in the water in which the effluents are released, the stabilization of a resistome derived principally from treated wastewaters (Corno et al., 2019), making the freshwater bodies reservoirs of ARGs (Di Cesare et al., 2015). The ability of *E. coli* to acquire and transfer exogenous DNA (Hasegawa et al., 2018; Sun, 2018), together with its capability to survive and thrive in different habitats (i.e., water, rhizospheric soil or human gut; Van Elsas et al., 2011; Raimondi et al., 2019), where the presence of ARGs has been reported (Du et al., 2020; Osińska et al., 2020) and HGT can be enhanced (i.e., rhizosphere, Chen et al., 2019), could pose a risk for the food safety and public

health (Krzeminski et al., 2019). This risk could be high for fresh products such as spinach, sprout, and lettuce, which are generally consumed as raw vegetables (Shen et al., 2019). Indeed, antibiotic resistant bacteria belonging to the pathogenic species *E. coli* and *Salmonella enterica* have been already reported in farm environments and fresh products, including lettuce and ready-to-eat food (Nüesch-Inderbinen et al., 2015; Araújo et al., 2017; Schierstaedt et al., 2019; Perera et al., 2020; Yang et al., 2020).

In the framework of "One Health" approach, this study aimed to (i) characterize the possible acquisition of exogenous DNA by an environmental strain of *E. coli* mimicking the conditions that may be encountered in the agri-food system, and to (ii) study the *E. coli* strain capacity to colonize plant rhizosphere, using soil potted lettuce as model system.

MATERIALS AND METHODS

Strains and Media

Escherichia coli strain ED1 was isolated from individuals of *Daphnia* sp. collected from a small rainwater-fed pond in the garden of the CNR-IRSA, Verbania, Italy. Thirty daphnids (in triplicates) were washed in sterile Milli-Q water, crushed and sonicated (3 times, 1 min each cycle with a shaking application by vortexing between cycles) in 1 ml of 2M NaCl. Serial 10-fold dilutions were prepared and filtered on nitrocellulose membrane filters (type GSWP, 25 mm diameter, 0.22 μm pore size, Millipore) which were placed onto agar plates of the selective medium mFC (Biolife) and incubated for 24–48 h at 37°C. Once colonies of presumptive *E. coli* (blue color on mFC agar) appeared on plates, they were purified by streaking three times and then stored in 25% glycerol solutions at −80°C. A small amount of the bacterial biomass was then introduced in 1 ml of 2M NaCl, centrifuged (5000 g, 10 min, 4°C), boiled 15 min, frozen for 2–4 h and finally centrifuged as before. One of the isolate, named ED1, was identified as an *E. coli* strain due to positive amplification of the *uid*A gene (Srinivasan et al., 2011) by PCR as described elsewhere (Sabatino et al., 2015).

Preparation of Transforming Exogenous DNA

Transformations were carried out by using pCR™II-TOPO® (Invitrogen) plasmid carrying ampicillin and kanamycin resistance genes. The plasmid was extracted from the strain *E. coli* Mach1™ T1 Phage-Resistant pCR™II-TOPO® using the QIAPrep® Spin Miniprep Kit (Qiagen, Milan, Italy) following the manufacturer instructions. The plasmid was quantified by measuring the optical density at 260 nm wavelength in a spectrophotometer (BIO RAD SmartSpec™ 3000).

Natural Transformation Protocol

Precultures of ED1 and DH5α strains were firstly grown in 25 ml of LB liquid medium overnight at 37°C with shaking. Then, 1 ml of cultures were diluted in 100 ml of LB and incubated at 37°C until the cells reached early exponential or stationary growth phases, i.e., at optical densities at 600 nm ($OD_{600\ nm}$) of 0.4–0.5 or 2, respectively. Forty milliliter of cells were then centrifuged twice with Milli-Q water for 10 min at 2700 g and finally resuspended in 500 μl of the same washing buffer. All centrifugation steps were performed at room temperature (RT) between 20 and 23°C. Four aliquots of 100 μl of cells were prepared, and the proper quantities of plasmidic DNA were added and gently mixed, without pipetting (the mixture is hereafter named as transformation mixture). The remaining 100 μl-cells aliquot was used as negative control (no DNA was added). Samples were incubated at RT for 1 h: three aliquots were then plated on LB plates added with ampicillin (100 μg/ml), while the fourth aliquot was serially diluted (from the undiluted sample to -8) and used to evaluate (in triplicate) the total cell count on LB agar plates without the antibiotic selection. Aliquot of negative control was plated as well on LB plates added with ampicillin (100 μg/ml). All the plates were kept at 37°C overnight. Experiments were performed with three biological replicates. Putative colonies of transformants, retrieved by ampicillin selection, were then streaked on LB plates added with kanamycin (100 μg/ml). Both ED1 and DH5α strains are sensitive to 100 μg/ml ampicillin, 100 μg/ml kanamycin and 50 μg/ml rifampicin. To further confirm the plasmid acquisition, kanamycin-resistant colonies were also subjected to PCR amplification. Transformation frequencies were calculated as the ratio between the number of transformants and the total number of culturable cells (about 10^9 cell/ml in case of cells harvested at the exponential phase and 10^{10} cell/ml in case of cells harvested at the stationary phase). Bacterial transformation was performed using 0.25, 0.5, 1, and 2 μg of plasmidic DNA.

Transformation protocols were then carried out using cells collected at the early exponential phase and exposing them to 2 μg of plasmidic DNA in two different types of water as washing and incubation buffers: besides Milli-Q water (pH 6.23) we used (i) artificial lake water (ALW, pH 7.69) prepared modifying the protocol of Zotina et al. (2003) in regard to the inorganic medium components (**Supplementary Table 1**), and (ii) water collected from the effluent of a WWTP located in Verbania (pH 6.84; water sampled on December 10th, 2019; **Supplementary Table 1**), serving 51,000 population equivalent and equipped with chlorination as disinfection process (Di Cesare et al., 2016). In order to reduce the presence of environmental bacteria, water samples were filtered through nitrocellulose membrane filters with 0.22 μm pore size (Millipore).

DNase sensitivity was tested by adding DNase I to the transformation mixture at different times, e.g., immediately after the transformation mixture preparation and after 1, 3, 4, 6, and 18 h from the preparation of the transformation mixture. Then, the transformation mixtures containing DNase I were incubated 1 h at RT before plating on LB agar plates added with ampicillin (100 μg/ml) (Sun et al., 2006).

To verify the acquisition of pCR™II-TOPO® plasmid, DNA was extracted from putative transformants by boiling lysis (Ferjani et al., 2015) and used as template to amplify a plasmid sequence fragment of about 250 bp with primer M13f (-20) (5′-GTA AAA CGA CGG CCA G-3′) and M13r (5′-CAG GAA ACA GCT ATG AC-3′) according to manufacturer's (Invitrogen) instruction. Thermal protocol was set up as follows: 94°C for

5 min, followed by 34 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min and the last step at 72°C for 10 min.

Generation of Rifampicin Resistant Mutants of *E. coli* Strains ED1 and DH5α

Rifampicin mutants of ED1 and DH5α strains were obtained by plating stationary-phase cultures on LB plates added with 50 μg/ml of rifampicin. Plates were then incubated at 37°C overnight. Upon appearance, rifampicin resistant (RIF-R) colonies were selected and initially re-streaked on LB added with 50 μg/ml rifampicin and, finally, on LB added with 100 μg/ml rifampicin.

Root Colonization by *E. coli* Strains

RIF-R ED1 and RIF-R DH5α strains were used for the bacterization of *Lactuca sativa* (var. Canasta) seedlings to verify their ability to colonize plant rhizosphere. Lettuce seeds were sterilized with 0.7% sodium hypochlorite for 5 min followed by 5 rinsing steps in sterile distilled water (Bonaldi et al., 2015) and grown in pots filled with non-sterile soil under greenhouse conditions. Three days after sowing, lettuce seedlings (n = 3 for each strain) were inoculated with 5 ml of bacterial suspensions obtained by growing the RIF-R ED1 and RIF-R DH5α strains in LB medium supplemented with rifampicin (100 μg/ml) for 24 h at 37°C, centrifuging twice the bacterial cultures at 4000 rpm for 10 min and re-suspending the pelleted cells in physiological solution (NaCl 0.9%) to obtain a final bacterial concentration of 10^8 cell/g of soil. Six lettuce seedlings were irrigated with 5 ml of distilled water and considered as negative control. One week after bacterization, lettuce seedlings were harvested and the rhizosphere soil was separated from the root by vortexing for 5 min the root system in physiological solution. To evaluate the number of colony-forming units (cfu) per gram of soil, rhizosphere samples (n = 3 for each strain; n = 6 for negative control) were serially diluted in physiological solution, plated in triplicate on LB medium supplemented with rifampicin (100 μg/ml) and cfu were counted after 24 h of incubation at 30°C. In order to confirm the identity of the isolates, after the visual check of colony morphology on the Petri dishes, 10 bacterial colonies isolated from the rhizosphere of each bacterized lettuce seedlings were picked. The DNA was extracted through boiling cell lysis and the 16–23S rRNA Intergenic Transcribed Spacer (ITS) region was amplified by ITS-PCR fingerprinting (Mapelli et al., 2013), comparing the ITS profiles of the bacteria re-isolated from the rhizosphere at the end of the experiment with those of RIF-R ED1 and RIF-R DH5α strains used for lettuce bacterization.

The colonization experiment was repeated to investigate the stability of ED1 and DH5α strains in the lettuce rhizosphere over time (14 days). For this experiment, lettuce seeds were sterilized as reported above and grown in soil previously sterilized through tindalization process. One week after sowing, lettuce seedlings were inoculated with 5 ml of bacterial suspensions (10^8 cell/g of soil) prepared as described above. The presence of RIF-R ED1 and RIF-R DH5α strains in lettuce rhizosphere was verified 1 week (t1) and 2 weeks after bacterization (t2). As previously described, rhizosphere soil samples (n = 3 for each strain and each experimental time) were serially diluted and plated in triplicate on LB medium supplemented with rifampicin (100 μg/ml). Assessment of cfu/g of soil and strain identity were performed as described above.

DNA Extraction, Genome Sequencing, and Analysis

Genomic DNA from *E. coli* strain ED1 was extracted from an overnight culture in LB liquid medium using the UltraClean Microbial DNA extraction kit (Qiagen), according to the manufacturer's protocol. DNA quantity was assessed using fluorometry (Qubit, Invitrogen) according to the manufacturer's protocol. Sequencing was performed on an Illumina NovaSeq platform using paired-end sequencing of 150 bp fragments at IGA Technologies (Udine, Italy). The genome was assembled as described by Cabello-Yeves et al. (2018): briefly, *Trimmomatic* was used for read trimming and filtering and *SPAdes* for the genome assembly, while a preliminary gene annotation was done using NCBI (Johnson M.et al., 2008). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAAWVB000000000. The version described in this paper is version JAAWVB010000000.

Genome assemblies of *E. coli* strains ED1 and K12 NEB DH5α (Accession Number CP017100; Anton and Raleigh, 2016) were submitted to the RAST Service[1] and compared taking advantage of the RAST function-based comparison tool. Plasmid presence in ED1 genome was investigated through the platform PlasmidFinder (Carattoli et al., 2014)[2]. VirulenceFinder 2.0 platform (Joensen et al., 2014)[3] was used to identify virulence genes in the genomes of *E. coli* strains ED1, K12 NEB DH5α, O157:H7 Sakai (Accession Number BA000007, Makino et al., 1999) and O157:H7 EDL933 (Accession Number AE005174, Perna et al., 2001). Genomic islands, insertion sequences (IS) and phage genome sequences were searched in ED1 and K12 NEB DH5α genomes by IslandViewer4 (Bertelli et al., 2017), ISfinder (Siguier et al., 2006) and PHASTER (Arndt et al., 2016). Details on RAST and NCBI annotation can be found in **Supplementary Table 2**.

Statistical Analyses

Statistical analyses were conducted with R 3.1.2 (R Core Team, 2013) through RStudio (RStudio Team, 2015) and with Calc Statistical Function of Microsoft® Office Excel. Linear model was applied to assess the relation between transformation frequency and quantities of DNA added during transformation protocols. Student's t-test was employed to verify differences between ED1 and DH5α strains concerning transformation frequencies (considering growth phase and types of water) and root colonization efficiency.

[1]http://rast.nmpdr.org/

[2]https://cge.cbs.dtu.dk/services/PlasmidFinder/

[3]https://cge.cbs.dtu.dk/services/VirulenceFinder/

RESULTS

Influence of Different Growth Phases on Transformation

The capability to acquire exogenous DNA by the environmental *E. coli* strain ED1, compared with the laboratory *E. coli* strain DH5α, was initially tested in pure water on resting cells harvested at different phases of the growth curve: Milli-Q water was used as washing and incubation buffer (to avoid the presence of interfering cations) and a large amount of transforming DNA plasmid (2 μg) was added to minimize any possible interference on transformation frequencies linked to a limiting quantity of DNA. First, we used cells harvested from early exponential phase cultures ($OD_{600\ nm}$ between 0.4 and 0.5) (**Supplementary Figure 1**), observing a transformation frequency of 4.26×10^{-8} ($\pm$ 2.26×10^{-8}) and 4.44×10^{-10} ($\pm$ 7.70×10^{-10}) for ED1 and DH5α strains, respectively (**Supplementary Table 3**). ED1 cells in early exponential growth phase demonstrated a significantly higher transformation frequency in comparison with DH5α cells (Student's *t*-test, $p = 0.032$, **Figure 1**). When cells were harvested at the stationary phase ($OD_{600\ nm}$ between 2.1 and 2.2), a transformation frequency of 3.95×10^{-9} ($\pm$ 3.91×10^{-10}) was obtained with ED1 strain, resulting however statistically higher than the value recovered for DH5α strain ($1.93 \times 10^{-10} \pm 1.56 \times 10^{-10}$; Student's *t*-test, $p = 0.0001$, **Figure 1** and **Supplementary Table 3**). While transformation frequencies of DH5α strain were not significantly different between both growth phases (Student's *t*-test, $p = 0.609$), statistical analysis indicated that ED1 natural competence is significantly higher in the early exponential phase than in the stationary one (**Figure 1**; Student's *t*-test, $p = 0.0415$). All the following transformation assays were therefore run with cells at the early exponential phase.

In order to confirm the occurrence of natural transformation (which is a DNase-sensitive mechanism differently from the DNase-resistant mechanisms i.e., conjugation and transduction), we checked the sensitivity of ED1 uptake of DNA to the addition of DNase I. Since no transformation events were retrieved, unveiling thus the DNase sensitivity of the mechanism, we confirmed ED1 cells' ability to uptake DNA by natural competence (Hasegawa et al., 2018).

Influence of Exogenous DNA Quantity on Transformation Frequency

Transformation frequencies of ED1 and DH5α strains were analyzed in Milli-Q water with increasing quantities of plasmid pCR® II-TOPO® as exogenous DNA, by adding 0.25, 0.5, 1, and 2 μg of plasmidic DNA to the cells harvested at the early exponential phase. As shown in **Supplementary Table 4**, transformation frequency for DH5α strain was estimated to be $\leq 4.44 \times 10^{-10}$, while increasing transformation frequencies were reported for ED1 strain, ranging from 5.48×10^{-9} to 4.26×10^{-8} when increasing quantities of plasmid from 0.25 to 2 μg, respectively, were added. Statistical analysis revealed a statistical difference for ED1 strain exposed to 2 or 0.25 μg of plasmidic DNA (Student's *t*-test, $p = 0.0480$ between 2 and 0.25 μg). As shown in **Figure 2**, transformation frequency of ED1 strain was significantly related to the amount of plasmid added (linear model: $t = 3.9$, $p = 0.003$), whereas this was not the case for DH5α strain (linear model: $t = 0.55$, $p = 0.6$) (Baur et al., 1996).

Bacterial Transformation in Different Types of Waters

Transformation of ED1 and DH5α strains was assessed in natural and artificial water solutions considered as representative of environmental habitats, i.e., the artificial lake water (ALW)

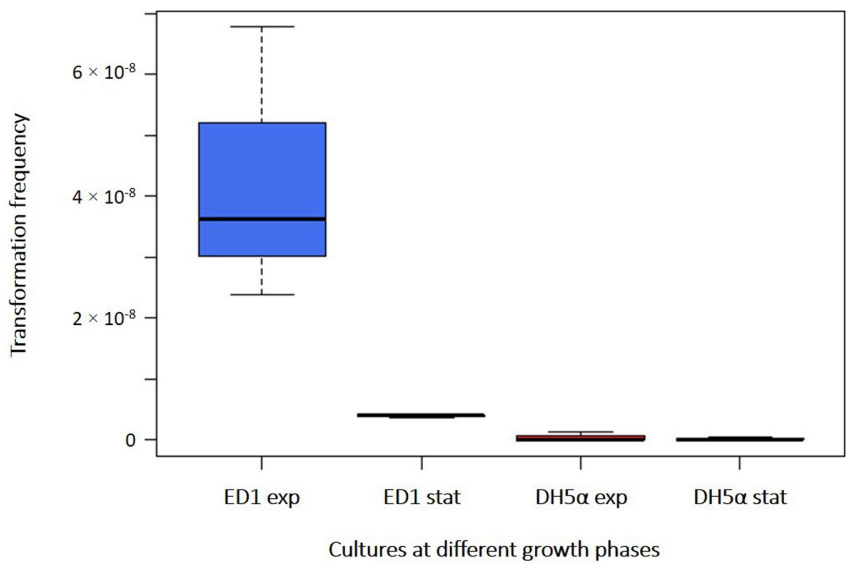

FIGURE 1 | Transformation frequencies of *E. coli* strains ED1 and DH5α with cells collected at early exponential ("exp") and stationary ("stat") phases. Transformations were performed in Milli-Q water with 2 μg of plasmidic DNA.

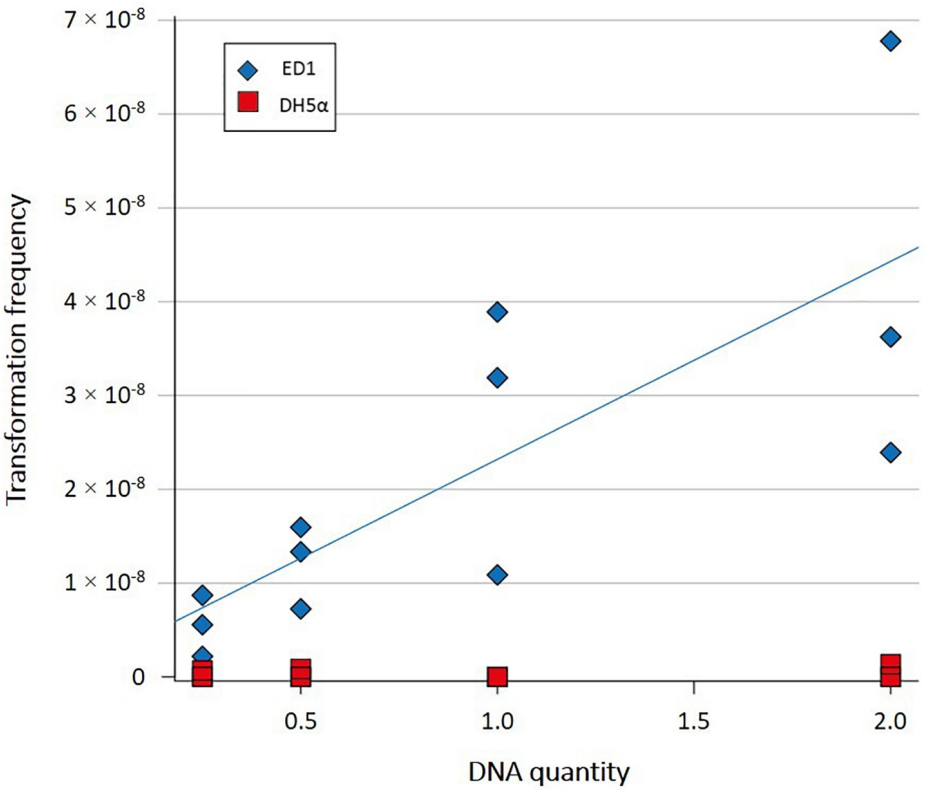

FIGURE 2 | Transformation frequencies of *E. coli* strains ED1 and DH5α in Milli-Q water with increasing quantities of plamidic DNA. Transformation frequency of ED1 strain was significantly related to the amount of plasmid added (linear model: $t = 3.9$, $p = 0.003$).

and the water collected from the effluent of Verbania WWTP. Milli-Q water was used as control and the transformations were carried out with a not limiting quantity of transforming DNA (2 μg). Statistical analysis showed that the transformation frequencies of ED1 strain were significantly higher than the ones observed for DH5α strain considering all the types of water used (Student's test; $p = 0.0295$, 0.0226, and 0.0364 with ALW, Milli-Q water and treated wastewater, respectively, **Figure 3**). Transformation frequencies $\leq 5.19 \times 10^{-9}$ were obtained for DH5α strain in the different types of water (**Supplementary Table 5**). Moreover, transformation frequencies of ED1 strain were significantly higher in ALW than in the other types of water (**Figure 3**; Student's *t*-test *p*-values: between Milli-Q water and ALW, $p = 0.029$; between ALW and treated wastewater, $p = 0.047$): specifically, we obtained for this strain transformation frequencies values of 1.06×10^{-7} ($\pm 5.26 \times 10^{-8}$) in ALW and 1.83×10^{-8} ($\pm 9.80 \times 10^{-9}$) in the effluent water released into the environment from Verbania WWTP, whereas for the control in pure water a value of 4.26×10^{-8} ($\pm 2.26 \times 10^{-8}$) was retrieved (**Supplementary Table 5**).

Plant Colonization by *E. coli* Strains

The ability of ED1 and DH5α strains added to the soil to colonize plants' rhizosphere was verified using the correspondent RIF-R strains and lettuce seedlings as model system. The experiment was firstly conducted in short term conditions in non-sterile soil to check the rhizocompetence of *E. coli* strains in presence of the competing soil dwelling microbial community. Seven days after *E. coli* addition to the 3 days-old plantlets surrounding soil, the rifampicin resistant bacteria re-isolated from the rhizosphere of the lettuce seedlings amounted to 1.59×10^9 ($\pm 8.29 \times 10^8$) cfu/g rhizospheric soil for RIF-R ED1 strain and resulted statistically higher in comparison to rifampicin resistant bacteria isolated from the rhizosphere of both non-bacterized lettuce seedlings ($1.97 \times 10^5 \pm 1.62 \times 10^5$ cfu/g soil; $p = 1.61 \times 10^{-7}$) and seedlings bacterized with RIF-R DH5α strain ($4.23 \times 10^8 \pm 4.45 \times 10^8$ cfu/g soil; $p = 1.14 \times 10^{-3}$), as shown in **Figure 4A**. Ten randomly picked colonies isolated from each bacterized plant ($n = 30$ per ED1 strain bacterization; $n = 30$ per DH5α strain bacterization) were subjected to ITS-PCR fingerprinting. The ITS profiles detected for all colonies corresponded to those of the *E. coli* strains used for plants bacterization, as shown in **Supplementary Figure 2A** and **Supplementary Figure 2B** for ED1 and DH5α, respectively. Although both the tested *E. coli* strains were able to colonize in 7 days the lettuce rhizosphere under non-sterile soil condition, the environmental *E. coli* strain ED1 showed a higher colonization performance of this microhabitat compared to the laboratory strain DH5α ($p = 1.92 \times 10^{-3}$).

Similar results were obtained when the experiment was repeated with lettuce plants older (7 days-old) than those

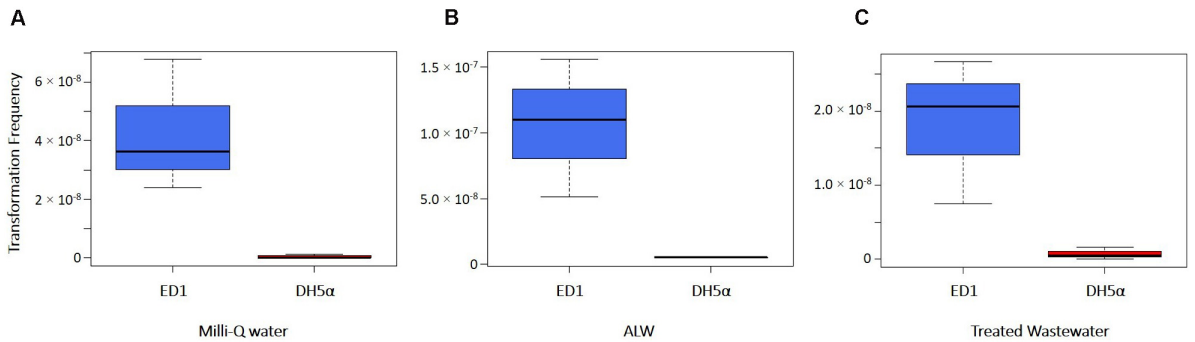

FIGURE 3 | Transformation frequencies of *E. coli* strains ED1 and DH5α using different types of water: **(A)** Milli-Q water, **(B)** ALW, and **(C)** treated wastewater (WW).

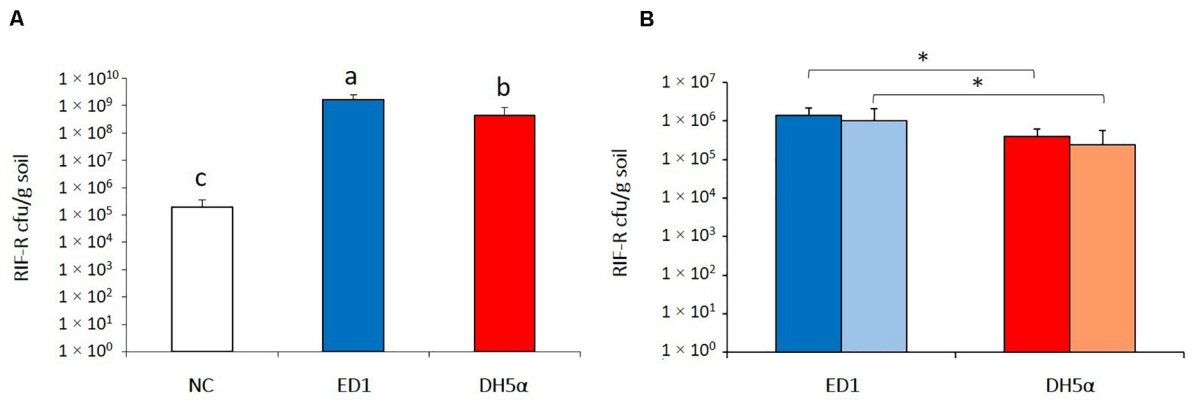

FIGURE 4 | Evaluation of *E. coli* strains ED1 and DH5α rhizocompetence. **(A)** Bacterial abundance (cfu/g soil) of rifampicin-resistant bacteria in the lettuce rhizosphere after a 1-week colonization experiment performed with non-sterile soil. **(B)** Assessment of the stability of the rifampicin-resistant bacteria abundance in the rhizosphere of lettuce seedlings grown in sterile soil. Dark- and light-colored columns indicate cfu/g of soil 1 and 2 weeks after the bacterization, respectively. NC, non-bacterized lettuce seedlings; ED1/DH5α, lettuce seedlings bacterized with ED1 and DH5α strains, respectively. Different letters or asterisks indicate statistically significantly differences according to Student's *t*-test ($p < 0.01$).

used in the first colonization assay in sterile soil and for a longer period, to verify the stability of the strains in the rhizosphere microhabitat, without any competition with the soil residing microbiota. As shown in **Figure 4B**, 1 week after plant bacterization with ED1 strain, 1.35×10^6 ($\pm$ 8.39×10^5) cfu/g soil of RIF-R cells were recovered, whereas plants exposed to DH5α strain led to isolate from the lettuce rhizosphere a significant lower RIF-R titer ($3.86 \times 10^5 \pm 2.38 \times 10^5$ cfu/g soil; $p = 0.0043$). The RIF-R isolated colonies in all the assays demonstrated to belong to the inoculated *E. coli* strains by evaluating their ITS-PCR fingerprinting on representative colonies (**Supplementary Figure 3**). Two weeks after bacterization the presence of both ED1 and DH5α *E. coli* strains remained stable in plants rhizosphere, amounting, respectively to 1.01×10^6 ($\pm$ 1.03×10^6) cfu/g soil and 2.44×10^5 ($\pm$ 3.33×10^5) cfu/g soil ($p = 0.048$; **Figure 4B**).

Genome Analysis

Taking advantage of RAST function-based comparison tool, genomes of *E. coli* strains ED1 and K12 NEB DH5α (Anton and Raleigh, 2016; Accession Number CP017100), composed of 5,159,712 and 4,583,637 bp, respectively, were compared. *E. coli* strain K12 NEB DH5α has been chosen as reference strain for genomic analysis since it is a fhuA2 derivative of *E. coli* K12 DH5α, the genome sequence of which is not currently available.

Both genomes shared a high percentage of metabolic functions: indeed, the presence of all the main basic metabolic functions, such as, for instance, carbohydrate metabolism or respiration, was assessed. Differences in the genomes' size are primary reflected in the fact that ED1 strain owns a larger number of genes (included in the below reported metabolic pathways) than K12 NEB DH5α strain. We detected the presence of the "propanediol metabolic pathway" and the "methylcytrate cycle" in ED1 genome, which were absent in K12 NEB DH5α genome (**Table 1**). Furthermore, in ED1 genome we retrieved genes encoding proteins related to the osmoregulatory choline-glycine betaine system e.g., the high-affinity choline uptake protein BetT, a choline dehydrogenase and a betaine aldehyde dehydrogenase (**Table 1**). Only in the genome of ED1 we found several genes classified by RAST as involved

TABLE 1 | Main subsystems revealed in the genome of *E. coli* strain ED1.

Role	CDS ID in ED1 genome*	ED1
IncF conjugative transfer genes	HBA78_21285-HBA78_21370, HBA78_21380-HBA_21390, HBA78_21405-HBA78_21425, HBA78_21435-HBA78_21455,	+
Propapendiol metabolism pathway	HBA78_16485-HBA78_16525, HBA78_16535-HBA_7816560, HBA78_16570-HBA78_16580	+
Type I secretion system LapB, C, E	HBA78_15000, HBA78_15005, HBA78_ 15015	+
CFA/I fimbriae encoding system	HBA78_08810, HBA78_08825	+
Type III secretion system	HBA78_04615, HBA78_04630, HBA78_04645, HBA78_04655-HBA78_ 04665, HBA78_04680, HBA78_04690, HBA78_04695, HBA78_20315, HBA78_ 20335	+
Choline and Betaine Uptake and Betaine Biosynthesis	HBA78_08680, HBA78_08695	+
Hydroxyaromatic non-oxidative decarboxylase protein	HBA78_20830, HBA78_20835	+

*"+" indicates presence of functions; *Details on RAST and NCBI annotation can be found in* ***Supplementary Table 2****.*

in the bacterial adhesion and secretory systems, i.e., CFA/I pili, the secretion system type I and the type III secretion injectosome (**Table 1**).

Considering genes related to the acquisition of exogenous DNA, the automatic annotation revealed in both genomes the presence of several genes homologous to those required for the DNA uptake in species that are known to be naturally competent: *pilQ/HofQ* (HBA78_15695 and NEB5A_17330; HBA78 code refers to ED1 strain, while NEB5A one refers to K12 NEB DH5α strain), encoding for a transmembrane channel allowing dsDNA to cross the outer membrane; *pilA* (HBA78_09875; NEB5A_00545), *pilB* (HBA78_09880 and NEB5A_00540), *pilC* (HBA78_09885; NEB5A_00535), related to the construction of the pseudopilus; *dprA* (HBA78_16130; NEB5A_16795), also called *smf*, responsible of the DNA processing and *ycaI/ComEC* (HBA78_03445; NEB5A_04210) related to the uptake of exogenous DNA (Chen and Dubnau, 2004; Cameron and Redfield, 2006; Sun, 2018). We detected in both genomes the presence of genes involved in one of the two *E. coli*-specific mechanisms of natural transformation, i.e., the general stress response regulator factor RpoS (HBA78_20820; NEB5A_05530) (Zhang et al., 2012; Sun, 2018), as well as the RpoS-regulated genes *ydcS* and *ydcV* (HBA78_24185 and HBA78_24200) in ED1; NEB5A_07355 and NEB5A_07370 in K12 NEB DH5α) (Sun, 2016). Additional analysis was performed submitting ED1 genome to the PlasmidFinder platform (Carattoli et al., 2014; Yang et al., 2015; **Table 1**): we found the presence of i) a IncFII plasmid replicon sequence (with an identity of 96.55% against the one of the reference sequence AY458016) and ii) a IncX1 plasmid replicon sequence (with an identity of 95.23% against the one of the reference sequence JN935898). The replicon sequences were located on two separate contigs of 79,647 and 25,889 bp, respectively, and allowed us to speculate the presence of two plasmids in ED1 chromosome.

In order to identify virulence factors, we further analyzed the genomes of strains ED1 and K12 NEB DH5α through the platform VirulenceFinder 2.0 (Joensen et al., 2014). We found a higher number of virulence factors in ED1 than in DH5α genome (**Table 2**). Both genomes showed the presence of the glutamate decarboxylase (GAD) system which contributes to acid resistance in the human gut (Vanaja et al., 2009). Conversely, we detected only in ED1 genome the presence of genes encoding the adhesin *air*, an enteroaggregative immunoglobulin repeat protein involved in bacterial aggregation and colonization (Sheikh et al., 2006), *astA*, a heat stable enterotoxin-1 (Yatsuyanagi et al., 2003) and *eilA*, a putative activator of the type three secretion system (T3SS), which contributes to the pathogenicity of enteroaggregative *E. coli* (EAEC) strains (Sheikh et al., 2006). Moreover, from the comparison with DH5α genome we found that ED1 genome lost *iss* virulence factor, defined as a serum survival gene (Johnson T.J. et al., 2008). When we included in our analysis the genomes of two pathogenic strains of *E. coli*, i.e., *E. coli* strains O157:H7 Sakai (Accession Number BA000007, Makino et al., 1999) and O157:H7 EDL933 (Accession Number AE005174, Perna et al., 2001), we could observe that a conspicuous higher number of virulence factors was retrieved in the latter than in ED1 or K12 NEB DH5α genomes (**Supplementary Table 6**). Whereas similar numbers of genomic islands are present in both genomes, the number of IS sequences predicted in ED1 genome is higher than the one retrieved for NEB DH5α genome. Moreover, we found more phage genomic sequences in the former than in the latter strain (**Supplementary Table 7**).

DISCUSSION

Several studies have revealed the modest capability of *E. coli* strains to acquire exogenous DNA by natural transformation and researchers have recently underlined the existence of a few peculiar DNA uptake mechanisms of natural transformation in this species (Sun et al., 2006; Guo et al., 2015; Hasegawa et al., 2018). *E. coli* laboratory strains, known for their high artificial transformation efficiency, demonstrated to undergo to natural transformation in experiments mimicking natural conditions, e.g., using freshwater or food extracts (Baur et al.,

TABLE 2 | Virulence genes revealed by the analysis of the genomes of *E. coli* strains ED1 and K12 NEB DH5α using the platform VirulenceFinder 2.0.

		ED1	K12 NEB DH5α
air	Enteroaggregative immunoglobulin repeat protein	+	−
astA	Heat stable enterotoxin-1	+	−
eilA	hilA-like regulator in enteroaggregative *E. coli*	+	−
gad	Glutamate decarboxylase	++	++
iss	Increased serum survival	−	+

"+" indicates presence of functions; "−" indicates absence of functions. Number of " + " indicates the number of sequences of virulence factors detected in genomes.

1996; Woegerbauer et al., 2002; Maeda et al., 2003, 2004), whereas a limited number of publications verified natural competence in *E. coli* strains isolated from human and warm-blooded animals (Tsen et al., 2002; Woegerbauer et al., 2002; Matsumoto et al., 2016). Environmental *E. coli* strains, to our knowledge, were never tested for natural competence. In this study, we investigated the ability of the environmental *E. coli* strain ED1, isolated form the crustacean *Daphnia* sp., to acquire exogenous DNA, comparing the results with the ones showed by the laboratory *E. coli* strain DH5α in relation to the cell growth phase, amount of transforming DNA and in environmental-mimicking conditions, i.e., exposed to lake water and WWTP effluents.

We ascertained a higher transformation frequency (10^{-8}–10^{-9}) for the environmental strain than for the laboratory one (10^{-10}), observing a higher number of transformation events when high quantities of plasmidic DNA were used, up to a saturation level (Baur et al., 1996). Values retrieved for ED1 strain underlined the modest capability of transformation in *E. coli* strains, especially if compared with other bacterial strains known to be naturally competent, such as *Acinetobacter baylyi* BD413 (Lorenz et al., 1992) and *Bacillus subtilis* 168 (Hauser and Kanamata, 1994). As reported by Baur et al. (1996), our results showed higher transformation frequencies for ED1 strain with cells grown at early exponential growth phase (0.4–0.6 $OD_{600\ nm}$) rather than at the stationary one. Log-phase cells were also used by Woegerbauer et al. (2002) who compared the transformation frequency and efficiency of laboratory and clinical isolates, revealing higher transformation rates for the former. In case of DH5α strain we recovered low values of transformation frequencies: we retrieved only two transformants in all the replicates in which Milli-Q water and 2 μg of DNA were applied. Nevertheless, other studies reported in case of DH5α strain higher numbers of transformants or transformation frequencies than the ones we obtained, likely due to differences of the adopted experimental protocols, which included, among the others, variations of the bacterial growth condition and growth phase (Woegerbauer et al., 2002; Sun et al., 2006).

The protocol we adopted in our experiments was conceived to mimic conditions feasible in the environment. To this aim, strains were subjected to a few manipulation procedures before incubation on selective agar plates and were exposed to different kinds of waters considered as representative of a few habitats (i.e., ALW and treated wastewater). Moreover, temperatures of 20–23°C, closer to environmental values than the ones usually used in laboratory procedures, were maintained during the transformation protocol (not for the incubation), differently from what reported in literature i.e., 37°C (Sun et al., 2006), 10°C or temperature shifts (Baur et al., 1996). Although it was reported that disinfection by-products in the WWTP effluents can enhance the rate of bacterial transformation, promoting the spread of extracellular ARGs (Augsburger et al., 2019; Mantilla-Calderon et al., 2019; Jin et al., 2020; Lu et al., 2020), ED1 strain showed higher transformation efficiency in presence of ALW than treated wastewater. This could be due, on one hand, to a water composition of ALW that was more similar to that of the original habitat of the bacterium; on the other, lower transformation frequencies detected for ED1 strain in presence of treated water than ALW could be related to the peculiar chemical composition of the sampled water (Pereira et al., 2015; Papageorgiou et al., 2016). Thus, we cannot rule out that experiments performed with water collected in different moments could bring the same results. Certain natural and anthropic environments could supply optimal conditions for natural transformation. An example are biofilms in which cell density is very high and cells can be exposed to high concentrations of free DNA (even higher than the ones routinely used in laboratory procedures) derived from the dead neighboring cells (Baur et al., 1996; Hasegawa et al., 2018); this condition can result in ARGs acquisition and spread in the bacterial communities, as characterized in several studies (Petrovich et al., 2018). Moreover, clinically relevant ARGs enter freshwater systems through the outflow of WWTPs (Zhang et al., 2018).

Gram-positive and Gram-negative bacteria that are known to be naturally transformable usually share a similar DNA uptake machinery linked to the Type IV pili and Type II secretion systems (Claverys and Martin, 2003) and both ED1 and K12 NEB DH5α showed the presence of these genes in their genomes. Taking into account the peculiar *E. coli* DNA uptake machineries (Sun, 2018), we found the presence of the genes encoding the transcriptional regulator RpoS that regulates *E. coli* natural transformation (Zhang et al., 2012), as well as the RpoS-regulated genes *ydcV* and *ydcS*, which are involved in the DNA internalization into the inner membrane (Sun, 2016). Although we retrieved in both *E. coli* strain genomes the presence of the above-mentioned genes, we demonstrated that ED1 transformation frequency was higher than DH5α one. Even though we observed an overall genomic function-based similarity between the strains (using the RAST function-based comparison tool), we cannot exclude the existence of some signaling-dependent or regulatory mechanisms that can favor natural transformation in ED1 rather than in DH5α strain. Natural transformation is known to be a very complex mechanism activated differently among species and strains (Lorenz and Wackernagel, 1994; Blokesch, 2016). For instance, in *Haemophilus influenzae* natural competence was demonstrated to be triggered by a lack of phosphotransferase system (PTS) sugars and purine precursors (Mell and Redfield, 2014). Furthermore, since only a DNA-based analysis has been performed in our study, we do not have information about the effective production of the proteins corresponding to the natural transformation-related genes.

Genomic analysis allowed to identify a larger number of genes encoding for metabolic pathways in ED1 genome rather than in the one of K12 NEB DH5α strain, e.g., we found in ED1 genome the propanediol utilization pathway, which allows *E. coli* to grow in anaerobic conditions using rhamnose as carbon source (Liu et al., 2007) and the genes of methilcytrate cycle, which allows microorganisms to use propionate as a carbon/energy sources, being especially useful in the propionate-rich environments such as the gastrointestinal tract (Upton and McKinney, 2007). Furthermore, we found several genes that may help ED1 to thrive in different habitats, i.e., genes encoding for proteins related

to the osmotic stress (involved in the synthesis and uptake of compatible solutes; Sim et al., 2014); genes involved in cell to cell aggregation and biofilm production, such as RTX that seems to be responsible for cell-surface adhesions, cells' aggregation and production of biofilm (Tchagang et al., 2018); CFA/I pili-related genes implicated in the bacterial adhesion through the production of fimbriae; and genes encoding the type III secretion injectosome (Diepold et al., 2011; Zheng et al., 2019; **Table 1**). Therefore, strain ED1 has different traits that may help it to thrive in the environment and that might be related to a high transformation rate success. Moreover, the higher total amount of the mobile genetic elements found in ED1 than in K12 NEB DH5α could be due to the fact that these elements are commonly found in bacteria exposed to a "horizontal gene pool", which can be easily found in several environments (Dobrindt et al., 2004). HGT is, indeed, known to contribute to bacterial adaptation to different habitats and, in the long term, to bacterial evolution (Lorenz and Wackernagel, 1994; Thomas and Nielsen, 2005; Vandecraen et al., 2017). This result is also in agreement with the data available on *Vibrio* species, the transformation proficiency of which appears to be more common in environmental strains than in clinical ones (Bernardy et al., 2016).

The environments where *E. coli* is known to survive include soil, water and manure besides several micro-habitats associated to plants, given the ability of some *E. coli* strains to colonize roots, leaf surfaces and endosphere (Van Elsas et al., 2011; Wright et al., 2017; Eissenberger et al., 2020). The capacity of an environmental and naturally transformable *E. coli* strain like ED1 to survive in soil and colonize the plant rhizosphere has relevant implications in the light of the antibiotic cycle and the One Health vision. The plant rhizosphere is indeed a well characterized, substrate-rich, hot spot for bacterial activity and abundance (Zhu et al., 2018), where naturally competent cells can find higher concentrations of free DNA and could, moreover, reach the growth phase in which transformation occurs with high frequency (Sørensen and Jensen, 1998; Mølbak et al., 2003; Ling et al., 2016; Zhu et al., 2018). Relevant concentrations of ARGs can reach the plant rhizosphere, e.g., through soil amended with manure, sewage sludge and treated wastewater (Chen et al., 2017; Riva et al., 2020; Wu et al., 2020). We selected lettuce as a model plant for the root system colonization experiments, as representative of raw-consumed vegetables of high economic importance in the ready-to-eat food industry. Our results showed that ED1 strain colonized efficiently the lettuce rhizosphere both in sterile and non-sterile soils and indicated that the rhizosphere colonization was stable over a period of 14 days. The ability of ED1 strain to acquire exogenous DNA in environmental mimicking conditions and to efficiently colonize the plant rhizosphere might represent a possible route of ARGs spread in the plant microbiome, potentially representing a risk for health through the consumption of raw vegetables (Nüesch-Inderbinen et al., 2015). In this perspective we analyzed the ED1 strain genome for the presence of virulence factors, revealing a higher number of virulence factors in this environmental and naturally competent strain than in the laboratory strain K12 NEB DH5α. Although further analyses are required to unveil any possible relation with human pathogenic *E. coli* strains, these data allow us to hypothesize a low and not relevant virulence for *E. coli* strain ED1 (**Supplementary Table 6**).

CONCLUSION

We demonstrated the ability of an environmental *E. coli* strain to acquire exogenous DNA by natural competence with relatively high frequency in exponential growth phase in environmental-like conditions, together with its capability, when applied to soil, to thrive in lettuce rhizosphere. These results confirm the importance to further investigate the possible spread of antibiotic resistant determinants through HGT in the environment and, particularly, in the rhizosphere of those plant species consumed as raw vegetables, to elucidate the related food and human safety risks. Further studies on environmental *E. coli* strains could allow to strengthen our results and to understand the spread of this phenomenon.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, JAAWVB000000000.

AUTHOR CONTRIBUTIONS

EC, FM, and SB designed the study. FR, VR, EE, NC, and AD carried out the experiments. FR, VR, FM, and EC analyzed the data. FM and SB supported the research. FR and EC wrote the first draft of the manuscript. All authors contributed to the manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.574301/full#supplementary-material

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Exopolysaccharide Features Influence Growth Success in Biocrust-forming Cyanobacteria, Moving From Liquid Culture to Sand Microcosms

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Land degradation in drylands is a drawback of the combined action of climate change and human activities. New techniques have been developed to induce artificial biocrusts formation as a tool for restoration of degraded drylands, and among them soils inoculation with cyanobacteria adapted to environmental stress. Improvement of soil properties by cyanobacteria inoculation is largely related to their ability to synthesize exopolysaccharides (EPS). However, cyanobacterial EPS features [amount, molecular weight (MW), composition] can change from one species to another or when grown in different conditions. We investigated the differences in growth and polysaccharidic matrix features among three common biocrust-forming cyanobacteria (*Nostoc commune, Scytonema javanicum,* and *Phormidium ambiguum*), when grown in liquid media and on sandy soil microcosms under optimal nutrient and water, in controlled laboratory conditions. We extracted and analyzed the released EPS (RPS) and sheath for the liquid cultures, and the more soluble or loosely-bound (LB) and the more condensed or tightly-bound (TB) soil EPS fractions for the sandy soil microcosms. In liquid culture, *P. ambiguum* showed the greatest growth and EPS release. In contrast, on the sandy soil, *S. javanicum* showed the highest growth and highest LB-EPS content. *N. commune* showed no relevant growth after its inoculation of the sandy soil. A difference was observed in terms of MW distribution, showing that the higher MW of the polymers produced by *P. ambiguum* and *S. javanicum* compared to the polymers produced by *N. commune,* could have had a positive effect on growth for the first two organisms when inoculated on the sandy soil. We also observed how both RPS and sheath fractions reflected in the composition of the soil TB-EPS fraction, indicating the role in soil stabilization of both the released and the cell attached EPS.

Our results indicate that the features of the polysaccharidic matrix produced by different cyanobacteria can influence their growth success in soil. These results are of great relevance when selecting suitable candidates for large-scale cyanobacteria applications in soil restoration.

Keywords: cyanobacteria liquid culture, sand inoculation, sandy soil microcosms, EPS monosaccharidic composition, EPS molecular weight distribution, semiarid soil

INTRODUCTION

Cyanobacteria are the oldest oxygenic photosynthetic organisms. They are widespread in aquatic and terrestrial ecosystems, and occupy almost every habitat on Earth thanks to their ability to adapt to a wide range of environmental conditions (Whitton and Potts, 2002). In dryland soils, cyanobacteria are found in close associations with other organisms such as bacteria, algae, lichens and mosses forming the so called biological soil crusts or biocrusts (Weber et al., 2016). As part of these communities, cyanobacteria play key roles in soil properties and functions. Cyanobacteria filaments bind soil aggregates and create a stable surface layer that facilitates the path for colonization by other biocrust organisms such as lichens and mosses (Deng et al., 2020). Cyanobacteria fix CO_2 (Miralles et al., 2018) and some species are able to fix N_2, increasing soil organic matter and nutrient content (Mager and Thomas, 2011). They also release a wide array of substances in the soil such as growth-promoting regulators, vitamins, amino acids, polypeptides, biotins, proteins, and sugars that contribute to soil fertility and act as biocontrol agents against plant pathogenic bacteria, fungi and micro-algae (Singh et al., 2016). Cyanobacteria have received special attention as bio-inoculants for ecological restoration of degraded lands (Rossi et al., 2017). Soil inoculation with cyanobacteria has been shown to lead to soil improvements in desertified natural soils (Park et al., 2017), mine (Muñoz-Rojas et al., 2018) and quarry substrates (Roncero-Ramos et al., 2019a,b), fire-affected soils (Acea et al., 2003; Chamizo et al., 2020) and agricultural lands (Maqubela et al., 2009). Exopolysaccharides (EPS) are among the most important compounds synthesized by cyanobacteria playing a vital role in soil functions. Cyanobacterial EPS consist of polymeric substances of high viscosity with varying biochemical composition and biophysical properties that are among the most structurally and functionally complex bacterial structures (Hill et al., 1994). These biopolymers form an envelope surrounding the cells called sheath, glycocalyx, capsule, or slime, depending on its consistency and localization (De Philippis and Vincenzini, 1998), which protect cells from physical and biological stresses (Costa et al., 2018).

In dryland soils, cyanobacterial EPS are implied in soil stabilization, nutrient provision and resistance to desiccation (Brüll et al., 2000; Hu et al., 2003; Mager and Thomas, 2011). Exopolysaccharides regulate the loss and uptake of water from cells (Potts, 1994; Mazor et al., 1996; Adessi et al., 2018) and protect cells from damage during swelling and shrinkage due to frequent desiccation-rehydration cycles (Liu et al., 2017). After rewetting, cyanobacteria can rapidly recover metabolic activities and repair cellular components (Billi and Potts, 2002). Cyanobacterial EPS also contain sunscreen pigments that protect cells against UV-A/B radiation (Scherer et al., 1988; Ehling-Schulz et al., 1997; Fleming and Castenholz, 2007). More soluble soil EPS fractions or loosely-bound (LB) EPS are thought to represent an important source of energy for heterotrophic activity, while more condensed soil EPS fractions or tightly-bound (TB) EPS are mainly involved in soil particle consolidation, contributing to soil stability (Chen et al., 2014; Chamizo et al., 2019). Consequently, changes in soil properties can be related, to a large extent, to soil polysaccharidic matrix features such as amount, molecular weight (MW) distribution and chemical composition of soluble and condensed EPS fractions (Chen et al., 2014; Colica et al., 2014; Mugnai et al., 2018a,b; Chamizo et al., 2019). In general, EPS synthesis by a given cyanobacterial strain mostly depends on the species and the cultivation conditions such as the source of nitrogen, light intensity, temperature, salinity, and phosphorus and potassium contents (De Philippis and Vincenzini, 1998; Nicolaus et al., 1999; Otero and Vincenzini, 2003). In the soil, EPS characteristics have been related to the soil type (Chamizo et al., 2019) and biocrust age or successional stage (Chen et al., 2014; Colica et al., 2015).

Cyanobacterial EPS properties may represent an important factor to be considered for the selection of suitable cyanobacteria candidates for soil restoration. Cyanobacterial EPS characterization has been mainly done in liquid cultures for isolated strains but less explored in cyanobacteria-inoculated soil trials. Besides, little is known of how EPS features might change for a specific strain when grown in liquid culture and in the soil. The paucity of information may due to the fact that it is still not clear whether the soluble fraction of the EPS in liquid cultures is the same soluble fraction than when the strain grows in soil, similarly for the sheath EPS and the more strongly attached soil EPS. Another important factor to take into account for selection of adequate cyanobacteria bio-inoculants is that some strains might exhibit a fast growth when cultured in liquid conditions but limited growth when inoculated on the soil and vice versa. Thus, viability for biomass growing of the selected cyanobacteria in liquid culturing systems as well as capability to successfully colonize the soil after inoculation are key issues to be considered, especially for scaling up of cyanobacteria applications with restoration purposes. Last, the inoculated cyanobacteria should be able to induce significant improvements in soil properties and functions, so that the desired objective of soil recovery is fulfilled. In this respect, it would be very useful to have some descriptors of the potential of the strains for successful restoration approaches before actually applying them to the soil. Characterization of cyanobacterial EPS features in terms of their macromolecular

distribution and monosaccharidic composition could provide valuable insights to this regard.

The main goal of this study was to examine whether different biocrust cyanobacterial strains showed contrasting performances when grown in liquid and solid medium and if so, if such differences could be linked to their EPS features. To achieve this goal, we analyzed the growth and polysaccharidic matrix features (amount, MW distribution and monosaccharidic composition) of three biocrust-forming cyanobacteria (*Nostoc commune, Scytonema javanicum,* and *Phormidium ambiguum*) when grown in liquid medium and after inoculation on a sandy soil under comparable optimal water and nutrient availability conditions. This can also help improve our understanding on how cyanobacterial performance and EPS traits might change when cyanobacteria are inoculated together with the nutrient media during their culturing, as a viable approach to improve biocrust performance and soil restoration success.

MATERIALS AND METHODS

Selection of Cyanobacteria Strains

We selected three terrestrial cyanobacterial strains available in the laboratory collection and isolated from biocrust samples: the N-fixing *Nostoc commune*, belonging to the order Nostocales, isolated from the Negev desert; the N-fixing *Scytonema javanicum* Bornet & Flahault NIES-1956, belonging to the order Nostocales, originally isolated from the Tsukuba Botanical Garden (Japan); and the non N-fixing *Phormidium ambiguum* Gomont NIES-2121, belonging to the order Oscillatoriales, originally isolated from an African soil. These three genera have been described as part of the cyanobacterial community in biocrusts from semiarid regions (Roncero-Ramos et al., 2020). Besides, *N. commune* has been reported in biocrusts worldwide (Büdel et al., 2016). *Nostoc* spp., *Scytonema* spp. and *Phormidium* spp. have been also employed as soil inoculants to reverse land degradation and promote soil recovery in degraded arid soils (Li et al., 2014; Park et al., 2017; Zhao et al., 2019). The selected cyanobacterial strains were used for the batch culture and sand-microcosm experiments described below. A scheme summarizing the experimental design and the variables measured on each experiment can be seen in **Figure 1**.

Batch Culture Experiments in Liquid Media

Biomass of the selected cyanobacterial strains was collected and used to set up the batch culture experiments (**Figure 1A**). The strains were transferred to aerated glass vertical columns (diameter 50 mm) with a working volume of 500 ml. BG-11_0 medium (Rippka et al., 1979) was used for the heterocystous strains, *N. commune* and *S. javanicum*, and BG-11 medium (with nitrate) for the non-heterocystous strain, *P. ambiguum*. The columns were placed in a thermostated water bath at a constant temperature of 30°C and were continuously illuminated on both sides, with a photon flux density (PFD) of 70 μmol m^{-2} s^{-1}. The strains were acclimated to these conditions during two weeks previous to perform growth curve measurements. Cultures were bubbled with filter-sterilized air supplemented with CO_2 (0.05%v/v.) in order to homogenize the culture and keep the pH close to 7.0. Three replicates were considered for *N. commune* and four replicates for *S. javanicum* and *P. ambiguum* due to their frequent cell aggregation which introduced higher variability of parameter measurements. Cultures were inoculated at an initial dry weight of 0.1 g L^{-1} and the growth curve for the three strains was followed for 9 days. Evaporation was compensated daily with sterilized distilled water. Every day, a sample of 30 mL was withdrawn from each culture to monitor the culture behavior and analytical measurements.

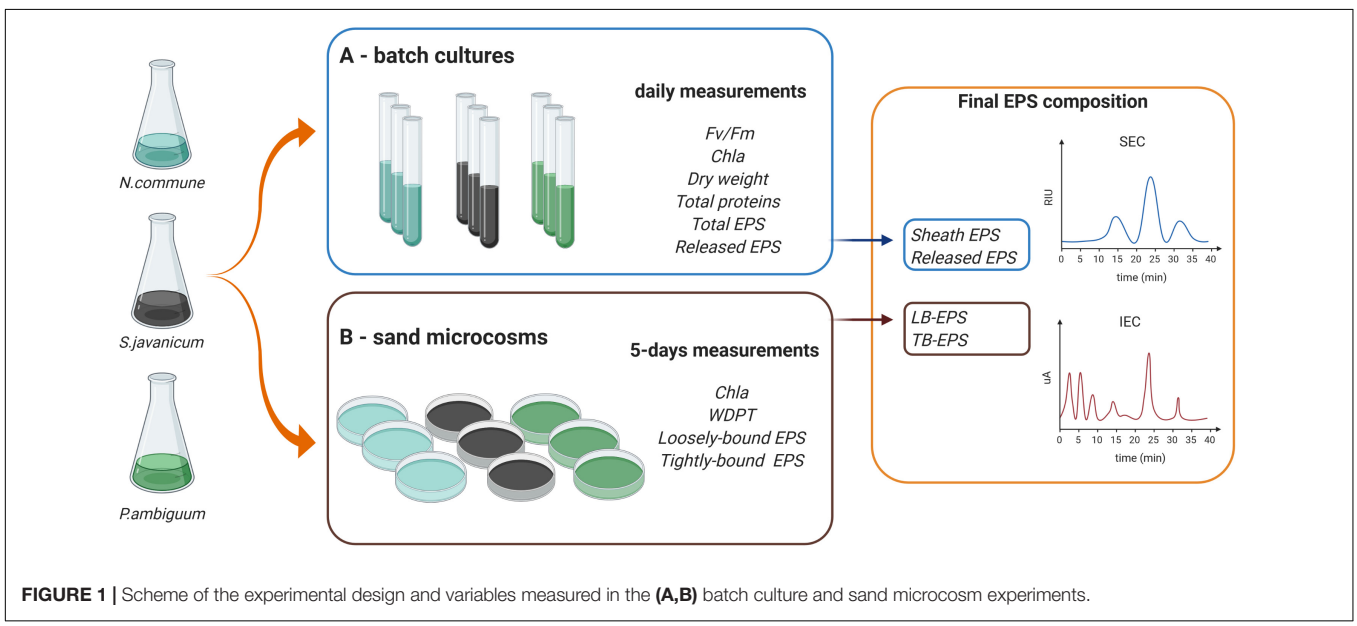

FIGURE 1 | Scheme of the experimental design and variables measured in the **(A,B)** batch culture and sand microcosm experiments.

Dry Weight and Chlorophyll a Concentration

Dry weight was measured by filtering 5–10 mL of culture on a pre-weighted filter (Whatman grade GF/F) 1 μm pore filters, and then dried at 105°C for 3 h. Chlorophyll *a* concentration was measured following the method by Singh et al. (2016). In brief, 5 mL of culture was centrifuged at 2500 × *g* at laboratory temperature for 7 min and the supernatant was thoroughly discarded, after which 5 mL of methanol was added and the sample was vortexed and heat at 70°C. Then, samples were centrifuged at 2500 × *g* and absorbance of the supernatant was measured in a spectrophotometer at 665 nm and 750 nm. Chlorophyll *a* content was determined according to the equation by Ritchie (2006).

Chla [μg/ml] = 12.9447 (A665 – A750) x Volume of methanol (mL)/Volume of sample (mL) (Eq. 1).

Fluorescence Parameters

The ratio between variable and maximum fluorescence, F_v/F_m, was measured to determine the maximum photochemical quantum yield of PSII, using a pulse-amplitude-modulation fluorimeter (PAM-2100, H. Walz, Germany). For this purpose, samples were taken from the cultures and incubated in the dark for 15 min to remove any energy-dependent quenching. Then, one far-red light (above 700 nm) pulse with a duration of 10 sec (10 W m^{-2}), supplied by the PAM-2100, was applied. This procedure was applied to attain a full oxidation of the plastoquinone pool (PQ). For comparison, measurements of F_v/F_m were also performed in the light using 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (10^{-5} M), and resulted in a lower value, therefore all the measurements were carried out using far red light. In addition, rapid light-response curves (RLCs) of cultures were measured daily using a 2 mL cell sample placed in a Liquid-Phase Oxygen Electrode Chamber (Hansatech, DW3) cuvette, thermostated at 25°C. A series of stepwise increasing irradiance intensities (LEDs, 0–636 μmol photons m^{-2} s^{-1}) provided by PAM-2100 were automatically applied at 20 s intervals to obtain the light-adapted fluorescence level F' (steady-state fluorescence yield in the light), and at the end of each step a saturating pulse (>6,000 μmol photons m^{-2} s^{-1}, 0.6 s duration) was triggered to reach the maximum fluorescence level F_m' (steady state maximum fluorescence in the light). The effective PSII photochemical quantum yield in the light, Y_{II}, was determined as (F_m'- F')/F_m' in the light-adapted state at respective irradiance level. The effective quantum yield of PSII was used to calculate the electron transfer rate (ETR). However, it must be pointed out that with cyanobacteria, this parameter can furnish only a relative measure of the ETR, since the PSI/PSII ratio is much higher than in leaves (Vermaas, 2001; Fraser et al., 2013), and the light absorption coefficient can affect the measurements (Szabó et al., 2014). Relative electron transport rates (rETR = μmol e- $m^{-2}s^{-1}$) were calculated as rETR = PFD x (F_m'- F')/F_m' x ETR factor (i.e., the fraction of light absorbed by the sample and distributed to PSII). An ETR factor of 0.42 was used, which takes into account the default setting for percentage of light absorbed (0.84) and that distributed to PSII (0.5). Analysis of RLCs was used to calculate changes in important parameters, that is, the maximum relative electron transport rate through PSII, $rETR_{max}$, the initial slope, α, of the rETR vs. PFD curve which is the quantum efficiency of the photosynthesis, the saturation irradiance, I_k, given as intercept between α, and $rETR_{max}$. The curves were fitted to the non-linear least-squares regression model by Eilers and Peeters (1988) using PamWin 3 software.

EPS Characterization

Total EPS content and released EPS (RPS hereafter) were also daily determined in the cultures. For total EPS content, 1 mL of the culture was taken and its carbohydrate content was quantified by means of the phenol-sulfuric acid assay (Dubois et al., 1956). RPS were extracted by centrifuging 5 mL of culture at 4000 × *g* for 30 min. Then, the supernatant was recovered and an aliquot of 1 mL was taken to quantify carbohydrate content using the phenol-sulfuric assay. When EPS amount was very high and/or there was interference of nitrate in BG-11 cultures, a 1:5 or 1:10 dilution was applied to the samples; v) Protein content of the cultures was measured by the Lowry method using bovine serum albumin as standard (Lowry et al., 1951).

Additionally, at the end of the growth curve, apparent molecular weight (MW) distribution and monosaccharidic composition of the cyanobacterial sheath and RPS of the three strains were determined. For this, the remaining culture of the strains at the end of the experiment and once the described measurements were done, was mixed and centrifuged to separate the RPS from the cell biomass. Then, the cyanobacterial sheath was recovered from the pellet by washing cyanobacterial cells with 5 mL of distilled water at 80°C for 1 h, centrifugation at 4000 × *g* for 30 min and recovery of the supernatant. This operation was repeated three times. In the cases in which the sheath was strongly attached to cells (e.g., *P. ambiguum*), the sheath-containing pellet was additionally mixed with a 1.5% NaCl solution and then extracted with 5 mL of distilled water at 80°C for 1 h (Rossi et al., 2018). Effective sheath extraction from cyanobacterial filaments was checked by optical microscope observations. MW distribution of RPS and cyanobacterial sheaths was determined by size exclusion chromatography (SEC). 1 mL of the sheath- and RPS-containing extracts was taken and injected in a Varian Pro-Star liquid chromatograph (Varian Inc., United States) equipped with a refractive index (RI) detector and two columns, PolySep-GFC-P 6000 and 4000 (Phenomenex, United States), connected in series. The columns (700 mm length and 7.8 mm internal diameter) had separation ranges of 100 kDa to 15 MDa and 0.3 to 400 kDa, respectively. Samples were analyzed with runs of 70 min using HPLC-grade water as eluent at a flow rate of 0.4 mL min^{-1}. Size classes were established according to retention times of known MW dextran standards (Sigma-Aldrich, United States). These MW classes were: >2000 kDa (0–25 min), 2000–1100 kDa (25–35 min), 1100–410 kDa (35–36 min), 410–150 kDa (36–38 min), 150–50 kDa (38–40 min) and <50 kDa (40–70 min). To obtain the% of the different MW classes, we performed the ratio of each peak area to the total area under the curve and assigned the resulting% area to the corresponding size class according to the retention time of the peak output.

The monosaccharide composition was analyzed using Ion Exchange Cromatography (IEC) following the procedures described in Mugnai et al. (2018a,b) and Chamizo et al. (2019). Before IEC analysis, 1 mL of the extracts (sheath or RPS) was mixed with 1 mL of 4 N trifluoroacetic acid (TFA) and heated for 120 min at 120°C. Afterward, the excess of TFA was removed by drying on a rotary evaporator and the dried extracts re-solubilized in deionized water, repeating this operation three times per sample. Monosaccharide composition was analyzed with a Dionex ICS-2500 ion exchange chromatograph (Dionex, United States) equipped with an ED50 pulsed amperometric detector operating with a gold working electrode (Dionex) and a CarboPac PA1 column of 250-mm length and 4.6-mm internal diameter (Dionex). We used as eluents HPLC-grade water (A), 0.185 M Na hydroxide (B), and 0.488 M Na acetate (C), at a flow rate of 1 mL min^{-1}. Single sugars were identified on the basis of the retention time of known standards. Results were expressed as molar ratio.

Sandy Soil Microcosms Experiments

Preparation and Inoculation of the Sandy Soil Microcosms

Cultures of the same cyanobacterial strains used for the batch culture experiments were used for the sand-microcosms experiment (**Figure 1B**). The sandy soil (92% sand, 1% silt and 7% clay) was collected from a semiarid area in Almeria province (SE Spain) and had an organic carbon content of 1.23 g kg^{-1} and nitrogen content of 0.21 g kg^{-1}. Small Petri dishes (12 mm height x 54 mm diameter) were filled with 30 g of sterilized sand (by autoclaving twice for 20 min at 120°C). Inoculation was done by adding 30 mg (dry weight) of cyanobacterial biomass on each Petri dish or sand microcosm, equivalent to 5 g m^{-2}, which has been found to be an optimal areal density to promote biocrust formation on sandy soils (Mugnai et al., 2020). In addition, chlorophyll *a* content of the inoculum solutions was measured following the procedure explained above for the liquid culture. Chlorophyll *a* concentrations of *N. commune*, *S. javanicum,* and *P. ambiguum* solutions were 10.4 ± 1.5, 11.0 ± 2.3 and 8.1 ± 1.0 μg mL^{-1}, respectively. Soil samples with non-inoculated sand were used as controls. Each treatment (control and sand inoculation with each strain) was done in triplicate. Sand microcosms were incubated in a plexiglass growth chamber with controlled temperature (30°C) and light intensity (70 μmol photons m^{-2} s^{-1}) for 30 days. Water and nutrients were added to the microcosms in order to provide optimal conditions for cyanobacteria growth, similar to that of liquid cultures. Microcosms were watered with 5 mm of distilled water five days a week. In addition, 5 mL of culture media (3 x concentrated) (BG11 for the sand inoculated with the non-heterocystous strain and $BG11_0$ for the sand inoculated with the heterocystous strains) was added to the soil samples every 5 days. The amount of nutrients applied to the soil after 30 days was equivalent to 0.5 L of culture media (similar to that of the liquid cultures). Addition of water and nutrients was also done on the control samples. Of the three control replicates, two were sprayed with BG11 and one with $BG11_0$. As no difference was found in the control sand provided either with BG11 or $BG11_0$, the average of the three replicates was considered for further comparisons with the inoculated sand treatments. Six samplings were carried out during the experimental period, so that a total of 72 samples (4 treatments x 3 replicates x 6 samplings) were prepared.

Soil Determinations

Every 5 days, 12 samples (three per treatment) were randomly selected and the surface crust (2 mm thick) was collected in order to determine chlorophyll *a* content, as a proxy of cyanobacterial growth, and EPS content. Previous to crust sampling, soil hydrophobicity was measured on the sand microcosms using the water drop penetration time (WDPT) test (Doerr, 1998). Five to seven drops of distilled water were dropped from a height of 1.5 cm on to the surface of the soil sample. The average time the drops remained on the surface was used as an index of the severity of water repellency. After this test, the crust was collected with a small putty knife and ground to a fine powder with mortar and pestle. Chlorophyll *a* content was determined after extraction with hot ethanol at 80°C for 5 min, centrifugation and measurement of supernatant absorbance at 665 nm (Castle et al., 2011). Chlorophyll *a* content was calculated according to Ritchie (2006) equation:

$$\text{Chlorophyll } a = (11.9035 \times A665_0 \times V) \text{ x (g soil}^{-1}\text{) x L} \quad \text{(Eq. 2)}$$

where V is the volume of solvent (mL) and L is the path length.

Soil EPS Characterization

The amount of two soil EPS fractions were determined in the crust: the more soluble and less condensed fraction, easily released into the environment or "loosely bound EPS" (referred to as LB-EPS), and the more condensed fraction, firmly attached to the cells and soil particles or "tightly bound EPS" (referred to as TB-EPS). LB-EPS were extracted with distilled water at room temperature for 20 min. The supernatant was recovered after centrifugation at 3500 × *g* for 30 min. This process was repeated three times for each sample and the three supernatants obtained were mixed together. TB-EPS were recovered from the resulting pellet using three extractions with 0.1 M Na_2EDTA and centrifugation at 3500 × *g* for 30 min. The three supernatants obtained after the three extractions were mixed together. The carbohydrate content of both LB-EPS and TB-EPS extracts was determined using the phenol-sulfuric acid assay.

At the end of the experiment (30 days), the apparent MW distribution and monosaccharide composition of the two soil EPS fractions were also determined. In the TB-EPS fraction, the excess of Na_2EDTA that could interfere with the chromatographic analysis was removed by dialyzing the extracts in nitrocellulose tubular membranes (14 kDa MW cutoff, Medicell International, United Kingdom) for 24 h in distilled water. Extracts were then dried and dissolved in deionized water, transferred to Eppendorf tubes, and clarified by ultracentrifugation at 13,000 × *g* in order to remove the coarse particulate. The MW distribution was analyzed by size exclusion chromatography (SEC) following the procedure explained above for the liquid culture extracts. For monosaccharidic composition determination, the extracts were hydrolyzed and purified following the same procedure explained above for the liquid culture and monosaccharide

composition was determined by IEC analysis following the previously described methodology.

Data Elaboration and Statistical Analysis

Differences in dry weight, chlorophyll *a* content, fluorescence parameters, and total and released EPS content among the three cyanobacterial strains grown in liquid culture were analyzed using one-ANOVA and the Tukey *post hoc* test. Variables were previously checked for normality and homogeneity of variance using the Shapiro-Wilk and Levene's test. When needed, data were log transformed before performing parametric analysis. In the case of the sandy soil microcosms, as variables did not meet normality assumptions, the effect of the inoculation treatment (control and sand inoculation with the three strains) and incubation time on chlorophyll *a*, LB-EPS and TB-EPS was analyzed using a permutational multivariate analysis of variance (PERMANOVA) based on Euclidean distances. Further differences among the strains were analyzed with paired-wise tests using a maximum of 9999 permutations and applying Monte Carlo correction. All the analyses were performed using Primer 7 and Permanova + (PRIMER-E Ltd., Plymouth, United Kingdom). The complexity of the monosaccharidic profiles was analyzed and interpreted calculating diversity indices. Thus, alpha-diversity of sugar residues of the different strains and conditions (sheath and RPS in liquid cultures, and LB-EPS and TB-EPS in the sand microcosms) were compared. Diversity indexes were calculated using the percentiles of a bootstrap distribution with 9999 repetitions, using the Past 4.0 software. Significance was established at $p < 0.05$.

RESULTS

Cyanobacteria Growth in Liquid Cultures

Batch cultures of the three cyanobacteria strains were monitored during 9 days through determination of dry weight and chlorophyll *a* concentration. The greatest growth was observed with *P. ambiguum* which reached a dry weight of 3.33 g L^{-1} after 9 days, while *N. commune* and *S. javanicum* showed a lower growth, 1.46 g $L^{-1}$and 1.75 g L^{-1}, respectively (**Figure 2A**). The higher growth showed by *P. ambiguum* was also reflected in a higher chlorophyll *a* increase, which reached 31.9 mg L^{-1} at day 7 and then progressively decreased until day 9 (**Figure 2B**). Chlorophyll *a* concentration increased in *N. commune* and *S. javanicum* over time and after 9 days of biomass culturing, reached values of 24.1 mg L^{-1} and 8.9 mg L^{-1}, respectively (**Figure 2B**). Chlorophyll increase with time showed a Gompertz curve fitting for *P. ambiguum* (r^2 = 0.9903) and an exponential curve for *N. commune* and *S. javanicum* (r^2 = 0.9667 and 0.9901, respectively). Protein content of the cultures at the end of the experiment reached 796.0 ± 135.5 mg L^{-1} for *N. commune*, 933.4 ± 306.1 mg L^{-1} for *S. javanicum* and 1128.2 ± 290.3 mg L^{-1} for *P. ambiguum*, representing, respectively, 55%, 53% and 34% of their respective dry weight.

During the whole period of culture, the F_v/F_m ratio remained rather constant or slightly increased in *N. commune,* indicating good physiological state of the cultures (**Figure 3**). The mean values of the F_v/F_m were 0.525 ± 0.039 in *N. commune*; 0.502 ± 0.014 in *S. javanicum*, and 0.434 ± 0.016 in *P. ambiguum* (**Table 1**). The mean values of the calculated parameters of RLCs, i.e., the $rETR_{max}$, the initial slope (α) of the rETR vs. PFD curves, and the saturation irradiance (I_k) are reported in **Table 1**. The mean $rETR_{max}$ gathered over the whole cultivation period resulted about 25% higher in *P. ambiguum*, while the initial slope, i.e., the quantum efficiency of photosynthesis, was higher in *N. commune* (0.228) than in *S. javanicum* (0.190) and *P. ambiguum* (0.174). The highest saturation irradiance of photosynthesis was found in *P. ambiguum* (**Table 1**).

Analysis of EPS production during cyanobacteria growth showed a similar trend to chlorophyll content. The amount of released and total EPS was significantly higher in *P. ambiguum* showing the highest values at day 6 and 7, after which it slightly decreased in both EPS fractions (**Figures 4A,B**), in coincidence with the pattern of chlorophyll content. *N. commune* and *S. javanicum* showed lower EPS contents and significant differences were found between them in the released EPS. While the total EPS amount was similar for both cultures (**Figure 4B**), *N. commune* showed a higher amount of released EPS than *S. javanicum* (**Figure 4A**).

Cyanobacteria Growth in Sandy Soil Microcosms

The cyanobacteria strain and incubation time, as well as interaction between them, had a significant effect on chlorophyll *a* content and LB-EPS and TB-EPS amounts ($p < 0.05$), indicating that change of these variables with time depended on the strain. Chlorophyll *a* content increased in the inoculated sand with *P. ambiguum* and *S. javanicum,* thus indicating a significant cyanobacterial growth over time. However, no significant increase was observed in the inoculated sand with *N. commune*, which showed a chlorophyll content close to zero and similar to control soils (**Figure 5**). In contrast to the pattern observed in batch cultures where *P. ambiguum* exhibited the highest growth, in the sand microcosms *S. javanicum* was the strain that showed a better performance. After 30 days of soil incubation, chlorophyll content resulted four times higher in the *S. javanicum*-inoculated sand than in the *P. ambiguum*-inoculated sand. The content of the two soil EPS fractions differed between the two strains. LB-EPS content was similar in the inoculated sand with *P. ambiguum* and *S. javanicum* during the first days after sand inoculation (**Figure 6A**) but it was significantly higher in *S. javanicum* from the fifteenth day onward (**Figure 6A**), also coinciding with a sharper increase in chlorophyll content. TB-EPS content increased in both inoculated soils over time and although the sand inoculated with *P. ambiguum* showed higher TB-EPS content than the sand inoculated with *S. javanicum*, the difference resulted not significant (**Figure 6B**). It is worth mentioning that a significant increase in soil hydrophobicity was found in the sand inoculated with *S. javanicum* from day 10 till the end of the experiment. While controls and the other inoculated soils showed no water repellence (WDPT < 5s), *S. javanicum*-inoculated sand showed

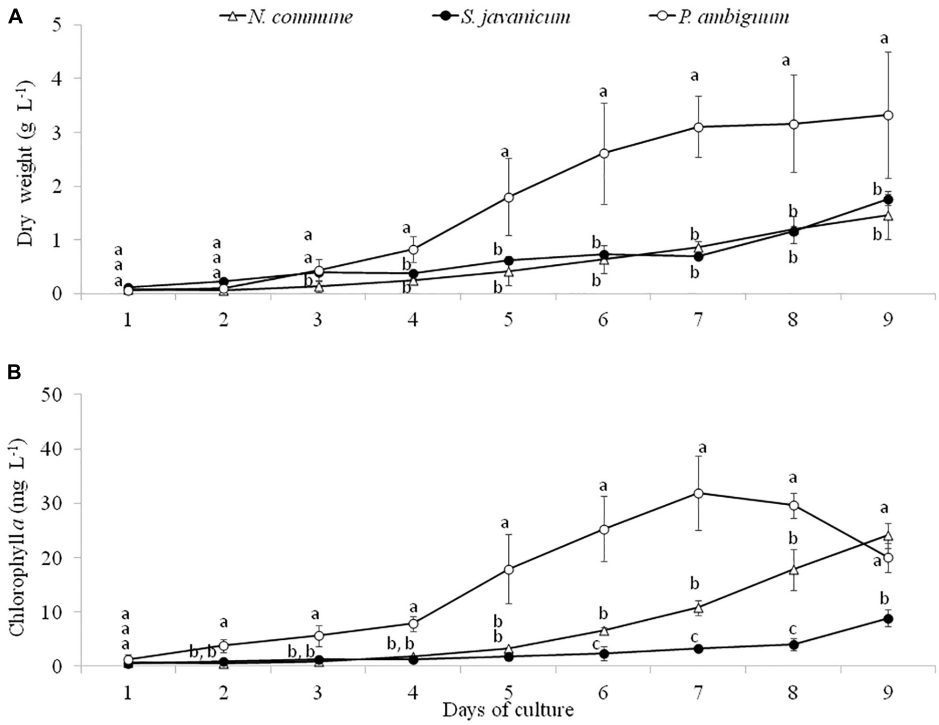

FIGURE 2 | Dry weight **(A)** and chlorophyll *a* concentration **(B)** during the cultivation period of the three cyanobacteria strains. Different letters indicate significant differences among the strains for each day.

WDPT > 60 s in most cases and some times lasting for several minutes, indicating severe hydrophobicity.

EPS Molecular Weight Distribution and Monosaccharidic Composition of the Cyanobacterial Strains in Liquid Culture and Soil Microcosms

The EPS extracted both from culture biomass and from the soil microcosms were analyzed in terms of molecular weight (MW) distribution and monosaccharidic composition. The EPS extracted from *N. commune* inoculated microcosms were insufficient for these in-depth analytical purposes (see Results in **Figure 6**), hence data are not shown in **Figures 7C,D**, **8C,D**. The MW distribution results (**Figure 7**) showed that the polymers were composed of four classes of MW, from >2MDa to <50 kDa, not equally represented among strains and among culturing conditions. In liquid cultures the highest MW class (>2MDa) could only be detected in *P. ambiguum* sheath (16.3%), while the lowest MW class (<50kDa) was always largely represented (ranging from 36.0% in *S. javanicum* sheath to 83.4% in *N. commune* sheath) (**Figure 7B**). The released EPS were mostly composed of low MW molecules <50KDa. However, *S. javanicum* showed also a high percentage of the MW class 2MDa-1.1MDa (56%), as well as *N. commune* which showed high percentages of the MW class 2MDa-1.1MDa (20%) and 1.1MDa–410 kDa (27%) (**Figure 7A**).

In the sandy soil microcosms (**Figures 7C,D**), the EPS MW distribution profiles of the two strains were similar, either for LB-EPS or TB-EPS: the only exception was the presence of the intermediate MW class (1.1MDa–410 kDa) in *P. ambiguum* LB-EPS. The highest MW class (>2MDa) was always represented both in LB-EPS (2.5 and 2.1% for *P. ambiguum* and *S. javanicum*, respectively) and in TB-EPS (6.0 and 5.7% for *P. ambiguuum* and *S. javanicum*, respectively), while the lowest MW class (<50kDa) was more represented in LB-EPS (50.5 and 67.0% for *P. ambiguum* and *S. javanicum*, respectively) than in TB-EPS (35.1 and 34.3% for *P. ambiguum* and *S. javanicum*, respectively).

The EPS monosaccharidic composition was heterogeneously dependent on the strain or on the culturing conditions (**Figure 8**, **Supplementary Table S1**). In almost all cases the most represented sugar residue was glucose, but many differences could be observed. Due to the complexity of the monosaccharidic profiles and the number of conditions to compare, for a clearer and informative interpretation of analytical data, we used an innovative approach for the description of monosaccharidic composition, as usually done in ecologic approaches, based on analysis of diversity indices of the samples. The results referring to the liquid cultures (**Figures 8A,B**, **Table 2**, and **Supplementary Table S1**) showed similar profiles of *N. commune* and *P. ambiguum* in terms of most represented sugars (a 3% threshold was arbitrarily set to define a significantly represented sugar): glucose, mannose, xylose, galactose, arabinose and glucuronic acid, in order from the most to the less represented residue in RPS; besides glucose,

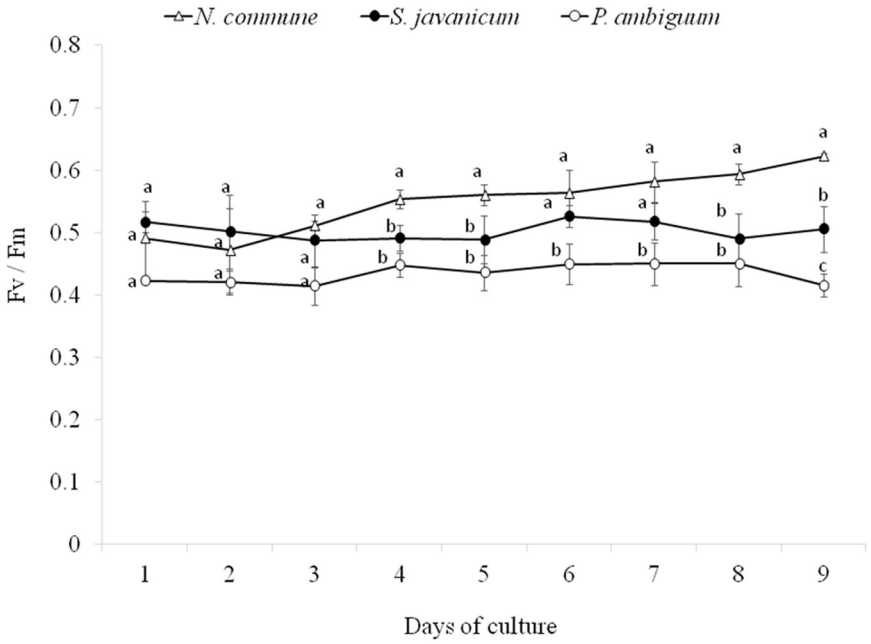

FIGURE 3 | Time course of the daily values of the F_v/F_m ratios measured during the cultivation period of the three cyanobacteria strains (mean ± SD, *n* = 9). Different letters indicate significant differences among the strains for each day.

TABLE 1 | Mean values of fluorescence parameters measured over a cultivation period of nine days in cultures of *N. commune, P. ambiguum, and S. javanicum*.

Fluorescence parameters	*Cyanobacteria strain*		
	N. commune	**S. javanicum**	**P. ambiguum**
$rETR_{max}$ (μmol e- m^{-2} s^{-1})	40.85 ± 10.25^{a}	40.0 ± 17.72^{a}	52.80 ± 33.0^{a}
I_k (μmol photons m^{-2} s^{-1})	178.62 ± 26.19^{b}	211.42 ± 89.28^{ab}	303.57 ± 32.68^{a}
α (μmol e- μ photons^{-1})	0.228 ± 0.031^{a}	0.190 ± 0.018^{b}	0.174 ± 0.033^{c}
F_v/F_m	0.525 ± 0.039^{a}	0.502 ± 0.014^{b}	0.434 ± 0.016^{c}

Data are the mean values ± SD (n = 9). Different letters indicate significant differences among the strains. $rETR_{max}$: maximum relative electron transport rate; I_k: saturation irradiance; α: initial slope of the rETR vs. PFD curve; F_v/F_m: ratio between variable and maximum fluorescence.

only rhamnose was present (>3%) in the sheath. On the contrary, *S. javanicum* showed glucose, xylose, galactose and fucose as the main RPS components, and glucose, mannose, ribose, fructose, and galactose composing the sheath. In general, the RPS composition showed a higher diversity than sheath composition, but some differences were observed among the strains. *S. javanicum* maintained the same diversity between RPS and sheath, while *P. ambiguum* and *N. commune* showed a lower diversity for the sheath compared to the RPS (**Table 2**).

In the sandy soil microcosms, comparison between *P. ambiguum* and *S. javanicum*-inoculated soils highlighted differences mostly in LB-EPS composition, while TB-EPS composition resulted similar between the two strains (**Figures 8C,D**, **Supplementary Table S1**). Glucose was the most abundant monosaccharide in LB-EPS and also galactose was found at a relatively high percentage in *S. javanicum*-inoculated sand (**Figure 8C**). TB-EPS was mostly composed of glucose, galactose, mannose, xylose, and uronic acids in both inoculated soils (**Figure 8D**, **Supplementary Table S1**). Noticeably, the monosaccharidic profile composing TB-EPS resulted similar or even higher than the respective sheath or RPS profiles, in terms of diversity (**Table 2**). In *P. ambiguum*-inoculated sand, mannose and xylose were present in TB-EPS, and the same were only present in RPS (and not in the sheath); same occurred for xylose in *S. javanicum*-inoculated microcosms. On the other hand, the TB-EPS extracted from *S. javanicum*-inoculated microcosms contained for example mannose, which was only present in the strain's sheath (and not in its RPS).

DISCUSSION

The current study allowed exploring the growth performance and amount and chemical features of EPS of three biocrust cyanobacterial strains when grown in liquid culture and on a sandy soil. Although direct extrapolation of results from one experimental setting to another cannot be done due to the intrinsic characteristics of the growing media (liquid and solid), which affect light distribution, cyanobacteria dispersion in the medium, and contrasting capabilities for the use of nutrients, among others, application of similar conditions for both experiments (light, temperature and addition of nutrient medium) facilitates, to the best extent, comparisons of results

among strains in the two growing conditions. Contrasting growth performances and EPS synthesis and chemical features of the cyanobacterial strains under the two growth conditions provide valuable insights for the screening of cyanobacteria candidates to be used in soil restoration.

Growth and EPS Characteristics of the Cyanobacterial Strains in Liquid Cultures

The three cyanobacteria strains showed a significant growth within 9 days, as shown by the increase of dry weight and chlorophyll concentration. At the end of the experiment, the dry weight surpassed 1 g L^{-1} in all the strains (**Figure 2**). *P. ambiguum* was the strain that showed the higher growth, reaching 3.33 g L^{-1} (dry weight). F_v/F_m remained stable during the whole cultivation period although at different levels. The highest value (0.525 ± 0.039) was found in *N. commune* (**Figure 3**). The F_v/F_m ratios, usually lower in cyanobacteria than in green algae, can be explained by different ratios of PSI/PSII, which in cyanobacteria varies from 2 to 3.5 (Vermaas, 2001; Fraser et al., 2013), and raises the level of minimum level of fluorescence, Fo. However, F_v/F_m ratios well mirrored those of initial slopes (α) in the three tested strains. The rETR values of the strains correlated well with the increase in biomass being higher in *P. ambiguum*. This cyanobacterium showed the lowest quantum yield and the highest saturation irradiance I_k (**Table 1**), meaning that this organism utilizes light with lower efficiency and requires more light to saturate photosynthesis. One consequence could be that this cyanobacterium, compared to *N. commune* and *S. javanicum* is more adapted to cope with high light exposure, a condition that could be found in arid zones.

In all the strains the increase in cyanobacterial biomass over time was accompanied by a parallel increase in both total EPS (cellular EPS + RPS) and RPS contents (**Figure 4**). An increase in the amount of RPS along with cell growth in batch cultures has been previously documented (Rossi and De Philippis, 2015). Total EPS content was up to 14 times higher than RPS content, highlighting the significant contribution of cell sugars content to total EPS amount. Parallelly to the higher dry weight and chlorophyll content in *P. ambiguum*, this strain also showed the highest amounts of total and released EPS. The greater growth and EPS synthesis by *P. ambiguum* in comparison to the other two strains can be attributed to the presence of a nitrogen source in the medium, which is a condition requiring lower energy for the assimilation of combined nitrogen compared with the energy needed for nitrogen fixation (Otero and Vincenzini, 2003).

In our experiments, with nutrient replete cultures, both RPS and sheath fractions showed the presence of very high MW polymers (> 1.1MDa). Previous studies have also confirmed that most cyanobacterial EPSs are characterized by the presence of high MW components (Pereira et al., 2009). However, a relevant presence of smaller macromolecules (<50kDa) was also found in both RPS and sheath (**Figures 7A,B**). This result is in contrast with that obtained by Mugnai et al. (2018a) who found for the strain *Schizothrix cf. delicatissima AMPL0116* that

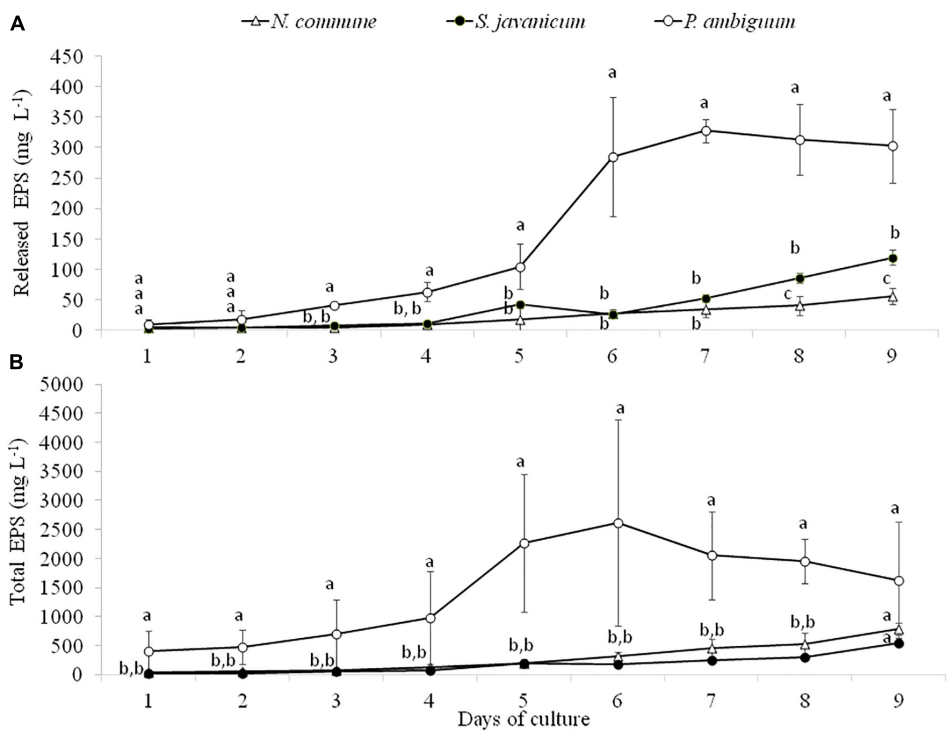

FIGURE 4 | Released **(A)** and total EPS **(B)** content during the cultivation period of the three cyanobacteria strains. Different letters indicate significant differences among the strains for each day.

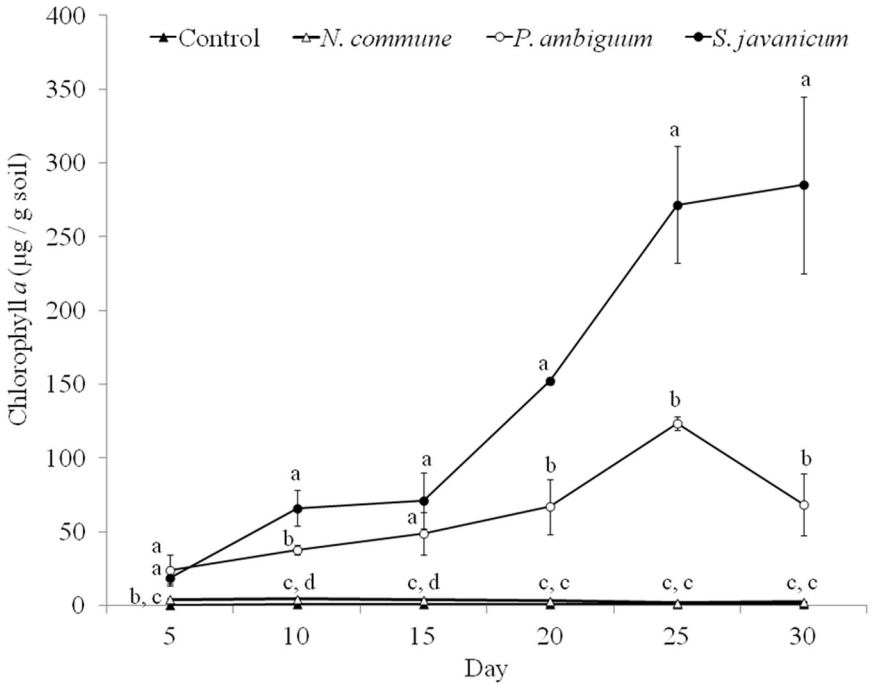

FIGURE 5 | Chlorophyll *a* content over time in the control and inoculated sandy soil microcosms. Different letters indicate significant differences among the control and cyanobacteria-inoculated soils for each sampling day.

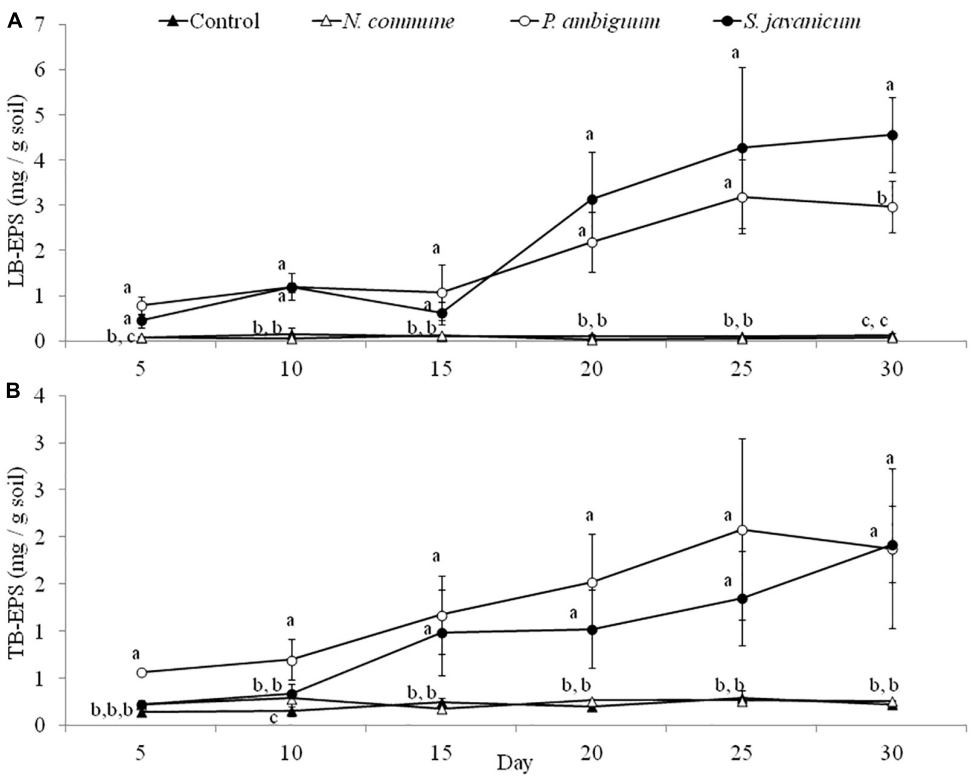

FIGURE 6 | LB-EPS **(A)** and TB-EPS **(B)** contents over time in the control and inoculated sandy soil microcosms. Different letters indicate significant differences among the control and cyanobacteria-inoculated soils for each sampling day.

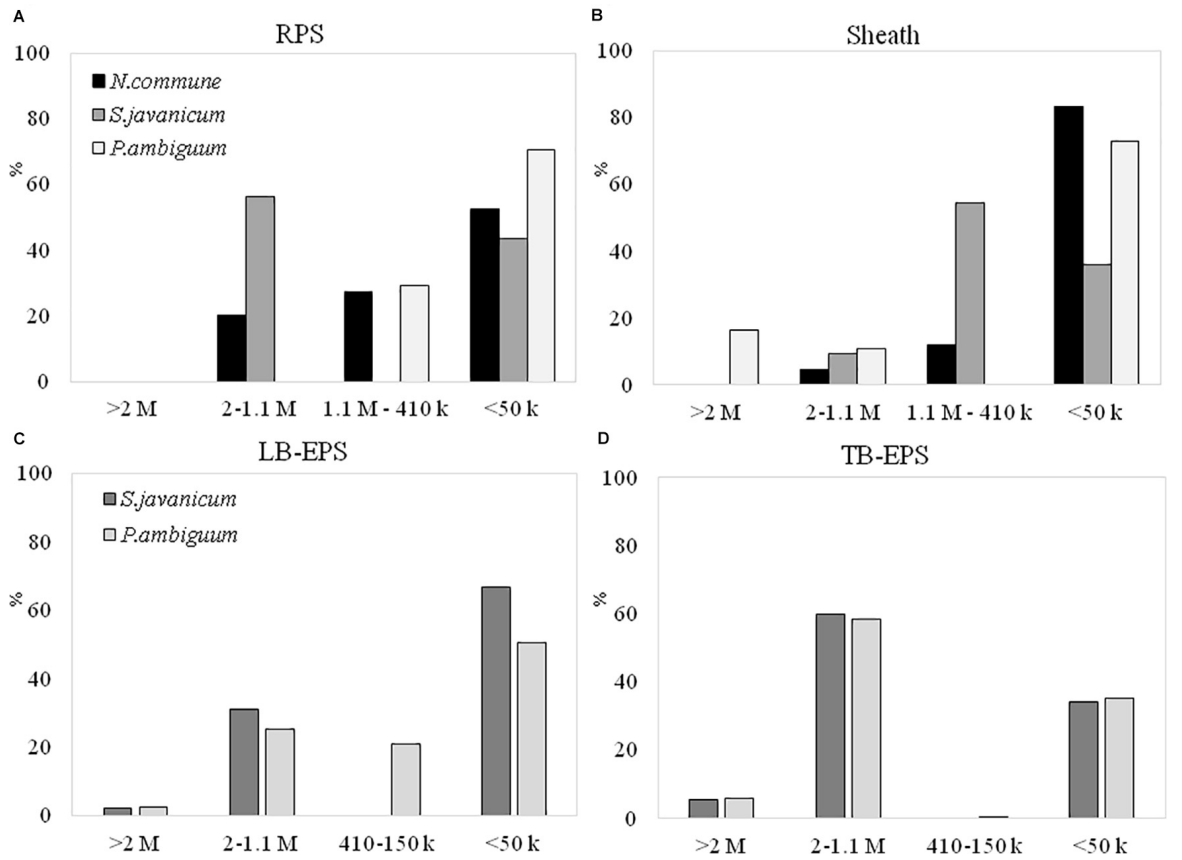

FIGURE 7 | MW distribution of the EPS extracted from liquid culture: **(A)** RPS, **(B)** sheath, and from sandy soil microcosms: **(C)** LB-EPS; **(D)** TB-EPS, of the cultured strains. Relative proportions (%) of MW classes are represented. No data are reported for *N. commune* in the sandy soil microcosms (**C,D**) because of the negligible amounts of EPS extracted (See **Figure 5**).

both RPS and sheath were mostly composed of macromolecules higher than 1 MDa.

EPS composition of the three cyanobacterial strains was characterized by a high complexity, and a high number of monosaccharides (up to 12, see **Figure 8**, **Table 2**, **Supplementary Table S1**) was identified. EPS composition of cyanobacterial sheaths and RPS showed different composition profiles. Within each EPS fraction, the composition was similar for *N. commune* and *P. ambiguum*, and different for *S. javanicum* (**Figures 8A,B**, **Supplementary Table S1**). The EPS released into the medium were mainly composed of glucose, and this monosaccharide was also the main component for *S. javanicum* and *P. ambiguum* sheaths (63.5 and 35.4%, respectively), while for *N. commune* the major sheath component was xylose (30.6%), and glucose as the second one (26.6%) (**Figures 8A,B**, **Supplementary Table S1**). This composition partially confirms the results of previous studies that reported the presence of different sugars in *N. commune* colonies such as glucose, galactose, xylose, and uronic acids (Helm et al., 2000). Hu et al. (2003) reported that glucose was the most abundant monosaccharide (44%) in *Nostoc* sp., with galactose and xylose being also found at a high molar percentage (21.5% and 20.9%, respectively). These authors also reported that most abundant monosaccharides in the EPS from *S. javanicum* were glucose (24.8%), galactose (23.4%) and mannose (22.9%), and for one species of the genus *Phormidium*, *P. tenue*, were arabinose (43.9%), glucose (32.5%) and rhamnose (10.4%).

Growth of the Cyanobacterial Strains and Characteristics of the Induced EPS Matrix in the Sand Microcosms

A different pattern in cyanobacteria growth among the three strains was observed in the sandy soil microcosms. When inoculated on the sand, *S. javanicum* exhibited the highest growth, followed by *P. ambiguum*, as shown by the increase in chlorophyll content after one month of soil incubation (**Figure 5**). Both EPS fractions LB-EPS and TB-EPS increased over time as chlorophyll increased (**Figure 6**). A similar trend has been described in previous studies that documented an increase in chlorophyll *a* in cyanobacteria inoculated soils accompanied by an equivalent increase in EPS (Mazor et al., 1996; Chamizo et al., 2018). No growth was observed for *N. commune,* which showed a chlorophyll *a* content close to zero and similar to the non-inoculated sand. In their natural habitat, *N. commune* forms macroscopic colonies in which the entangled filaments are

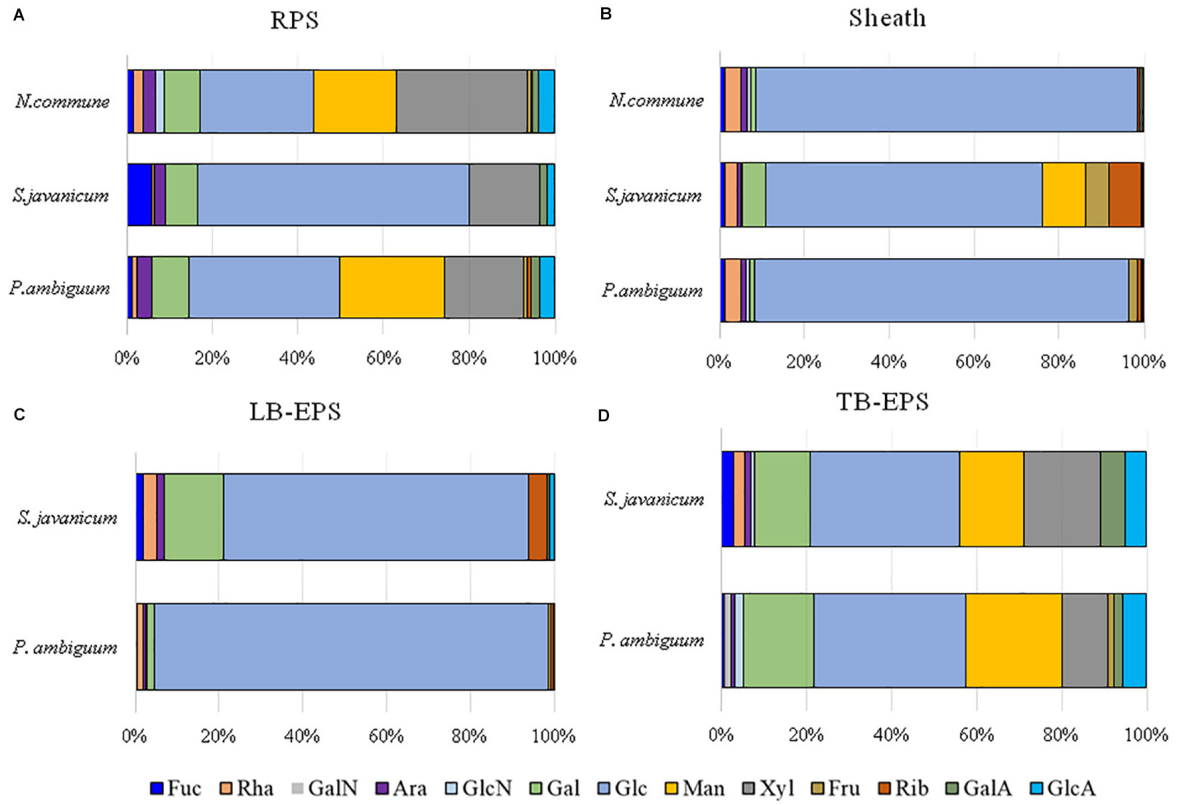

FIGURE 8 | Monosaccharidic composition of the EPS extracted from liquid culture: **(A)** RPS, **(B)** sheath, and from sandy soil microcosms: **(C)** LB-EPS; **(D)** TB-EPS, of the cultured strains. Molar percentages (%) of single sugars are represented (expressed as moles of the single monosaccharide divided by the total amount of moles of monosaccharides in the EPS × 100). No data are reported for *N. commune* in the sandy soil microcosms (**C,D**) because of the negligible amounts of EPS extracted. Abbreviations: Fuc fucose, Rha rhamnose, GalN galactosamine, Ara arabinose, GlcN glucosamine, Gal galactose, Glc glucose, Man mannose, Xyl xylose, Fru fructose, Rib ribose, GalA galacturonic acid, GlcA glucuronic acid.

embedded in massive polysaccharidic structures which are crucial in the stress tolerance of this species to drought and frequent desiccation-rewetting (Potts, 1994) and freezing-thawing cycles (Tamaru et al., 2005). The chemical analysis on its RPS and sheath showed a higher percentage of low MW polymers (<50 kDa), compared to the two other strains, despite a very similar composition in monosaccharides to *P. ambiguum* EPS. The presence of smaller polymers may have reduced *N. commune* capability of forming stable aggregates in the sandy soil for growth sustain. Previous studies have shown the efficiency of this cyanobacterium to grow and lead to stable biocrusts over fine-textured soils (<70% sand) (Román et al., 2018; Roncero-Ramos et al., 2019a,b). However, in coarse soils, the ability of this cyanobacterium to bind sand grains and form stable organo-mineral layer could be reduced, as has been also reported for other cyanobacterial strains such as *Microcoleus vaginatus* (Rozenstein et al., 2014) or *Leptolyngbya ohadii* (Mugnai et al., 2020) on coarse sand. Of the two strains that showed a significant growth on the sandy soil, *S. javanicum* showed higher chlorophyll *a* (**Figure 5**) and LB-EPS contents than *P. ambiguum* (**Figure 6A**), regardless the latter strain was supplied with nitrogen-rich medium (BG11).

Under natural stressing conditions, cyanobacteria are considered to produce compositionally simpler EPS compared to liquid cultures, where they experience optimal abiotic conditions and excess of nutrients (Brüll et al., 2000). In the current study, under optimal conditions of water and nutrient availability, the soil EPS matrix was still characterized by a high number of monosaccharides (7–11), being almost always glucose largely the most abundant (**Figure 8**, **Supplementary Table S1**). While LB-EPS was compositionally simpler, TB-EPS showed a grater sugar diversity (**Table 2**) and besides glucose, galactose, mannose, xylose, and uronic acids were also relatively abundant. In induced biocrusts of different ages, mannose and glucose resulted the sugars present at the highest molar percentages in the LB-EPS fraction, while mannose, glucose, galactose and galacturonic acid were present at the highest molar percentages in the TB-EPS fraction (Chen et al., 2014). In inoculated sand with *S. javanicum* and *P. ambiguum,* but in the absence of nutrient supply and subjected to low water additions, the most abundant monosaccharides were glucose and galactose in the two soil EPS fractions, with also relatively high abundances of other sugars such as mannose, xylose, rhamnose and fructose in the TB-EPS fraction

TABLE 2 | Diversity indices of the EPS fractions extracted from the three strains in the two culturing conditions.

Culture condition	EPS fraction	Strain	Number of sugar residues S	Dominance D	Shannon H	Evenness e^H/S	Equitability J	Fisheralpha
Liquid	RPS	*N. commune*	12	0.21	1.81	0.51	0.73	1.35
culture		*S. javanicum*	8	0.44	1.21	0.42	0.58	0.85
		P. ambiguum	11	0.23	1.74	0.52	0.72	1.22
	Sheath	*N. commune*	9	0.82	0.50	0.18	0.23	0.98
		S. javanicum	11	0.45	1.28	0.33	0.53	1.22
		P. ambiguum	11	0.44	0.59	0.32	0.52	1.22
Sandy soil	LB	*S. javanicum*	7	0.56	0.95	0.37	0.49	0.74
microcosms		*P. ambiguum*	8	0.88	0.33	0.17	0.16	0.89
	TB	*S. javanicum*	10	0.21	1.84	0.63	0.80	1.10
		P. ambiguum	11	0.23	1.75	0.50	0.72	1.28

(Chamizo et al., 2019). According to Brüll et al. (2000), the heterogeneous sugar composition detected in our experiment could be due to the availability of nutrients. However, the experiment previously cited by Chamizo et al. (2019) led to a complex monosaccharidic profile even without nutrient addition, suggesting that in the natural conditions to which Brüll et al. (2000) refer, some other abiotic factors may influence the composition of the EPS.

When EPS features of the inoculated sandy soils were compared with the EPS profile of liquid cultures, we found that the soil TB-EPS fraction reflected both the MW distribution and monosaccharidic composition of the sheath but also the RPS fraction. Accordingly, Mugnai et al. (2018b) reported that EPS from the cyanobacterium *Schizothrix cf. delicatissima AMPL0116* was significantly different in soil growth compared to liquid cultures, both in composition and MW distribution, affecting these changes mainly the more condensed TB-EPS fraction, while no alterations were observed for the more soluble LB-EPS fraction. Their results showed that TB-EPS fraction was composed of a higher number of sugars than the LB-EPS fraction, and they attributed this difference to the different composition between sheath (composed of a higher number of sugar residues) and RPS (less heterogenous than the sheath). Our results, in turn, show that the TB-EPS fraction was very heterogeneous, resembling, on the contrary, the RPS fraction as witnessed by its high diversity compared to the sheath and by the presence of RPS-exclusive sugar residues both for *S. javanicum* and *P. ambiguum*. Moreover, the diversity describing the composition of the TB-EPS fraction resulted even higher than the RPS fraction alone (**Table 2**). It is indeed hard to dissect between two possible phenomena that may have occurred, that is, between the possibility that organisms produced polymers composed of different monosaccharides when growing on a different substrate, or that the complexity observed in TB-EPS composition was the result of the extraction procedure that removed a mixture of RPS and sheath EPS. The high diversity of TB-EPS leads us to believe that when the RPS get in contact with soil particles, the various chemical features of the sugars residues make the polymers bind tightly to the particles, so that they only become extractable with Na_2-EDTA treatment (TB-EPS extraction), while only the very soluble glucose-based polymers can be extracted with water (LB-EPS extraction). This can easily be related to the fact that the growth and high EPS production capability of cyanobacteria in soil favor the formation of aggregates (Issa et al., 2001; Mager and Thomas, 2011).

Implications for Soil Restoration

Production of EPS by cyanobacteria inoculation on the soil induces changes in soil properties important for a number of processes occurring at the soil surface. Cyanobacterial filaments together with their sticky EPS bind sand grains (Mugnai et al., 2018b), forming a cohesive and stable layer that contributes to reducing soil erosion (Kheirfam et al., 2017; Fattahi et al., 2020), one of the most important processes accelerating land degradation in drylands (Ravi et al., 2010). Experiments carried out in sand dunes in China have shown effective soil stabilization after inoculation with *Microcoleus vaginatus* and *S. javanicum*, encouraging soil colonization by other biocrust organisms and accelerating biocrust succession (Lan et al., 2014; Park et al., 2017). EPS also increase water retention capacity of the surface and thus play an important role in the maintenance of moisture (Mazor et al., 1996; Adessi et al., 2018). However, on very sandy soils, EPS have been also reported to retard water movement and reduce the amount of water that can penetrate into the sand (Mazor et al., 1996) thus decreasing hydraulic conductivity (Colica et al., 2014). We found that soil hydrophobicity was increased in the sandy soil inoculated with *S. javanicum*, probably associated to a higher growth and EPS release (especially of the LB-EPS) by this strain compared to *P. ambiguum*. In a previous study (Chamizo et al., 2019) we found no increase in hydrophobicity by inoculation of sandy soil with this strain possibly explained by the lower EPS amount (∼1 mg/g soil) under more water stressing conditions compared to the higher EPS amount (∼6.5 mg/g soil) recorded in this study where sandy soils were supplied with water and nutrients. Nevertheless, an increase in soil hydrophobicity by cyanobacteria inoculation on sandy soils could have as advantage a decrease in the time for runoff start and an increase in runoff yield, providing surplus water to downslope vegetation patches and favoring plant survival in drylands.

On the other hand, our results also showed that the monosaccharidic features of the released EPS and of sheath

don't represent *per se* an advantage for surviving in a sandy environment, as suggested by the scant growth of *N. commune*, despite the strain showed a polymer composition very similar to the EPS of *P. ambiguum*. Indeed, it might have been the smaller dimension of the secreted polymers that contributed in preventing *N. commune* growth in the sandy soil. These may be relevant information when planning the selection of cyanobacteria candidates for soil restoration purposes. In addition, our findings suggest that inoculation of the soil with the cyanobacterial biomass as well as their RPS can provide additional advantages derived from: (1) the supply of low MW molecules contained in the RPS fraction that could be easily hydrolyzed and used as carbon sources for soil heterotrophic microorganisms; (2) addition of a medium characterized by a diverse monosaccharidic composition, likely with different traits and contributing to increasing soil microbial heterogeneity. Indeed, in the attempt to extrapolate soil restoration techniques based on cyanobacteria inoculation from lab to the field, our findings point out the need of further considering the importance of the characteristics of the EPS released during culture growth in providing successful results, by improving conditions for cyanobacteria survival and growth and help them cope with abiotic stresses.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SC conceived the idea, designed the experiment, performed the laboratory analyses, analyzed the data, and wrote the manuscript. AA designed the experiment, performed the laboratory analyses, analyzed the data, and wrote the manuscript. GT designed the experiment, analyzed the data, and improved manuscript editing. RD conceived the idea, designed the experiment, and improved manuscript editing. All authors contributed with constructive comments to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.568224/full#supplementary-material

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Applications of Wild Isolates of *Saccharomyces* Yeast for Industrial Fermentation: The Gut of Social Insects as Niche for Yeast Hybrids' Production

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In the industry of fermented food and beverages, yeast cultures are often selected and standardized in order to ensure a better control of fermentation and a more stable product over time. Several studies have shown that the organoleptic characteristics of fermented products reflect geographic variations of the microbial community composition. Despite investigations of the worldwide distribution and genetic diversity of *Saccharomyces cerevisiae*, it is still unclear how and to what extent human intervention has shaped the brewer's yeast population structure. The genotypic and phenotypic characterization of environmental yeast populations and their potential application in the fermentative processes can significantly enrich the industrial fermentation products. Social insects have proven to be closely associated to the yeasts ecology. The relationships between yeasts and insects represent a fundamental aspect for understanding the ecological and evolutionary forces shaping their adaptation to different niches. Studies on phylogenetic relationships of *S. cerevisiae* populations showed genetic differences among strains isolated from gut and non-gut environments (i.e., natural sources and fermentation). Recent evidences showed that insect's gut is a reservoir and an evolutionary niche for *Saccharomyces*, contributing to its survival and evolution, favoring its dispersion, mating and improving the inter-specific hybrids production during hibernation. Here, we discuss the potential use of social insects for production of a wide range of hybrid yeasts from environmental *Saccharomyces* isolates suitable for industrial and biotechnological applications.

Keywords: *Saccharomyces cerevisiae*, social insects, yeast–insect association, hybrids, wine, beer, biotechnological application, ecology

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HISTORY OF FERMENTATION AND YEAST DOMESTICATION

Many species of Saccharomycetes and non-Saccharomycetes are essential components of human production of fermented food and beverages. *Saccharomyces cerevisiae*, known as the brewer's or bakery's yeast, has been widely used for its fermentative capacity for thousands of years (Legras et al., 2007) (**Figure 1**). The fermentation process has allowed preservation of perishable food and made bioavailable nutrients and microelements, improving the quality of foods (Hatoum et al., 2012). The oldest traces of yeast and fermented foods have been found in the tomb of U-j

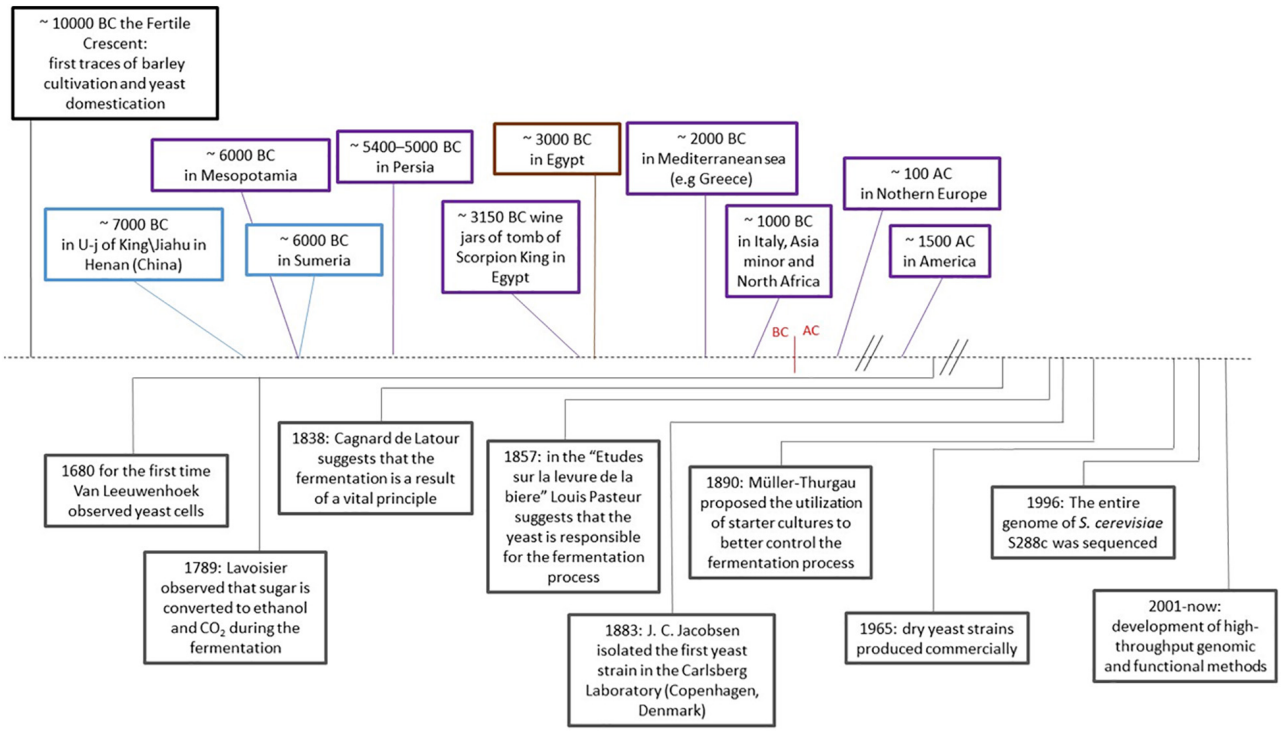

FIGURE 1 | History of fermentation and yeast domestication. *S. cerevisiae* and human activities related to the production of fermented products. In the timeline: on the top of the dashed line, the oldest traces of *S. cerevisiae* across the human history and geography. Blue: fermented products; purple: wine; brown: bakery products. Below the dashed line, events related to *S. cerevisiae* in the history of fermentation and scientific advancements.

of King/Jiahu in Henan in China (7,000 B.C.) (McGovern et al., 2004), in Iran (6,000 B.C.) (Fatahi et al., 2003), in wine jars of tomb of Scorpion King in Egypt (3,150 B.C.) (Cavalieri et al., 2003), and in Mesopotamia (about 4,000 to 3,100 B.C.) (McGovern, 2003), demonstrating that fermentation of cereals, honey and fruit, has been carried out since the dawn of civilization (McGovern et al., 2004; Kupfer, 2013, 2015). Successively, viticulture spread in Asia Minor and northern Africa, and around 1,000 B.C. arrived in Mediterranean countries (Legras et al., 2007). Beer production is supposed to be almost as ancient as wine and came from the Middle East, subsequently acquired by Germanic and Celtic populations around 1st century A.C. (Legras et al., 2007).

The long lasting association of *S. cerevisiae* with fermentative processes has led to propose the idea that its wide use caused its domestication as adaptation to different fermented products (Legras et al., 2007; Sicard and Legras, 2011). Yet the effect of domestication on trait selection has been proven only in the yeast strains used for the beer production (Piskur et al., 2006; Berlowska et al., 2015; Gallone et al., 2016, 2018).

The art of fermentation had been developed empirically from generation to generation. Scientific awareness related to biochemical transformations during fermentation started around the end of the 18th and in 19th century (Gay-Lussac, 1815; Kützing, 1837; Cagniard-Latour, 1838). In 1883, the first pure yeast culture was created by Emil Christian Hansen for beer production (**Figure 1**). Hansen had been brought to work from Copenhagen University to the Carlsberg's Laboratories by Jacobsen, the founder of the Carlsberg Empire, to standardize the quality of beer by isolating and stabilizing the microorganism used in the brewing process. Subsequently in 1890, Hermann Mueller-Thurgau planned the process for a better control and repeatability of wine fermentations with starter cultures (Marsit and Dequin, 2015) (**Figure 1**). The rise of the industry of fermented products and the application of innovative biotechnological methods for selection of enhanced yeast strains or production of hybrids suitable for different types of fermentable substrates enables a much more defined control of the fermentation process than the generally used strains.

SOCIAL INSECTS AND YEASTS OF NATURAL ENVIRONMENTS

The ability of yeasts to metabolize sugars by producing ethanol through anaerobic fermentation even when oxygen is available (the Crabtree effect) (Chambers and Pretorius, 2010) and in presence of high glucose concentrations (Otterstedt et al., 2004) has enabled *S. cerevisiae* to gain an evolutionary advantage over other microorganisms (Albergaria and Arneborg, 2016). Ethanol production and acidification of the growth medium have always kept bacteria and other aerobic molds under control. The microbial communities, including yeasts, can play a key role in triggering fermentation processes conferring a typical bouquet,

thanks to volatile compounds (El-Sayed et al., 2005; Christiaens et al., 2014). *S. cerevisiae* produces several aromatic esters: ethyl acetate (varnish, nail polish, and fruity aroma), isoamyl acetate (banana and pear), isobutyl acetate (banana), phenylethyl acetate (fruity and flowery), ethyl hexanoate (apple, banana, and violets), ethyl octanoate (pineapple and pear), and ethyl decanoate (floral) (Ruiz et al., 2019).

The question of the natural environmental niche for *S. cerevisiae* was debated for a long time (Goddard and Greig, 2015). Environmental *S. cerevisiae* strains are subjected to harsh conditions and they developed survival strategies, which are not retained when laboratory or industrial strains are cultured under most favorable conditions. *S. cerevisiae* has been isolated from different natural environments, such as oak trees (Sampaio and Goncalves, 2008; Zhang et al., 2010; Hyma and Fay, 2013), maize (Hayford and Jespersen, 1999; Halm et al., 2004), various fermentations and other substrates, including soil (van der Aa Kuhle et al., 2001; Oba et al., 2011; Dunn et al., 2012; Kubo et al., 2014).

Saccharomyces cerevisiae has adapted itself and evolved to different environmental niches (Goddard and Greig, 2015; Peter et al., 2018). Evidences indicated that, although *S. cerevisiae* is found in abundance in environments, such as wineries, it does not originate from grapevines or grape berries. Mortimer and Polsinelli (Mortimer and Polsinelli, 1999) demonstrated that *S. cerevisiae* was found at very low frequency on unripe and intact grape berries (0.05% on average), while during grape maturation it was present on average with a frequency of 25% on broken berries (Mortimer et al., 1994; Polsinelli et al., 1996; Mortimer and Polsinelli, 1999). Thus, ripe and crushed grape berries (with high concentration of fermentable sugars), represent a suitable environment for *S. cerevisiae*. In a recent study (Taylor et al., 2014) the rare presence of *S. cerevisiae* in intact grapes was confirmed: one *S. cerevisiae* cell was found among 20,000 cells of other fungi by metagenomic approach.

Researchers wondered how *S. cerevisiae* could be carried on the grapes. The agents proposed to play a role in spreading of microorganisms in the environment are animal vectors (e.g., insects and birds) (Mortimer and Polsinelli, 1999; Goddard et al., 2010; Stefanini et al., 2012). Unlike bacteria and fungal spores that can easily be dispersed by other means (e.g., air/wind) (Madden et al., 2018), yeast spores are not adapted for wind-borne transmission. Many decades ago (Grace and Collins, 1976), testing the dispersion rate of bacteria and yeasts spores using a wind tunnel, demonstrated that the wind is unable to disperse *S. cerevisiae* cells when these are adhered to the leaf surface.

Evidences showed that insects have a mutualistic relationship with yeasts (Belisle et al., 2012; Stefanini et al., 2012, 2016; Madden et al., 2018) and play a key role for yeast dispersion in natural environments. *Saccharomyces* spp., in particular *S. cerevisiae*, were detected in different insects worldwide (Stefanini et al., 2012; Buser et al., 2014; Jimenez et al., 2017; Meriggi et al., 2020; YeastFinder[1]; **Table 1**).

Social insects represent a fulcrum in the yeast ecology and evolution. At the same time, volatile compounds produced by yeasts attract insects that preferentially foraged nectar sources, and influence their behavior and physiology (Becher et al., 2012; Babcock et al., 2017; Stefanini, 2018).

In our previous studies (Stefanini et al., 2012, 2016), we demonstrated not only that wasps contribute to dispersion of yeast strains into the environment, but also that they can host yeasts in their gut, contributing to their survival and biodiversity. *Polistes dominula* allows the transmission of yeasts to the progeny (Stefanini et al., 2012), ensuring the presence of yeast in the colony and in the foraging area. This make a flow of yeast cells in the environments (in the vineyard, on grapes) from autumn to spring. These observations allow to explain where yeasts can reside during the winter and how reappear during the spring and summer, an unsolved question up until few years ago.

In spite these evidences, specific factors that select natural yeasts in the insect gut have not yet been found. Our recent findings showed that yeast has evolved strategies to adapt to the gastrointestinal tract of insects (Stefanini et al., 2012, 2016; Ramazzotti et al., 2018). The wall of the ascospore is able to resist to the insects gastric digestion, allowing survival within the host gut (Coluccio et al., 2008; Stefanini et al., 2012, 2016). More recently, we demonstrated that host's gut, not only of insect but also human, is a potential reservoir for yeasts (Ramazzotti et al., 2018). Genetic and phenotypic differences, including peculiar cell wall composition, different ability to sporulation and to induce host immune response were discovered between strains isolated from human and insect's gut and non-gut environment, suggesting the existence of gut-specific features that could represent a selective advantage for survival and expansion in the gut environment (Ramazzotti et al., 2018).

Based on our previous studies (Stefanini et al., 2012, 2016), the insect's gut is an advantageous ecological niche for

[1]www.stefaninilab.com/tools/

TABLE 1 | Isolation of *Saccharomyces* spp. in the insects' gut and geographical distribution.

Insect order	Insect common name	Yeast species	Geographical distribution	References
Hymenoptera	Wasps Honeybees	*Saccharomyces cerevisiae* *Saccharomyces ludwigii*	North America, Brasil, Europe, and New Zeland	Batra et al. (1973), Sandhu and Waraich (1985), Stefanini et al. (2012), Jimenez et al. (2017), and Meriggi et al. (2019)
Diptera	Fruit flies Flies	*Saccharomyces cerevisiae* *Saccharomyces ludwigii*	New Zeland, Australia, Taiwan, Seychelles Islands, Brasil, and Europe	Phaff and Knapp (1956), Kircher et al. (1982), Broderick and Lemaitre (2012), Buser et al. (2014), and Meriggi et al. (2019)
Coleoptera	Beeteals	*Saccharomyces cerevisiae*	North America, Asia, and Africa	Suh et al. (2005) and Stefanini (2018)

S. cerevisiae, favoring the intra− and inter−species mating of yeast cells, and allowing increased fitness of hybrids, thus representing an environment favoring the generation of yeast genetic biodiversity.

The current used industrial strains represent a small fraction of the natural biodiversity (Liti et al., 2009). The nature could provide unknown strains with relevant characteristics that may enhance the industrial fermentations. Specific strategies could help to transfer these properties to industrial strains or create novel strains with best performance for fermentation processes. Here, we propose and discuss the use of wasp's gut for accelerated selection and production of hybrids suitable for different types of fermentable substrates.

YEAST STRAINS AND FERMENTATION INDUSTRY: HYBRIDS DO IT BETTER

Recent genomic studies provided a comprehensive overview of the biodiversity of wild and industrial *Saccharomyces* strains (Liti et al., 2009; Schacherer et al., 2009). Diversity within *S. cerevisiae* population structure was at least in part associated to its industrial application. According to the domestication hypothesis, this long-term process has resulted in different strains with specific characteristics suited for industrial fermentation, clustering differently from wild populations (Fay and Benavides, 2005; Liti et al., 2009; Schacherer et al., 2009; Sicard and Legras, 2011). Several studies reported genome-wide signatures of clonal expansion of yeast strains, as well as convergent evolution of industrially relevant traits in separate lineages (Liti et al., 2009; Gallone et al., 2016; Parapouli et al., 2020). Yet the only *bona fide* evidence for selection on genomic regions associated to domestication was reported by Gallone et al. (2019) that studied the whole-genome of more than 200 industrial yeasts showing that about 25% consisted of interspecific hybrids derived from *S. cerevisiae*, *S. kudriavzevii*, *S. eubayanus*, and *S. uvarum*. Langdon et al. (2019) analyzed the genomes of 122 interspecies hybrids and introgressed strains in *Saccharomyces* genus revealing three domesticated lineages, including wild lineages from Europe and Northern continents of the world. These evidences show that industrial yeasts are the result of selection following clonal expansion and adaptation of specific strains, shaped by genetic drift caused by bottlenecking.

Beer's yeasts present the strongest and maybe only genetic and phenotypic signatures of domestication (Gallone et al., 2019; Langdon et al., 2019). The strong selective pressure imposed over many generations allowed to obtain desirable phenotypes, but has also dramatically affected the genomic structure and stability of domesticated yeasts (Legras et al., 2007; Gallone et al., 2016, 2018). Hybrid strains are preferred in the industrial fermentation because they show phenomena, such as "vigor of the hybrid" that confer better fermentation capacity in terms of speed, use of alternative sugar source and an enriched pattern of aromatic compounds (Bellon et al., 2011; Piotrowski et al., 2012; Bellon et al., 2013; Gamero et al., 2013; Snoek et al., 2015).

An example of yeast's domestication trait is the ability to ferment maltotriose. This trait evolved independently and through different genetic pathways in the two main beer lineages, such as ale (by the top-fermenting *S. cerevisiae*) and lager (by the bottom – fermenter *S. pastorianus*, a interspecific hybrid *S. cerevisiae* × *S. eubayanus*), suggesting strong selection pressure (Gallone et al., 2016, 2018). *S. pastorianus* can ferment at lower temperatures than *S. cerevisiae* (Dunn and Sherlock, 2008; Libkind et al., 2011). This hybridization process has combined the efficiency of *S. cerevisiae* in the sugar metabolism and the cryoprotective capacities of *S. eubayanus* (Hebly et al., 2015; Krogerus et al., 2015). The cryoprotective capacity was demonstrated to be related with the mitochondrial genome inheritance. *S. eubayanus* (Baker et al., 2019) and *S. uvarum* (Li et al., 2019) mitochondrial genome provided with low-temperature tolerance to the interspecies hybrids compared when the same hybrids inherited the mitochondrial genome of *S. cerevisiae*. In addition, hybrids between natural strains of *S. kudriavzevii* × *S. cerevisiae*, selected to confer diverse flavors, are used for the production of Trappist beers (a subgroup of ale) (Gonzalez et al., 2008; Peris et al., 2018; Gallone et al., 2019; Langdon et al., 2019).

On the other hand, several authors suggested that during centuries of wine production, *S. cerevisiae* acquired remarkable resistance/tolerance to high sugar concentrations and nitrogen metabolic activity, through adaptive horizontal gene transfer and copy number variations (Fay and Benavides, 2005; Aa et al., 2006; Ezov et al., 2006; Ruderfer et al., 2006; Legras et al., 2007; Stefanini and Cavalieri, 2018). These events potentially conferred competitive advantages during must fermentation (Almeida et al., 2017), and production of a wide spectrum of aromatic profiles (González et al., 2007). Triple hybrid *S. cerevisiae* × *S. kudriavzevii* × *S. uvarum* has shown to be able to use fructose more efficiently than *S. uvarum* strain and to restart fermentation (Christ et al., 2015), avoiding the upper hand of bacteria and the consequent spoilage of the must. Inactive or stuck fermentations are detrimental to wine/beer production. These events have been reduced by the commercial availability of selected yeast strains used as starter. However, this practice limits the developing of wild yeasts during fermentation (Parapouli et al., 2020). Camarasa et al. (2011) investigated the fermentative efficiency of a set of *S. cerevisiae* strains, observing that strains isolated from sugar-rich environments were able to complete the fermentation process, while the laboratory or environmental strains were unable. The extensive genetic diversity of environmental *S. cerevisiae* isolates, in particular of interspecific hybrids, could be an extremely significant source of innovation for biotechnological applications (Parapouli et al., 2020).

BIOTECHNOLOGICAL APPLICATIONS OF INSECTS AS YEAST HYBRIDS' PRODUCERS

The quest for increased and improved productivity and adaptability to changing consumer preferences lead to the study and development of industrial strains with novel and desired properties.

Non-genetically modified organisms (non-GMO) and GMO techniques can be used for selection of yeast strains with suitable traits and industrially relevant phenotypes. Jan Steensels et al. (2014) extensively described these approaches, indicating their advantages and limitations. Non-GMO techniques have been developed to create performant yeast variants, that can be freely used in industrial fermentations, without encounter any problems with legislation and/or consumer acceptance.

Currently there are four main approaches for generate artificial diversity in yeast strain using sexual hybridization: (i) direct mating – crossing of two haploid cells or spores of opposite mating types; (ii) rare mating – crossing of strains without sporulation by occasional and rare homothallic mating-type switch; (iii) mass mating – crossing of multiple parental strains or a heterogeneous population of the same parental strain; and (iv) genome shuffling of multiple strains. In addition, asexual hybridization includes cytoduction (a method that transfer cytoplasmically inherited traits) and protoplast fusion (asexually merging of cells after cell wall distruption in osmotic medium). Overall, the above mentioned approaches could have important impact in the optimization of industrial processes, at technical and economic level. Our previous studies demonstrated that the insects' gut represents the environment where *S. cerevisiae* mates and interspecific hybrids arise spontaneously. After 2 and 4 months of hibernation in the wasp's gut, *S. paradoxus* can survive only in hybrid shape with *S. cerevisiae*. The rate of inbreeding in *S. cerevisiae* spores increases up to ten times when inoculated in the insect gut. The high frequency of outbreeding coincides with the rates of mosaicism and genetic diversity in yeast strains (Reuter et al., 2007).

In last years, the interest for insects for food and applicative purposes is growing. The insects breeding is a sustainable method with low energy impact and represents a great opportunity for large-scale industrial applications. Social insects could play a role in the evolution and genetic recombination of yeasts. They seems to be a perfect niche in which the formation of hybrids occurs naturally and much more efficiently than in any other wild place, without employing artificial strategies of genetic manipulation, as recently showed by Peris et al. (2020) making synthetic hybrids by six yeast species. Wasps could provide yeast communities with the level of genetic variation required in time to adapt to a changing environment. Given the fast pace at which climate change is affecting the man made, "ersatz" environment where fermented products are produced, maintaining the genetic variation force driven by the presence of insects could be very important to support the adaptability of yeast to these changes. Considering that many yeasts used in the fermentation industry are interspecific hybrid strains, we could speculate that insects have been the breeders of the past, having likely provided a great deal to the evolution of brewing and wine making. The need of product diversification present in the fermented beverages field could look with great interest at the potential of using wasps as breeding places to produce yeasts of biotechnological interest, including yeasts producing beverages with a reduced ethanol content.

CONCLUSION

Optimization of current strategies and novel technologies such as *next-generation sequencing*, together with a better understanding of complex phenotypes allows to create yeasts with more variants and better adapted to the industrial goals. In the near future, the fermented beverages industry could benefit significantly from the possibility to breed insects massively and use them as a forge for accelerated selection and production of hybrids suitable for different types of fermentable substrates, further modeling these according to biotechnological requirements.

We can conclude that the yeasts–insect association certainly goes beyond the simple link between vectors and transported. In a future perspective, a better understanding of the ecology and relationships between insects and yeasts can play a key role in producing fermented beverages meeting the needs of tomorrow's consumers.

AUTHOR CONTRIBUTIONS

MDP, DC, and NM ideated the review. MDP and NM gathered the data. MDP, NM, and DC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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