A MULTIDISCIPLINARY LOOK AT *STENOTROPHOMONAS MALTOPHILIA*: AN EMERGING MULTI-DRUG-RESISTANT GLOBAL OPPORTUNISTIC PATHOGEN

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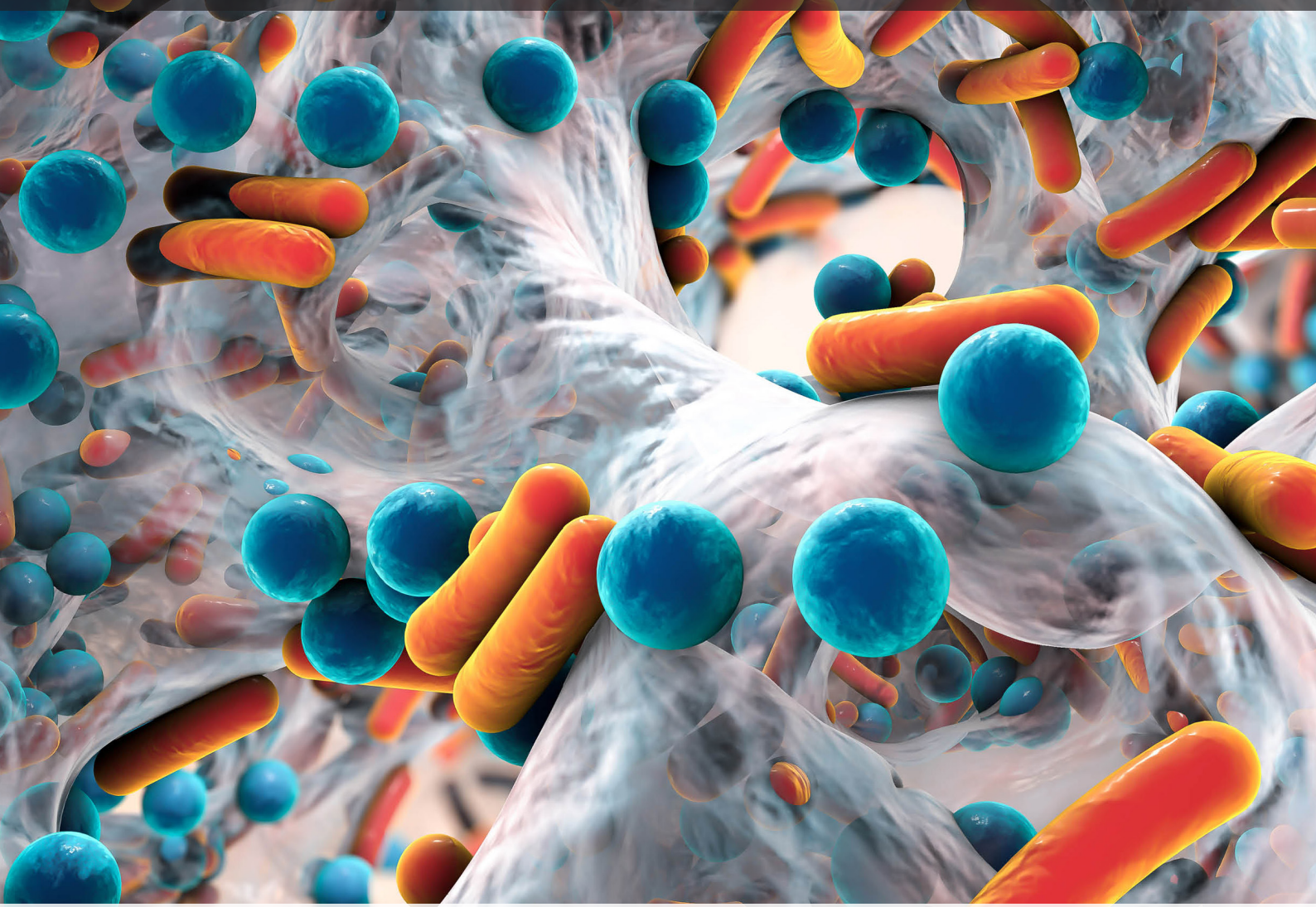

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A MULTIDISCIPLINARY LOOK AT *STENOTROPHOMONAS MALTOPHILIA*: AN EMERGING MULTI-DRUG-RESISTANT GLOBAL OPPORTUNISTIC PATHOGEN

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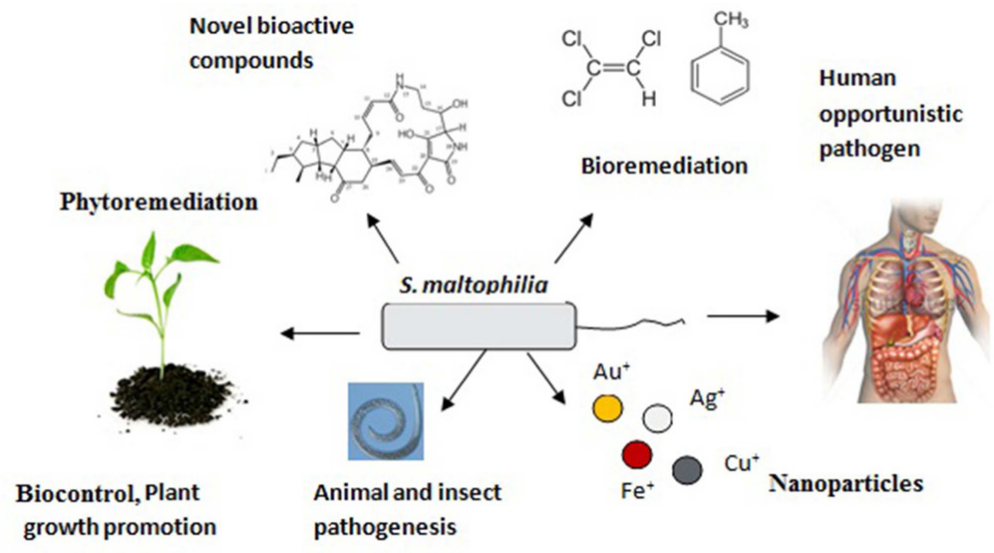

Multidimensional role of *Stenotrophomonas maltophilia* in our environment signifies its substantial genetic, metabolic, and residential diversity. Figure from *Front. Microbiol.* 7:967. doi: 10.3389/fmicb.2016.00967

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Stenotrophomonas maltophilia is a Gram-negative bacterium found in water, plant rhizospheres, animals, and foods. It is associated with a variety of infections in humans, involving respiratory tract (most common), soft tissue and bone, blood, eye, heart, and brain. This opportunistic pathogen is of serious concern to the immunocompromised patient population, and it is also being isolated with increasing frequency from the respiratory tract of individuals with cystic fibrosis. The observed increase worldwide in antibiotic resistance and the ability of this organism to make biofilms on epithelial cells and medical devices make it difficult for health-care personnel to treat infections caused by this pathogen. Recently, several genomes of *S. maltophilia* have

been sequenced, revealing high genetic diversity among isolates. This pathogen uses a variety of molecular mechanisms to acquire and demonstrate resistance to an impressive array of antimicrobial drugs. Research has also focused on the pathogenesis of *S. maltophilia* in animal models and the resulting host immune response. *S. maltophilia* is recognized as an important organism in the plant microbiome. This environmental bacterium uses a diffusible signal mechanism for controlling its colonization and interaction with other bacteria and plants. *S. maltophilia* has also gained considerable research interest for its biotechnological applications, with recent studies on enzyme production, anti-biofilm strategies, biodegradation, and bioremediation. This e-book focuses on the latest developments in the areas of physiology, genomics, infection and immunity, host-pathogen interaction, pathogenesis, antimicrobial resistance and therapy, molecular epidemiology, applied and environmental microbiology, bioremediation and biotechnology.

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EDITORIAL
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Editorial: A Multidisciplinary Look at *Stenotrophomonas maltophilia*: An Emerging Multi-Drug-Resistant Global Opportunistic Pathogen

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Editorial on the Research Topic

A Multidisciplinary Look at *Stenotrophomonas maltophilia*: An Emerging Multi-Drug-Resistant Global Opportunistic Pathogen

Stenotrophomonas maltophilia is a human opportunist pathogen, with an environmental origin. Members of the species are common inhabitants of water and soils, including rhizosphere. *S. maltophilia* can be a plant endosymbiont and is found in animals and washed foods. This Gram-negative bacterium has intrinsic resistance to various classes of antimicrobials. Within the *S. maltophilia* genome, genes encoding antibiotic inactivating enzymes, multidrug efflux pumps, and a quinolone resistance gene contribute to its reduced antibiotic susceptibility. Although a low virulence pathogen, *S. maltophilia* can cause various infections in susceptible patients. In addition, *S. maltophilia* isolates present important biotechnological properties, which can be carefully taken into consideration given the pathogenic potential of this microorganism. This research topic examines *S. maltophilia* from different perspectives, and it includes 11 articles: 1 commentary, 7 primary research articles, and 3 review articles.

In the first article, Berg and Martinez provide a global perspective on the two faces of *S. maltophilia*: a bacterial pathogen as well as an organism that can be used as a biocontrol agent or stress protective agent in crop production, and in bioremediation. They highlight the difficulties distinguishing between beneficial and harmful strains of *S. maltophilia*, and suggest *Stenotrophomonas rhizophila* as a close relative and harmless alternative for use in biotechnology. *S. rhizophila*, is a plant-associated organism that grows only at a lower temperature than *S. maltophilia*, suggesting its inability to cause disease in humans.

GENOMES AND BIOLOGY

The genetic and metabolic diversity of *S. maltophilia* reflects its diverse habitats. In their review, Mukherjee and Roy describe the intra- and inter-species genetic diversity of *S. maltophilia*

strains. The metabolic diversity of *S. maltophilia* is responsible for production of novel bioactive compounds including biocontrol agents against microbes and insects, enzymes and nanoparticles used in medicinal, industrial, and bioremediation applications. This article reviews the use of *S. rhizophila* in phyto- and rhizo-remediation.

Pompilio et al. examine the phenotypic and genotypic diversity of *S. maltophilia* during a decade-long colonization in the lungs of a cystic fibrosis (CF) patient. Two distinct groups are present among the 13 temporally isolated *S. maltophilia* strains. The study demonstrates that *S. maltophilia* adapts to the CF lung with increased growth rate and antibiotic resistance, but with lowered biofilm formation and decreased pathogenicity. During chronic CF lung infection, *S. maltophilia* develops new phenotypes, likely due to genetic or epigenetic changes.

ANTIBIOTIC RESISTANCE AND PATHOGENESIS

S. maltophilia has intrinsic resistance to various antibiotics. Sanchez in her review provides updated information about the molecular mechanisms underlying resistance: efflux pumps, low membrane permeability, antibiotic-modifying enzymes, or the quinolone resistance gene. *S. maltophilia* can acquire drug resistance via mutations and horizontal gene transfer. The understanding of the intrinsic resistome of *S. maltophilia* is currently limited. Sanchez reports on phenotypic resistance (without genotypic changes) that can occur in *S. maltophilia* including biofilm production. This review highlights the need for new drugs to thwart *S. maltophilia* infections.

In their review, Chang et al. provide information about prevalence rates of infection due to *S. maltophilia*, coming from nationwide and worldwide surveillance. They report on the prevalence of *S. maltophilia* in intensive care units, pediatric populations and in community-acquired infections. The antimicrobial susceptibility of *S. maltophilia* is presented, as well as molecular resistance mechanisms and current drug treatment strategies.

To investigate how quinolone resistance in *S. maltophilia* can occur through molecular mechanisms other than mutation of genes encoding bacterial topoisomerases, Bernardini et al. used a transposon mutagenesis approach. They observe that when a gene encoding for RNase G is inactivated, this *S. maltophilia* mutant displays reduced susceptibility to quinolones. Complementation of the mutant with the wild type RNase G gene restores quinolone sensitivity. Inactivation of RNase G results in increased expression of genes involved in *S. maltophilia* heat shock response. The study shows that heat shock induces a transient phenotype of quinolone resistance in *S. maltophilia*. This study further demonstrates that additional molecular mechanisms are used to acquire antimicrobial resistance.

Roscetto et al. found that *S. maltophilia* strains (from CF and non CF patients and from the environment) demonstrate heterogeneity in their ability to internalize and replicate within human monocyte-derived dendritic cells (DCs). All strains activated DCs, as measured increases in surface maturation markers and proinflammatory cytokines (TNFα and IL-12) were observed. No significant differences in the maturation of immature DCs were observed between the strains.

The virulence of *S. maltophilia* is controlled by iron availability, probably using the Fur system, as described by Garcia et al. This study reports that biofilm formation, oxidative stress response, outer membrane proteins expression, and diffusible signal factor (DSF) production are mediated by iron availability. Confocal laser scanning microscopy shows that iron depletion stimulated biofilm formation resulting in low reactive oxygen species production. This study shows that iron also negatively regulates DSF production in *S. maltophilia* and that a spontaneous *fur* mutant is more virulent in a *Galleria mellonella* infection model.

Huedo et al. have investigated the genetic and functional diversity of the DSF quorum-sensing machinery in *S. maltophilia*. The *rpf* cluster controls this system in *S. maltophilia*. Two variants, *rpf*-1 and *rpf*-2, are used to distinguish between two *S. maltophilia* groups which may have originated through horizontal exchange. The *rpf*-1 strains make DSF whereas the *rpf*-2 strains produce DSF only in the presence of DSF-producers. The production of DSF is mediated by temperature, culture media composition, and fatty acid supplementation. This article suggests that DSF is produced through cross-talk between *rpf*-1 and *rpf*-2 strains and using a positive-feedback mechanism. The *rpf*-1 and *rpf*-2 strains act synergistically to promote virulence in the zebrafish infection model.

It has been suggested that in co-culture biofilms, *S. maltophilia* modulates the virulence of *Pseudomonas aeruginosa* (Pompilio et al.). To further understand the interactions of these bacteria, each pathogen was co-isolated from one chronically infected CF patient. Each strain was assessed during planktonic growth, adhesion and biofilm formation, motility, and gene expression in mixed biofilms. *P. aeruginosa* inhibits *S. maltophilia*'s growth planktonically and in biofilm. *S. maltophilia* affects *P. aeruginosa*'s ability to adhere to CF bronchial cells and induces alginate over-production by *P. aeruginosa*. The alginate over-production may have been responsible for the decreased susceptibility of *S. maltophilia* to tobramycin in mixed culture biofilms. Understanding these interspecies interactions can lead to development of novel therapeutic strategies against these pathogens.

S. MALTOPHILIA AND BIOTECHNOLOGY

Mukherjee and Roy present *S. maltophilia* as a useful biocontrol agent against fungi, bacteria, and insects. In their review, they note its ability to produce various enzymes for biotechnological applications, including the degradation of keratin, atrazine, trichloroethylene, dichlorodiphenyltrichloroethane (DDT), and its use in metal bioremediation, and its colonization of plant roots. A safer non-pathogenic alternative to using *S. maltophilia* is suggested, *S. rhizophila* (Berg and Martinez).

S. maltophilia demonstrates antimicrobial activity. Dong et al. found that *S. maltophilia* produces a phage-tail like bacteriocin, maltocin P28 with broad activity against Gram-positive and

Gram-negative bacteria. The activity of purified maltocin P28 is stable across different temperatures and pH, and it causes lysis of the target cell without EDTA treatment. Maltocin P28 demonstrates lytic activity against several pathogenic bacteria.

AUTHOR CONTRIBUTIONS

JB wrote the manuscript which was then reviewed by GDB, GB, and JM.

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PERSPECTIVE
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Friends or foes: can we make a distinction between beneficial and harmful strains of the *Stenotrophomonas maltophilia* complex?

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Stenotrophomonas maltophilia is an emerging multi-drug-resistant global opportunistic pathogen of environmental, mainly plant-associated origin. It is also used as a biocontrol or stress protecting agent for crops in sustainable agricultural as well as in bioremediation strategies. In order to establish effective protocols to distinguish harmless from harmful strains, our discussion must take into consideration the current data available surrounding the ecology, evolution and pathogenicity of the species complex. The mutation rate was identified as one of several possible criteria for strain plasticity, but it is currently impossible to distinguish beneficial from harmful *S. maltophilia* strains. This may compromise the possibility of the release and application for environmental biotechnology of this bacterial species. The close relative *S. rhizophila*, which can be clearly differentiated from *S. maltophilia*, provides a harmless alternative for biotechnological applications without human health risks. This is mainly because it is unable to growth at the human body temperature, 37°C due to the absence of heat shock genes and a potentially temperature-regulated suicide mechanism.

Keywords: ***Stenotrophomonas maltophilia*, *S. rhizophila*, biocontrol, bioremediation, risk assessment**

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Introduction

In recent years, the number of human infections caused by opportunistic pathogens has increased dramatically. Plant organs, especially the rhizosphere (root) as well as the endosphere (inner tissues) are natural reservoirs of emerging opportunistic pathogens. Various bacterial genera including *Burkholderia, Enterobacter, Herbaspirillum, Ochrobactrum, Pseudomonas, Ralstonia, Staphylococcus*, and *Stenotrophomonas* contain plant-associated strains that can encounter dual interactions with both plant and human hosts (Parke and Gurian-Sherman, 2001; Berg et al., 2005). Opportunistic human pathogens with saprophytic phases or pathogens residing in environmental reservoirs, such as those provided by plants, are also referred to as environmental pathogens (Morris et al., 2009).

The mechanisms responsible for the colonization of plant and human tissues are similar (Berg et al., 2005). Further, multiple antibiotic resistances are not only found amongst clinical strains, but also within strains isolated from plants and often caused by multidrug efflux pumps

(Berg et al., 1999; Martinez et al., 2009). High levels of competition, the occurrence of diverse antibiotics and secondary antimicrobial plant metabolites, and enhanced horizontal gene transfer and mutation rates in the microenvironment contribute to the development of high levels of natural resistance (Martínez, 2013; García-León et al., 2014). Congruently, these factors contribute favorably to the enormous potential application of these inhabitants as biocontrol or stress protecting agents in sustainable agricultural practices (Hirsch and Mauchline, 2012; Berg et al., 2013). Although recent research has elucidated the impact of pathogen ecology in environmental reservoirs on the evolution of novel or enhanced pathogen virulence (Morris et al., 2009), less is known about features that differentiate between pathogens and beneficial bacteria. Moreover, the question of how we can distinguish beneficial from harmful strains still remains, and it will be addressed here as it relates to the historical body of *Stenotrophomonas* research.

Plant-Associated *Stenotrophomonas* Strains Occupy New Niches and Hosts

For a long time after its description in 1961 as *Pseudomonas maltophilia* (Hugh and Ryschenko, 1961), *Stenotrophomonas* belonged to the *Xanthomonadaceae*, a yellow pigmented bacterial family strongly associated with plants (Swings et al., 1983). Indeed, most of the species were able to cause diseases in plants, and in order to substantiate this taxonomic concept, the non-pathogenic *Xanthomonas maltophilia* was excluded from the genus. A new genus was established by Palleroni and Bradbury (1993). At that time, *Stenotrophomonas* was mainly known for its occurrence in plants, and many different plant species were reported as hosts including diverse crops, e.g., oilseed rape, maize, potato, cabbage, mustard, and beet (Juhnke and des Jardin, 1989; Berg et al., 1996). It was interesting to observe that especially in plants with extraordinary secondary metabolisms (*Brassicaceae*, eucalyptus) living in extreme habitats (dune environments) *Stenotrophomonas* belonged to the dominant bacterial inhabitants (De Boer et al., 2001; Ribbeck-Busch et al., 2005). Many of them showed an endophytic life style, representing a highly intimate interaction with its host (Krechel et al., 2002; Ryan et al., 2009). Many reports showed the enormous potential for agricultural biotechnology: strains of *Stenotrophomonas maltophilia* were able to promote plant germination and growth and to suppress plant pathogens (Berg and Ballin, 1994; Kobayashi et al., 1995; Nakayama et al., 1999; Dunne et al., 2000; Suckstorff and Berg, 2003; Messiha et al., 2007; Jin et al., 2011). *S. maltophilia* was used as an efficient biocontrol agent, and up until the 1980s, no significant risk to human health was reported. Use of the species in the decontamination of soil (bioremediation) has attracted considerable interest because of its capacity to degrade a wide range of xenobiotic compounds by a broad spectrum of unique enzymes (Binks et al., 1995; Ribitsch et al., 2012). Interestingly, *S. maltophilia* strains, e.g., OS4, was able to reduced silver nitrate ($AgNO_3$) to generate cuboid and homogenous nanoparticles (AgNPs) with antimicrobial but without cytotoxic effects (Oves et al., 2013).

On the other hand, *S. maltophilia* has been reported since the early 1980s as a new pathogen in hospitals, and now it is a global pathogen and one of the most common opportunistic pathogens in hospitals (Ryan et al., 2009; Brooke, 2012). Although *S. maltophilia* does not usually infect healthy hosts (community infection), this bacterial species produce at hospitals bacteraemia and these infections are often associated with high mortality rates (reviewed in Brooke, 2012). *S. maltophilia* strains are characterized by multi-resistance to many antibiotics. In agreement with this situation, antibiotic treatment and the basal situation of the host (immunocompromised, cystic fibrosis) constitute the main risk factors for fatal *Stenotrophomonas* infections (Sanchez et al., 2009; Hernández et al., 2011; Brooke, 2012).

Due to their beneficial interactions with plants on one hand and on the other hand their facultative pathogenic interactions with humans, *Stenotrophomonas* strains challenge us to find differentiating features. Differentiation of features is of critical importance in terms of further applications in the field of biotechnology and in our understanding of the risks of infections and related epidemiological questions. The prediction of human health risks is currently one of the main challenges facing environmental biotechnology. The assessment of potential risk factors is the main obstacle in registration procedures, especially within the European Union (Ehlers, 2011).

Stenotrophomonas: Diversity and Properties

Stenotrophomonas maltophilia was well-known for its intraspecific heterogeneity, which is already described in the type description by Palleroni and Bradbury (1993). This heterogeneity was confirmed in physiological studies at a phenotypic level (Swings et al., 1983; Van den Mooter and Swings, 1990) as well as at a genotypic level (Gerner-Smidt et al., 1995; Moore et al., 1997; Berg et al., 1999; Hauben et al., 1999). In the 1990s molecular fingerprinting methods were applied to distinguish species and find infection routes. These studies did not always result in clear conclusions, and in some of them a reservoir of infection was found in hospitals, e.g., in ice machines and in ventilators, however, other studies identified highly diverse strain patterns. By contrast, patient to patient transmission was found to be a rare event. In addition, antibiotic resistance patterns were monitored, and although the pattern varied, most of the analyzed strains were multi-resistant. It was originally assumed that multi-resistance was acquired in hospitals as *S. maltophilia* is naturally competent to acquire foreign DNA; however, strains isolated from plants also showed multiple antibiotic resistances (Berg et al., 1999). Multilocus sequence typing (Maiden, 2006) was applied for a highly diverse inter-continental selection of 70 *Stenotrophomonas* strains of various ecological origins (Kaiser et al., 2009). Interestingly, also in this study the heterogeneity was confirmed while on the other side geno-groups, which contained only isolates of strictly environmental including the *S. maltophilia* type strain, were identified. Gherardi et al.

(2015) provide an overview of various typing methods including proteome-based bacterial identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) for clinical epidemiology of *S. maltophilia* and suggest novel Web-based platforms for rapid data processing for outbreak investigations and surveillance studies in routine clinical practices.

We applied diverse typing strategies with the aim of differentiating clinically relevant strains from environmental ones (Berg et al., 1999; Minkwitz and Berg, 2001). From the 16S rDNA sequencing analysis, the isolates could be separated into three genomovars, two of which consisted of isolates originating from the environment (especially rhizosphere isolates; E1 and E2), and one which contained clinical and aquatic strains (C). In contrast to previous investigations (Denton and Kerr, 1998), most of the strains could be grouped according to their sources of isolation (Minkwitz and Berg, 2001), in spite of the fact that the antibiotic resistance profile of *S. maltophilia* isolates, and their ability to colonize plant roots, did not correlate with their origin (Suckstorff and Berg, 2003). However, it was possible to establish a new, clearly plant-associated species *S. rhizophilia* DSM14405^T (Wolf et al., 2002) with antagonistic activity toward fungal plant pathogens that comprised the isolates of the E1 cluster, and could be further distinguished from *S. maltophilia* isolates by (i) growth temperatures, (ii) xylose assimilation, and (iii) osmolyte production. Additionally, the *smeD* gene which is part of the genes coding for the multidrug efflux pump smeDEF from *S. maltophilia* was identified as a further genetic marker, (Ribbeck-Busch et al., 2005). In contrast to *S. maltophilia*, no pathogenicity to humans is known for plant-associated *S. rhizophila,* and fortunately there have been no reports of the species being associated with human infections or clinic environments to date.

Can *S. rhizophila* Provide an Alternative for Biotechnological Applications?

Stenotrophomonas rhizophila is a species closely related both phylogenetically and ecologically to *S. maltophilia* (Wolf et al., 2002), and therefore a careful risk assessment for any biotechnological use is necessary. *S. rhizophila* is a model bacterium for a plant-competent, salt-tolerant plant growth promoting rhizobacterium (PGPR) with an endophytic lifestyle (Ryan et al., 2009; Berg et al., 2010). Plant growth promotion by the *S. rhizophila* strain DSM14405^T (synthesis strain e-p10) was observed under greenhouse conditions (Schmidt et al., 2012) and in the highly salinated soils of Uzbekistan (Egamberdieva et al., 2011). The differences between *S. maltophilia* and *S. rhizophila* were analyzed by comparative genomics (Alavi et al., 2014). Despite the notable similarity in potential factors responsible for host invasion and antibiotic resistance, other factors including several crucial virulence factors and heat shock proteins were absent in the plant-associated *S. rhizophilia* DSM14405^T. Instead, *S. rhizophila* DSM14405^T possessed unique genes responsible for the synthesis and transport of the plant-protective spermidine, plant cell-wall degrading enzymes, and high salinity tolerance. In addition, spermidine and osmoprotectant production (glucosylglycerol and trehalose) was the main response of *S. rhizophilia* DSM14405^T to rhizosphere exudates in a transcriptomic study, which suggested the involvement of these substances in the mode of interaction with plants (Alavi et al., 2013b). Moreover, the capability of bacteria for growing at 37°C was identified as a very simple criterion in differentiating between pathogenic and non-pathogenic *S. maltophilia* and *S. rhizophila* isolates. DSM14405^T is not able to grow at that temperature, most likely in great part due to the absence of heat shock genes and perhaps also because of the up-regulation at increased temperatures of several genes involved in a suicide mechanism (Alavi et al., 2014). The conclusion of these studies is that *S. rhizophila* currently does indeed provide an alternative to biotechnological applications without posing any risks to human health. The main reason for this conclusion is the demonstration of a lack of any growth at 37°C and the identified underlying mechanisms, which should prevent or disallow colonization of the human body. It was suggested that *S. rhizophila* can be used as a biocontrol and stress protecting agent (Alavi et al., 2013b), however, as we have learned, host–microbe interaction is a co-evolutionary process, and the outcome of interaction can be changed by many factors, which suggest a need for preventive genomic monitoring.

An Evolutionary Concept to Explain *Stenotrophomonas* Diversity and Plasticity

Within three decades *S. maltophilia* developed from a typical plant-associated species into a serious human pathogen. How was this possible? *S. maltophilia* belongs to the bacterial group of *r*-selected species that places an emphasis on a high growth rate, and typically exploiting less-crowded ecological niches producing a high number of bacteria in a short time.

In contrast to the reported heterogeneity of the *S. maltophilia* complex, the diversity of pathogenicity and interaction factors seems to be low. Proteases, siderophores, and biofilm formations are reportedly regulated by the DSF quorum sensing system (Alavi et al., 2013a). Multiple efflux systems are responsible for the resistance to antibiotics, toxins, and metals (Ryan et al., 2009). Anbitiotics and volatiles were shown to be involved in the anti-eukaryotic activity (Jacobi et al., 1996; Kai et al., 2007). However, clinical and environmental *S. maltophilia* strains presented comparable distribution of identified potential virulence genes thus far (Adamek et al., 2014) and they harbor the same resistance determinants, which make them highly resistant to antibiotics currently in use at clinical settings.

Altogether, this suggests that the main reason for the recent emergence of *S. maltophilia* as a relevant pathogen may reside in the host itself more than in a process of bacterial evolution. As above stated, *S. maltophilia* is a prototype of highly resistant microorganism. Debilitated patients at hospitals are more prone to infection than healthy people. In this situation, the main factor impeding infection is antibiotic prophylaxis or treatment. In this situation of high antibiotic load, organisms highly resistant as *S. maltophilia* should have higher chances

for surviving and hence produce infection. Virulence factors and resistance elements, likely acquired in the field for plant colonization may have allowed *S. maltophilia* to become an infective bacterium, just in debilitated patients with underlying diseases.

This does not mean, however, that *S. maltophilia* cannot further evolve during infection. These bacteria are characterized by a high rate of genomic re-arrangements and hypermutator activity, which allow rapid adaptation to new niches. We were able to confirm the latter for *S. maltophilia* strains: clinical strains belonged exclusively to the hypermutators, whereas environmental strains showed a broader spectrum of mutation rates (Turrientes et al., 2010). This indicates that the mutation rate is an important criterion of assessing the probability that a bacterial strain can occupy new niches and hosts. The rate and effects of mutations is one of the main ecological and genetic factors that may affect the likelihood of emergence of a pathogen (Gandon et al., 2013). In a long-term study analyzing *S. maltophilia* strains from chronically colonized cystic fibrosis patients, Vidigal et al. (2014a) was able to demonstrate that different genotypes with different mutation rates including 31.2% strong hypermutators exist. As a sign of adaption their mutation status switches over time to a less mutator phenotype without increasing resistance, which suggests that *S. maltophilia* attempts to sustain its biological fitness as a mechanism for long-term persistence. Horizontal gene transfer is another mechanism by which pathogenicity islands can be acquired. The natural capacity for DNA uptake of *S. maltophilia* was shown by strains carrying pathogenicity island from other species, e.g., from *Staphylococcus aureus* (Ryan et al., 2009). All of these factors contribute to the high intra-specific heterogeneity and genomic plasticity of *Stenotrophomonas,* which allow them not only to colonize new hosts but also to develop new genotypes and species.

Conclusion

The current established theory of opportunistic pathogens is that the ancestors of virulent bacteria as well as the origin of virulence and resistance determinants are most likely to originate from environmental microbiota (Martínez, 2013). *S. maltophilia* is an appropriate model, which fits into this theory. It is currently impossible to distinguish between beneficial and harmful *Stenotrophomonas* strains. This evidence may compromise the possibility of any application for environmental biotechnological purposes. Although bacterial strains adapt to its specific niches and then develop new properties, *S. rhizophila* has been clearly differentiated from the *S. maltophilia* complex and seems not to pose a risk.

Future Aspects

In recent years, we have deciphered parts of a puzzle in our efforts to understand the evolutionary process within the *Stenotrophomonas* complex. We have gained new insights into the lifestyle, the physiology, and potential risk factors as antibiotic resistance elements or virulence determinants of *Stenotrophomonas* through the application of genomics and transcriptomic techniques. Having identified a completely different methylation rate for different *Stenotrophomonas* strains, interpretation of epigenetic analyses is the current challenge that we are confronted with. This opens up new possibilities for the manipulation of the bacteria, while also requiring an understanding of the physiology and ecology of the bacteria. However, there are still taxonomical problems to be solved, problems which led to misidentifications in the past as in the case of the perceived overlap between *Stenotrophomonas* and *Lysobacter*. The latter is also a biotechnologically relevant genus because of its micropredatory activity (Hayward et al., 2009). Brooke (2014) reviewed new strategies for prevention and treatment of *Stenotrophomonas* infections in great detail. Therefore, we would like to suggest alternative strategies which require a thorough rethinking of the entire approach to microbiome management (Arnold, 2014). *Stenotrophomonas* strains closely interact with phages; they carry different phages in their genome but lytic phages have also been identified (Hagemann et al., 2006; García et al., 2008). This may explain a fast evolution by selection pressures on bacteria and open new possibilities for phage therapies for multi-resistant *Stenotrophomonas* (Vos et al., 2009). Maltocin P28, the first identified phage tail-like bacteriocin from *S. maltophilia* is a promising therapeutic substitute for antibiotics for *S. maltophilia* infections (Liu et al., 2013). In addition to phages, probiotic bacteria demonstrating antagonistic activity toward *S. maltophilia* would be an interesting alternative to consider in the prevention of infections. Natural products like the green tea compound epigallocatechin-3-gallate have also showed a promising activity against *S. maltophilia* (Vidigal et al., 2014b).

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REVIEW
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Genomic Potential of *Stenotrophomonas maltophilia* in Bioremediation with an Assessment of Its Multifaceted Role in Our Environment

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The gram negative bacterium *Stenotrophomonas* is rapidly evolving as a nosocomial pathogen in immuno-compromised patients. Treatment of *Stenotrophomonas maltophilia* infections is problematic because of their increasing resistance to multiple antibiotics. This article aims to review the multi-disciplinary role of *Stenotrophomonas* in our environment with special focus on their metabolic and genetic potential in relation to bioremediation and phytoremediation. Current and emerging treatments and diagnosis for patients infected with *S. maltophilia* are discussed besides their capability of production of novel bioactive compounds. The plant growth promoting characteristics of this bacterium has been considered with special reference to secondary metabolite production. Nano-particle synthesis by *Stenotrophomonas* has also been reviewed in addition to their applications as effective biocontrol agents in plant and animal pathogenesis.

Keywords: *Stenotrophomonas*, nosocomial, immunocompromised, multidisciplinary, bioremediation

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INTRODUCTION

Stenotrophomonas maltophilia is an uncommon, aerobic, non-fermentative, gram negative bacterium; motile due to polar flagella, catalase-positive, oxidase-negative slightly smaller (0.7–1.8 × 0.4–0.7 μm; which distinguishes them from most other members of the genus) and have a positive reaction for extracellular DNase. While *S. maltophilia* is an aerobe, it can still grow using nitrate as a terminal electron acceptor in the absence of oxygen (Crossman et al., 2008). *S. maltophilia* strains are found to be ubiquitously distributed in the environment with regard to habitat and geography: often associated with roots of many plant species (Ryan et al., 2009).

Growth of *S. maltophilia* studied in presence of different carbon sources: trichloroethylene (TCE), toluene, phenol, glucose, chloroform, and benzene with 0.1% peptone revealed an interesting growth pattern. Growth in presence of TCE, benzene, and chloroform was almost the same, whereas comparatively less growth was seen in presence of toluene and no growth in phenol even in presence of peptone (Mukherjee and Roy, 2013c). Pompilio et al. (2011), observed the mean growth rate of *S. maltophilia* obtained from clinical [cystic fibrosis (CF) and non-CF patients] and environmental isolates. CF isolates showed higher mean generation time compared to non-CF ones (3.5 ± 0.5 h vs. 3.1 ± 0.6 h, respectively; $p < 0.001$). Environmental isolates grown at 37°C exhibited a significantly lower generation time compared to that observed at 25°C (2.5 ± 0.6 h vs. 3.2 ± 0.4 h, respectively; $p < 0.05$).

Stenotrophomonas maltophilia have specific flagella like structures. The flagella filaments are composed of a 38-kDa subunit, SMFliC, and analysis of its N-terminal amino acid sequence showed considerable sequence identity to the flagellins of *Serratia marcescens* (78.6%), *Escherichia coli*, *Proteus mirabilis*, *Shigella sonnei* (71.4%), and *Pseudomonas aeruginosa* (57.2%; de Oliveira-Garcia et al., 2002). de Oliveira-Garcia et al. (2003), were the first to characterize fimbriae in this genus. All so far studied *S. maltophilia* strains contain multidrug efflux pumps – RND family: SmeABC, SmeDEF, SmeGH, SmeIJK, SmeMN, SmeOP, SmeVWX, and SmeYZ; ABC family: SmrA, MacABCsm; MFS family: EmrCABsm. Evenmore, the intergenic region smet-smeD is considered as a *S. maltophilia* phylogenetic marker (Alonso and Martínez, 2000, 2001; Alonso et al., 2000; Zhang et al., 2001; **Figure 1**).

Xenobiotic-degrading *S. maltophilia* have tremendous potential for bioremediation but new modifications are required to make such microorganisms effective and efficient in removing these compounds, which were once thought to be recalcitrant. Metabolic engineering through genomic manipulations might help to improve the efficiency of degradation of toxic compounds by *S. maltophilia.* However, efficiency of naturally occurring *Stenotrophomonas* sp. for field bioremediation could be significantly improved by optimizing certain factors such as bioavailability, adsorption and mass transfer. Chemotaxis and microbe–plant interactions could also have an important role in enhancing biodegradation of pollutants.

The great genetic and metabolic diversity within *S. maltophilia* makes it a "Wonder-bug." **Figure 2** below, describes the multifaceted role of *S. maltophilia* in our environment:

WHAT ARE THE RAPID *S. maltophilia* SPECIFIC DIAGNOSTIC PROCEDURES?

Stenotrophomonas maltophilia may cause nosocomial infections in immune-compromised patients (Elting et al., 1990). Infection is usually facilitated by the presence of prosthetic material (colonizing breathing tubes, endotracheal tubes, or urinary catheters) and removal of the infected prosthesis is sufficient to cure the infection: antibiotics are required if prosthesis cannot be removed. This suggests that its level of invasivity is likely low. A considerable mortality rate (up to 37.5%) can be attributed to *S. maltophilia* infection (Falagas et al., 2009). The median time to hematological diagnosis and *S. maltophilia* identification was 2.5 days as reported by Chaplow et al. (2010) in 37 patients with *S. maltophilia* bacteraemia in a blood and marrow transplant (BMT) and non-BMT hematology conditions, treated with co-trimoxazole or ceftazidime with ciprofloxacin. The median duration of organism-specific treatment was 9 days.

Stenotrophomonas maltophilia infections are laboratory diagnosed using standard techniques as per LiPuma et al. (2007). Gram negative selective medium (GNSA) was developed by Moore et al. (2003) for rapid isolation of gram negative strains like *S. maltophilia*. Culture based identification is a time taking process and may give false positive results leading to incorrect identification of strains. The availability of the whole DNA sequence of the *S. maltophilia* strain K279a was utilized to set up fast and accurate PCR-based diagnostic protocols (Emanuela et al., 2008). A novel internally controlled 5-plex real-time PCR nucleic acid diagnostics assay (NAD), was utilized for rapid (<5 h), quantitative detection and identification of *S. maltophilia*

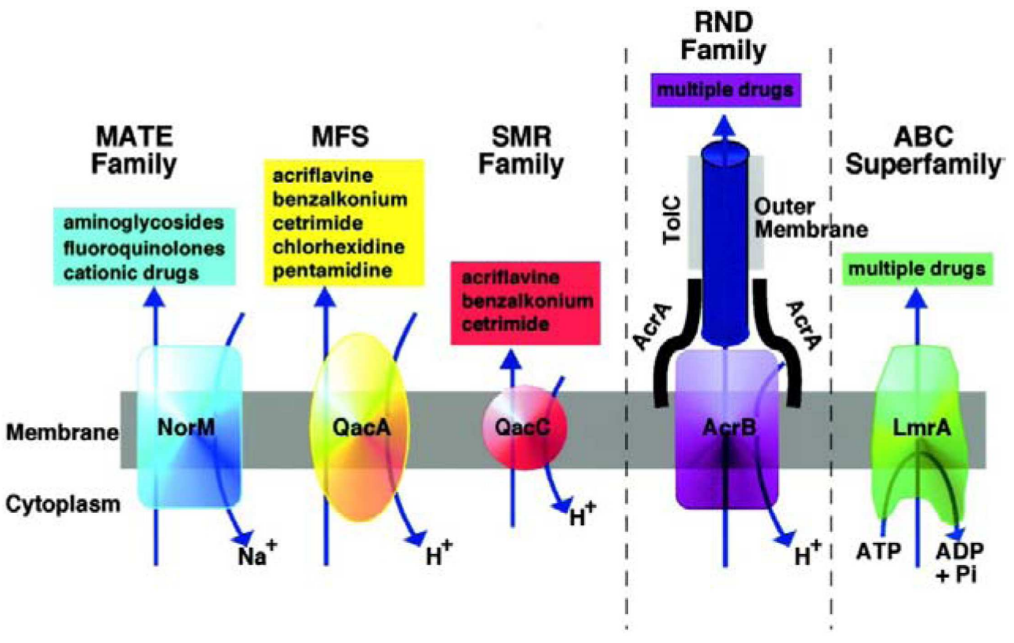

FIGURE 1 | Diagrammatic representation of the five families of MDR efflux pumps in bacteria are the resistance nodulation division (RND) family, the major facilitator superfamily (MFS), and the staphylococcal multiresistance (SMR), and multidrug and toxic compound extrusion (MATE) families. A role for ABC (ATP binding cassette) MDR transporters in MDR of clinically relevant bacteria has yet to be established (Piddock, 2006). Reproduced with permission.

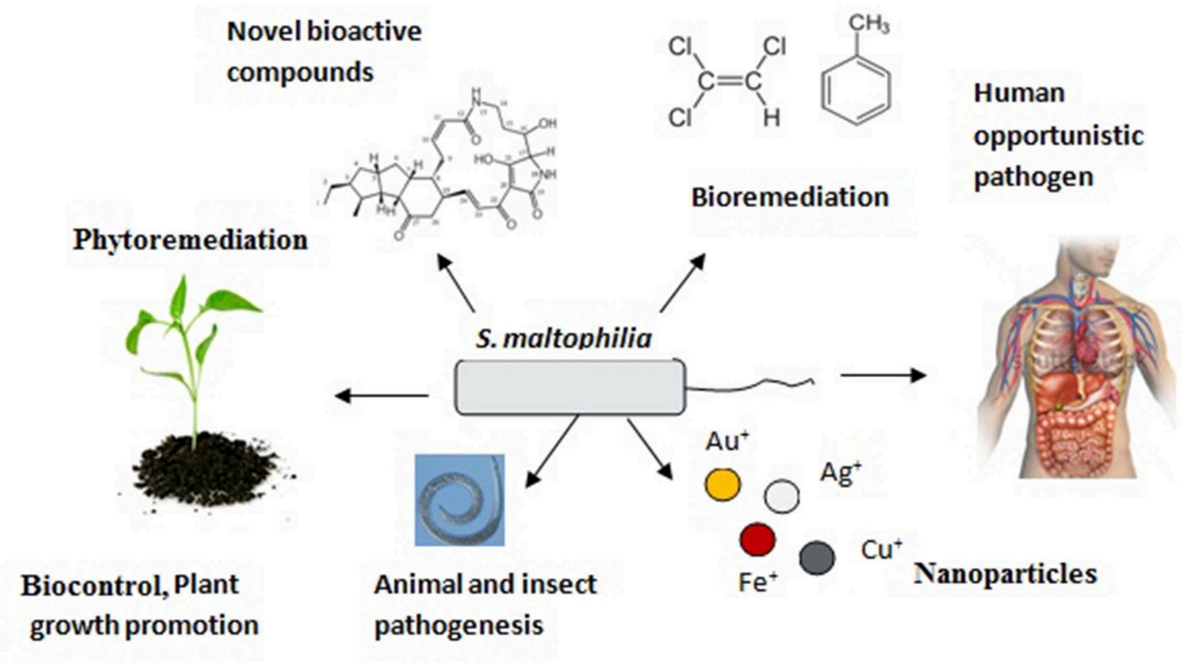

FIGURE 2 | Multidimensional role of *Stenotrophomonas maltophilia* in our environment signifies its substantial genetic, metabolic, and residential diversity.

(Minogue et al., 2015). Yang et al. (2014), first described a loop-mediated isothermal amplification (LAMP) method for the rapid detection (<60 min) of metallo-β-lactamase (bla_{L1}) in clinical samples with sensitivity 100-fold greater than that of conventional PCR. Kinoshita et al. (2015) also reported LAMP based rapid detection of *S. maltophilia*. Another method is target enriched multiplex PCR (TEM-PCR) that was applied for the detection of bloodstream pathogens like *S. maltophilia* (Stalons et al., 2014). Development of a novel peptide nucleic acid (PNA) probe for *S. maltophilia* identification by fluorescence *in situ* hybridization (FISH) was reported (Hansen et al., 2014). PNA FISH is a very fast (less than 90 min) and reliable molecular identification method.

Considering the importance of rapid and accurate diagnosis, PNA probe based identification seems to have some advantages compared to DNA probe based diagnosis methods involving RAPD/NAD/multiplex PCR. PNA probes are small in size with a non-charged polyamide backbone that renders them easy to hybridize and increases the binding strength compared to DNA probes (Pellestor et al., 2008). PNA probes have better sensitivity and specificity, show improved penetration into cells and through biofilm matrices and are not susceptible to bacterial endonucleases which may be present in clinical samples (Bjarnsholt et al., 2009). FISH is comparatively useful for *in situ* detection of this microorganism directly in clinical samples and mixed bacterial populations without prior cultivation.

The preferred clinical diagnostic protocol of choice depends on several factors. Blood cultures are preferred in most cases though their lengthy incubation time and lack of consistency. For rapid and accurate detection in local hospital laboratory setting, the authors propose PCR to be the tool of choice though FISH in combination with Flow Cytometry is more preferable.

ARE NEW TREATMENT STRATEGIES BEING DEVELOPED TO OVERCOME *S. maltophilia* INFECTIONS?

Stenotrophomonas maltophilia are naturally resistant to many broad spectrum antibiotics such as cephalosporins, carbapenems, and aminoglycosides. This means that treatment options are relatively limited According to the World Health Organization (WHO), *S. maltophilia* is one of the leading drug-resistant pathogens in hospitals worldwide (WHO; Public health importance of antimicrobial resistance[1]; Brooke, 2014). The treatment of choice for *S. maltophilia* is trimethoprim-sulfamethoxazole (SXT; Wang et al., 2014). Several combinations of novel agents are currently under investigation, including a β-lactam and dual β-lactamase inhibitor combination (Page et al., 2011) and MD3 (a novel synthetic inhibitor of peptidases) plus colistin (Personne et al., 2014). Most data is collected from case reports; compelling clinical evidence for combination therapies is lacking. Additional published cases and clinical trials are required to formulate a more evidence-based approach for the treatment of patients with *S. maltophilia* infections.

[1]www.who.int/drugresistance/AMR_Importance/en/

Quorum sensing (QS) is a bacterial cell–cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers. *S. maltophilia* has a diffusible signal factor (DSF) that controls cell–cell communication and many functions such as motility, extracellular protease production and microcolonies formation in artificial sputum medium. This DSF signaling also mediates interspecies interactions between *S. maltophilia* and *P. aeruginosa* such as susceptibility to polymixin and its influence on biofilm formation. QS-inhibition based drugs need to be developed (Boyen et al., 2009) but DSF-signaling has more potential as drug target for this species. Production and detection of DSF are governed by the *rpf* cluster, which encodes the synthase RpfF and the sensor RpfC, among other components. Structural analogs of DSF like *cis*-2-decenoic acid may have a role in control of virulence factor synthesis in different pathogens. Such molecules may represent lead compounds for new drugs. Also, DSF signaling is normally finely balanced during the disease process and such a fine balance can be readily disrupted by either degradation or over-production of the signal (Ryan et al., 2015). Iron, probably through the Fur system, negatively regulates DSF production in *S. maltophilia* (García et al., 2015). Emodin (an active component of Chinese traditional medicines) was found to inhibit biofilm formation in *S. maltophilia* and induced proteolysis of the QS signal receptor TraR in *E. coli* (Ding et al., 2011).

An alternative approach has been to utilize the inherent specificity of immunoglobulins to inhibit the pathogenic functions in *S. maltophilia*. Another approach has involved screening combinatorial libraries of random peptides. In a study, antibodies to *S. maltophilia* iron regulated outer membrane proteins (TROMP) were developed that reduced the uptake of iron by blocking the binding of ferric complexes resulting in the inhibition of *S. maltophilia* proliferation. Growth inhibition studies gave positive results with polyclonal antibodies recovered from rabbits immunized with *S. maltophilia* membrane associated polypeptides and monoclonal antibodies were also produced using mouse hybridoma technology model (Freeman, 2009).

In another study, *in vitro* and *in vivo* activities of epigallocatechin-3-gallate (EGCG), a green tea component, against *S. maltophilia* isolates from cystic fibrosis patients were analyzed (Vidigal et al., 2014). Essential oils from plants (e.g., orange, bergamot, cinnamon, clove, cypress, eucalyptus, fennel, lavender, lemon, mint, rosemary, sage, and thyme) were investigated and found to demonstrate antibacterial activity against *S. maltophilia* (Fabio et al., 2007). A surfactant-stabilized oil-in-water nanoemulsion (NB-401) has shown antimicrobial activity against planktonic and biofilm-associated cells of *S. maltophilia* (LiPuma et al., 2009). This nano-emulsion consists of emulsified cetylpyridinium chloride, poloxamer 407, and ethanol in water with super-refined soybean oil. The interaction of the nano-emulsion with the cell was suggested to result in the fusion of the outer membrane with the nano-emulsion, leading to cell lysis. Bismuth-thiols (BTs) can prevent the formation of microbial biofilms as well as eradicate established biofilms at uncommonly low concentrations. BTs are comprised of a central bismuth atom that is chelated by organic molecules known as thiols. The ability of thiols to chelate bismuth and other metals, has led to long, successful history as antidotes for treatment of heavy metal poisoning. The resulting low toxicity of BTs in mammals and the low cost of production and their stability, make them ideal candidates for development as prescription drugs and as anti-infective medical device coatings (Domenico et al., 2000, 2003; Wu et al., 2002). A device (Podhaler device) that delivers new inhalational tobramycin [tobramycin inhalation powder (TIP)] and attains high drug levels to the lung may be able to exceed current high MICs of tobramycin in *S. maltophilia* (Ratjen et al., 2015). Waters (2012), suggested a potential role of inhaled aztreonam lysine in the treatment of *S. maltophilia* pulmonary infection. A Monte Carlo pharmacokinetic/pharmacodynamic simulation was performed that suggested that minocycline may be a proper choice for treatment of HAP caused by *S. maltophilia*, while tigecycline, moxifloxacin, and levofloxacin may not be optimal as monotherapy (Wei et al., 2015).

The use of phage therapy may be an alternative to the use of antibiotics to treat *S. maltophilia* infections. A novel giant *S. maltophilia* phage ΦSMA5 was isolated from sputum samples, pleural effusions, and catheter tips (Chang et al., 2005). This phage was tested against 87 *S. maltophilia* strains isolated from hospitals and was found to have a narrow host range. A recent review suggested that the use of phages to treat biofilms has potential (Donlan, 2009). To the best of my knowledge, phage therapy is not used in ordinary clinical practice for the treatment of *S. maltophilia* infections. Together, the observations from the studies described above suggest that it is possible that a cocktail of surfactant, antimicrobial peptides, and phage may provide a suitable alternative to the administration of antibiotics.

Many alternative remedies including biofield energy treatment have recently found their way into the medical mainstream and is widely accepted by most of the healthcare professionals. A change in sensitivity pattern of amikacin from resistant to intermediate along with changes in sensitivity of trimethoprim/sulfamethoxazole and chloramphenicol was observed on biofield treatment (Clarke et al., 2015; Trivedi et al., 2015).

WHAT IS THE INTRA- AND INTER-SPECIES GENETIC DIVERSITY IN *S. maltophilia*?

With the advance in molecular biology tools and sequencing methods, large repertoires of *S. maltophilia* strains are easily accessible through NCBI. Molecular dendogram or phylogenetic tree (drawn with Clustalx and MEGA5 softwares), among different *Stenotrophomonas* groups of bacteria suggests the genetic diversity among the different strains (**Figure 3**).

Stenotrophomonas maltophilia was grouped in the genus *Xanthomonas* by Swings et al. (1983). However, the proposed reclassification of *P. maltophilia* as *Xanthomonas maltophilia* did not meet with universal approval (Palleroni, 1984), and the controversy about the taxonomic status of this bacterium in the genus *Xanthomonas* remained unresolved (Bradbury, 1984).

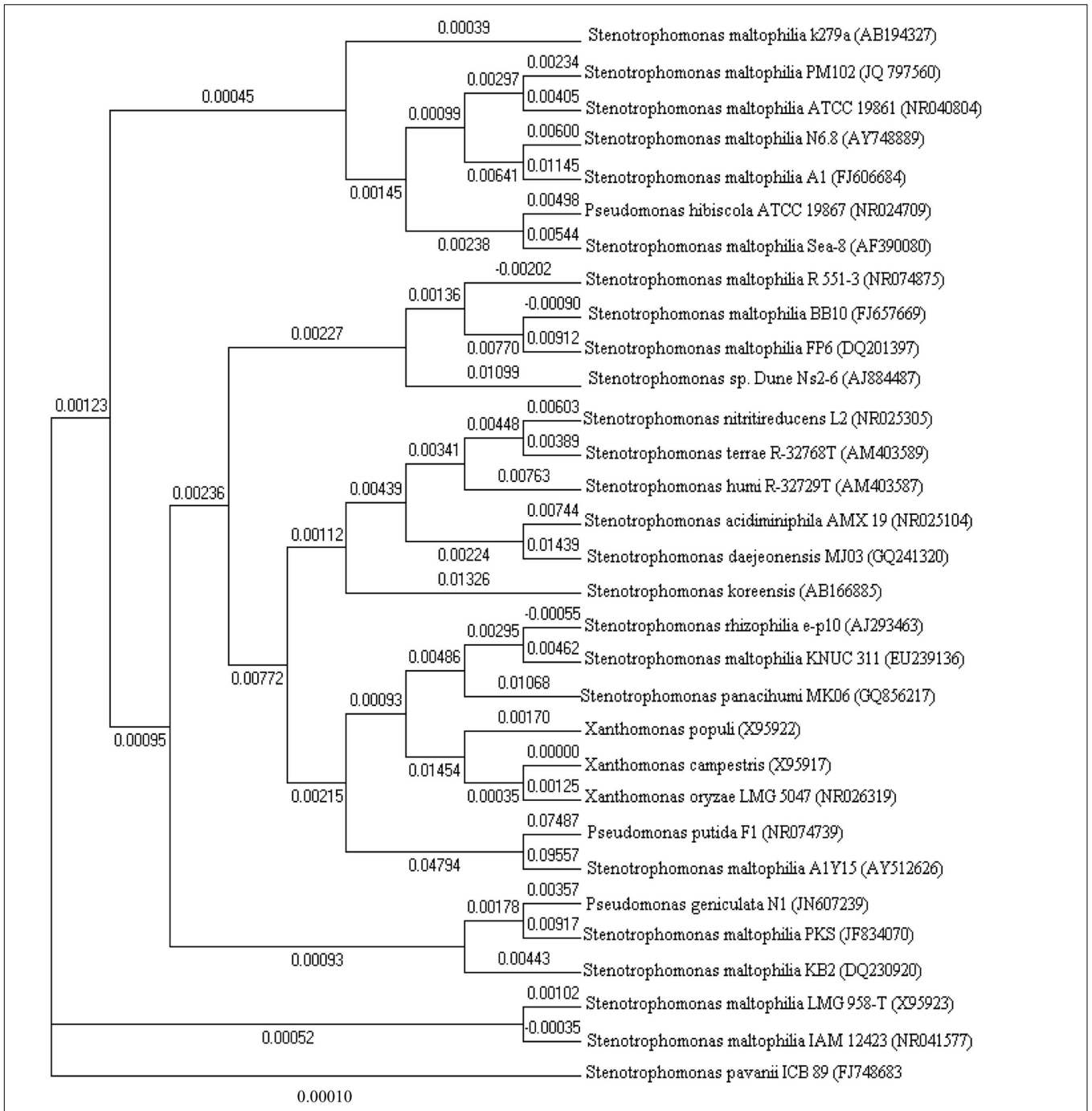

FIGURE 3 | Phylogenetic tree constructed with 16S rDNA sequences of various *Stenotrophomonas* bacteria retrieved from NCBI GenBank.

Several factors requested a reinterpretation of the taxonomic position of *X. maltophilia* (Van Zyl and Steyn, 1992). When a *Xanthomonas*-specific 16S rDNA sequence as the primer for PCR was used, a single 480-bp PCR fragment was seen for Xanthomonads; however, *X. maltophilia* strains produced additional PCR fragments, leading the author to conclude that *X. maltophilia* does not belong in the genus *Xanthomonas* (Maes, 1993). Continuing dissatisfaction with the classification of this organism finally gave rise to the proposal in Palleroni and Bradbury (1993) to create the new genus *Stenotrophomonas*. A proteome driven clustering correctly groups all well-annotated *S. maltophilia* genomes correctly from the *Xanthomonas* species.

Neighbor joining tree revealed *S. maltophilia* clustered homologs were *Pseudomonas hibiscola*, *Stenotrophomonas rhizophilia, Pseudomonas geniculata,* and *Pseudomonas putida* F1. *S. pavanii* seems to be the most distant in terms of homology.

DNA sequence identity values among the *S. maltophilia* strains ranged from 100 to 98.9%. The dendrogram in this paper reveals 10 different species of the genus *Stenotrophomonas* and depicts the closest homolog of *S. maltophilia* PM102 (JQ797560; that was isolated and characterized in our laboratory; being the first report of a *Stenotrophomonas* species in uptake of trichloroethylene as the sole carbon source) to be *S. maltophilia* ATCC 19861 (NR040804).

Total genome sequencing of few *S. maltophilia* strains like AU12-09, k279a, and R551-3 have been undertaken (**Figure 4**). AU12-09 genome consists of 129,784,052 bp of DNA (GenBank APIT00000000; Zhang et al., 2013). The genome of K279a is 4,851,126 bp and of high G+C content. The sequence reveals an organism with a remarkable capacity for drug and heavy metal resistance: nine resistance-nodulation-division (RND)-type putative antimicrobial efflux systems are present and several mobile DNA segments code for pili/fimbrae involved in adhesion and biofilm formation that contributes to increased antimicrobial drug resistance (Crossman et al., 2008). *S. maltophilia* R551-3 (Accession no. PRJNA17107) was isolated from the poplar *Populus trichocarpa*. In the presence of cadmium (Cd), it accumulates cysteine as a reducer in order to undergo chelation, and form CdS, or cadmium sulfide in order to avoid lethal toxicity (Pages et al., 2008). Conchillo-Solé et al. (2015) reported the draft genome sequence of *S. maltophilia* UV74, isolated from a vascular ulcer. In this isolate, the DSF-mediated QS system is regulated by a new *rpf* cluster variant (Huedo et al., 2014). Comparative genomic and Transcriptomic approaches have been used to identify significant borders between the MDR *S. maltophilia* and non-pathogenic plant-associated *S. maltophilia* R551-3 and *S. rhizophila* DSM14405. Although, there was significant similarity in host invasive and antibiotic resistance genes, several crucial virulence factor and heat shock protein genes were absent in plant-associated strains (Alavi et al., 2014). Furthermore, an environmental strain of *S. maltophilia* named BurA1 showed absence of RND pumps (SmeABC) but presence of another MDR RND efflux pump named EbyCAB on a genomic island acquired via Horizontal gene transfer (Youenou et al., 2015).

WHAT ARE THE NOVEL BIOACTIVE COMPOUNDS AND NANOPARTICLES SYNTHESIZED BY *Stenotrophomonas maltophilia*?

Stenotrophomonas maltophilia have been documented as a potential source for several novel bioactive compounds. These natural chemicals are not only used as bio control agents due to their antifungal, antibacterial and insecticidal properties but also have widespread applications as plant growth promoting substances (PGPR). Rhizobacteria introduced in the rhizosphere of tomato, pepper, melon or bean were found to increase growth of roots/shoots (Elad et al., 1987). **Table 1** is listed with the various bioactive compounds from *S. maltophilia* with their varied applications.

Additionally, *S. maltophilia* strains have been shown to produce enzymes that play important role in synthesis of compounds with medicinal applications. Production of 2-arylpropanoic acid (NSAID compounds: non-steroidal anti-inflammatory analgesics) was done using lipase obtained from *S. maltophilia* (Sharma et al., 2001). An hghly thermostable xylanase was reported from *Stenotrophomonas*. According to recent research from Lucknow, India, a novel psychro-tolerant *S. maltophilia* (MTCC 7528) with an ability to produce extracellular, cold-active, alkaline and detergent stable protease was isolated from soil of Gangotri Glacier, Western Himalayas, India (Kuddus and Ramteke, 2009).

A novel strain of *S. maltophilia* was isolated from actual gold enriched soil (Singhbhum gold mines in Jharkhand state of India). After incubation for 8 h in gold chloride ($HAuCl_4$), monodisperse preparation of gold nanoparticles was obtained (Nangia et al., 2009). Another strain of *S. maltophilia*,

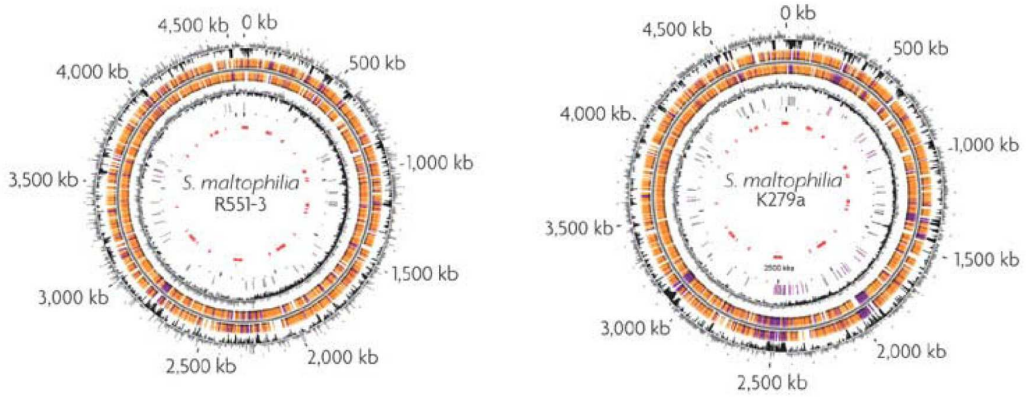

FIGURE 4 | Genome maps of the poplar endophyte *S. maltophilia* R551-3 and of the opportunistic pathogen *S. maltophilia* k279a. From the outside-in, the circles represent coordinates in kilobase pairs (kbp), % GC content, predicted open reading frames (ORFs) in the clockwise and anticlockwise orientations, GC skew [(G-C and G+C) in a 1000-bp window], transposable elements (pink) and pseudogenes (gray) and the putative *S. maltophilia* k279a genomic islands (red; Ryan et al., 2009). Reproduced with permission.

TABLE 1 | Bioactive compounds produced by *Stenotrophomonas maltophilia*.

Organism	Source	Compound	Activity	Reference
Stenotrophomonas maltophilia R3089	Rhizosphere of rape plants (*Brassica napus* L.)	Maltophilin	Antifungal	Jakobi et al., 1996
S. maltophilia	Oil contaminated soil	Bio surfactant Rhamnolipid	Mosquito larvicidal	Korade et al., 2014
S. maltophilia	Nematicidal plants	Hydrolytic enzymes and HCN, phenol oxidation	Anti-trichodorid nematode density on potato	Insunza et al., 2002
S. maltophilia N5.18		Enhance antioxidant activity	Improved sprouts quality in Soybean	Algar et al., 2013
Stenotrophomonas sp. strain SB-K88	Rhizosphere of sugar beet	Xanthobaccins A, B, and C	Suppresses damping-off disease	Nakayama et al., 1999
Stenotrophomonas sp.	Deep sea invertebrates	Antimicrobial activity	Hemolysis of fungus	Romanenko et al., 2007
S. maltophilia S1	Soil bacteria from Japan	Alkaline serine protease	Hydrolyses zein: major protein in maize seeds	Miyaji et al., 2005
S. maltophilia SSA	Roots of *Solanum surrattense* Burm	Phytohormones: IAA, gibberellic acid, *trans*-zeatin riboside, abscisic acid	Enhance growth of *Zea mays* seedlings	Naz and Bano, 2012
S. maltophilia		Dipeptidyl aminopeptidase IV	Substrate with hydro-xyproline residue	Nakajima et al., 2008
S. maltophilia PML168	Temperate intertidal zone	Class B Flavoprotein	Catalytic activity	Willetts et al., 2012
S. maltophilia D457	Laboratory collection (Alonso and Martínez, 2001)	3,5-dihydroxy benzoic acid and the α- phenyl benzenethanethioic acid	Antimicrobial activity against *E. coli, S. aureus, P. aeruginosa*, *Bacillus* spp.	Ricardo and Dionísio, 2014
S. maltophilia MUJ	Rhizosphere	Chitinase	Antifungal: *Rhizoctonia, Fusarium, Alternaria*.	Jankiewicz et al., 2012
S. maltophilia AVP27	Chili rhizosphere soil	IAA, ammonia, phosphatise, HCN	Promote growth of chili plant	Kumar and Audipudi, 2015
S. maltophilia W81	Sugarbeet rhizosphere	Chitinase, protease	Inhibit growth of *Pythium ultimum*	Dunne et al., 1997
S. maltophilia PD3533	Eggplant rhizosphere	Chitinase/protease	Suppress potato brown rot fungus	Messiha et al., 2007
S. maltophilia	Rhizosphere of oilseed rape	Lytic enzymes	Antifungal: *Rhizoctonia solani*; *Verticillium dahliae*	Berg et al., 1996
X. maltophilia	Cucumber root and bark media	Antifungal activity	*Rhizoctonia* and *Trichoderma*	Kwok et al., 1987
X. maltophilia	Maize rhizosphere in France Pyrrolnitrin		Antifungal: *P. ultimum*; *F. culmorum*	Lambert et al., 1866

isolated from Indian marine origin could synthesize both silver and gold nanoparticles (Malhotra et al., 2013). Role of this bacterium in nanoparticle synthesis (gold and silver being the most important) implicates their importance in biology and medicine (Sperling et al., 2008; Abou El-Nour et al., 2010). In another report, *S. maltophilia* SELTE02 showed promising transformation of selenite to elemental selenium, accumulating selenium granules in cell cytoplasm or extracellular space (Iravani, 2014). Another *S. maltophilia* isolated from soil rhizosphere of *Astragalus bisulcatus* could completely reduce selenite (Vallini et al., 2005). A novel bacterial strain OS4 of *S. maltophilia* (GenBank: JN247637.1) was isolated. At neutral pH, this Gram negative bacterial strain significantly reduced hexavalent chromium, an important heavy metal contaminant found in the tannery effluents and minings (Oves et al., 2013). Not much is known regarding the mechanism of metal nanoparticle synthesis by bacteria although different hypothesis have been suggested. A promising mechanism for the biosynthesis of gold NPs by *S. maltophilia* and their stabilization via charge capping was suggested, which involves an NADPH-dependent reductase enzyme that reduces Au^{3+} to Au^{0} through electron shuttle enzymatic metal reduction process (Nangia et al., 2009).

GENOMIC POTENTIAL OF *Stenotrophomonas maltophilia* TO LOCATE ENZYMES INVOLVED IN BIOREMEDIATION

The first genomic tool applied when bioremediation is investigated is 16s rDNA sequencing that is applied to identify the organism involved in bioremediation. Isolation of pure cultures and their identification by 16s rDNA analysis is the most important step in the study of biodegradation. A metagenomic approach can also be undertaken in mass identification of environmental samples involved in xenobiotic degradation. The next approach is Genomic analysis to identify the enzymes involved in bioremediation, Genomic analysis showed how various *Stenotrophomonas* strains are able to make enzymes that play vital role in dechlorination of polychlorinated hydrocarbons or heavy metal uptake by turning the genes on and off as the organism detects something appetizing. Whole genome sequences take this task of enzyme identification a step up further. The draft genome of *S. maltophilia* strain ZBG7B assembled into 145 contigs with an N_{50} of 50,104 bp. (Chan et al., 2015) contains a repertoire of biodegradation-related genes:

xylosidase, xylanase, xylose isomerise. *Stenotrophomonas* sp. has been found to play important role in biodegradation of keratin (Yamamura et al., 2002), RDX (Binks et al., 1995), geosmin (Zhou et al., 2011), atrazine (Rousseaux et al., 2001), *p*-nitrophenol (Liu et al., 2007) and monocyclic hydrocarbons (Urszula et al., 2009), phenanthrene (Gao et al., 2013) and styrene.

Keratin degradation was an outcome of the cooperative action of two types of extracellular proteins: proteolytic (serine protease) and disulfide bond-reducing (disulfide reductase). The best evidence for the pathway of geosmin degradation by bacteria has been provided by Saito et al. (1999), who identified four possible biodegradation products of geosmin (Hoefel et al., 2009). Two of these products were identified as 1, 4a-dimethyl-2, 3, 4, 4a, 5, 6, 7, 8- octahydronaphthalene and enone. Strains belonging to the *C. heintzii*, *A. aminovorans*, *S. maltophilia,* and *A. crystallopoietes* species are capable of mineralizing or degrading atrazine. Atrazine is catabolized in three enzymatic steps to cyanurate, which can be further metabolized by ring cleavage to carbon dioxide and ammonia. The first enzyme converts atrazine to hydroxyatrazine. Two additional hydrolases continue the process by removing the ethylamine and isopropylamine groups (de Souza et al., 1998; Smith et al., 2005). While some organisms possess all of the required enzymes, other communities degrade atrazine by a community-approach, where different organisms have some of the enzymes, and the intermediates in the pathway are passed between the organisms. These methods undertake a co-metabolic approach of enzyme activity. On the other hand, compounds utilized as sole carbon source involve novel enzymes that are coded by set of genes characteristic of pure cultures. A novel strain PM102 of *S. maltophilia* was shown to be able to degrade and utilize trichloroethylene as the sole carbon source (Mukherjee and Roy, 2012). A copper enhanced monooxygenase was characterized from the PM102 strain to be involved in the biotransformation of TCE (Mukherjee and Roy, 2013b). Phenanthrene utilization as sole carbon source involved dioxygenation on 1,2-, 3,4-, and 9,10-C, where the 3,4-dioxygenation and subsequent metabolisms were most dominant. The metabolic pathways were further branched by *ortho*- and *meta*-cleavage of phenanthrenediols. The KEGG genome map can be retrieved from DBGET integrated genome retrieval system that maps the KEGG pathway off styrene degradation by *S. maltophilia* K279a.

Stenotrophomonas maltophilia strain (SeITE02) was capable of resistance to high concentrations of selenite [SeO_3^{2-}, Se (IV)], reducing it to nontoxic elemental selenium under aerobic conditions (Antonioli et al., 2007). Various enzymatic systems, such as nitrite reductase, sulfite reductase, and glutathione (GSH) reductase (GR), have been proposed the reduction of selenite in bacteria, under anaerobic conditions as predicted from the whole genome sequencing of SelTE02. Under aerobic conditions, nitrite reductase was found to play no role but glutathione had some contribution. A study involving bioremediation of copper using copper-resistant bacteria, *S. maltophilia* PD2 has been reported (Ghosh and Saha, 2013). Metagenomic sequencing has also been used to profile the genes present in microbiomes from Cu-contaminated mining area. A list of genes coding heavy metal uptake efflux pumps could be mapped like P-type ATPases linked to Cu uptake, *mdt*C in Zn efflux and *cus*A and *ybd*B forming cationic efflux systems for Cu and Ag respectively (Van Rossum et al., 2016). Shotgun sequencing of DNA from biofilm samples has facilitated identification of genes that encode for efflux pumps, cell wall components, and metabolic processes for metals tolerance as well. *Stenotrophomonas* sp. C21 carries plasmids containing the Cu-resistance *copA* genes. *S. maltophilia* RSV-2 strain could degrade mixed textile dyes up to 2100 ppm within 67 h and 58% decolorization was obtained through acclimatization study (Rajeswari et al., 2013). Laccase activity of this bacterium has been shown to play a role in dye removal (Galai et al., 2009). copA gene was traced to encode the multicopper oxidase with laccase activity by degenerative PCR; cloned and homologous recombination was used to construct copA mutant strain to confirm laccase activity and dye decolorization of *cop*A *in vitro*. The biosorption of Pb(II), Zn(II), and Ni(II) from industrial wastewater using *S. maltophilia* was investigated (Wierzba, 2015). *S. maltophilia* has been reported to acquire genes involved in heavy metal resistance from Gram-positive bacteria (Alonso et al., 2000).

Stenotrophomonas maltophilia has also been found to play important role in the bioremediation of chlorinated pesticides like Chloropyrifos (Dubey and Fulekar, 2012) and endosulfan (Barragaán-Huerta et al., 2007; Kumar et al., 2007). Chloropyrifos uptake and degradation was mapped to *mpd* genes of *S. maltophilia* MHF ENV20. Gene mapping is carried out by PCR amplification using pre-designed primers. In our laboratory, predesigned *todc* gene primers of *Pseudomonas putida* were used to map the tce300 and tce350 genes of *S. maltophilia* PM102 (Mukherjee and Roy, 2013a) and the *tce 1* gene of *Bacillus cereus* 2479 (Mitra and Roy, 2011). The *tce300* and *tce350* genes were cloned in *E. coli* and their TCE degradation ability was confirmed by IPTG induction of the recombinant clones.

Soil isolates of *Stenotrophomonas* degraded dichlorodiphenyl-trichloroethane (DDT) to l, l-dichloro-2, 2-bis (p-chlorophenyl) ethane (DDD; Mwangi et al., 2010). The three main enzyme families implicated in pesticide degradation are esterases, glutathione *S*-transferases (GSTs) and cytochrome P450 (Ortiz-Hernández et al., 2013). *S. maltophilia* ZL1 was able to convert steroid hormone: estradiol (E2) to estrone (E1) and finally to amino acid tyrosine (Li et al., 2012). Enzymes involved in protein and lipid biosyntheses were observed to be particularly active. *S. maltophilia* OG2, isolated from the body microflora of cockroaches, could grow on synthetic pyrethroid insecticides (Chen et al., 2011). Tallur et al. (2008) proposed the pathway of α-cypermethrin biodegradation. It was speculated that the pathway of α-cypermethrin biodegradation in *S. maltophilia* OG2 is similar. A new feather degrading *S. maltophilia* DHHJ strain was isolated that produced keratinolytic enzymes (Wu et al., 2012). Thus, *S. maltophilia* have been shown to possess intrinsic resistance mechanisms against heavy metals and signaling or metabolic pathways as an outcome of their genomic potential toward various environmental pollutants.

Most isolates are considered Biosafety Level 1 (BSL-1) organisms. However, there are notable exceptions, including some commonly studied isolates, which are considered Biosafety Level 2 (BSL-2) pathogens. As a result of the hazard assessment

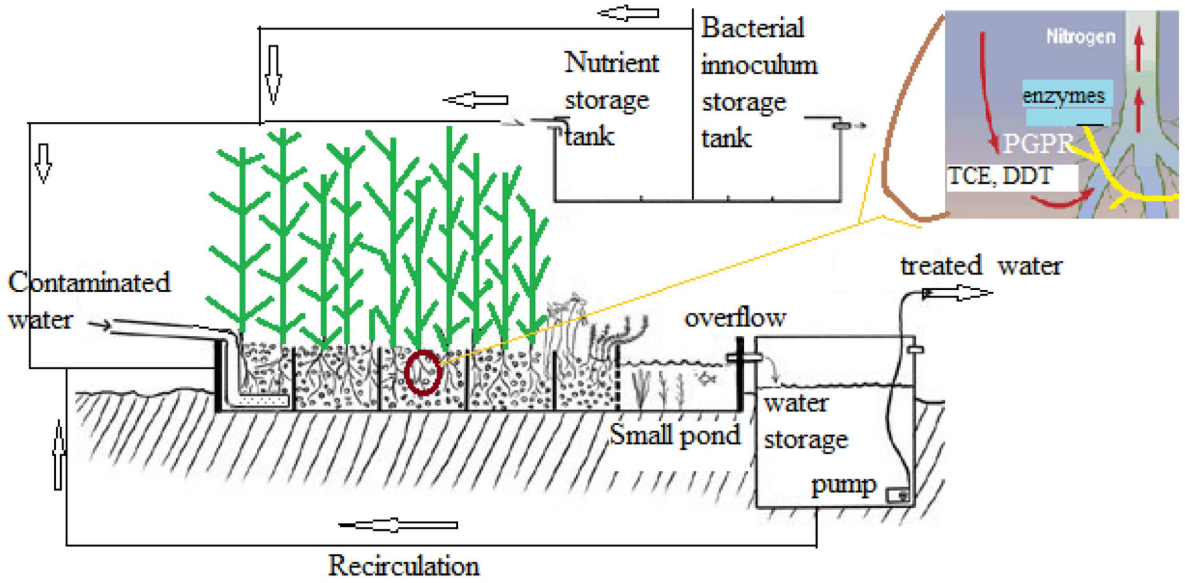

FIGURE 5 | Model for phytoremediation in contaminated water treatment.

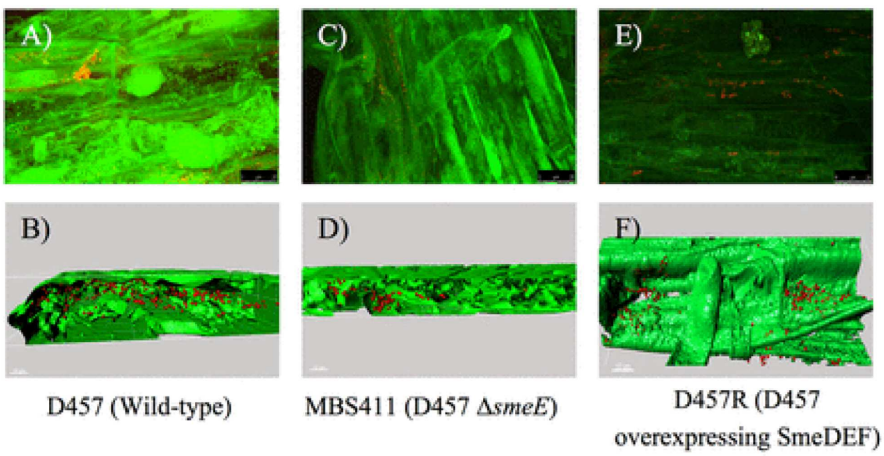

FIGURE 6 | Colonization of oilseed rape roots by *S. maltophilia* visualized by FISH. (A) Wild-type strain D457. **(C)** sme-E-defective mutant strain MBS411. **(E)** D457R mutant that overexpresses SmeDEF. 3D analysis of the CLSM stacks of D457 **(B)**, MBS411 **(D)**, D457R, **(F)** was performed with Imaris 7.0 software (Guillermo et al., 2014). Reproduced with permission.

the application of microbes in bioremediation is categorized into one of three risk estimates (Slovic, 1987, 1997): high risk – severe, enduring, or widespread adverse effects are probable; medium risk – adverse effects predicted for probable exposure scenarios may be moderate and self-resolving and low risk – adverse effects predicted for probable exposure scenarios are rare, or mild and self-resolving. Assessment of these risk factors impose an obstacle in registration procedures for release of microbes into the environment for bioremediation purposes in Europe and USA, though in Australia the limit on microbial bioremediation applications is not so tight.

It is much easier and simpler to apply the plant associated naturally occurring *S. rhizophilia* in bioremediation or rhizoremediation approaches, as pathogenicity to humans for plant-associated *S. rhizophila* has not been heard of till date. Rather, the association of endophytic bacteria with their plant hosts has been shown to have a growth-promoting effect for many plant species. Endophytic bacteria have several mechanisms by which they can promote plant growth and health on marginal, polluted soils. These include the production of phytohormones or enzymes involved in growth regulator metabolism such as ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, auxins, indoleacetic acid (IAA) or cytokinins. In addition, endophytic bacteria can help their host plants to overcome the phytotoxic effects caused by environmental contamination.

Stenotrophomonas maltophilia R551-3 was isolated from *Populus trichocarpa* and characterized to improve the growth and phytoremediation potential of poplar on marginal, contaminated soils (Nordberg et al., 2014). Endophytic bacteria equipped with the tom toluene degradation pathway could significantly improve the *in planta* degradation of BTEX and TCE in poplar, resulting in reduced phytotoxicity and release. Integrated microbial genomes were studied to identify metabolic functions (Markowitz et al., 2006). In a study, an attempt to determine the effect of aromatic compounds of plant origin on nitrophenols degradation by *S. maltophilia* KB2 strain was made. *S. maltophilia* KB2 used in this study is known to metabolize broad range of aromatic compounds including phenol, some chloro and methylphenols, benzoic acids, catechols, and others (Gren et al., 2010). A model system for selenium rhizofiltration from the rhizosphere soil of *Astragalus bisulcatus*, a legume based on plant–rhizobacteria interactions has been proposed (Vallini et al., 2005). **Figure 5** represents a model system for the *in situ* application of plants in the bioremediation of contaminated water and other pollutants like trichloroethylene (TCE).

In another study *Pennisetum pedicellatum* rhizosphere associated degrading strain MHF ENV20 of *S. maltophilia* were evaluated for chlorpyrifos remediation (Dubey and Fulekar, 2012). This genus was among several other bacteria isolated as an endophyte in coffee seeds (Vega et al., 2005). Lata et al. (2006) found endophytic bacteria belonging to *Stenotrophomonas* associated with Echinacea plants. Endophytic bacteria (*Stenotrophomonas*) associated with sweet potato plants [*Ipomoea batatas* (L.) Lam] were isolated, identified and tested for their ability to fix nitrogen, produce indole acetic acid (IAA), and exhibit stress tolerance (Khan and Doty, 2009). The multidrug-efflux-pump SmeDEF in *S. maltophilia* has been shown to play vital role in root colonization in oilseed rape plant (cv. Californium; Kwizda, Austria) rather than its role in resistance toward antibiotic quinolones (**Figure 6**). Although, naturally occurring plant–microbe interactions are routinely applied in the field of phytoremediation, transgenic plants are also being reported in literature: *Brassica juncea* for phytoremediation of heavy metals from soil (Dushenkov et al., 1995), *Helianthus anus* (Dushenkov et al., 1995), *Chenopodium amaranticolor* (Eapen and D'Souza, 2003) for rhizofiltration of uranium and pumpkin plants for remediation of trichloroethylene. Dicotyledon plant species can be genetically engineered using the *Agrobacterium* vector system, while most monocotyledon plants can be transformed using particle gun or electroporation techniques.

CONCLUSION

The advent of new Molecular diagnostic techniques for *S. maltophilia,* has created the potential to improve clinical outcomes. However, further validation and investigation of clinical correlates (viable bacterial load, antibiotic susceptibility profiles, virulence factor expression and clinical outcomes) is required before routine application. Genome sequencing of various *S. maltophilia* strains will help to identify the virulence factors, proteins and genes specific for MDR and pave the way for targeted drug delivery in treating *S. maltophilia* infections. Despite of its initial discovery as a human opportunistic pathogen, the different applications of *S. maltophilia* has not been left unexplored, although the question of biosafety remains. Non-pathogenic strains can be applied in various environmental issues: being a natural soil bacterium, *Stenotrophomonas* strains have wide applications in agriculture as potential biocontrol agents in treating fungal infections and plant growth promotion. *S. rhizophilia* is non-pathogenic and widely associated with plant roots and holds great promise in phytoremediation or rhizoremediation of contaminated ground water. Transgenic plants application to *in situ* bioremediation is not clear till date as adequate field studies have not been reported. Impact of transgenics to environment like competiveness of transgenic to wild type plants, effect on birds or insects and possibility of gene transfer to other natural plants through pollination are points to be considered. *S. maltophilia* are ubiquitously distributed in our environment. Pathogenic *S. maltophilia* might be applied to our environment provided proper measures are taken to eradicate its pathogenicity first. This will involve long term research to identify the genes or proteins of *S. maltophilia* involved in pathogenicity and MDR. Recombinant *S. maltophilia* with knockout of such genes might be applied but the problem in changes in present MDR properties through genetic mutation needs to be kept in mind. Lot of work has been done on applications of *S. maltophilia* in various domains. Still, it is quite impossible to separate these domains and the attitude "favorable" cannot be truly related to pathogenic strains though comparative genomics and transcriptomics helps detect significant borders between pathogenic strains and non-pathogenic plant-associated strains.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Stenotrophomonas maltophilia Phenotypic and Genotypic Diversity during a 10-year Colonization in the Lungs of a Cystic Fibrosis Patient

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The present study was carried out to understand the adaptive strategies developed by *Stenotrophomonas maltophilia* for chronic colonization of the cystic fibrosis (CF) lung. For this purpose, 13 temporally isolated strains from a single CF patient chronically infected over a 10-year period were systematically characterized for growth rate, biofilm formation, motility, mutation frequencies, antibiotic resistance, and pathogenicity. Pulsed-field gel electrophoresis (PFGE) showed over time the presence of two distinct groups, each consisting of two different pulsotypes. The pattern of evolution followed by *S. maltophilia* was dependent on pulsotype considered, with strains belonging to pulsotype 1.1 resulting to be the most adapted, being significantly changed in all traits considered. Generally, *S. maltophilia* adaptation to CF lung leads to increased growth rate and antibiotic resistance, whereas both *in vivo* and *in vitro* pathogenicity as well as biofilm formation were decreased. Overall, our results show for the first time that *S. maltophilia* can successfully adapt to a highly stressful environment such as CF lung by paying a "biological cost," as suggested by the presence of relevant genotypic and phenotypic heterogeneity within bacterial population. *S. maltophilia* populations are, therefore, significantly complex and dynamic being able to fluctuate rapidly under changing selective pressures.

Keywords: cystic fibrosis, lung infections, *Stenotrophomonas maltophilia*, chronic infection, biofilm, virulence, antibiotic-resistance

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INTRODUCTION

Stenotrophomonas maltophilia is one of the most common emerging multi-drug resistant pathogens found in the lungs of people with cystic fibrosis (CF) where its prevalence is increasing (Amin and Waters, 2014; Green and Jones, 2015; Salsgiver et al., 2016). Nevertheless, it is unclear whether *S. maltophilia* simply colonizes the lungs of people with CF without adverse effect or

Abbreviations: CF, cystic fibrosis; TSB, Trypticase soy broth; MHA, Mueller-Hinton agar; PFGE, pulsed-field gel electrophoresis; MGT, mean generation time; SBF, specific biofilm formation index; MOI, multiplicity of infection.

causes true infection leading to pulmonary inflammation and clinical deterioration. Clinical studies reported conflicting results on the correlation between the presence of this microorganism and lung damage (Karpati et al., 1994; Goss et al., 2002). It has recently been shown that chronic infection with *S. maltophilia* in people with CF is an independent risk factor for pulmonary exacerbation requiring hospitalization and antibiotics and was associated with a systemic immune response to *S. maltophilia* (Waters et al., 2011).

In a series of studies, we found evidence highly suggestive of the pathogenic role of *S. maltophilia* in CF patients. This microorganism can grow as biofilm not only on abiotic surfaces (Di Bonaventura et al., 2004, 2007a,b; Pompilio et al., 2008) but also on CF-derived epithelial monolayer (Pompilio et al., 2010), probably because of a selective adaptation to CF airways (Pompilio et al., 2011). Furthermore, in a murine model of acute respiratory infection we observed that *S. maltophilia* significantly contributes to the inflammatory process resulting in compromised respiratory function and death (Di Bonaventura et al., 2010).

In the diseased CF lung, pathogens are exposed to a complex range of selection pressures including host physiological factors, oxygen tension, immune responses, therapeutic antimicrobials, and competing microorganisms. Together, these are thought to drive genetic and phenotypic diversity in the pathogen over time. Consequently, various airway-specific adaptations are postulated to favor persistence and lead to host-tolerant clonal lineages that are less cytotoxic, better at evading the immune system, more resistant to antimicrobials and less metabolically active than their ancestral strains (Hill et al., 2005; Bragonzi et al., 2009; Behrends et al., 2013). These studies have been largely focused on *Pseudomonas aeruginosa* (Hogardt and Heesemann, 2010; Hauser et al., 2011). In comparison, the adaptation of *S. maltophilia* in the CF lung has been investigated rarely (Vidigal et al., 2014), and is largely unknown.

In order to understand the adaptive strategies developed by *S. maltophilia* for chronic colonization of the CF lung, we systematically characterized 12 temporally isolated strains from a single CF patient over a 10-year period. We studied their relative growth rate, biofilm formation, motility, mutation frequencies, antibiotic resistance spectrum, virulence, and pathogenicity. We report for the first time that chronic *S. maltophilia* displays unusual adaptive plasticity by modulating its virulence and pathogenicity, yet exacerbating antibiotic resistance and other factors that augment its fitness in the CF lungs.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Thirteen *S. maltophilia* isolates, collected during 11 year-period (2004–2014) from sputum of a CF patient (ethically coded "ZC") at the CF Unit of "Bambino Gesù" Children's Hospital and Research Institute of Rome, were investigated in this study. One strain per year was considered, except than for 2012 and 2013 when two strains were obtained during the same year. The patient was selected owning to clinically defined chronic infection with *S. maltophilia*, which mandates at least 50% of samples must be positive in the preceding 12 months (Pressler et al., 2011). *S. maltophilia* was co-cultured with *P. aeruginosa* in 2010, 2011, and 2014 only. Each strain was identified by the Vitek automated system (bioMérieux Italia SpA; Florence, Italy), then stored at −80°C until use, when it was grown at 37°C in Trypticase Soy broth (TSB; Oxoid SpA; Garbagnate M.se, Milan, Italy) or Mueller-Hinton agar (MHA; Oxoid) plates. *S. maltophilia* ATCC13637 reference strain, and *S. maltophilia* Sm111, knock-out for *fliI*-gene (Pompilio et al., 2010), were used as controls in mutation frequency and motility assays, respectively.

Bacterial Genotyping

The epidemiological relatedness of the strains was studied by pulsed-field gel electrophoresis (PFGE), as previously described (Pompilio et al., 2011). Agarose-embedded DNA was digested with the restriction enzyme *XbaI*, and then separated with 6 V/cm for 20 h at 12°C, with pulse times 5–35 s and an included angle of 120°. PFGE profiles were analyzed by visual inspection and isolates were considered as belonging to the same PFGE cluster if they differed by ≤3 bands (Gherardi et al., 2015). Isolates with indistinguishable PFGE profiles belonged to the same pulsotype.

Growth Rate

Overnight cultures in TSB were corrected with fresh TSB to an OD_{550} of 1.00, corresponding to about 1–5 × 10^9 CFU/ml. This suspension was diluted 1:100 in fresh TSB, then 200 µl were dispensed in each well of a microtiter plate (Kartell SpA; Noviglio, Milan, Italy), and incubated at 37°C, under static conditions, in a microplate reader (Sinergy H1 Multi-Mode Reader; BioTek Instruments, Inc., Winooski, VT, USA). OD_{550} readings were taken every 30 min for 24 h. Considering the exponential growth phase selected on a graph of ln OD_{550} vs. time (t), mean generation time (MGT) was calculated as follows: $MGT = \ln 2/\mu$, where μ (growth rate) $= (\ln OD_t - \ln OD_{t0})/t$.

Biofilm Formation

Biofilm formation was assayed as described by Pompilio et al. (2008). Two-hundred microliters of the 1:100 diluted inoculum (prepared as described in "Growth Rate") were dispensed to each well of a flat bottom 96-well polystyrene tissue culture-treated plate (Falcon BD; Milan, Italy), and incubated in static culture at 37°C for 24 h. Samples were washed twice with PBS (pH 7.3; Sigma-Aldrich Co., Milan, Italy), then crystal violet-stained biomass was quantified by measuring the optical density at 492 nm (OD_{492}). Biofilm biomass was normalized on the growth rate by calculating the "Specific Biofilm Formation" (SBF) index as follows: SBF = biofilm biomass (OD_{492})/growth rate (μ).

Motility

Swimming, swarming, and subsurface twitching assays were performed as described by Rashid and Kornberg (2000), with modification. Swimming and swarming were assessed by surface inoculating a single colony onto swimming agar (10 g/l tryptone, 5 g/l NaCl, 3 g/l agar) or into swarming (8 g/l nutrient broth, 5 g/l dextrose, and 5 g/l agar) agar. After incubation at 37°C for 24 h, the growth zone was measured in millimeters. Twitching was

measured by inoculating a single colony to the bottom of Petri dish containing 1% TSB solidified with 1% agar. Twitch zones were stained with crystal violet after 72 h of incubation at 37°C, and measured in millimeters.

Mutation Frequencies

Mutation frequency of each strain was assessed according to Oliver et al. (2000), with modification. For each sample, three tubes containing 20 ml of Mueller-Hinton broth (Oxoid) were inoculated with one independent colony, obtained from overnight-growth on MHA plate, and incubated overnight with agitation (130 rpm). Samples were centrifuged (4500 rpm, 10 min, 4°C) and pellets resuspended in 1 ml of Mueller-Hinton broth. Ten-fold dilution of each sample was seeded onto MHA plates (controls) and onto MHA added with rifampin (Sigma-Aldrich) 250 μg/ml. Colony counts were performed after 24 h of incubation of the MHA plates and after 48 h of incubation of the MHA-rifampin plates. Mutation frequency was calculated as the number of rifampin-resistant colonies in proportion to the total viable count. Strains were classified into four categories based on mutation frequency (f) (Turrientes et al., 2010): hypo-mutators ($f \leq 8 \times 10^{-9}$), normo-mutators ($8 \times 10^{-9} < f < 4 \times 10^{-8}$), weak-mutators ($4 \times 10^{-8} \leq f < 4 \times 10^{-7}$), and strong-mutators ($f \geq 4 \times 10^{-7}$).

Virulence Assays

The virulence potential of *S. maltophilia* strains was evaluated both *in vivo* in *Galleria mellonella* larvae, and *in vitro* on human A549 alveolar basal epithelial cells. (i) *Galleria mellonella* infection assays were performed as described by Betts et al. (2014), with minor modifications. Overnight cultures of *S. maltophilia* grown in TSB were washed and resuspended in PBS. Twenty larvae were inoculated with each *S. maltophilia* strain at doses of 10^3, 10^4, 10^5, and 10^6 CFU/larva, or PBS only (controls). Ten microliters of the bacterial suspension or PBS were injected directly into the hemocoel of the wax moth via the right proleg using 10-μL Hamilton syringe (Hamilton Co., Nevada, USA). Larvae were incubated in the dark at 37°C and checked daily for survival until 96 h. Larvae were considered dead if they failed to respond to touch. A "pathogenicity score" was assigned to each strain, considering both time and dose needed to achieve LD_{50}. The higher the score, the higher the virulence.

(ii) *S. maltophilia* co-culture infection assays on human respiratory epithelial cells were performed according to Karaba et al. (2013), with minor modifications. Human A549 alveolar basal epithelial cells (ATCC CCL-185) were seeded at 10^5 cells/ml in 24-mm diameter cell culture polyester inserts in 6-well Transwell™ plates (Corning; USA). Monolayers were grown overnight (37°C, 5% CO_2) in DMEM (high glucose) with 1% penicillin, streptomycin, amphotericin (HiMedia; Mumbai, India), and supplemented with 10% fetal bovine serum (Invitrogen, USA). Each *S. maltophilia* strain, grown in TSB medium, was added to each well at Multiplicity-Of-Infection (MOI) of 500 on the apical surface of the monolayer insert. The co-culture sets were incubated for 24 h at 37°C in 5% CO_2, gently washed to remove suspended bacteria and dead epithelial cells, and then subjected to Live/Dead™ assay (ThermoFisher Scientific; Rodano, Milan, Italy). Cell death, cell rounding, and loss of adherence were studied. Images were acquired on Olympus FLUOVIEW FV1000 confocal laser scanning microscope (excitation: 488 and 543 nm; emission: 505–526 and 612–644 nm, respectively). Quantitative image analysis was performed using FV1000 Viewer-1.7 for fluorescence intensity, and the percent cell death was calculated against total cell population in the respective set.

MIC Determination

The *in vitro* susceptibility of *S. maltophilia* strains to trimethoprim-sulfamethoxazole, minocycline, ciprofloxacin, levofloxacin, ticarcillin-clavulanate, ceftazidime, piperacillin-tazobactam, amikacin, and chloramphenicol was assessed by MIC-Test Strip (Liofilchem; Roseto degli Abruzzi, Italy), according to CLSI guidelines [Clinical Laboratory Standards Institute (CLSI), 2016]. In the case of piperacillin-tazobactam, amikacin, and ciprofloxacin, because no breakpoints are available for *S. maltophilia*, we used those established for *P. aeruginosa* [Clinical Laboratory Standards Institute (CLSI), 2016]. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were chosen as quality control strains in each batch of tests.

Statistical Analysis

Each test was performed in triplicate and repeated on two different occasions. Statistical analysis was performed using Prism 6 for Windows software (version 6.01; GraphPad Software Inc., La Jolla, USA). Gaussian distribution was evaluated by Kolmogorov–Smirnov-test with Dallal-Wilkinson-Lille for p-value. Differences were measured using both parametric (one-way ANOVA-test followed by Tukey's multiple comparison post-test), and non-parametric (Mann–Whitney-test; Kruskal–Wallis ANOVA-test followed by Dunn's multiple comparison post-test) tests. Linear regression analysis was used to assess the significance of a trend. Spearman correlation coefficient was calculated for correlation analysis. MIC-values were considered as discordant for discrepancies ≥ 2 $\log_2$ concentration steps. Statistical significance was set at 0.05.

RESULTS

Bacterial Genotyping

We first assessed the clonality of strains isolated from patient ZC at different time points during the course of chronic infection over 11 years. Based on PFGE patterns, two distinct PFGE groups, with four different pulsotypes, were identified among *S. maltophilia* isolates according to the previously described interpretative criteria (**Figure 1**). PFGE group 1 encompassed two related PFGE subtypes, namely pulsotype 1.1, consisting of two strains (ZC2005 and ZC2011), and pulsotype 1.2, consisting of three strains (ZC2010, ZC2012-STM1, and ZC2013-STM1); PFGE group 2 comprised two related PFGE subtypes, pulsotype 2.1, consisting of six strains (ZC2006, ZC2008, ZC2009, ZC2012-STM2, ZC2013-STM2, and ZC2014); and pulsotype 2.2, consisting of ZC2007 strain only. Strains isolated both in 2012 (ZC2012-STM1 and ZC2012-STM2) and 2013 (ZC2013-STM1

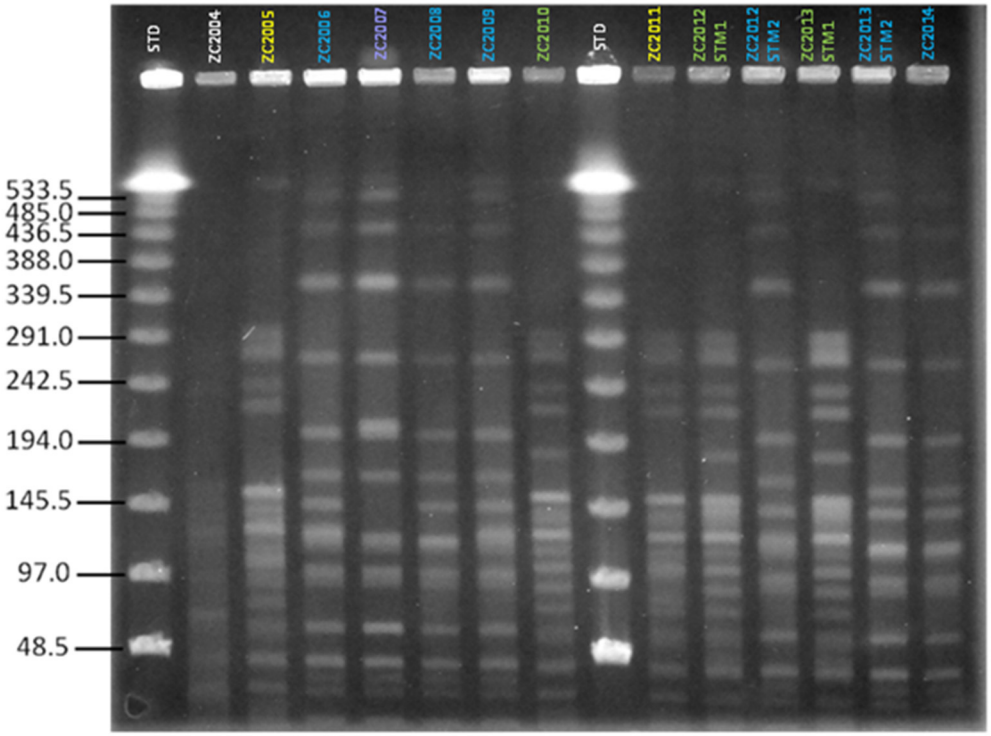

FIGURE 1 | Clonal relatedness of *S. maltophilia* strains, as assessed by PFGE analysis. The similarity of PFGE profiles was visually assessed, and considered as follows: (i) isolates with identical PFGE patterns were assigned to the same PFGE type and subtype; (ii) isolates differing by one to three bands were assigned to the same PFGE type and were considered genetically related; while (iii) isolates with PFGE patterns differing by more than four bands were considered genetically unrelated and were assigned to different PFGE types. Four pulsotypes were observed: 1.1 (yellow), 1.2 (green), 2.1 (blue), and 2.2 (purple). STD, molecular weight standard. The profile exhibited by ZC2004 strain could not be interpreted because of poor resolution and, consequently, was not assigned to a pulsotype and not enrolled in the study.

and ZC2013-STM2) belonged to different pulsotypes of unrelated PFGE types. The profile exhibited by ZC2004 strain could not be interpreted because of lack of resolution in the high molecular weight zone of the gel and, therefore, was not further studied.

Growth Rate

Growth rate values exhibited by each *S. maltophilia* strain were spectrophotometrically assessed, and results are summarized in **Figures 2A**, **3A**.

S. maltophilia strains isolated over time significantly differed for growth rate ($p < 0.0001$, Kruskal–Wallis-test), showing a significant upward trend ($p < 0.01$; **Table 1**).

Significant differences were observed within each pulsotype (**Figure 2A**). With regard to strains belonging to pulsotype 1.1, ZC2011 showed a growth rate higher compared to ZC2005 (median: 0.402 vs. 0.217, respectively; $p < 0.01$). With regard to pulsotype 1.2, ZC2010 strain showed a median growth rate significantly higher than ZC2012-STM1 (median: 0.429 vs. 0.316, respectively; $p < 0.05$). Among pulsotype 2.1 strains, ZC2013-STM2 grew significantly faster than ZC2006 and ZC2009 strains (median: 0.465 vs. 0.292 and 0.279, respectively; $p < 0.01$).

Considering each pulsotype as a whole, no statistically significant differences were observed (**Figure 3A**). The kinetics of changes in growth rate showed that both pulsotypes 1.1 and 2.1 significantly increased over the study-period ($p < 0.0001$ and 0.05, respectively), while pulsotype 1.2 remained generally unchanged (**Table 1**).

Biofilm Formation

The results concerning biofilm biomass formed by each strains tested were normalized on growth rate and expressed as SBF, as shown in **Figures 2B**, **3B**. SBF-values were statistically related to non-normalized biofilm OD_{492}-values (data not shown; Spearman *r*: 0.986; $p < 0.0001$).

S. maltophilia strains significantly differed for efficacy in forming biofilm ($p < 0.0001$, Kruskal–Wallis + Dunn post-test). Significant differences were found among strains belong to each pulsotype (**Figure 2B**). With regard to pulsotype 1.1 strains, ZC2005 produced significantly more biofilm compared to ZC2011 (median: 16.19 vs. 0.34, respectively; $p < 0.0001$). Among strains belonging to pulsotype 1.2, ZC2010 formed significantly more biofilm biomass than other strains (median: 2.23 vs. 1.02, and 1.06, respectively, for ZC2010, ZC2012-STM1, and ZC2013-STM1 strains; $p < 0.0001$). With regard to pulsotype 2.1, ZC2012-STM2 produced higher biofilm biomass compared to most of other strains (median: 1.79 vs. 0.76, 1.06, and 0.19; respectively, for ZC2012-STM2, ZC2008, ZC2013-STM2, and ZC2014 strains; $p < 0.0001$).

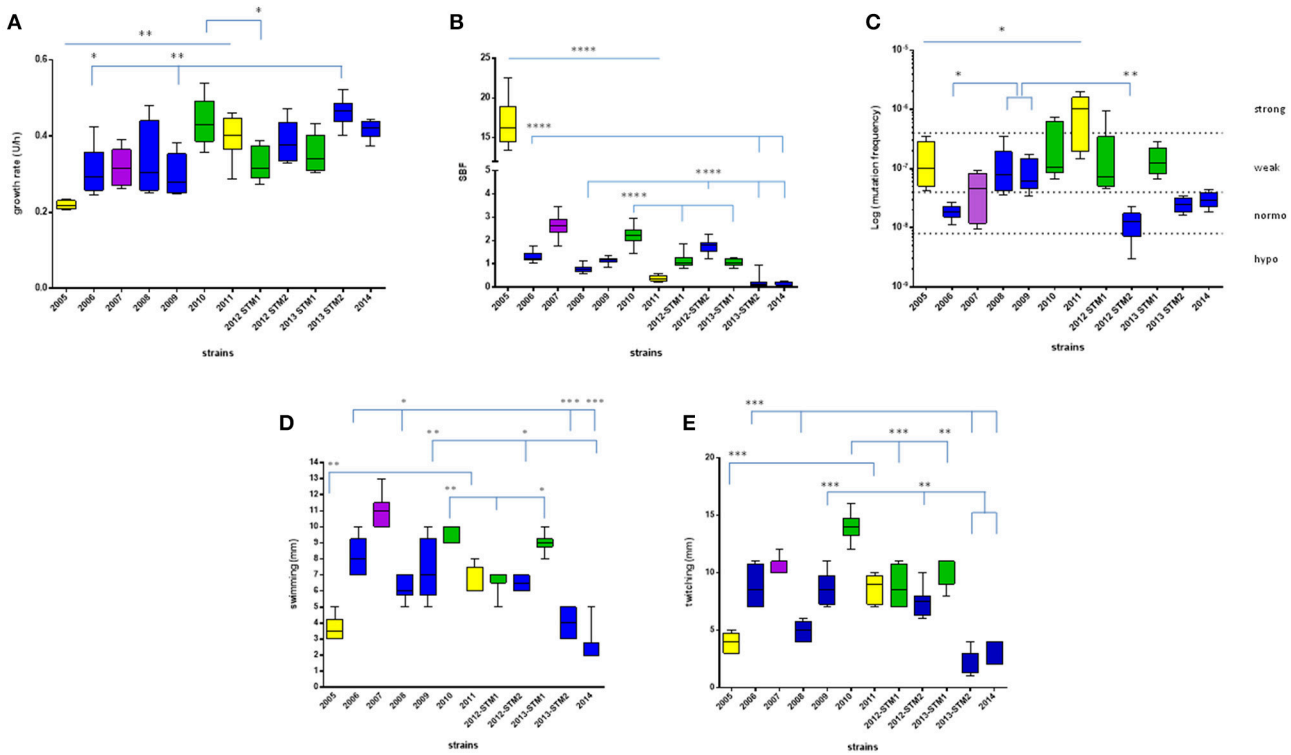

FIGURE 2 | Phenotypic traits exhibited by *S. maltophilia* strains. Colors indicate the pulsotypes: 1.1 (yellow, $n = 2$ strains), 1.2 (green, $n = 3$ strains), 2.1 (blue, $n = 6$ strains), and 2.2 (purple, $n = 1$ strain). **(A)** Growth rate, expressed as generation time (U/h). **(B)** Biofilm biomass formation, normalized on growth rate and expressed as specific biofilm formation (SBF) index. **(C)** Mutation frequency; strains were classified into four categories based on mutation frequency (f): hypo-mutators ($f \leq 8 \times 10^{-9}$), normo-mutators ($8 \times 10^{-9} < f < 4 \times 10^{-8}$), weak-mutators ($4 \times 10^{-8} \leq f < 4 \times 10^{-7}$), and strong-mutators ($f \geq 4 \times 10^{-7}$). **(D)** Swimming motility, expressed as diameter of growth zone (mm). **(E)** Twitching motility, expressed as diameter of twitch zone (mm). Results are shown as box and whiskers ($n = 6$, for each strain): the box always extends from the 25th to 75th percentiles, while the line in the middle of the box is plotted at the median. Statistical analysis: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, Mann–Whitney-test (pulsotype 1.1) or Kruskal–Wallis followed by Dunn's multiple comparison post-test (pulsotypes 1.2 and 2.1).

Pulsotypes significantly differed for biofilm formation ($p < 0.0001$, Kruskal–Wallis-test; **Figure 3B**). In particular, pulsotype 1.1 produced significantly more biofilm than 2.1 and 2.2 (median: 6.99 vs. 0.93 and 2.63, respectively; $p < 0.01$ and 0.05, respectively), pulsotype 2.2 formed a biofilm biomass significantly higher than 1.2 (median: 1.2; $p < 0.01$) and 2.1 ($p < 0.001$), while pulsotype 1.2 produced higher biofilm amount than 2.1 ($p < 0.01$; **Figure 3B**).

The kinetics of biofilm biomass formed during the study-period showed a significant downward trend for pulsotypes 1.1 and 2.1 ($p < 0.0001$; **Table 1**).

Mutation Frequency

Variations in the frequency of mutation exhibited by *S. maltophilia* strains isolated over 10 years are summarized in **Figures 2C**, **3C**.

Mutation frequency significantly differed among *S. maltophilia* strains ($p < 0.0001$, Kruskal–Wallis-test), while no significant trend was observed (**Table 1**). Significant differences were found among strains belong to each pulsotype (**Figure 2C**). Among pulsotype 1.1 strains, ZC2005 showed a mutation frequency significantly lower than ZC2011 (median: 9.9×10^{-8} vs. 1.0×10^{-6}, respectively; $p < 0.05$). With regard to pulsotype 2.1, strains ZC2008 and ZC2009 exhibited higher mutation frequency (median: 7.9×10^{-8} and 6.2×10^{-8}, respectively) compared to ZC2006 and ZC2012-STM2 (median: 1.8×10^{-8} and 1.2×10^{-8}, respectively; $p < 0.05$ and 0.01, respectively). Strains belonging to pulsotype 1.2 did not change significantly over the study-period.

According to mutation frequency, most of the strains were weak-mutators (7 out of 12, 58.3%), followed by normo-mutators (4 out of 12, 33.3%), while only one strain resulted to be a strong-mutator (8.4%). No hypo-mutators were found (**Figure 2C**).

Considering the pulsotypes as a whole, pulsotype 1.1 showed higher frequency compared to pulsotype 2.1 and 2.2 (median: 2.3×10^{-7} vs. 2.7×10^{-8}, and 4.6×10^{-8}, respectively; $p < 0.0001$ and 0.05, respectively; **Figure 3C**). The only hyper-mutator strain belonged to pulsotype 1.1, while pulsotype 1.2 consisted of weak-mutators only. The strains belonging to pulsotype 2.1 were mainly normo-mutators (66.6%), while the remaining ones were weak-mutators. No significant changes were observed among strains belonging to each pulsotype.

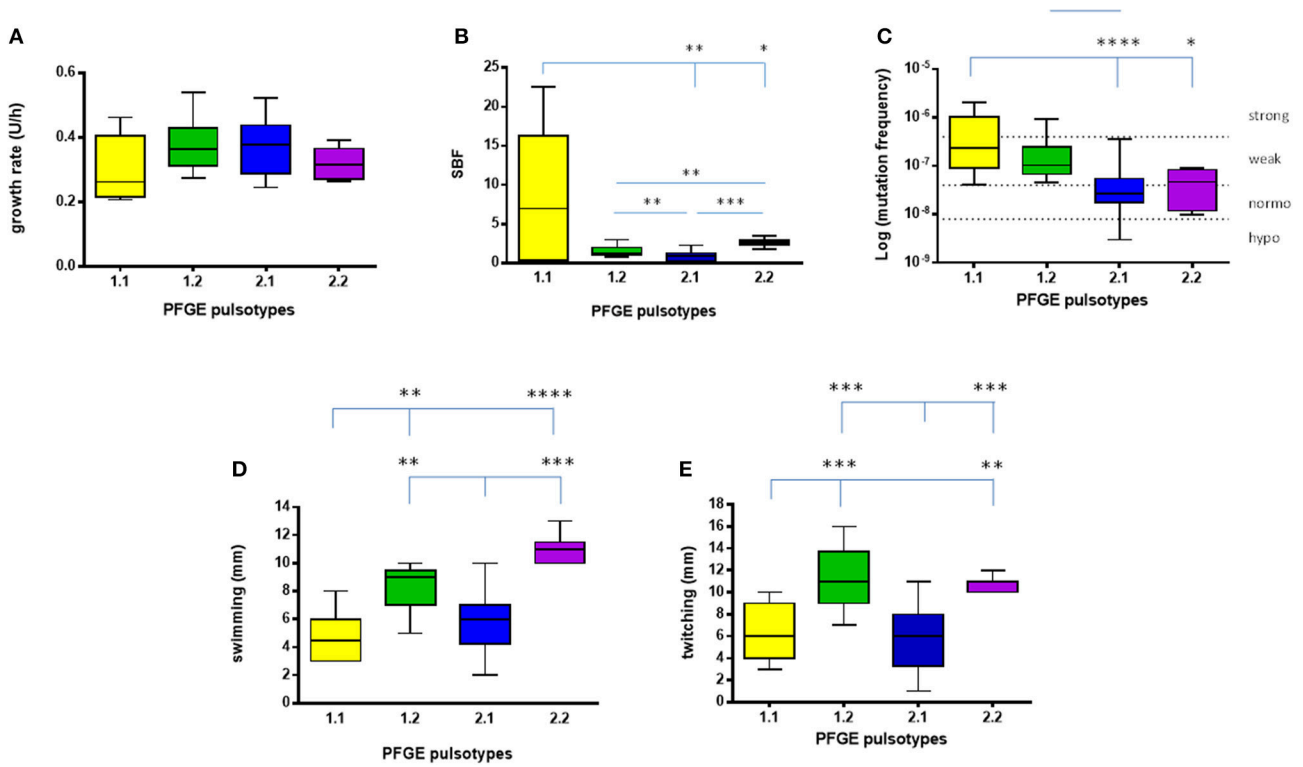

FIGURE 3 | Phenotypic traits exhibited by *S. maltophilia* strains. Results ($n = 6$, for each strain) are stratified according to the pulsotype: 1.1 (yellow, $n = 2$ strains), 1.2 (green, $n = 3$ strains), 2.1 (blue, $n = 6$ strains), and 2.2 (purple, $n = 1$ strain). **(A)** Growth rate. **(B)** Biofilm biomass formation, normalized on growth rate (SBF; specific biofilm formation). **(C)** Mutation frequency; strains were classified into four categories based on mutation frequency (f): hypo-mutators ($f \leq 8 \times 10^{-9}$), normo-mutators ($8 \times 10^{-9} < f < 4 \times 10^{-8}$), weak-mutators ($4 \times 10^{-8} \leq f < 4 \times 10^{-7}$), and strong-mutators ($f \geq 4 \times 10^{-7}$). **(D)** Swimming motility. **(E)** Twitching motility. Results are shown as box and whiskers: the box always extends from the 25th to 75th percentiles, while the line in the middle of the box is plotted at the median. Statistical analysis: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, Kruskal–Wallis followed by Dunn's multiple comparison post-test.

TABLE 1 | *S. maltophilia* trends in expression over time across all traits.

Pulsotype (*n*)	Trait							
	Growth rate	Biofilm formation	Mutation frequency	Swimming motility	Twitching motility	*G. mellonella* pathogenicity	A549 cells pathogenicity	Antibiotic resistance
1.1 (2)	↗	↘	↗	↗	↗	↘	↘	↗
1.2 (3)	—	—	—	—	—	—	↘	↗
2.1 (6)	↗	↘	—	↘	—	↘	↘	↗
Overall ($n = 12$)	↗	—	—	—	—	—	↘	—

The statistical significance of a trend was assessed by Mann–Whitney-test (pulsotype 1.1) or linear regression analysis (pulsotypes 1.2 and 2.1). Pulsotype 2.2 was not considered as composed of one strain only. Arrows indicate significant trends: increased (red) or decreased (blue). Trend in antibiotic resistance was measured by considering mean variation in MIC-values over time, considering only ≥ 4-fold changes.

The kinetics of the median mutation frequency showed that in pulsotype 1.1 only strains increased ($p < 0.05$) their mutation frequency over the study-period, shifting from weak (ZC2005)- to strong (ZC2011)-mutator phenotype. No particular trend was observed for other pulsotypes (**Table 1**).

Motility

Swimming and twitching motility levels exhibited by *S. maltophilia* strains are summarized in **Figures 2D,E**, **3D,E**, respectively. None of strains showed swarming motility.

Significant differences were found among strains both for swimming and twitching ($p < 0.0001$). Particularly, the motility exhibited by ZC2011 strain was significantly higher compared to that of ZC2005 (swimming, median: 6.0 vs. 3.5 mm, respectively; $p < 0.01$; twitching, median: 9 vs. 4 mm, respectively; $p < 0.001$). With regard to pulsotype 2.1, the motility observed for ZC2006 strain was significantly higher than ZC2008, ZC2013-STM2, and ZC2014 strains (swimming: 8 vs. 6, 4, and 2 mm, respectively; $p < 0.05$; twitching: 8.5 vs. 5, 3, and 2 mm, respectively; $p < 0.001$).

Among strains belonging to pulsotype 1.2, ZC2012-STM1 strain showed a swimming motility significantly lower than strains ZC2010 and ZC2013-STM1 (7 vs. 10 and 9 mm, respectively; $p < 0.05$; **Figure 2D**). Contrarily, twitching motility was significantly higher in ZC2010 strain, compared to ZC2012-STM1 and ZC2013-STM1 strains (14.0 vs. 8.5, and 9 mm, respectively; $p < 0.001$; **Figure 2E**).

With regard to each pulsotype, a similar trend was found for both swimming and twitching motilities (**Figures 3D,E**). In particular, a comparable trend for both swimming and twitching motilities was observed for the strains belonging to pulsotypes 1.1 and 2.1.

Pulsotype 1.1 showed significantly lower motility, compared to pulsotypes 1.2 and 2.2 (swimming: 4.5 vs. 9, and 11, respectively; $p < 0.01$; twitching: 6 vs. 11 and 11 mm, respectively; $p < 0.01$), whereas motility exhibited by pulsotype 2.1 (6 mm, for both swimming and twitching) was significantly lower compared to pulsotypes 1.2 and 2.2 ($p < 0.01$).

The kinetics of changes in swimming and twitching motilities over the study-period showed a significant upward trend in pulsotype 1.1 ($p < 0.01$), whereas a trend toward decreased swimming motility was found in pulsotype 2.1 strains ($p < 0.05$; **Table 1**).

Virulence Assays

(i) *G. mellonella* infection assay. The kinetics of *G. mellonella* survival monitored over 96 h following infection with *S. maltophilia* showed that the killing activity was generally dose-dependent, regardless of strain or pulsotype considered (**Figure 4**).

To comparatively evaluate the pathogenicity of tested strains, a "pathogenicity score" was assigned to each strain (**Table 2**). ZC2005 was the most virulent strain (score: 8), causing the killing of all infected larvae already at 24 h and at the lowest dose used (10^3 CFU/larva). Other strains showed striking differences in virulence, except for ZC2010, ZC2012-STM1, ZC2013-STM1, and ZC2014 strains that resulted to be not virulent (score: 1), not being able to kill at least 50% of infected larvae following 96 h-exposure to the highest dose (10^6 CFU).

Pulsotypes 1.1 and 2.1 showed comparable virulence (mean score: 5, and median score: 5.5, respectively), significantly higher than pulsotype 1.2 (median score: 1).

The same trend in virulence was observed, over time, in strains belonging to pulsotypes 1.1 and 2.1: pathogenicity score in fact significantly decreased from 8 (ZC2005) to 2 (ZC2011), and from 7 (ZC2006) to 1 (ZC2014), respectively. No change was observed in pulsotype 1.2 strains (**Table 1**).

(ii) A549 cells co-culture assay. *S. maltophilia* pathogenicity was also assessed on human A549 alveolar cells using Live/Dead^{TM} cell viability staining (ThermoFisher Scientific; **Figure 5**).

Generally, strains significantly differed for pathogenicity level ($p < 0.0001$), although a downward trend was observed over the time (**Figure 5A**; **Table 1**). With regard to pulsotype 1.1, the damage caused by ZC2005 strain was significantly higher compared to that of ZC2011 (mean percentage ± *SD*: 46.7 ± 10.0 vs. 17.7 ± 2.1, respectively; $p < 0.05$). Among pulsotype 1.2 strains, damage significantly decreased from ZC2010 toward 2012-STM1 and ZC2013-STM1 (mean ± *SD*: 70.4 ± 1.8, 45.8 ± 1.1, and 10.0 ± 1.2, respectively; $p < 0.001$). Striking significant differences were also found among strains belonging to pulsotype 2.1, where pathology rate significantly decreased over time from 89.8 ± 3.5 (ZC2006) to 9.1 ± 1.9 (ZC2014) ($p < 0.001$). Linear regression analysis confirmed the existence of a negative trend in each pulsotype (**Table 1**).

No significant differences were found in cellular damage among pulsotypes.

However, subtler differences between the temporal profiles within a pulsotype were observed with regard to non-lethal effects, including cell rounding and detachment (**Figure 5B**). Cells exposed to ZC2010 exhibited unusually high rounding comparable to ZC2006, whereas ZC2008 consistently showed high detachment of the epithelial monolayer. The early colonizers like ZC2005 provoked all three effects, whereas ZC20014 strain caused minimal damage.

A positive, although statistically not significant, trend was observed between *G. mellonella* and A549 assays, considering results either as a whole (Spearman *r*: 0.395) or stratified to pulsotypes 1.1 and 2.1 (Spearman *r*: 0.550).

Antibiotic Susceptibility

The susceptibility patterns of the sequential *S. maltophilia* strains under study were determined by the MIC-test strip method, and results are shown in **Figure 6**.

Considering the strains as a whole, MIC of each antibiotic significantly varied over the study-period: 16 to ≥256 μg/ml (piperacillin-tazobactam), 0.5 to ≥32 μg/ml (levofloxacin), 12 to ≥256 μg/ml (amikacin), 0.094–0.75 (trimethoprim-sulfamethoxazole), 0.19–4 μg/ml (minocycline), 1 to ≥256 μg/ml (ticarcillin-clavulanate), 3–96 μg/ml (chloramphenicol), 3 to ≥32 μg/ml (ciprofloxacin), and 4 to ≥256 μg/ml (ceftazidime).

Pulsotype 1.1 strains significantly increased their MIC for levofloxacin (from 0.75 to 32 μg/ml), amikacin (from 4 to

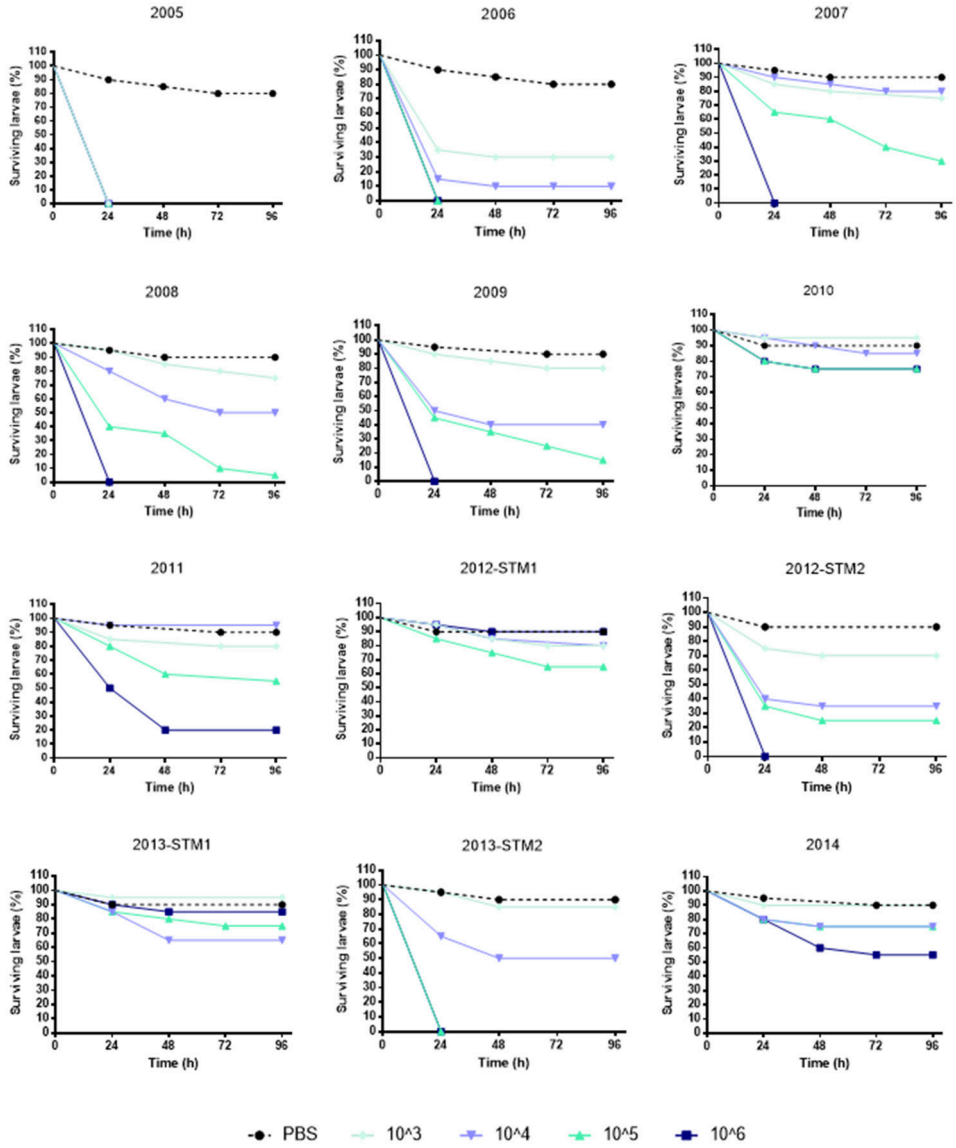

FIGURE 4 | Survival of *Galleria mellonella* over 96 h following infection. Each strain was used at the following infective doses prepared in PBS: 10^6, 10^5, 10^4, and 10^3 CFU/larva. Uninfected control larvae were exposed to PBS only (black dotted line). Larvae were incubated at 37°C for 96 h and checked daily for survival, considering dead those not reactive to touch. Results are shown as mean + *SD*.

32 μg/ml), cotrimoxazole (from 0.94 to 0.5 μg/ml), minocycline (from 0.19 to 4 μg/ml), chloramphenicol (from 4 to 48 μg/ml), and ciprofloxacin (from 3 to 32 μg/ml), shifting toward resistant class in the case of chloramphenicol. The mean increase in MIC-values over time was 18.1-fold.

MIC-values exhibited by the strains belonging to pulsotype 1.2 significantly increased over the study-period, in the case of levofloxacin (from 1 to 12 μg/ml), chloramphenicol (from 4 to 96 μg/ml), and ciprofloxacin (from 3 to 32 μg/ml). This resulted in susceptible-to-resistant transition in the case of levofloxacin and chloramphenicol, but not for ciprofloxacin whose MICs always indicated resistance. In contrast, MICs significantly decreased of at least 80-fold for ceftazidime and ticarcillin/clavulanate (from 250 to 3 μg/ml, and from 256 to 1 μg/ml, respectively), and of 16-fold for piperacillin-tazobactam (from 256 to 16 μg/ml), switching in all cases from resistant to susceptible class. Trimethoprim-sulfamethoxazole MIC increased as well, although the range was within susceptibility breakpoint (from 0.094 to 0.75 μg/ml). The mean increase in MIC-values over time was 13.6-fold.

Strains belonging to pulsotype 2.1 exhibited increased MICs for levofloxacin only, passing from susceptible to resistant class (from 1 to 3 μg/ml). The mean increase in MIC-values over time was 2.8-fold.

TABLE 2 | Pathogenicity of 12 *S. maltophilia* strains, isolated from the same CF patient over 10 year-period, as assessed in *G. mellonella*.

Strains	Time (h) required to obtain LD_{50} at the following infective doses (CFU/larva)				Pathogenicity score
	10^3	10^4	10^5	10^6	
ZC2005	<24	<24	<24	<24	8
ZC2006	24	24	<24	<24	7
ZC2007			72	<24	3
ZC2008		72	24	<24	4
ZC2009		24	24	<24	6
ZC2010					1
ZC2011				24	2
ZC2012-STM1					1
ZC2012-STM2		24	24	<24	6
ZC2013-STM1					1
ZC2013-STM2		48	<24	<24	5
ZC2014					1

Each strain was inoculated, at different doses (10^3, 10^4, 10^5, and 10^6 CFU/larva), in 20 larvae. Following infection, the larvae mortality was assessed at different times (24, 48, 72, and 96 h), and LD_{50} (the dose required to kill 50% of the infected larvae) was recorded. For each strain, the "pathogenicity score" was calculated assigning decreasing values from the highest (score: 8) to the lowest (score: 1) virulence, as assessed considering both dose and time necessary to obtain LD_{50}. In some cases, 24 h-exposure to S. maltophilia caused the killing of all larvae (LD_{50} < 24 h). Pulsotypes are indicated by colors: 1.1 (yellow), 1.2 (green), 2.1 (blue), and 2.2 (purple).

The only hyper-mutator strain ZC2011 showed the highest number of antibiotic resistances. Interestingly, a trend toward multiple antibiotic resistance among hyper- (4), weak- (2.6 ± 0.97), and normo-mutator (2.3 ± 0.5) strains was noted, although this was not statistically significant.

Mutator phenotypes showed higher mean MIC-values compared to non-mutator phenotype in the case of levofloxacin (mean ± *SD*: 1.6 ± 1.1 vs. 6.8 ± 10.9, respectively), chloramphenicol (mean ± *SD*: 5.3 ± 2.2 vs. 32.8 ± 41.8, respectively), and ciprofloxacin (mean ± *SD*: 5.0 ± 2.0 vs. 14.1 ± 14.8, respectively). However, these differences were not statistically significant probably due to high *SD*-values.

In pulsotype 2.1 strains, increased antibiotic resistance was observed against fluoroquinolones following antibiotic treatment cycles with levofloxacin or ciprofloxacin.

Trends in *S. maltophilia* Adaptive Phenotypes

The phenotypic traits significantly changed in each *S. maltophilia* pulsotype over the study-period are summarized in **Table 1**.

The pattern of evolution followed by *S. maltophilia* was dependent on pulsotype considered. Following long-lasting *S. maltophilia* infection in CF lung, strains belonging to pulsotype 1.1 resulted to be the most adapted, being significantly changed in all traits considered. Pulsotype 2.1 strains showed variations in all traits but mutation frequency and twitching motility, sharing with pulsotype 1.1 the same trend for growth rate, biofilm formation, pathogenicity, and antibiotic resistance, while an opposite one was observed for swimming motility. All pulsotypes were affected in A549 pathogenicity and antibiotic susceptibility. The same temporal trends were confirmed using non- normalized biofilm OD_{492}-values (data not shown).

Considering the strains as a whole, we found several relationships among the phenotypic traits considered (**Figure 7**). Swimming and twitching motilities were positively correlated (Spearman $r = 0.876$; $p < 0.001$), whereas a negative correlation was observed between growth rate and biofilm formation (Spearman $r = -0.720$; $p < 0.05$). Pathogenicity, as assessed in *G. mellonella* model, was negatively correlated both with growth rate (Spearman $r = -0.591$; $p < 0.05$) and levofloxacin MIC (Spearman $r = -0.698$; $p < 0.01$), whereas a positive association was found with biofilm formation (Spearman $r = 0.624$; $p < 0.05$). The mortality observed in A549 cells was negatively associated with ciprofloxacin MIC (Spearman $r = -0.684$; $p<0.01$). Susceptibility to levofloxacin, ciprofloxacin and chloramphenicol were positively correlated each other.

DISCUSSION

In contrast to *P. aeruginosa* CF lung colonization, where genetic adaptations leading to phenotypic variation are well known (Clark et al., 2015), the adaptive characters in *S. maltophilia* that drive its persistence in CF lungs is largely unknown. It is, therefore, interesting to understand trait changes during *S. maltophilia* persistence in CF lungs and to evaluate whether there is a typical phenotypic profile related to chronic infection. With that aim, the approach followed in this study was to combine genotypic profiling with phenotypic characterization to compare sequential *S. maltophilia* isolates recovered from a chronically infected CF patient.

The main conclusion arising from our results is the evidence that during the course of chronic lung colonization *S. maltophilia* develops new phenotypes and modulates its pathogenicity, probably reflecting genetic or epigenetic changes.

S. maltophilia population structure within CF patient airway is highly dynamic during long-term chronic infection. Our findings in fact showed that 2 distinct PFGE genotypes, with 4 different pulsotypes, colonize CF lung over time, and multiple lineages are present at the same time, as observed during years 2012 and 2013. Further, we observed replacements of infecting genotypes that, from an ecological perspective, could suggest the existence of a competition among different genotypes for the same specific niches of the CF patient lung. The patient harbored pulsotypes significantly different for isolation frequency over time. A similar high degree of strain diversity was found by Vidigal et al. (2014) in *S. maltophilia* consecutively isolated from chronically colonized CF patients. A more diverse community reflects niche separation within the host making bacterial subpopulations better able to resist an external stress such as exposure to antibiotics or the human defense system. Our results cannot provide information if strains come from an environmental source rather than acquired from another patient, although we

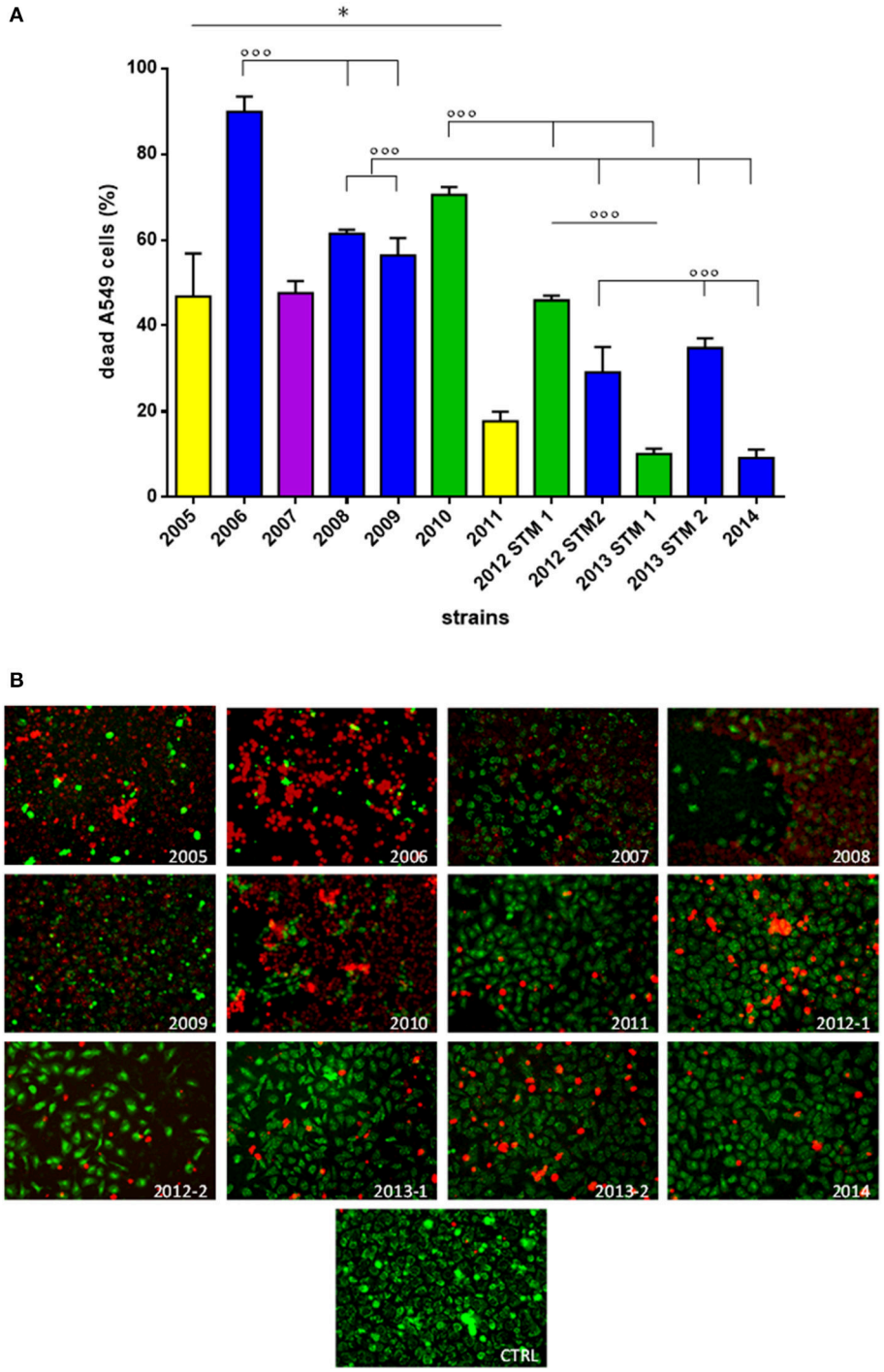

FIGURE 5 | Effect of *S. maltophilia* exposure on human A549 alveolar cells. Cell monolayers were exposed for 24 h to each *S. maltophilia* strain at MOI 500. The co-culture sets were washed, and then stained with Live/Dead^{TM} assay. Images were acquired by confocal laser scanning microscope, and quantitative image analysis was performed for fluorescence intensity. **(A)** The percent cell death was calculated compared to uninfected control samples (CTRL; cell death, 0%). Bar colors indicate the pulsotypes: 1.1 (yellow, $n = 2$ strains), 1.2 (green, $n = 3$ strains), 2.1 (blue, $n = 6$ strains), and 2.2 (purple, $n = 1$ strain). Results are expressed as mean + *SD* ($n = 6$). $^{*}p < 0.05$, unpaired *t*-test; $^{°°°}p < 0.001$, ANOVA followed by Tukey's multiple comparison post-test. **(B)** CLSM micrographs of infected A549 cell monolayers, stained with Syto-9 (green fluorescence, indicating live cells), and propidium iodide (red fluorescence, indicating dead cells). Representative microscopic fields are shown. Magnification, 100x.

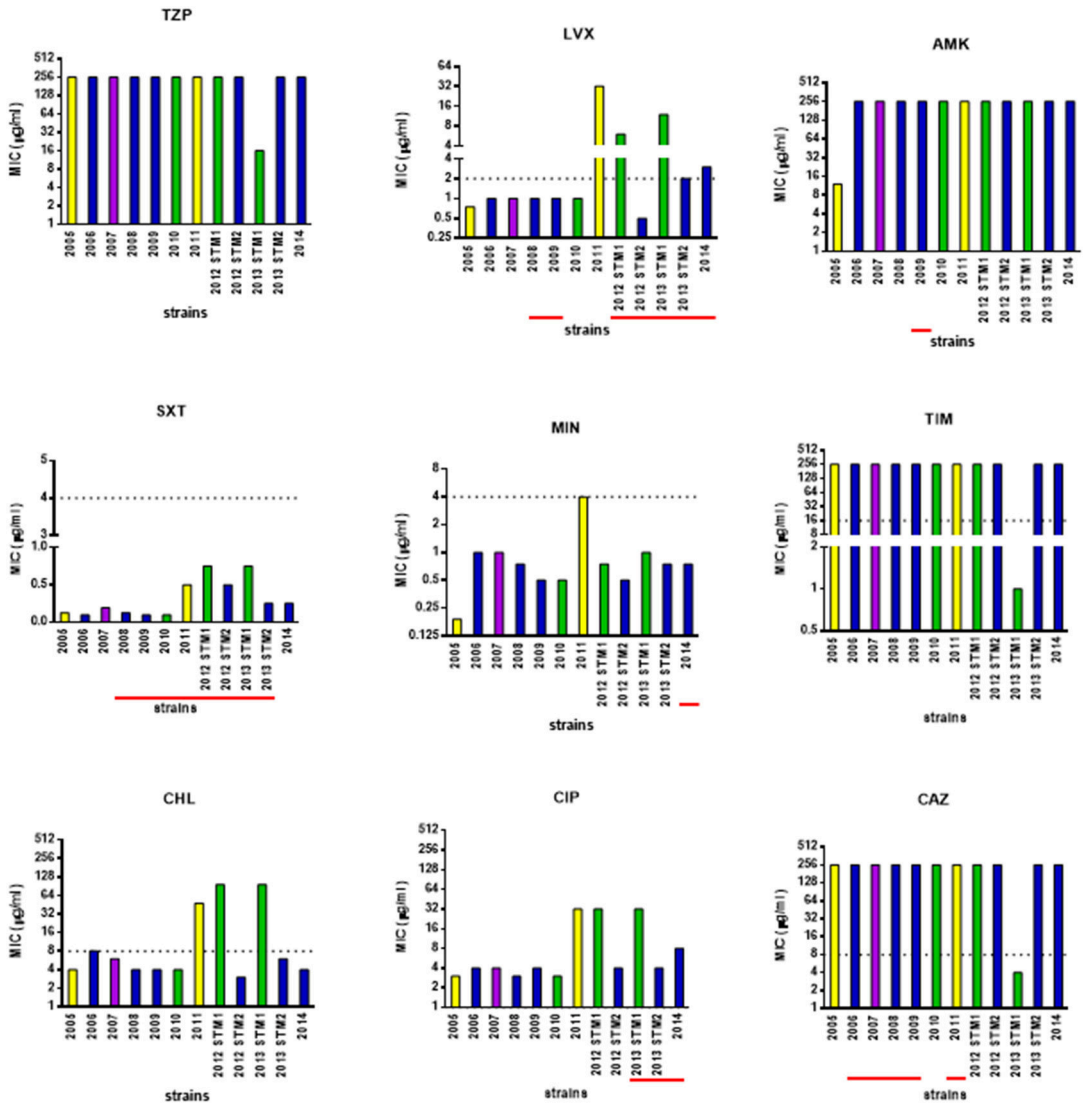

FIGURE 6 | Antimicrobial susceptibility of *S. maltophilia* strains. Bar colors indicate the pulsotypes: 1.1 (yellow, $n = 2$ strains), 1.2 (green, $n = 3$ strains), 2.1 (blue, $n = 6$ strains), and 2.2 (purple, $n = 1$ strain). MIC-values were determined, using the MIC-test strip method, for the following antibiotics: piperacillin/tazobactam (TZP), levofloxacin (LVX), amikacin (AMK), cotrimoxazole (SXT), minocycline (MIN), ticarcillin/clavulanate (TIM), chloramphenicol (CHL), ciprofloxacin (CIP), and ceftazidime (CAZ). The dotted line indicates the breakpoint MIC for susceptibility [Clinical Laboratory Standards Institute (CLSI), 2016], when available. Underlined in red, the years when the antibiotic was therapeutically administered to patient. Information on antibiotic therapy was available for all years but 2005.

recently found that both scenarios are plausible (Pompilio et al., 2011).

Growth rate is an accepted measure of adaptation and has been previously used to evaluate fitness deficits associated with antibiotic resistance (Pope et al., 2008). It has been suggested that diminished growth rate of *P. aeruginosa* in the sputum of chronically infected CF patients is due to the low PMN-related availability of O_2 within the mucus (Kragh et al., 2014) or, alternatively, to genetic adaptations (Rau et al., 2010). In contrast, we observed that *S. maltophilia* growth rate, as calculated under our experimental setting (TSB medium, 37°C, static conditions), increased over time, considering strains both as a whole and stratified on pulsotype. Our findings suggest that *S. maltophilia*, contrarily to *P. aeruginosa*, might predominantly colonize respiratory zone (oxygenated due to continuous O_2 supply from the venous blood passing alveoli), rather than the anoxic infectious mucus in the bronchi of the conducting zone. We also found that growth rate negatively correlated with biofilm formation. This is consistent with the finding that slow bacterial growth enhances extracellular polymeric substance matrix production, therefore allowing stratification of the bacterial community to form biofilms (Sutherland, 2001). However, future studies are needed to evaluate if *S. maltophilia* fitness could be dependent on medium and conditions used.

A

p-values	growth rate	SBF	mutation frequency	swimming	twitching	in vivo pathogenicity (*G. mellonella*)	in vitro pathogenicity (A549 cells)	levofloxacin MIC	chloramphenicol MIC	ciprofloxacin MIC
growth rate		**0,0106**	0,9739	0,7448	0,9687	**0,0379**	0,1340	0,1685	0,8775	0,3924
SBF	**0,0106**		0,9560	0,4185	0,3966	**0,0354**	0,3789	0,0717	0,6338	0,5064
mutation frequency	0,9739	0,9560		0,7358	0,1321	0,1401	0,7830	0,1458	0,2745	0,7366
swimming	0,7448	0,4185	0,7358		**0,0003**	0,4199	0,1511	0,8235	0,3200	0,9297
twitching	0,9687	0,3966	0,1321	**0,0003**		0,2260	0,3536	0,6084	0,2267	0,6782
in vivo pathogenicity (*G. mellonella*)	**0,0379**	**0,0354**	0,1401	0,4199	0,2260		0,2018	**0,0063**	0,1525	0,0536
in vitro pathogenicity (A549 cells)	0,1340	0,3789	0,7830	0,1511	0,3536	0,2018		0,0632	0,5075	**0,0081**
levofloxacin MIC	0,1685	0,0717	0,1458	0,8235	0,6084	**0,0063**	0,0632		**0,0051**	**0,0026**
chloramphenicol MIC	0,8775	0,6338	0,2745	0,3200	0,2267	0,1525	0,5075	**0,0051**		**0,0152**
ciprofloxacin MIC	0,3924	0,5064	0,7366	0,9297	0,6782	0,0536	**0,0081**	**0,0026**	**0,0152**	

B

Spearman rank coefficients	growth rate	SBF	mutation frequency	swimming	twitching	in vivo pathogenicity (*G. mellonella*)	in vitro pathogenicity (A549 cells)	levofloxacin MIC	chloramphenicol MIC	ciprofloxacin MIC
growth rate		**-0,7203**	-0,0140	-0,1018	0,0141	**-0,5919**	-0,4615	0,4278	0,0509	0,2724
SBF	**-0,7203**		-0,0210	0,2561	0,2681	**0,6204**	0,2797	-0,5076	-0,1128	-0,1620
mutation frequency	-0,0140	-0,0210		0,1088	0,4621	-0,4315	-0,0909	0,4496	0,3421	0,1104
swimming	-0,1018	0,2561	0,1088		**0,8761**	-0,2290	0,4421	-0,0364	0,3123	0,0296
twitching	0,0141	0,2681	0,4621	**0,8761**		-0,3507	0,2928	0,1646	0,3763	0,1337
in vivo pathogenicity (*G. mellonella*)	**-0,5919**	**0,6204**	-0,4315	-0,2290	-0,3507		0,3958	**-0,6988**	-0,3860	-0,5068
in vitro pathogenicity (A549 cells)	-0,4615	0,2797	-0,0909	0,4421	0,2928	0,3958		-0,5221	-0,1710	**-0,6847**
levofloxacin MIC	0,4278	-0,5076	0,4496	-0,0364	0,1646	**-0,6988**	-0,5221		**0,7698**	**0,8016**
chloramphenicol MIC	0,0509	-0,1128	0,3421	0,3123	0,3763	-0,3860	-0,1710	**0,7698**		**0,6897**
ciprofloxacin MIC	0,2724	-0,1620	0,1104	0,0296	0,1337	-0,5068	**-0,6847**	**0,8016**	**0,6897**	

FIGURE 7 | Correlation matrix of phenotype-phenotype associations, as determined by Spearman rank correlation coefficient. (A) *p*-values: red values in bold indicate a significant ($p < 0.05$) correlation between any given phenotype pair. **(B)** Spearman rank coefficients. Red values in bold indicate a significant ($p < 0.05$) correlation between any given phenotype pair: positive and negative values indicate direct and inverse correlations, respectively.

The ability of *S. maltophilia* cells to form biofilm was reduced during the late stages of a chronic infection. We recently observed a reduced efficiency in forming biofilm by *S. maltophilia* CF isolates, compared to non-CF ones, probably secondary to the bacterial adaptation to a stressed environment such as CF lung (Pompilio et al., 2011). Similarly, *P. aeruginosa* isolates from chronically infected patients are often impaired in forming biofilms (Head and Yu, 2004).

Conditions in CF airways consistently select against bacterial functions deemed essential for biofilm formation during *in vivo* bacterial evolution (Wilder et al., 2009). At early stage of disease, it is more suitable to increase biofilm formation to gain benefits including superior access to nutrients and resistance to environmental insults, such as phagocytosis and antibiotic treatment. During long-term persistence in the airways of CF patients, the increased lung damage, a higher prevalence of co-colonizing pathogens, and increase of neutrophils make necessary to impair biofilm formation to disseminate in new, ecologically more favorable, airways locations (Nadell and Bassler, 2011; Steenackers et al., 2016).

The adaptation of bacterial population to new or challenging environments normally results in spontaneous generation of hypermutable strains which display higher mutation frequencies than their normal counterparts, as a result of defects in the DNA repair system or proof reading systems. Antibiotics—as well as host environment—select for these variants, as these undergo more genetic mutations and are better able to adapt and survive under the antimicrobial pressures *in vivo* (Rodriguez-Rojas et al., 2013).

In CF lung the selection for hypermutable *P. aeruginosa* strains becomes more frequent in later stages or chronic infection in CF patients, suggesting that genetic and phenotypic diversification plays an essential role in the adaptation of *P. aeruginosa* to the hostile and diverse CF lung environment, probably by selecting for less virulent phenotypes (Hogardt et al., 2007; Oliver, 2010).

Interestingly we did not observe hypermutability in our panel of *S. maltophilia* strains. Half of *S. maltophilia* strains we tested were in fact weak mutators, while only one strain (8.4%) was hypermutator. These observations are similar to that reported by O'Neill and Chopra (2002) in *S. aureus* clinical isolates, but contrary to findings by Vidigal et al. (2014) who observed comparable frequency of strong- (31.2%) and weak-mutators (27.7%), and a lower frequency of hypomutators (17.7%), in 90 *S. maltophilia* isolates collected from the sputum of 19 CF patients considered chronically colonized. However, in agreement with this study, we found that mutation rates of the most clonally related genotypes varied over time with the tendency to become less mutable, except for pulsotype 1.1 that significantly increased mutation frequency over time. We are tempted to hypothesize that mutation frequency does not contribute significantly to the adaptation of *S. maltophilia* population to CF lung. However, this discrepancy could be also due to the small number of strains we tested, therefore warranting further studies on larger populations.

The bacterial colonization of CF airways is mediated by the adhesion of cell appendages such as flagellum and type IV pili to host epithelial cell surface. Our findings revealed that in *S. maltophilia* motility changes during long-term colonization depend on the pulsotype considered. Swimming motility is well described as an adaptive trait in *P. aeruginosa* infections in CF whereby in contrast to initially infecting motile strains, chronic ones are characterized by the lack of swimming motility due to the loss of the flagellum (Huse et al., 2013). We observed a similar trend for pulsotype 2.1 strains whose swimming motility significantly decreased over study-period. The potential reason for this phenotypic selection *in vivo* is that the decreased flagellar motility may enable *S. maltophilia* to better evade immune recognition and airway clearance by phagocytosis. Several studies have in fact shown the inability of macrophages to phagocytose non-flagellated *P. aeruginosa* isolates (Mahenthiralingam et al., 1994), and the reduced inflammasome activation and antibacterial IL-1β host response following the loss in motility (Patankar et al., 2013).

Inversely, strains belonging to pulsotype 1.1 significantly increased swimming and twitching motilities during chronicization. In agreement with our findings, *Burkholderia cenocepacia* complex isolates from chronic infections were found not to lose swimming motility (Zlosnik et al., 2014), even showing increased expression in genes associated with flagella assembly and adhesion during the late stage of infection (Mira et al., 2011). We do not know the significance of this observation, although this trend could be due to the small number of strains ($n = 2$) belonging to pulsotype 1.1.

In partial agreement with our previous findings (Pompilio et al., 2011), swimming and twitching motility were positively correlated, but neither associated with biofilm formation. Although it is generally agreed that motility and biofilm development are mutually exclusive events (Belas, 2013), flagella are not only required as a mechanical device for propulsion, but also play a critical role in the initial stages of surface adhesion that leads to the formation of a biofilm, therefore representing attractive therapeutic targets (Erhardt, 2016).

Accumulating evidences support that *S. maltophilia* exhibits plethora of pathogenic determinants to exert its association with human respiratory epithelium. These determinants are unique in context of this pathogen's limited virulence that limits its invasive potential in comparison to other evolved pathogens like *P. aeruginosa*. However, *S. maltophilia* is increasingly known to employ multifactorial determinants like extracellular proteases (Karaba et al., 2013), host cell actin modifiers (MacDonald et al., 2016), quorum signaling molecules (Huedo et al., 2015), and highly evolved efflux-pumps (Chang et al., 2015), which independently or together offer formidable recalcitrance and pathogenic fitness.

P. aeruginosa adaptation in CF airways selects patho-adaptive variants with a strongly reduced ability to cause acute infection processes in a host-independent way (Hoboth et al., 2009; Folkesson et al., 2012; Lorè et al., 2012). Consistent with these findings, our data clearly showed that the virulence potential of *S. maltophilia* plays little if any role in its ability to persist in CF airways. Pathogenicity, as measured with similar trends both in *G. mellonella* and human lung epithelial cells, was in fact severely reduced over time. This result indicates a host-pathogen relationship that results in attenuated virulence and pathogenicity during the establishment of chronic infection.

Virulence factors or determinants are in fact often non-essential to the pathogen and, consequently, are lost (Brown et al., 2012). Further studies are needed to evaluate whether reduced virulence in *S. maltophilia* is itself adaptive in terms of helping bacterial cells to go unnoticed by host immune system (Gama et al., 2012) or resist antibiotic therapy (Malone et al., 2010), or if it is a pleiotropic cost associated with other within-host adaptation.

However, despite decreased virulence *S. maltophilia* might retain the ability to contribute to disease pathogenesis in CF lung by inducing high proinflammatory cytokine and adhesion molecule expression, as described in *P. aeruginosa* (Hawdon et al., 2010). In this regard, we observed that *S. maltophilia* biofilm formation efficiency, although decreased over time, is directly associated with mortality rate in *G. mellonella*, a finding supported elsewhere for *Candida albicans* and *Cryptococcus neoformans* (Cirasola et al., 2013; Benaducci et al., 2016).

Chronic respiratory infections by *S. maltophilia* are very difficult to treat due to bacterial intrinsic resistance to a wide number of antibiotics, and ability to develop high-level resistance during antibiotic treatment and to adapt to and resist other adverse environmental conditions (Ryan et al., 2009; Chang et al., 2015). Although we observed variable resistance profiles along the study period, as a general trend evolution toward lower levels of susceptibility to antibiotics was observed over time, in terms of mean increase in MIC-values and accumulation of resistances. Interestingly, in the case of levofloxacin and chloramphenicol, MIC changes even resulted in susceptible-to-resistant category transition. All strains were susceptible to cotrimoxazole and mynocycline, although a genotype-dependent trend toward higher MIC-values was observed over time for both antibiotics. Our findings could have significant implications in the management of CF patients since these drugs are considered first-line therapeutic choices for *S. maltophilia* infections (Wei et al., 2016).

Fluoroquinolones are commonly used to treat infections due to *S. maltophilia*. However, their overuse worldwide has resulted in higher resistance rates in many kinds of pathogenic bacteria, including *S. maltophilia* (Pien et al., 2015). In this respect, following administration of parental or inhaled fluoroquinolones therapy reduced susceptibility to both ciprofloxacin and levofloxacin was observed in pulsotype 2.1 strains. The ability of *S. maltophilia* to develop resistance during antibiotic treatment is in agreement with the generalized idea that this adaptive mechanism is among the important features contributing to persistent infection.

No correlation could be established between antibiotic resistance and the amount of the biofilm formed, indicating that other relevant mechanisms might also contribute to the increased resistance registered toward several antimicrobials of different classes. In this regard, contrarily to Vidigal et al. (2014), we found higher MIC-values in mutator strains compared to non-mutator ones. A correlation between *S. maltophilia* mutators and increasing antibiotic resistance was found namely for ciprofloxacin, levofloxacin, and chloramphenicol. It is thought that the genetic and phenotypic changes that confer resistance also result in concomitant reductions in *in vivo* virulence (Cameron et al., 2015). For the first time, the present work described in *S. maltophilia* a direct relationship between the development of resistance to fluoroquinolones and reduced pathogenicity.

Taken together, our results show that *S. maltophilia* is a versatile pathogen which can adapt successfully to a highly stressful environment such as CF lung. To this, *S. maltophilia* pays a "biological cost," as suggested by the presence of relevant genotypic and phenotypic heterogeneity within a bacterial population chronically infecting the CF lung. A number of social traits are in fact changed over time, probably as a result of evolution within a lineage, or by displacement of one by another lineage. Although adaptation occurred with selection of substantially different *S. maltophilia* phenotypes, depending on genotype considered, it was possible to detect a general trend of adaptation toward less virulence and increased antibiotic resistance in our investigated isolates.

S. maltophilia adaptation, measured as number of changed traits, is associated with length of persistence. In addition, the establishment of a highly heterogeneous bacterial population, suggestive for niche separation in the host by different strains, indicates that populations are significantly more complex and dynamic than can be described by the analysis of any single isolate and can fluctuate rapidly to changing selective pressures.

Although the differences at the genetic or epigenetic level giving rise to phenotypic variability in CF isolates are not yet known, our results gained new insights into the behavior of *S. maltophilia* during persistence in CF lung that will hopefully help to identify vulnerabilities and potential targets for the development of treatment strategies directed at chronic infection.

The main limitation of the present study is that having considered only one chronically infected patient does not allow us to evaluate if the adaptation process may relate to the complexity of the individual host niche. In future investigations, we plan to expand the number of patients in order to: (i) study the precise microenvironmental pressures driving diversification we observed among phenotypic traits within *S. maltophilia* populations in the CF lung; (ii) identify specific genetic determinants contributing to such diversity, by using of whole genome sequencing of large numbers of isolates coupled with phenotypic characterization and genome-wide association analyses; and (iii) evaluate other phenotypic adaptations classically involved during progression from acute to chronic infection (i.e., exopolysaccharide production, quorum sensing, expression of virulence factors associated with chronic infection).

AUTHOR CONTRIBUTIONS

AP, VC, DG, MC, GG, and LV performed analyses. EF collected and processed clinical specimens, and provided clinical expertise for discussion of results. AP, DG, and GD statistically evaluated results, drafted the manuscript and defined the study design. All authors read, reviewed, and approved the final manuscript.

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Antibiotic resistance in the opportunistic pathogen *Stenotrophomonas maltophilia*

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Stenotrophomonas maltophilia is an environmental bacterium found in the soil, associated with plants and animals, and in aquatic environments. It is also an opportunistic pathogen now causing an increasing number of nosocomial infections. The treatment of *S. maltophilia* is quite difficult given its intrinsic resistance to a number of antibiotics, and because it is able to acquire new resistances via horizontal gene transfer and mutations. Certainly, strains resistant to quinolones, cotrimoxale and/or cephalosporins—antibiotics commonly used to treat *S. maltophilia* infections—have emerged. The increasing number of available *S. maltophilia* genomes has allowed the identification and annotation of a large number of antimicrobial resistance genes. Most encode inactivating enzymes and efflux pumps, but information on their role in intrinsic and acquired resistance is limited. Non-typical antibiotic resistance mechanisms that also form part of the intrinsic resistome have been identified via mutant library screening. These include non-typical antibiotic resistance genes, such as bacterial metabolism genes, and non-inheritable resistant phenotypes, such as biofilm formation and persistence. Their relationships with resistance are complex and require further study.

Keywords: *Stenotrophomonas maltophilia*, antibiotic resistance, multidrug resistance, intrinsic resistome, phenotypic resistance

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Stenotrophomonas maltophilia is an opportunistic nosocomial pathogen that has caused an increasing number of infections in recent years (Brooke, 2012). It is associated with a number of clinical syndromes, such as endocarditis, urinary infections, and respiratory infections, including pneumonia in patients with cystic fibrosis and the immunocompromised (Falagas et al., 2009; Looney et al., 2009).

S. maltophilia shows low susceptibility to many antibiotics, including those commonly used to treat the infections it causes. It is therefore crucial that new antibiotic targets be found, and the appearance of resistance during treatment be predicted. The analysis of resistance mechanisms and the identification of antibiotic resistance genes can help in this. Bioinformatic studies have identified genes showing homology to known antibiotic resistance genes, although their exact functions remain to be confirmed. In recent years, deep sequencing technologies have allowed the complete sequencing of two clinical *S. maltophilia* strains, K279a and D457 (Accession numbers: NC_010943.1 and NC_017671.1) (Crossman et al., 2008; Lira et al., 2012), and two environmental strains, R551-3 and JV3 (Accession numbers: NC_011071.1 and CP002986.1). The assembly of the sequences of several other strains is currently underway, and results should be available in the near future. Genome sequencing has revealed much of the genome to be conserved across different *S. maltophilia* strains (Rocco et al., 2009; Alavi et al., 2014). In addition, most of the genes associated

with resistance in *S. maltophilia* have been found present in all strains examined. However, the genomes show also sequence variability, associated to normal evolution (mutation frequency) or induced in some situations. Antibiotic pressure increases the sequence variability in resistance or related genes, as regulators. The use of quinolones in *S. maltophilia* allows the appearance of mutants overexpressing efflux pumps, first SmeDEF, and when this is not present, SmeVWX. In both cases the overexpression is mainly associated to mutations in their regulators, SmeT and SmeRv, respectively (Garcia-Leon et al., 2014b).

The analysis of transposon mutant libraries allows the identification of genes which, if deleted, alter susceptibility to antibiotics, including genes that might appear to have no clear relationship with antibiotic resistance. The drawback of this type of analysis is that, since essential genes cannot be deleted, their putative involvement in antibiotic susceptibility cannot be confirmed. This methodology has, however, been successfully used with *Pseudomonas aeruginosa* and *Escherichia coli* (Girgis et al., 2009; Alvarez-Ortega et al., 2010). When *S. maltophilia* was thus examined, genes involved in biofilm formation appeared as being related to antibiotic resistance (Huang et al., 2006; Kang et al., 2015).

Transformation with genomic libraries allows the effect of gene overexpression to be investigated; genes whose overexpression (usually in heterologous microorganisms) exerts a direct effect on antibiotic susceptibility can therefore be identified. Only those genes which exert a direct effect on antibiotic susceptibility identified in this way. The use of a *S. maltophilia* D457R chromosomal DNA library allowed the identification of genes related to antibiotic resistance, and the subsequent cloning and characterization of the SmeDEF efflux pump (Alonso and Martinez, 2000; Alonso et al., 2000).

Evolution assays can provide information on the genes involved in acquired resistance. Such information could be useful for the rational design of better treatments since it might help predict the appearance of resistance.

Antibiotic Resistance Mechanisms in *S. maltophilia*

The reduced susceptibility of *S. maltophilia* to antibiotics has been associated with intrinsic resistance factors common to all *S. maltophilia* strains, such as low membrane permeability, the presence of multidrug resistance (MDR) efflux pumps, antibiotic-modifying enzymes, and the quinolone resistance gene Sm*qnr* (Crossman et al., 2008; Sanchez et al., 2009) (**Table 1**). Resistance can also be acquired via the acquisition of mutations or resistance genes through horizontal gene transfer (HGT). Microorganisms sharing the same environment can provide these exogenous genes. It has been postulated that other unknown mechanisms may also help account for the *S. maltophilia* antibiotic resistance phenotype. The intrinsic resistome has been defined as the group of chromosomal genes involved in the intrinsic resistance present in the strains of a bacterial species prior to exposure to an antibiotic and which is not due to HGT (Fajardo et al., 2008). The intrinsic resistome involves known and unknown genes related to antibiotic resistance, which might include genes involved in cell metabolism (Olivares et al., 2013). To date, most studies have focused on classical antibiotic resistance genes, such as those coding for efflux pumps or modifying enzymes (intrinsic resistance), and on the appearance during clinical treatment of mutants showing low susceptibility to antibiotics (acquired resistance). Our knowledge of the *S. maltophilia* intrinsic resistome is, however, limited.

Like all Gram negative bacteria, *S. maltophilia* shows low membrane permeability—the consequence of it having two cell membranes and a peptidoglycan wall. The outer membrane is an efficient barrier. Mutants showing altered outer membrane permeability or which have a different lipopolysaccharide structure show modified susceptibility to antibiotics (Vaara, 1993; Rahmati-Bahram et al., 1996).

Low susceptibility to antibiotics is often related to the presence of active efflux pumps. Such pumps have been identified in *S. maltophilia* K279a, including eight MDR efflux pumps belonging to the putative resistance nodulation cell division (RND) family, two belonging to the major facilitator superfamily (MFS), and two ATP-binding cassette (ABC) pumps (Crossman et al., 2008). In Gram negative bacteria, RND efflux pumps are composed of three proteins: an inner membrane protein, which binds the substrate, an outer membrane protein (porin), and a membrane fusion protein (MFP), which binds the outer and inner proteins in the periplasmic space. In general, the genes coding for the porin, MFP and inner protein are located in the same operon. Some exceptions in which there is no porin-coding gene have been identified. In addition, MDR efflux pumps are modulated by a regulator protein encoded by a gene located upstream and divergently transcribed from the efflux pump operon. In general, most efflux pump machinery is expressed at low levels (Li et al., 2002; Lin et al., 2014a). Overexpression is associated with low antibiotic susceptibility, and is sometimes related to mutations in regulator genes. Such mutations have been identified both *in vitro* and *in vivo* (Alonso and Martinez, 2001; Cho et al., 2012; Gould et al., 2013; García-León et al., 2015), supporting the idea that *in vitro* evolution studies may be able predict mutations appearing *in vivo* during the treatment of patients.

All the proteins of the efflux pumps SmeABC, SmeDEF and SmeVWX, which belong to the RND family, are encoded in the same operon following the typical genomic arrangement. The roles of these efflux pumps in intrinsic and acquired resistance have been extensively characterized (Alonso and Martinez, 2000; Li et al., 2002; Chen et al., 2011). SmeABC is involved in acquired resistance to β-lactams, aminoglycosides and quinolones, but has no influence on intrinsic resistance. The deletion of the *smeC* gene (porin) affects susceptibility to several antibiotics (Li et al., 2002), suggesting its possible relationship with other efflux pumps. SmeDEF is involved in both intrinsic and acquired resistance to chloramphenicol, tetracycline and quinolones, as well as acquired resistance to non-antibiotic compounds such as triclosan (Sanchez et al., 2005; Hernandez et al., 2011). SmeVWX has a role in acquired resistance to the same antibiotics (Alonso and Martinez, 2001; Zhang et al., 2001; Chen et al., 2011; Garcia-Leon et al., 2014b). In acquired resistance, the overexpression of the SmeDEF and SmeVWX efflux pumps is related to mutations

TABLE 1 | Summary of known antibiotic resistances genes in *S. maltophilia,* antibiotic resistance profile and their role in intrinsic and acquired resistance.

Gene(s)	Product	Antibiotic resistance phenotype	Intrinsic/Acquired antibiotic resistance	Reference
smeABC	RND-type efflux pump	Aminoglycosides, β-lactams, and quinolones	No/Yes	Li et al., 2002
smeDEF	RND-type efflux pump	Chloramphenicol, tetracycline and quinolones	Yes/Yes	Alonso and Martinez, 2000; Zhang et al., 2001
smeGH	RND-type efflux pump	Unknown	ND/ND	Crossman et al., 2008
smeIJK	RND-type efflux pump	Aminoglycosides, tetracycline and ciprofloxacin	Yes/Yes	Crossman et al., 2008; Gould et al., 2013
smeMN	RND-type efflux pump	Unknown	ND/ND	Crossman et al., 2008
smeOP	RND-type efflux pump	Aminoglycosides, nalidixic acid, doxycycline, macrolides	Yes/No	Lin et al., 2014a
smeVWX	RND-type efflux pump	Chloramphenicol and quinolones	No/Yes	Chen et al., 2011; Garcia-Leon et al., 2014b
smeYZ	RND-type efflux pump	Aminoglycosides	Yes/yes	Crossman et al., 2008; Gould et al., 2013
emrCABsm	MFS-type efflux pump	Nalidixic acid and CCCP	No/Yes	Huang et al., 2013a
smlt0032	MFS-type efflux pump	Unknown	ND/ND	Crossman et al., 2008
smtcrA	MFS-type efflux pump	Tetracycline	No/Yes	Chang et al., 2011
smrA	ABC-type efflux pump	Fluoroquinolones, tetracycline, doxorubicin	ND/Yes	Al-Hamad et al., 2009
macABCsm	ABC-type efflux pump	Macrolides, aminoglycosides and polymyxins	Yes/ND	Lin et al., 2014b
L1	β-lactamase	β-lactams	Yes/Yes	Hu et al., 2008; Okazaki and Avison, 2008
L2	β-lactamase	β-lactams	Yes/Yes	Hu et al., 2008; Okazaki and Avison, 2008
aph (3′)-IIc	Aminoglycoside phosphotransferase	Aminoglycosides	Yes/Yes	Okazaki and Avison, 2007
aac (6′)-Iz	N-Aminoglycoside acetyltransferase	Aminoglycosides	Yes/Yes	Li et al., 2003
Sm*qnr*	Pentapeptide Repeat Proteins	Quinolones	Yes/Yes	Sanchez and Martinez, 2010; Chang et al., 2011

ND not determined

in the regulators SmeT and SmeRv, respectively (Sanchez et al., 2002; Garcia-Leon et al., 2014b).

Other *S. maltophilia* efflux pumps have recently been studied, including SmeIJK and SmeYZ, which also belong to the RND family. Both have a role in intrinsic and acquired resistance, SmeJK to aminoglycosides, tetracycline and ciprofloxacin, and SmeZ to aminoglycosides (Crossman et al., 2008). In addition, their overexpression provides resistance to levofloxacin (Gould et al., 2013). Neither of these efflux pumps has a known associated porin. The efflux pump SmeOP, another RND family member, confers low susceptibility to aminoglycosides, nalidixic acid, doxycycline, macrolides and certain not antibiotic compounds, such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP), crystal violet, sodium dodecyl sulfate (SDS), and tetrachlorosalicylanilide (TCS). In the acquired resistance setting, however, it provides protection only against CCCP and TCS (Lin et al., 2014a). The TolCsm porin has been associated with the SmeOP efflux pump. The *tolCsm* gene is located upstream of the *smeOP* operon, in another operon known as *smeRo-pcm-tolC*. The Δ*tolCsm* phenotype increases susceptibility to several compounds (Huang et al., 2013b), although no correlation is seen with the Δ*smeOP* phenotype. This suggests that the TolCsm porin is not exclusive to the SmeOP efflux pump (Huang et al., 2013b; Lin et al., 2014a).

Bioinformatic analyses have also identified two putative MFS-type tripartite efflux transporters (Crossman et al., 2008). One of these, *emrCABsm*, shows high homology with *emrAB* of *E. coli* (Lomovskaya and Lewis, 1992). In *S. maltophilia*, this pump is encoded by an operon of four genes that cover the three efflux pump components and a MarR-type regulator, *emrRsm,* which is expressed in the same direction. Δ*emrRsm* mutants show low susceptibility to nalidixic acid and CCCP due to the overexpression of the efflux pump, indicating *emrRsm* to act as a repressor (Huang et al., 2013a).

Although ABC-type transporters play a major role in Gram positive bacteria, they have also been found in Gram negative organisms, e.g., MsbA and MacAB in *E. coli*, VcaM in *Vibrio cholerae*, MacAB in *Neisseria gonorrhoeae*, and SmdAB in *Serratia marcescens* (Lin et al., 2014b). Two ABC efflux pumps, SmrA and MacABCsm, have also been described in *S. maltophilia* (Al-Hamad et al., 2009; Lin et al., 2014b). The SmrA pump has only been studied in the heterologous microorganism *E. coli*, in which it provides resistance to fluoroquinolones, tetracycline, doxorubicin and multiple dyes; its role in *S. maltophilia* remains unknown (Al-Hamad et al., 2009). The other ABC-type efflux pump, MacABCsm, is associated with intrinsic resistance to macrolides, aminoglycosides and polymyxins. Interestingly, Δ*macCsm* mutant bacteria show lower susceptibility to polymyxins, aminoglycosides and macrolides than Δ*macAB* mutants, suggesting that the MacABCsm efflux pump uses an alternative, still-unidentified porin (Lin et al., 2014b). This efflux pump is constitutively expressed, contributing toward oxidative and envelope stress tolerances and biofilm formation. The original function of the MacABCsm

efflux pump may therefore have seen it involved in metabolism or adaptation to environmental changes (Lin et al., 2014b).

Bioinformatic analyses have predicted the existence of additional pumps. For example, the Sm*tcrA* gene, which codes for a putative MFS pump, has been associated with tetracycline resistance. Whether any other components are required to make this pump work remains unknown (Chang et al., 2011).

The presence of active extrusion mechanisms cannot, however, explain the low susceptibility of *S. maltophilia* to all antibiotics, and indeed the *S. maltophilia* genome codes for several modifying enzymes responsible for ß-lactam and aminoglycoside resistance phenotypes. *S. maltophilia* possesses two inducible ß-lactamases: L1, a Zn^{2+}-dependent metalloenzyme which can hydrolyze nearly all classes of β-lactams (though not monobactams), and L2, a serine active-site cephalosporinase (Avison et al., 2001). The expression of both enzymes is regulated by *ampR* (a LysR type regulator located upstream of the L2 gene), and induced by the presence of ß-lactam antibiotics. AmpR acts as a weak repressor of the L2 gene in the absence of the inducer, and as an activator in its presence. With respect to L1, AmpR is required both for basal and induced expression (Lin et al., 2009). Other expression-regulating mechanisms also influence one or the other of the two enzyme genes, without affecting the regulated expression of the other. However, the exact mechanism of this additional regulation system remains to be elucidated (Avison et al., 2002; Okazaki and Avison, 2008). Finally, the expression of these enzymes in *S. maltophilia* is also subject to a complex regulation network. The deletion of the *ampN-ampG* operon, which encodes a permease transporter, prevents the induction of β-lactamases (Huang et al., 2010), while the inactivation of *mrcA*, which is predicted to encode penicillin-binding protein 1 (PBP1a), or of $ampD_1$, which encodes a cytoplasmic N-acetyl-muramyl-L-alanine amidase, causes the hyperproduction of L1/L2 β-lactamase (Yang et al., 2009; Lin et al., 2011).

S. maltophilia also encodes two aminoglycoside modifying enzymes, conferring low susceptibility to aminoglycoside antibiotics (with the exception of gentamicin). Gene *aph* (3′)-*IIc* encodes an aminoglycoside phosphotransferase (Okazaki and Avison, 2007), while *aac (6′)-Iz* codes for an N-aminoglycoside acetyltransferase. The latter has three alleles—*aac(6′)-Iz, aac(6′)-Iaz* and *aac(6′)-Iam*—that show more than 80% similarity (Li et al., 2003; Tada et al., 2014). The presence of other inactivating enzymes (2′N-acetyltransferase, streptomycin 3″phosphotransferase/kinase, spectinomycin phosphotransferase, and chloramphenicol acetyltransferase) might be responsible for the susceptibility phenotype of *S. maltophilia* (Crossman et al., 2008). More studies are needed, however, to confirm their function.

S. maltophilia shows low susceptibility to synthetic antibiotics such as quinolones. Mutations in topoisomerases, the quinolone target, have been related to the main quinolone resistant mechanism in all bacteria. However, no topoisomerase mutations have ever been identified in *S. maltophilia* (Ribera et al., 2002; Valdezate et al., 2002; Garcia-Leon et al., 2014b). In contrast, *S. maltophilia* quinolone resistance is owed to efflux pumps (Alonso and Martinez, 2000; Li et al., 2002; Chen et al., 2011; Garcia-Leon et al., 2014b) and to the quinolone resistance protein SmQnr. This protein has been associated with both intrinsic and acquired resistance in *S. maltophilia* by a still unknown mechanism (Sanchez and Martinez, 2010; Chang et al., 2011). Qnr forms a dimer with a structure similar to double stranded DNA (Vetting et al., 2006; Xiong et al., 2011). Then, it has been proposed that SmQnr would bind topoisomerases protecting them, similarly to what was described for Qnr encoded in plasmid (Tran and Jacoby, 2002; Tran et al., 2005).

There is limited information on the antibiotic resistance mechanisms operating in clinical *S. maltophilia* strains. To date, overexpression of efflux pumps SmeABC, SmeDEF and SmeVWX, and the presence of class 1 integrons with antibiotic resistance genes have been associated with low susceptibility in clinical strains (Alonso and Martinez, 2001; Liaw et al., 2010; Cho et al., 2012).

Phenotypic Resistance

In addition to the antibiotic resistance genes described, microorganisms may possess a non-inheritable resistance mechanism known as phenotypic resistance. Some (or indeed all) of a bacterial population, may temporarily appear less susceptible to an antibiotic, without the appearance of any genomic differences. The factors responsible for phenotypic resistance might be good targets for novel treatments. However, our knowledge of the genes responsible for phenotypic resistance remains limited.

Biofilms are complex structures composed of an exopolysaccharide matrix, DNA and proteins, in which bacteria lie. They often affect clinical equipment such as catheters and other devices, from which they can be difficult to remove. The reduction in susceptibility to antibiotics afforded by biofilms is due to the difficulty of making the antibiotic come into contact with the bacteria, and these bacteria having a metabolic status different to that of their non-biofilm counterparts (a consequence of differences in the availability of nutrients and oxygen, etc.).

Different factors involved in biofilm formation have been studied in *S. maltophilia*. The deletion of different genes related to the regulation and structure of flagella, and to exopolysaccharide synthesis, affects biofilm formation (Huang et al., 2006; Kang et al., 2015). Other biofilm components, such as extracellular DNA, have been analyzed in microorganisms such as *P. aeruginosa* and *Salmonella enterica* (Mulcahy et al., 2008; Johnson et al., 2013), but the literature contains no information for *S. maltophilia*.

Bacteria showing low susceptibility to antibiotics but which are genetically identical to the susceptible strain can become persistent. Different genes involved in *E. coli* persistence include those coding for toxin/antitoxin systems and the PhoU regulator (Olivares et al., 2013). Although *S. maltophilia* persistence increases in chronic infections (Brooke, 2012), the mechanisms responsible for this phenotype remain unknown.

Post-transcriptional and post-translational regulation or modification can also alter antibiotic resistance. In *S. maltophilia*, however, little is known about this kind of regulation. Non-coding small RNAs (sRNA) and the RNA-binding Hfq protein

have been related to post-transcriptional gene expression, and in *E. coli* an sRNA and Hfq have been associated with antibiotic susceptibility (Moon and Gottesman, 2009). In *S. maltophilia*, 60 sRNA candidates and a *hfq* gene have been identified, and a Δ*hfq* mutant has been associated with changes in antibiotic susceptibility, biofilm production, motility and the expression of several sRNAs (Roscetto et al., 2012). However, further studies are needed to determine the role of sRNAs in antibiotic susceptibility.

Intrinsic Resistome

The study of the intrinsic resistome could provide novel antibiotic targets and help predict events during treatment. The intrinsic resistome has been studied in *P. aeruginosa* (Fajardo et al., 2008) and *E. coli* (Tamae et al., 2008; Girgis et al., 2009; Liu et al., 2010), and many genes whose deletion affects the antibiotic susceptibility phenotype have been identified. Recently, the screening of an *S. maltophilia* insertion mutant library identified *smeT*, which codes for a well-known regulator of the SmeDEF efflux pump, and *mutS*, which has a role in the DNA mismatch repair system. Other genes, e.g., the 23S gene, with no obvious role in antibiotic susceptibility may however influence it (Bernardini, 2014). Further analyses are required to precisely determine the role and mechanism of action of these genes in *S. maltophilia* antibiotic resistance.

The presence of this great number of influencing genes and antibiotic resistance mechanisms in *S. maltophilia* renders the treatment of its infections complicated. Several antibiotics are currently in use, including synthetic antibiotics and antibiotic combinations, that help prevent the appearance of resistant mutants. A trimethoprim/sulfamethoxazole (cotrimoxazole) combination is used as a last treatment option. Cotrimoxazole resistance in *S. maltophilia* has been associated with the genes *sul1* and *sul2*. These have been linked to the presence of class 1 integrons in plasmids in the main, but also in the chromosomal genome (Barbolla et al., 2004; Toleman et al., 2007). The presence of these genes, however, cannot explain all the cases of cotrimoxazole resistance recorded. Porin TolCsm deletion also increases cotrimoxazole susceptibility (Huang et al., 2013b), but further studies are required to determine whether other porins or efflux pumps are also involved.

How to avoid the antibiotic resistance? This has been a problem since the beginning of antibiotics use. The search of new antibiotics, inhibitors of efflux pumps (Leitner et al., 2011), new targets among genes form intrinsic resistome or use of combination of known antibiotics, as trimethoprim/sulfamethoxazole (described above), are some of the new strategies to avoid not only resistant strains but also the appearance of resistant mutants.

In summary, *S. maltophilia* possesses a great many antibiotic resistance mechanisms. Most of the genes involved were present in *S. maltophilia* before any use of antibiotics. For example, the efflux pump SmeDEF is associated with the ability of *S. maltophilia* to colonize plants, and its regulator SmeT is induced by plant-produced flavonoids (Garcia-Leon et al., 2014a); thus, the main function of the genes encoding them is unlikely to be the provision of antibiotic resistance. Other mechanisms might appear in the future, depending on antibiotic pressure, the emergence of mutations, and gene acquisition events. While resistance may benefit bacteria in the presence of antibiotics, in other situations it could impair growth, as has been described for the overexpression of the efflux pump SmeDEF (Alonso et al., 2004). Further, the fitness cost of acquired resistance in *S. maltophilia* determines whether new mechanisms are kept.

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Update on infections caused by *Stenotrophomonas maltophilia* with particular attention to resistance mechanisms and therapeutic options

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Stenotrophomonas maltophilia is a Gram-negative, biofilm-forming bacterium. Although generally regarded as an organism of low virulence, *S. maltophilia* is an emerging multi-drug resistant opportunistic pathogen in hospital and community settings, especially among immunocompromised hosts. Risk factors associated with *S. maltophilia* infection include underlying malignancy, cystic fibrosis, corticosteroid or immunosuppressant therapy, the presence of an indwelling central venous catheter and exposure to broad spectrum antibiotics. In this review, we provide a synthesis of information on current global trends in *S. maltophilia* pathogenicity as well as updated information on the molecular mechanisms contributing to its resistance to an array of antimicrobial agents. The prevalence of *S. maltophilia* infection in the general population increased from 0.8–1.4% during 1997–2003 to 1.3–1.68% during 2007–2012. The most important molecular mechanisms contributing to its resistance to antibiotics include β-lactamase production, the expression of *Qnr* genes, and the presence of class 1 integrons and efflux pumps. Trimethoprim/sulfamethoxazole (TMP/SMX) is the antimicrobial drug of choice. Although a few studies have reported increased resistance to TMP/SMX, the majority of studies worldwide show that *S. maltophilia* continues to be highly susceptible. Drugs with historically good susceptibility results include ceftazidime, ticarcillin-clavulanate, and fluoroquinolones; however, a number of studies show an alarming trend in resistance to those agents. Tetracyclines such as tigecycline, minocycline, and doxycycline are also effective agents and consistently display good activity against *S. maltophilia* in various geographic regions and across different time periods. Combination therapies, novel agents, and aerosolized forms of antimicrobial drugs are currently being tested for their ability to treat infections caused by this multi-drug resistant organism.

Keywords: ***Stenotrophomonas maltophilia*, prevalence, susceptibility, surveillance, treatment**

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Stenotrophomonas maltophilia is a Gram-negative, aerobic, glucose non-fermenting, motile bacillus. *S. maltophilia* was first isolated from pleural effusion in 1943 and initially named *Bacterium bookeri*. The organism was reclassified as a member of the genus *Pseudomonas* in 1961, *Xanthomonas* in 1983, and then *Stenotrophomonas* in 1993 (Al-Anazi and Al-Jasser, 2014). It survives on almost any humid surface and has been isolated from a wide variety of aquatic sources, such as suction tubing, nebulizers, endoscopes, hemodialysis dialysate samples, plant rhizosphere, faucets, sink drains, and shower heads (Brooke, 2012). S. *maltophilia* is characterized by its ability to form biofilms on various abiotic and biotic surfaces, including lung cells (de Oliveira-Garcia et al., 2003; Pompilio et al., 2010), and by its resistance to a broad array of antimicrobial agents. The World Health Organization recently classified S. *maltophilia* as one of the leading multidrug resistant organisms (MDROs) in hospital settings (Brooke, 2014).

S. maltophilia is generally regarded as an organism of low virulence and therefore an opportunistic pathogen, especially in immunocompromised hosts. The risk factors associated with acquiring *S. maltophilia* infections are well-known and include underlying malignancy (especially hematologic malignancy), organ transplantation, human immunodeficiency virus (HIV) infection, cystic fibrosis, prolonged hospitalization, intensive care unit (ICU) admission, mechanical ventilation, indwelling catheters (vascular, urinary, biliary), corticosteroid or immunosuppressant therapy, and recent antibiotics treatment (Al-Anazi and Al-Jasser, 2014). These risk factors reflect specific features of *S. maltophilia,* such as its ability to survive on almost any humid surface, its propensity to form biofilm and colonize humid surfaces, and its employment of several mechanisms that confer resistance to a number of antimicrobial agents.

S. maltophilia causes a wide range of infections including respiratory tract infections (RTI), blood stream infections (BSI) and, less commonly, skin and soft tissue infections (SSTI), bone and joint infections, biliary tract infections, urinary tract infections, endophthalmitis, endocarditis, and meningitis (Falagas et al., 2009a; Looney et al., 2009). The correlations between *S. maltophilia* infection and structural abnormalities with or without obstruction or procedural manipulation are well documented. Biliary tract infections caused by obstruction due to hepatobiliary neoplasms (Papadakis et al., 1995; Chang et al., 2014) or post-operative anastomotic strictures of the gastrointestinal tract (Perez et al., 2014) have been reported in patients with biliary *S. maltophilia* sepsis. Pleural infections caused by post-surgical/tube thoracostomy or fistula (broncho-/esophageal-/bilio-) (Lee et al., 2014), post-neurosurgical meningitis (Sood et al., 2013; Lai et al., 2014b), complicated urinary tract infections (Vartivarian et al., 1996), and obstructive lung cancer (Fujita et al., 1996; Vartivarian et al., 2000) have all been reported to create a milieu for *S. maltophilia* infection. In addition, although commonly perceived as nosocomial pathogens, community-acquired infections appear to be on the rise (Falagas et al., 2009a; Chang et al., 2014).

Prevalence

There were few data before 1970 regarding the prevalence or clinical characteristics of *S. maltophilia* (previously *Pseudomonas maltophilia* or *Xanthomonas maltophilia*) because of its rarity and relative clinical insignificance. It was in the 1980s when *S. maltophilia* became more frequently reported as an emerging nosocomial pathogen (Jang et al., 1992; Victor et al., 1994), especially in patients with post-chemotherapy neutropenia (Kerr et al., 1990; Labarca et al., 2000) and in those with indwelling central venous catheters (CVC) (Victor et al., 1994; Lai et al., 2006; Chen et al., 2014). Beginning in the late 1990s worldwide surveillance programs and multi-center studies began to provide more comprehensive information about the pathogenicity of *S. maltophilia*. Of the global surveillance programs, the SENTRY Antimicrobial Surveillance Program initiated in 1997 and the Study for Monitoring Antimicrobial Resistance Trends (SMART) initiated in 2002 are the most well-known (Jean et al., 2015). A number of nationwide and antimicrobial agent-targeted projects were also launched during the late 1990s, including the Canadian Ward Surveillance Study (CANWARD), the Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE) study, the British Society for Antimicrobial Chemotherapy (BSAC) Resistance Surveillance Project, the Taiwan Surveillance of Antimicrobial Resistance (TSAR) study, and the Tigecycline Evaluation Surveillance Trial (TEST).

Despite the massive scale of these surveillance studies, there are still limited integrated data on the prevalence and susceptibility patterns of *S. maltophilia*. The heterogeneity among the studies stems from the diverse patient demographics, geographic differences, and the ratio of the isolates collected from different sources, making inter-literature comparison difficult. To add to the complexity, there are no worldwide guidelines on susceptibility testing methodology and breakpoints for *S. maltophilia* (Nicodemo et al., 2004; Hombach et al., 2012), which results in different or absence of susceptibility breakpoints for some antibiotics. The lack of universal references for evaluating resistance of *S. maltophilia* to antimicrobial agents leads to confusion and complications when interpreting clinical data.

Table 1 shows the prevalence rates of infection due to *S. maltophilia*, categorized by sources of infection, reported by worldwide and nationwide surveillance projects as well as multi-center studies. Specific patient groups such as the critically ill in intensive care units (ICUs) and the pediatric population are presented separately in **Table 1**. By comparing data gathered by large surveillance studies over time we can estimate longitudinal change in prevalence of *S. maltophilia* infection in the general population. The frequency of occurrence among isolates from all sources ranged from 0.8 to 1.4% in five SENTRY studies during 1997∼2003 (Fluit et al., 2001a; Gales et al., 2001a; Sader et al., 2004; Sader and Jones, 2005; Fedler et al., 2006b). During 2007–2012, the CANWARD surveillance study (Zhanel et al., 2011, 2013; Walkty et al., 2014) and the SENTRY antimicrobial surveillance program (Farrell et al., 2010b; Sader et al., 2013) reported prevalence rates ranging from 1.3 to 1.68%. These data indicate that there is an increasing trend in infections due to *S. maltophilia* in the general population.

TABLE 1 | Prevalence of *S. maltophilia* in worldwide surveillance and multicenter studies.

Country[a]	Study[b]	Year	Number of isolates	Prevalence and/or ranking	References
All regions[c]	SENTRY	1997–1999	Total isolates:70067 SM[d] isolates: 842	Among: all pathogens: 1.2%	Gales et al., 2001a
EU	SENTRY	1997–1998	Total/SM isolates in: BSI[e]: 9194/82 RTI: 2052/54 SSTI: 2320/13 UTI: 2138/3	Among: all pathogens: 1.0% BSI: 0.89%, ranking: 19th RTI: 2.63%, ranking: 9th SSTI: 0.56%, ranking: 19th UTI: 0.14%, ranking: 25th	Fluit et al., 2001a
LA	SENTRY	1997–2001	Total isolates: 19547 SM isolates: 166	Among: all pathogens: 0.8%	Sader et al., 2004
All regions	SENTRY	1997–2001	NFGNB[f] isolates: 18569 SM isolates: 1488	Among: NFGNB: 8%	Jones et al., 2003
All regions	SENTRY	1997–2003	Total isolates: 221084 NFGNB[f] isolates: 25305 Uncommon NFGNB[g]: 3509 SM isolates: 2076	Among: all pathogens: 0.94% NFGNB: 8.20% Uncommon NFGNB: 59.16%	Sader and Jones, 2005
All regions	SENTRY	2001–2004	GNB isolates: 54731 NFGNB isolates: 13808 SM isolates: 1256	Among: GNB: 2.29% NFGNB: 9.10%	Gales et al., 2006
Canada	CANWARD	2007–2009	Total isolates: 18538 GNB isolates: 8949[h] SM isolates: 245	Among: all pathogens: 1.3%, ranking: 17th GNB: 2.7%	Zhanel et al., 2011
AP	SENTRY	2008	Total isolates: 5759 SM isolates: 97	Among: all pathogens: 1.68%	Farrell et al., 2010b
Canada	CANWARD	2008	Total isolates: 5282 SM isolates: 57	Among: all pathogens: 1.1%, ranking: 17th	Zhanel et al., 2010
France	MTC	2008–2009	Total isolates: 46400 Uncommon NFGNB isolates: 158 SM isolates: 61	Among: all pathogens: 0.13% Uncommon NFGNB: 39%	Fihman et al., 2012
Canada	CANWARD	2007–2011	Total isolates: 27123 SM isolates: 378	Among: all pathogens: 1.4%, ranking 16th	Zhanel et al., 2013
All regions	SENTRY	2011	Total isolates: 22005 SM isolates: 362	Among: all pathogens: 1.6%	Sader et al., 2013
Canada	CANWARD	2011–2012	Total isolates: 6593 SM isolates: 104	Among: all pathogens: 1.6%	Walkty et al., 2014
BSI					
USA	SCOPE	1995–1996	NFGNB isolates: 270 SM isolates: 18	Among: NFGNB: 6.7%	Jones et al., 1997
USA, Canada	SENTRY	1997	Total isolates:5058 SM isolates: 40	Among: all pathogens: 0.8%, ranking: 15th In USA: 0.7%, in Canada: 1.1%	Pfaller et al., 1998
NA, LA	SENTRY	1997	Total isolates: 9519 GNB isolates: 4267 SM isolates: 69	Among: all pathogens: 0.7% GNB: 1.6%	Diekema et al., 1999

(Continued)

TABLE 1 | Continued

Country[a]	Study[b]	Year	Number of isolates	Prevalence and/or ranking	References
EU	SENTRY	1997–1998	Total isolates: 9194 SM isolates: 82	Among: all pathogens: 0.89%, ranking: 19th	Fluit et al., 2001a
All regions	SENTRY	1997–1999		Among all pathogens in: AP: 0.9%, Canada: 0.6% EU: 0.9%, LA: 0.8%, USA: 0.7%	Gales et al., 2001a
LA	SENTRY	1997–2000	NA	Among: all pathogens: 0.7% 1997: 0.9%, 1998: 0.8%, 1999: 0.6%, 2000: 0.3%	
LA	SENTRY	1997–2001	Total isolates: 9058 SM isolates: 86	Among: all pathogens: 0.95%	Sader et al., 2004
Worldwide	MTC	2000–2004	All isolates: 26474 SM isolates: 203	Among: all pathogens: 0.8%	Sader et al., 2005b
RTI					
NA	SENTRY	1997	Total isolates: 2757 SM isolates: 99	Among: all pathogens: 3.6%, ranking: 8th In USA: 3.5%, in Canada: 3.7%	Jones et al., 2000
LA	SENTRY	1997	Total isolates: 556 SM isolates: 13	Among: all pathogens: 2.3%, ranking: 8th	Sader et al., 1998
NA	SENTRY	1998	Total isolates: 2773 SM isolates: 114	Among: all pathogens: 4.1%, ranking: 8th In USA: 3.7%, in Canada: 5.9%	Mathai et al., 2001
EU	SENTRY	1997–1998	Total isolates: 2052 SM isolates: 54	Among: all pathogens: 2.63%, ranking: 9th	Fluit et al., 2001a
All regions	SENTRY	1997–1999		Among all pathogens in: AP: 2.8%, Canada: 5.2% EU: 3.2%, LA: 1.8%, USA: 3.3%	Gales et al., 2001a
LA	SENTRY	1997–2000	Total isolates: 2505 SM isolates: 41	Among: all pathogens: 1.6%	Gales et al., 2002
LA	SENTRY	1997–2001	Total isolates: 3346 SM isolates: 60	Among: all pathogens: 1.8%	Sader et al., 2004
NA	SENTRY	2000	SM isolates: 94	Among: all pathogens: 3.5%	Hoban et al., 2003
NA, LA, EU	SENTRY	2004–2008	Isolates from HABP and VABP[l] Total cases: 31436	Regional incidence: all regions: 3.1% USA: 3.3%, LA: 2.3%, EU: 3.2%	Jones, 2010
Canada	CANWARD	2008	Total isolates: 1612 SM isolates: 42	Among: all pathogens: 2.6%, ranking: 9th	Zhanel et al., 2010
USA and EU	SENTRY	2009–2012	Total isolates: 12851 GNB isolates: 8201	Among all pathogens in: USA: 4.4%, ranking: 6th EU: 3.2%, ranking:9th GNB: 6.02%	Sader et al., 2014a
USA and EU	MTC	2012	Total isolates: 2968 SM isolates: 186	Among: all pathogens:6.3%	Farrell et al., 2014
UTI					
NA	SENTRY	1997	Total isolates: 1698 GNB isolates: 80% SM isolates: 6	Among: all pathogens: 0.35% GNB: 0.44%	Jones et al., 1999b

(Continued)

TABLE 1 | Continued

Country[a]	Study[b]	Year	Number of isolates	Prevalence and/or ranking	References
EU	SENTRY	1997–1998	Total isolates: 138 SM isolates: 3	Among: all pathogens: 0.14%, ranking: 25th	Fluit et al., 2001a
All regions	SENTRY	1997–1999		Among all pathogens in: AP: 0.2%, Canada: 0.0% EU: 0.2%, LA: 0.0%, USA: 0.3%	Gales et al., 2001a
LA	SENTRY	1997–2001	Total isolates: 1961 SM isolates: 0	Among: all pathogens: 0%	Sader et al., 2004
AP region	SMART[j]	2009–2010	Total GNB isolates: 1762	Among all GNB in: China: 1.3%, Thailand: 3.3%	Lu et al., 2012
USA	SMART	2009–2011	Total GNB isolates: 2135 SM isolates: 6	Among: all GNB: 0.28%	Bouchillon et al., 2013
IAI					
China	SMART	2002–2009	Total GNB isolates: 3420 SM isolates: 50	Among: all GNB: 1.5% NFGNB: ranking: 3rd	Yang et al., 2010
AP region	SMART	2003–2010	Total GNB isolates: 20710 NFGNB isolates: 2252 SM isolates: 204	Among: all GNB: 1.0% NFGNB: 9.1%	Liu et al., 2012
Taiwan	SMART	2006–2010	Total GNB isolates: 2417 SM isolates: 28	Among: all GNB: 1.2%	Lee et al., 2012
Africa and middle east	TEST	2007–2012	Total isolates of cSSSI[14] and IAI from TEST: 1990 and 255 GNB isolates from IAI: 225 SM isolates rom IAI: 16	Among: all pathogens in IAI: 6.3% GNB in IAI: 7.3%	Renteria et al., 2014
SSTI					
NA	SENTRY	1997	Total isolates: 1562 SM isolates: 15	Among: all pathogens: 0.96%	Doern et al., 1999
EU	SENTRY	1997–1998	Total isolates: 2320 SM isolates: 13	Among: all pathogens: 0.56%, ranking: 19th	Fluit et al., 2001a
All regions	SENTRY	1997–1999		Among all pathogens in: USA: 1.0%, Canada: 1.1% AP: 0.1%, EU: 0.6%, LA: 0.4%,	Gales et al., 2001a
LA	SENTRY	1997–2001	Total isolates: 1780 SM isolates: 7	Among: all pathogens: 0.39%	Sader et al., 2004
ICU					
EU	SENTRY	1997–1998	Total isolates from ICU: 3981	Among: all pathogens: 1.6%, ranking: 14th BSI: 1.1%, ranking: 15th RTI: 3.0%, ranking: 8th UTI: 0.0%	Fluit et al., 2001b
LA	SENTRY	1997–2001	Total isolates: 19547 SM isolates: 166	Among all pathogens in: ICU: 2.77%	Sader et al., 2004
NA	SENTRY	2001	Total isolates from ICU: 1321 SM isolates: 40 Respiratory source: 89.0%	Among: all pathogens: 3.0%, ranking: 10th	Streit et al., 2004
NA, LA, EU, Asia-Australia area	MTC	2000–2004	Isolates from ICU patients Total isolates: 9093 SM isolates: 131	Among: all pathogens: 1.4%	Sader et al., 2005a

(Continued)

TABLE 1 | Continued

Country[a]	Study[b]	Year	Number of isolates	Prevalence and/or ranking	References
German	SARI	2003–2004	Isolates collected from 39 German ICUs Total isolates:28266 GNB isolates: 12234	Among all pathogens: Median percentage: 1.7%	Meyer et al., 2006
Canada	CAN-ICU	2005–2006	Isolates from ICU patients Total isolates:4180 SM isolates: 108	Among: all pathogens: 2.6%	Zhanel et al., 2008
Korea	MTC	2008–2009	Respiratory tract isolates from patient with HABP in ICUs Total isolates: 372 CRGNB isolates: 82 SM isolates: 10	Among: all pathogens: 2.7% CRGNB[k]: 11.6%	Kim et al., 2014
EU	MTC(27) 9 countries	Published in 2011	Respiratory tract isolates from patient with HABP in ICUs Total isolates: 495 SM isolates: 13	Among: all pathogens: 2.6%	Magret et al., 2011
PEDIATRIC POPULATION					
NA	SENTRY	1998–2003	Total isolates: 59826 Total isolates from pediatric patients <7 years: 4641 SM isolates: 166	Among: all pathogens in: all ages: 1.4%, pediatric: 1.2%, both ranking: 10th	Fedler et al., 2006b
NA, LA, EU	SENTRY	2004	Total isolates from pediatric patients ≤18 years: 3537 SM isolates: 53	Among: all pathogens: 1.5%, ranking: 15th all regions combined	Fedler et al., 2006a
COMMUNITY-ACQUIRED					
USA, Canada, LA	SENTRY	1997	BSI SM isolates: 69	CA[l]/N/unknown: 23/28/18 CA: 33.3%	Diekema et al., 1999
UK and Ireland	BSAC	2001–2006	BSI SM isolates: 165	C/N: 31/66 CA: 33%	Livermore et al., 2008
AP region	SMART	2003–2010	IAI SM isolates: 204	CA/N: 26/125 CA: 17.2%	Liu et al., 2012
France	MTC	2008–2009	All sources SM isolates: 61	CA/N: 9/29 CA: 23.7%	Fihman et al., 2012
Taiwan	SMART	2006–2010	IAI SM isolates: 28	CA/N[i]: 3/18 CA: 14.3%	Lee et al., 2012

[a] *NA, North America; LA, Latin America; EU, Europe; USA, the United States of America; UK, United Kingdom; AP, Asia-Pacific.*

[b] *SENTRY, The SENTRY Antimicrobial Surveillance Program; SMART, Study for Monitoring Antimicrobial Resistance Trends; CAN-ICU, The Canadian Intensive Care Unit Surveillance Study; CANWARD, The Canadian Ward Surveillance Study; SARI, Surveillance of Antibiotic Use and Bacterial Resistance in ICUs(German); BSAC, The British Society for Antimicrobial Chemotherapy Resistance Surveillance Project; TEST, Tigecycline Evaluation Surveillance Trial; TIST, Tigecycline In Vitro Surveillance in Taiwan; TSAR, Taiwan Surveillance of Antimicrobial Resistance; SCOPE, Surveillance and Control of Pathogens of Epidemiologic Importance (USA); MTC, multicenter studies.*

[c] *The SENTRY Antimicrobial Surveillance Program has monitored the predominant pathogens and antimicrobial resistance in 5 geographic regions: Asia-Pacific, Europe, Latin America, Canada, and the United States (Gales et al., 2001a).*

[d] *SM, Stenotrophomonans maltophilia.*

[e] *BSI, bloodstream infection; RTI, respiratory tract infection; IAI, intra-abdominal infection; UTI, urinary tract infection; SSTI, skin and soft tissue infection.*

[f] *NFGNB, non-fermentative Gram-negative bacilli*

[g] *Uncommon NFGNB, Acinetobacter spp. and Pseudomonas aeruginosa excluded.*

[h] *Of the 18538 organisms collected, the 20 most common represented 16780 (90.5%) of the isolates and underwent susceptibility testing, which included 8949 (53.3%) Gram-negative bacilli.*

[i] *HABP, hospital-acquired bacterial pneumonia; VABP, ventilator-associated bacterial pneumonia.*

[j] *SMART is a global surveillance program that has monitored the in vitro susceptibility patterns of clinical Gram-negative bacilli to antimicrobial agents collected worldwide from intra-abdominal infections since 2002 and urinary tract infections since 2009 (Morrissey et al., 2013).*

[k] *CRGNB, carbapenem-resistant Gram-negative bacteria.*

[l] *C, community-acquired (collected within 48 h of hospitalization); N, nosocomial (collected more than 48 h after hospitalization).*

It has been observed in the general population (Gales et al., 2001a) and in ICUs (Fluit et al., 2001b) alike that *S. maltophilia* is most frequently associated with respiratory tract infections (RTIs), followed by bloodstream infections (BSIs), and, rarely, skin and soft tissue infections (SSTIs) and urinary tract infections (UTI) (Gales et al., 2001a). The prevalence of RTIs due to *S. maltophilia* is generally higher than that of other infections caused by that pathogen, but varies widely among countries and continents, ranging from 1.6 to 6.3% during the period 1997–2012 (Sader et al., 1998, 2004, 2014a; Jones et al., 2000; Fluit et al., 2001a; Gales et al., 2001a, 2002; Mathai et al., 2001; Hoban et al., 2003; Jones, 2010; Zhanel et al., 2010; Farrell et al., 2014). The United States has the most consecutive records regarding RTI isolates collected by the SENTRY program. Based on data from four SENTRY studies (Gales et al., 2001a; Hoban et al., 2003; Jones, 2010; Sader et al., 2014a), the prevalence rates increased from 3.3–3.5% during 1997–2004 to 4.4% during 2009–2012. During that 15-year period, *S. maltophilia* went from being the eighth to the sixth most common cause of RTI. In a large study on 2968 RTI isolates collected from 59 medical centers in the USA and 15 centers in European countries in 2012, 6.3% of the pathogens were *S. maltophilia* (Farrell et al., 2014). These observations suggest an increasing frequency of occurrence of respiratory tract infections due to *S. maltophilia*.

S. maltophilia is less frequently isolated from patients with BSIs, UTIs, or SSTIs than from patients with RTIs, with reported isolation rates ranging from 0.7 to 1.1% for BSIs (Jones et al., 1997; Pfaller et al., 1998; Diekema et al., 1999; Fluit et al., 2001a; Gales et al., 2001a; Sader et al., 2004, 2005b), 0–0.3% for UTIs (Pfaller et al., 1998; Jones et al., 1999b; Fluit et al., 2001a; Gales et al., 2001a; Sader et al., 2004, 2005b), and 0.39–0.96% for SSTIs (Diekema et al., 1999; Fluit et al., 2001a; Gales et al., 2001a; Sader et al., 2004). SMART studies have also shown that isolation of *S. maltophilia* from intra-abdominal infections (IAIs) is also fairly uncommon, with rates ranging from 1 to 1.7% (2002–2010) (Guembe et al., 2008; Yang et al., 2010; Lee et al., 2012; Liu et al., 2012). However, data from African and Middle Eastern countries collected as part of the Tigecycline Evaluation Surveillance Trial during 2007–2012 (Renteria et al., 2014) revealed an uncommonly high rate of isolation (6.3%) of *S. maltophilia* from patients with IAIs. In addition, the results from a SMART study surveying UTIs in the Asian-Pacific region during 2009–2010 disclosed higher rates of *S. maltophilia* isolated from patients with UTIs in China (1.3%) and Thailand (3.3%) than in other countries (Lu et al., 2012), although the rates were not as high as those in certain countries in Africa and the Middle East.

Gram-negative Bacilli (GNB) and Non-fermenting Gram-negative Bacilli (NFGNB)

The worldwide rate of isolation of *S. maltophilia* among GNB pathogens ranges from 2.29 to 2.7% according to a SENTRY study (2001–2004) (Gales et al., 2006) and a CANWARD surveillance study (2007–2009) (Zhanel et al., 2011). In the US state of Texas, however, a study at the M. D. Anderson Cancer Center revealed an increasing trend in the ratio of *S. maltophilia* among GNB isolates obtained from cancer patients during 1986–2002 (from 2% in 1986 to 7% in 2002) (Safdar and Rolston, 2007).

Among NFGNB, *S. maltophilia* has been reported to be the third most commonly isolated pathogen after *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. In a large survey conducted as a part of the SENTRY program, 221,084 GNB isolates were collected worldwide, including 25,305 (11.5%) NFGNB isolates, of which *Acinetobacter* spp. and *P. aeruginosa* accounted for the vast majority (87.7%). The remaining 3509 isolates were deemed unusual NFGNB species. Of them, *S. maltophilia* was the most frequently isolated ($n = 2076$, 59.16%) (Sader and Jones, 2005). A similar finding was reported in a prospective multi-center study involving nine teaching hospitals in France, in which *S. maltophilia* was the most commonly isolated NFGNB among all unusual NFGNB species (39%) (Fihman et al., 2012). Other surveillance studies, namely SCOPE (Jones et al., 1997), SENTRY (Jones et al., 2003; Gales et al., 2006), and SMART (Liu et al., 2012) showed a steady increase in isolation of *S. maltophilia* among all NFGNB pathogens during the period 1995–2010 (6.7% in 1995–1996, 8.0% in 1997–2001, and 9.1% in 2001–2010). These findings show that *S. maltophilia* is not an insignificant pathogen among disease-causing GNB and NFGNB species.

Intensive Care Units, Pediatric Population, and Community-acquired Infections

As expected, the prevalence of infections due to *S. maltophilia* is higher in intensive care units (1.4–3.0%) than in the general population (Fluit et al., 2001b; Sader et al., 2004; Streit et al., 2004; Sader et al., 2005a; Meyer et al., 2006; Zhanel et al., 2008; Magret et al., 2011; Kim et al., 2014).

There is limited information on the worldwide prevalence of *S. maltophilia* infections in the general pediatric population. SENTRY studies conducted during 1998–2003 (Fedler et al., 2006b) and in 2004 (Fedler et al., 2006a) showed that the prevalence of infections due to *S. maltophilia* was 1.2% among children $\leq$ 7 years and 1.4% among children $\leq$ 18 years old. The rates are similar to those in the adult population. A comparison of two single-center studies in China and the USA revealed markedly different incidence rates of ventilator-associated pneumonia due to *S. maltophilia* among pediatric patients in ICUs. Ning et al. reported a rate of 20.3% among patients aged 2 months to 16 years in a pediatric ICU in China (Ning et al., 2013) whereas Arthur et al. found that the rate of infection due to *S. maltophilia* among infants aged 0–6 months in a cardiac ICU in the USA was only 0.8% (Arthur et al., 2015).

Several recent studies have shown that *S. maltophilia* is also an emerging opportunistic pathogen in community settings (Falagas et al., 2009a; Chang et al., 2014). Results of a worldwide SENTRY study in 1997 (Diekema et al., 1999) and the British Society for Antimicrobial Chemotherapy Resistance surveillance project conducted during 2001–2006 (Livermore et al., 2008)

showed that 33.3 and 32%, respectively, of *S. maltophilia* isolates were collected within 48 h after admission (defined as community-acquired in these studies) from patients with bloodstream infections. The results from two recent SMART studies revealed that 14.3–17.2% of isolates from patients with community-acquired IAI (also defined by a 48-h time frame within admission) during 2003–2010 were *S. maltophilia* (Lee et al., 2012; Liu et al., 2012). Another recent study on the prevalence of community-acquired *S. maltophilia* BSI in Taiwan, which specifically divided the patients into three categories based on whether they had community-acquired (excluding patients hospitalized within 90 days before admission, cared in a nursing facility, etc.), healthcare-associated or hospital-acquired infections, reported that 17.6% of all community-acquired bloodstream infections were due to *S. maltophilia* (Chang et al., 2014). A similar study in France revealed that 23.7% of all community-acquired BSIs were due to *S. maltophilia* (Fihman et al., 2012). These studies show that community-acquired *S. maltophilia* infections are far less rare than previously thought.

Risk Factors of Mortalty

A number of risk factors for death due to *S. maltophilia* infections have been reported. Paez et al. (Paez and Costa, 2008) reviewed the literature from 1985 to 2008 and found that BSI and pneumonia, shock, thrombocytopenia, and Acute Physiological Assessment and Chronic Health Evaluation (APACHE) score >15 are independent risk factors associated with outcome. In addition, underlying hematological malignancy and admission to ICU are independent risk factors for cancer patients. The impact of appropriate antimicrobial treatment and removal of CVC on mortality were concluded to require further clinical studies (Paez and Costa, 2008). The conclusion of the review corresponds to the aforementioned studies. Falagas et al. analyzed 15 articles for attributable mortality of *S. maltophilia* infections. Only four studies provided relevant data regarding inappropriate antibiotic treatment, and three out of the four studies found significantly higher mortality when compared with initial appropriate therapy (Falagas et al., 2009b).

Antimicrobial Susceptibility

There are limited antimicrobial options for infections due to *S. maltophilia* because of its extensive resistance to most antibiotics, including β-lactam antibiotics, cephalosporins, macrolides, aminoglycosides, and carbapenems. Interpretive breakpoints for susceptibility are available only for ticarcillin/clavulanate, ceftazidime, minocycline, levofloxacin, trimethoprim/sulfamethoxazole (TMP/SMX), and chloramphenicol (CLSI, 2015). **Table 2** shows the rates of susceptibility of *S. maltophilia* to antimicrobial agents reported in the studies presented in **Table 1**. TMP/SMX is recognized as the drug of choice (Wang et al., 2014a). Resistance rates vary geographically but are generally less than 10% (Chung et al., 2013). However, high and various rates of resistance to TMP/SMX have been reported in patients with cancer (Vartivarian et al., 1994; Micozzi et al., 2000), cystic fibrosis (Saiman et al., 2002; Cantón et al., 2003; San Gabriel et al., 2004; Valenza et al., 2008), and in several countries, including Taiwan, Japan, Korea, Thailand, Spain, Mexico, Saudi Arabia, Turkey, and Canada (16–78.8%) (Valdezate et al., 2001; del Toro et al., 2002; Lai et al., 2004; Gülmez and Hasçelik, 2005; Memish et al., 2012; Wu et al., 2012; Rattanaumpawan et al., 2013; Rhee et al., 2013; Zhanel et al., 2013; Flores-Treviño et al., 2014; Hotta et al., 2014; Walkty et al., 2014; Wang et al., 2014a). In the present review, global surveillance data for the period 1997–2012 show that *S. maltophilia* continues to be highly susceptible to TMP/SMX (**Table 2**). Over that 15-year period, the susceptibility rates reported in worldwide SENRTY studies (Gales et al., 2001a; Jones et al., 2003; Gales et al., 2006; Sader et al., 2013, 2014a), a BSAC surveillance study (Livermore et al., 2008), and three large-scale multi-national studies (Sader et al., 2005b; Farrell et al., 2010a, 2014) ranged from 90 to 100%.

Ceftazidime and ticarcillin/clavulanate used to be the most effective among β-lactam drugs against *S. maltophilia*. However, recent studies have demonstrated resistance rates of more than 30% and a trend in decreasing susceptibility with ceftazidime (47–75% during 1997–1999 to 30.5–36.8% during 2009–2012) (**Table 2**) (Gales et al., 2001a; Farrell et al., 2010a; Sader et al., 2014b). The same is true for ticarcillin/clavulanate. During 1997–1998, the rates of susceptibility of *S. maltophilia* to that combination ranged from 71–90% but dropped to 27–46.1% during 2003–2008.

New fluoroquinolones exhibit better potency against *S. maltophilia* than ceftazidime or ticarcillin/clavulanate and have become reasonable alternatives. Nonetheless, a comparison of data from worldwide SENTRY studies reveals a decrease in sensitivity of *S. maltophilia* to levofloxacin, from 83.4% during the period 2003–2008 (Farrell et al., 2010a) to 77.3% in 2011 (Sader et al., 2013). Low susceptibility rates ranging from 64–69.6% have also been reported in Canada (Zhanel et al., 2013), China (Yang et al., 2010; Tan et al., 2014), and Korea (Chung et al., 2013). Few multi-center studies have investigated the efficacy of fluoroquinolones against *S. maltophilia* in patients with UTIs. In a SMART study conducted in the Asia-Pacific region, isolates of *S. maltophilia* from patients with UTIs showed exceptionally high rates of resistance to levofloxacin (33.3%) (Lu et al., 2012). Two recent reports showed low MIC_{50} (minimum inhibitory concentration)values (0.5 mg/L and 0.5 mg/L) and low MIC_{90} values (8 and 4 mg/L) for moxifloxacin against *S. maltophilia* (Zhanel et al., 2008; Chung et al., 2013), indicating that moxifloxacin could be considered an effective alternative. Data from a number of studies demonstrate that ciprofloxacin has poor activity against *S. maltophilia*, with susceptibility rates averaging lower than 50% (**Table 2**).

Minocycline, doxycycline, and tigecycline have consistently displayed good potency against *S. maltophilia* in studies with various time periods, sources of specimens, and geographic regions (Sader et al., 2005b, 2013, 2014b; Gales et al., 2008; Chen et al., 2012; Wu et al., 2012; Chung et al., 2013). A TSAR surveillance study conducted in Taiwan tested 377 isolates of *S. maltophilia* obtained over a 10-year period (1998–2008) and revealed low MIC_{50} (0.25 mg/L) and MIC_{90} values (1 mg/L) for

TABLE 2 | Susceptibility of *S. maltophilia* to various antimicrobial agents in worldwide surveillance and multicenter studies.

Country	Study	Year/ subgroups	TMP/SMX[d]	LEVO[d]	CIP[d]	CAZ[d]	T/C[d]	MCN[d]	TGC[d]	$TGCMIC_{50/90}$	PB[d]	References
NA, LA	SENTRY	1997		78.0	20.9							Jones et al., 1999a
All regions	SENTRY	1997–1999										Gales et al., 2001a
		AP	92		51	47	71					
		CAN	98		47	60	85					
		EU	90		79	72	86					
		LA	98		57	75	87					
		USA	95		55	67	90					
LA	SENTRY	1997–2001										Sader et al., 2004
		2001	98	98.6	55.7	54.3	45.7				59.2	
		4 years	97.1	88	43.4	57.8	56				NA	
		BSI	95.3	88.4	50	73.3	64					
		RTI	100	90	38.3	46.7	51.7					
All regions	SENTRY	1997–2001	92	86	32	54	86					Jones et al., 2003
All regions	SENTRY	1997–2003	95.3	86.1	30.9	52.9	55.7				67.6	Sader and Jones, 2005
All regions	SENTRY	2001–2004	97	86.9		52.4	47.6				72.4	Gales et al., 2006
NA, LA, EU, AP	MTC	2003–2008										Farrell et al., 2010a
		NA	97.6	82.5		51.0	46.1		94.5[a]	0.5/2	73.2	
		EU	98.9	83.7		45.2	42.7		95.3		72.6	
		AP	90.8	78.0		32.6	27.0		96.1		33.4	
		LA	95.5	91.3		48.8	36.7		96.5		76.4	
		ALL	96.0	83.4		44.8	39.1		95.5		64.6	
All regions	SENTRY	2011										Sader et al., 2013
		CLSI	94.5	77.3		36.7			92.3[a]	0.5/2		
		EUCAST	95	NA		NA			79.8			
BSI												
NA, LA	SENTRY	1997	90.9%	81.8	27.3	27.3	90.9					Diekema et al., 1999
NA	SENTRY	1998	73.9	87.0	52.2	65.2	55.7				73.9[c]	Gales et al., 2001b
All regions	MTC	2000–2004	98.0		29.6	56.9			93.1[a]	1/2	84.6	Sader et al., 2005b
UK and Ireland	BSAC	2001–2006	100						89[b]			Livermore et al., 2008

[a] *Tigecycline breakpoints of ≤2 μg/mL for susceptibility and ≥8 mg/L for resistance were used for comparison purposes only, as defined by the USFDA.*
[b] *Susceptibility to tigecycline at the breakpoint of 1 mg/L used for Enterobacteriaceae and Acinetobacter spp.*
[c] *Resistant strains with colistin and polymyxin B MICs of ≥4 mg/L.*
[d] *Antibiotics abbreviations: TMP/SMX, trimethoprim/sulfamethoxazole; LEVO, levofloxacin; CIP, ciprofloxacin; CAZ, ceftazidime; T/C, Ticarcillin/Clavulanate; PB, polymyxin B; TGC, tigecycline; MCN, minocycline.*

tigecycline (Wu et al., 2012). Similar results were demonstrated in several large-scale worldwide surveillance studies as well. A recent SENTRY study conducted during 2009–2012 (494 isolates) (Sader et al., 2014a) revealed a susceptibility of 96% and a recent TEST study conducted during 2007–2012 (2245 isolates) (Renteria et al., 2014) demonstrated low MIC_{50} (0.25 mg/L) and MIC_{90} (1 mg/L) values.

Molecular Mechanisms in Antimicrobial Resistance

S. maltophilia has several molecular mechanisms contributing to its extensive antimicrobial resistance. The mechanisms are summarized in **Table 3**. Detailed descriptions of the major mechanisms are elaborated as follows.

TABLE 3 | Molecular mechanisms of antimicrobial resistance in *S. maltophilia*.

Mechanisms	Associated determinants	Related antimicrobial resistance
β-lactamases 1. L1, L2 (chromosomally and plasmid encoded) 2. TEM-2 (on a Tn*1*-like transposon)	*ampR*-dependent (involving *ampR, ampN-ampG* operon, $ampD_I$ *and mrcA*)	β-lactamases
Class 1 integrons and IS*CR* elements	*sul1*, *sul2*, *dfrA*	TMP/SMX
Multidrug efflux pump	**RND family**: SmeABC, SmeDEF, SmeGH*, SmeIJK, SmeMN*, SmeOP, SmeVWX, and SmeYZ **ABC family**: SmrA, MacABCsm **MFS family:** EmrCABsm	Summarized in **Table 4**
Qnr	Sm*qnr*	Quinolones and tetracycline
Antibiotic-modifying enzymes	AAC(6′)-Iz, APH(3′)-IIc, AAC(6′)-Iak	Aminoglycoside
Lipopolysaccharide (LPS)	SpgM (phosphoglucomutase)	Aminoglycosides, polymyxin B, ticarcillin/clavulanic acid and piperacillin/tazobactam
Mutations of bacterial topoisomerase and gyrase genes		
Reduction in outer membrane permeability		

**not yet characterized.*

β-Lactamases

S. maltophilia has two chromosomal-mediated inducible β-lactamases, namely L1 and L2. L1 is a molecular class B Zn^{2+}-dependent metallo-β-lactamase and L2 is a molecular class A clavulanic acid-sensitive cephalosporinase. The L1 and L2 β-lactamases are simultaneously regulated by AmpR, a transcriptional regulator in the L2 upstream region (Okazaki and Avison, 2008). The *ampR*-L2 module is homologous to the *ampR-ampC* systems, which are widely distributed in some members of the family *Enterobacteriaceae* and in *P. aeruginosa* (Lodge et al., 1990). The regulation of chromosomal *ampR-ampC* systems has been well studied in *Citrobacter freundii*, where the AmpC β-lactamase induction is linked to peptidoglycan recycling and involves several regulatory genes, such as as *ampR*, *ampG*, and *ampD* (Lindberg et al., 1985). A similar induction mechanism was proposed for the *ampR-ampC* and the *ampR*-L2 modules (Okazaki and Avison, 2008). But unlike *P. aeruginosa*, the permease system in *S. maltophilia* requires an intact *ampN-ampG* operon for the induction of β-lactamase (Huang et al., 2010). Two *ampD* homologs, $ampD_I$ and $ampD_{II}$, were found in *S. maltophilia,* but only $ampD_I$appears to be relevant to the regulation of β-lactamase (Yang et al., 2009).

Penicillin-binding proteins (PBPs) participate in peptidoglycan biosynthesis and the inactivation of PBP4 in *P. aeruginosa* has been shown to confer AmpC overexpression and β-lactam resistance (Moya et al., 2009). The inactivation of a putative PBP1a gene, *mrcA*, recently was found to cause basal-level L1/L2 β-lactamase hyperproduction in *S. maltophilia* KJ. The inactivation of *mrcA* only affects basal L1/L2 production β-lactamase, which is *ampR*- and *ampN-ampG*-dependent, and does not augment their induction (Lin et al., 2011). The universality of disruption of $ampD_I$ or *mrcA* in β-lactamase-hyperproducing *S. maltophilia* mutants and clinical isolates has been proved by the existence of wild-type $ampD_I$ and *mrcA* genes. The result implicates mutation of at least one additional gene in this phenotype (Talfan et al., 2013).

Efflux Pumps

Efflux pumps in microorganisms mediate the extrusion of drugs and are classified into five families, namely the resistance-nodulation-cell-division (RND) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the ATP binding cassette (ABC) family, and the multidrug and toxic compound extrusion (MATE) family (Putman et al., 2000). Two ABC-type (SmrA, MacABCsm), one MFS-type (EmrCABsm), a fusaric acid extrusion efflux pump (FuaABC), and six out of the eight postulated RND-type efflux systems have been characterized in *S. maltophilia* (Alonso and Martinez, 2000; Li et al., 2002; Crossman et al., 2008; Al-Hamad et al., 2009; Chen et al., 2011; Hu et al., 2012; Gould et al., 2013; Huang et al., 2013a; Lin et al., 2014a,b). The six characterized RND-type efflux pumps in the *S. maltophilia* genome are SmeABC, SmeEF, SmeIJK, SmeOP, SmeVWX, and SmeYZ (including SmeGH and SmeMN). **Table 4** provides a summary of antimicrobial resistance associated with the abovementioned efflux pumps.

SmeABC

The overexpression of *smeABC* genes confers resistance to aminoglycosides, β-lactams, and fluoroquinolones. SmeC was identified to function independently of SmeAB, while deletions in *smeC* but not *smeB* compromised the antimicrobial resistance (Li et al., 2002).

TABLE 4 | Genetic determinants of efflux pumps.

Efflux pumps	Associated antibiotic resistance
RND FAMILY	
SmeABC	Quinolones, ß-lactams and aminoglycosides
SmeDEF	Quinolones, tetracyclines, macrolides, chloramphenicol, novobiocin and trimethoprim/sulfamethoxazole
SmeIJK	Ciproxin, levofloxacin, tetracycline and minocycline
SmeOP-TolCsm	Trimethoprim/sulfamethoxazole, aminoglycosides, macrolides, doxycycline, chloramphenicol, and nalidixic acid
SmeVWX	Quinolones, chloramphenicol and tetracyclines
SmeYZ	Trimethoprim/sulfamethoxazole and aminoglycosides
ABC FAMILY	
SmrA	Fluoroquinolones and tetracycline
MacABCsm	Aminoglycosides, macrolides and polymyxins
MFS FAMILY	
EmrCABsm	Nalidixic acid and erythromycin
FUSARIC ACID TRIPARTITE EFFLUX PUMP	
FuaABC	fusaric acid

SmeDEF

SmeDEF is a complex formed by an efflux pump located on the inner membrane (SmeE), an outer membrane protein (SmeF) and a periplasmic membrane fusion protein (SmeD). It is involved in resistance to quinolones, tetracyclines, macrolides, chloramphenicol and novobiocin (Alonso and Martinez, 2000). Expression of the *smeDEF* operon is regulated by the SmeT repressor (Hernandez et al., 2009). A recent study showed that the SmeDEF efflux pump is associated with plant root colonization by *S. maltophilia*, and that deletion of the *smeE* gene impairs this function (García-León et al., 2014a).

SmeVWX

The SmeVWX pump, encoded by a five-gene operon (*smeU1*, *smeV*, *smeW*, *smeU2*, and *smeX*), was identified and characterized in a multidrug-resistant mutant of *S. maltophilia* KJ. Overexpression of the SmeVWX pump resulted in increased resistance to chloramphenicol, quinolones, and tetracyclines but increased aminoglycoside susceptibility (Chen et al., 2011).

SmeYZ and SmeJK

The *smeZ*, *smeJ*, and *smeK* genes were identified in *S. maltophilia* K M5, a selected mutant derivative. SmeZ contributes to elevated aminoglycoside MICs. SmeJ and SmeK jointly elevate tetracycline, minocycline, and ciprofloxacin MICs and confer resistance to levofloxacin (Gould et al., 2013). In addition to drug extrusion, the SmeIJK pump has been reported to play a physiologic role in the maintenance of cell membrane integrity (Huang et al., 2014). A recent study further elucidated the physiologic significance of the SmeYZ pump and demonstrated its correlation with virulence-related functions, including swimming, flagella formation, oxidative stress susceptibility, biofilm formation, and protease secretion (Lin et al., 2015).

SmeOP

A *pcm-tolCsm* operon was recently verified in *S. maltophilia* KJ2. The *tolCsm* gene is involved in the resistance of several antimicrobial agents, including aminoglycosides, macrolides, β-lactams, chloramphenicol, nalidixic acid, doxycycline and TMP/SMX. The deletion of *pcm* was shown to result in decreased expression of *tolCsm*, which compromised the pathogen's resistance to amikacin and gentamicin (Huang et al., 2013b). A very recent study characterized a five-gene cluster efflux pump (*tolCSm-pcm-smeRo-smeO-smeP*) in *S. maltophilia*. The study showed that SmeOP requires TolCSm for efflux pump function and suggested that TolCSm is the cognate outer membrane protein (OMP) for the SmeOP pump. The SmeOP-TolCSm efflux pump was shown to be associated with resistance to nalidixic acid, doxycycline, amikacin, gentamicin, erythromycin, and leucomycin (Lin et al., 2014a).

ABC Family: SmrA and MacABCsm

SmrA, the first ABC-type efflux pump identified in *S. maltophilia*, has been shown to confer resistance to fluoroquinolones and tetracycline (Al-Hamad et al., 2009). The MacABCsm efflux pump in *S. maltophilia* was recently shown to confer intrinsic resistance to antimicrobials (aminoglycosides, macrolides, and polymyxins) and to play an important role in regulating oxidative and envelope stress tolerance and biofilm formation (Lin et al., 2014b).

MFS Family: EmrCABsm

Only one MFS-type efflux pump, EmrCABsm, has been characterized so far. It is involved in the extrusion of hydrophobic compounds, including the antibiotics nalidixic acid and erythromycin (Huang et al., 2013a).

Fusaric Acid Tripartite Efflux Pump

A novel tripartite fusaric acid efflux pump was found in *S. maltophilia*, namely FuaABC, which may constitute a new subfamily of the tripartite efflux pump. The *fuaABC* operon was demonstrated to be induced by fusaric acid and to contribute to fusaric acid resistance when overexpressed (Hu et al., 2012).

Trimethoprim/Sulfamethoxazole (TMP/SMX) Resistance Mechanisms

The *sul1* gene carried by class 1 integrons and the *sul2* gene, which is linked to insertion sequence common region (ISCR) elements, are known to be responsible for resistance to TMP/SMX in *S. maltophilia* (Barbolla et al., 2004; Toleman et al., 2007; Chung et al., 2015). The *dfrA* gene cassettes, which are located in class 1 integrons and encode for the dihydrofolate reductase enzyme, have also been reported to confer high-level resistance to TMP/SMX (Hu et al., 2011). Moreover, SmeDEF, TolCsm, and SmeYZ efflux pumps have recently been reported to be associated with TMP/SMX resistance (Huang et al., 2013b; Lin et al., 2015; Sánchez and Martínez, 2015).

Quinolones Resistance Mechanisms

Two mechanisms are associated with resistance of *S. maltophilia* to quinolones: efflux pumps and a chromosomally encoded *qnr* gene (Sm*qnr*) that protects both gyrase and topoisomerase IV from quinolones (Sanchez et al., 2009). Unlike other bacteria, clinical isolates of quinolone-resistant *S. maltophilia* do not present mutations in topoisomerases (Valdezate et al., 2005). To date, the characterized genetic determinants involving resistance to quinolones are *smeDEF*, *smeIJK*, *smeABC*, *smeVWX*, and Sm*qnr* genes, among which *smeDEF* and Sm*qnr* are the best-described. Sm*qnr* belongs to the *qnr* family. It confers low-level resistance and contributes to intrinsic resistance to quinolones in *S. maltophilia* (Sánchez et al., 2008; Shimizu et al., 2008; Sánchez and Martínez, 2010). García-León et al. further elucidated the interplay between intrinsic and acquired resistance to quinolones in *S. maltophilia*. Their study demonstrated that the capacity to develop mutation-driven antibiotic resistance is highly dependent on the intrinsic resistome. Their findings indicate that the most prevalent cause of acquired quinolone resistance in *S. maltophilia* is the overproduction of multidrug efflux pumps, among which SmeDEF efflux pump plays the most important role (García-León et al., 2014b). In addition, a more recent report by García-León et al. confirmed that overexpression of *smeVWX* in clinical isolates of *S. maltophilia* is associated with high-level quinolone resistance (García-León et al., 2015).

Aminoglycosides Resistance Mechanisms

The aminoglycoside-resistant mechanisms in *S. maltophilia* primarily involve aminoglycoside-modifying enzymes and efflux pumps. The reported enzymes to date include AAC(6′)-Iz (an aminoglycoside acetyltransferase) (Li et al., 2003), APH(3′)-IIc (an aminoglycoside phosphotransferase) (Okazaki and Avison, 2007) and a novel AAC(6′)-Iak, which was recently identified in a MDR strain from Nepal (Tada et al., 2014). Efflux pumps including SmeABC, SmeYZ, SmeOP-TolCsm, and MacABCsm are associated with resistance as described above.

Antimicrobial Treatment Studies

Trimethoprim/sulfamethoxazole (TMP/SMX) remains the most effective antimicrobial agent against *S. maltophilia*, with an overall susceptibility rate higher than 90% (Falagas et al., 2008). A recent study investigated the efficacy of sulfametrole/trimethoprim, an alternative sulphonamide/trimethoprim combination available in several Europe countries against non-fermenters (40 *S. maltophilia* included) and found that the activity of the alternative combination was similar to that of TMP/SMX (Livermore et al., 2014). Other common options include ceftazidime, ticarcillin-clavulanate, fluoroquinolones, and tetracyclines such as tigecycline, minocycline, and doxycyclines. As previously mentioned, resistance rates to ceftazidime and ticarcillin-clavulanate are high and rising and are, therefore, unreliable choices. Fluoroquinolones are now popular alternatives because of their less prominent side effects compared to TMP/SMX and their greater potency compared to β-lactams.

Fluoroquinolones

Fluoroquinolones (FQs) are commonly used to treat infections due to *S. maltophilia* (Nicodemo and Paez, 2007). However, their overuse worldwide has resulted in higher resistance rates in many kinds of pathogenic bacteria, including *S. maltophilia* (Chang et al., 2014; Pien et al., 2015).

To evaluate the effectiveness of FQs in this era of high FQ resistance, a retrospective study published in 2014 compared the outcomes of patients with *S. maltophilia* infections treated with TMP/SMX and those of patients treated with FQs monotherapy (Wang et al., 2014b). A total of 38 adults received TMP/SMX and 63 adults received FQs (levofloxacin $n = 48$ and ciprofloxacin $n = 15$). The overall microbiological cure rate was 63% (65% in the TMP/SMX group and 62% in the FQ group), and the overall clinical success rate was 55% (61% in the TMP/SXT group and 52% in the FQ group). The antibiotic regimens were equally effective in both groups. Another retrospective study compared the effectiveness of TMP/SMX ($n = 51$) with that of levofloxacin ($n = 35$) in treating *S. maltophilia* bacteremia and revealed no significant differences in treatment outcome between the two groups, including 30-day mortality, length of hospital day, and antibiotic withdrawal (Cho et al., 2014). However, the rate of adverse events was significantly lower in the levofloxacin group (0%) than in the TMP/SXT group (23.5%, $p = 0.001$).

Several new quinolones have been developed and some of them have recently been approved for clinical application, including nemonoxacin (Huang et al., 2015) and delafloxacin (Bassetti et al., 2015). Oral nemonoxacin, a novel nonfluorinated quinolone antibiotic, has been shown to have good activity against Gram-positive bacilli, such as methicillin-resistant *Staphylococcus aureus* (Huang et al., 2015). However, *in vitro* susceptibility assays on 32 clinical isolates of *S. maltophilia* revealed high MIC_{90} (32 mg/L) and MIC_{50} (8 mg/L) values (Lai et al., 2014a). Regarding delafloxacin, a phase II study published in 2009 that compared two doses of delafloxacin to tigecycline in adults with complicated skin and skin structure infections found that only one patient was infected with *S. maltophilia* and was treated successfully with delafloxacin (O'Riordan et al., 2015). More *in vivo* studies are needed to better understand the effectiveness of these new quinolones in treating *S. maltophilia* infections.

Tetracyclines

Tetracyclines such as tigecycline, minocycline, and doxycycline are some of the most active antimicrobial agents against *S. maltophilia* other than TMP/SMX, even in the cystic fibrosis population (Cantón et al., 2003; San Gabriel et al., 2004; Gülmez et al., 2010; Milne and Gould, 2012; Castanheira et al., 2014). The results agree with our aforementioned observation that these antibiotics consistently exhibited good activity against *S. maltophilia* in global surveillance studies (**Table 2**).

Tigecycline, a derivative of minocycline, has broad-spectrum antimicrobial activity (Stein and Babinchak, 2013) and is an alternative agent against *S. maltophilia* infections. A recent study from Brazil showed that the MIC_{50} and MIC_{90} values of tigecyline for *S. maltophilia* isolates, including isolates resistant

to levofloxacin and/or TMP/SMX harboring *sul-1, sul-2*, and *qnrMR*, were 1 and 4 μg/ml, respectively (Rizek et al., 2015). However, tigecycline has great bio-distribution after intravenous injection, which leads to lower serum drug levels. So, there are concerns about its efficacy for the treatment of bacteremia due to *S. maltophilia* (Stein and Babinchak, 2013). In a recent study, a high dose of tigecycline was effective at treating *S. maltophilia* bacteremia (Wu and Shao, 2014), although in another study, high-dose tigecycline treatment was associated with significant adverse effects (Falagas et al., 2014). In addition, a 3-year clinical therapeutic study that compared the effectiveness of TMP/SMX and tigecycline in treating nosocomial *S. maltophilia* infections revealed no significant differences in mortality or clinical response rates between the two regimens. Clinical improvement rates on the 14th day were 69.2% in the TMP/SMX group and 68.4% in the tigecycline group ($P = 0.954$) and mortality rates on the 30th day were 30.8% in the TMP/SMX group and 21.1% in the tigecycline group ($P = 0.517$) (Tekce et al., 2012). Therefore, tigecycline might be an alternative for patients who are unable to tolerate TMP/SMX. In addition to monotherapy, a combination regimen with tigecycline might be a better option for severe infections, especially for nosocomial infections (Samonis et al., 2012).

In a recent large collection of resistant organisms from the SENTRY program during 2007–2011 (1706 *S. maltophilia* included), minocycline was shown to be significantly more active than other tetracyclines against *S. maltophilia*. The rate of susceptibility of *S. maltophilia* to minocycline exceeded 97% across all geographic regions, and the potency was 2-fold higher than doxycycline ($MIC_{50/90}$: 0.5/2 μg/mL) (Castanheira et al., 2014). A study evaluated 53 multidrug resistant isolates of *S. maltophilia*, including 48 that were resistant to levofloxacin and/or TMP/SMX, and found that minocycline exhibited excellent activity against *S. maltophilia*. However, the clinical experience is still anecdotal (Rizek et al., 2015). A patient with pneumonia was reportedly successfully treated with minocycline (Irifune et al., 1994), and the combination of minocycline with TMP and ticarcillin/clavulanate has been suggested to be effective (Vartivarian et al., 1994).

Polymyxins and Fosfomycin

Polymyxins and fosfomycin are being reconsidered as alternatives or "last-resort" options because of the increasing emergence of multidrug-resistant organisms. Unfortunately, interpreting the susceptibility rates of *S. maltophilia* to polymyxins is problematic because of the discordance between different testing methods (Nicodemo et al., 2004; Gülmez et al., 2010; Moskowitz et al., 2010; Betts et al., 2014). A SENTRY surveillance program study conducted during 2001–2004 assessed the antimicrobial activity of polymyxin B among 54731 isolates of GNB and 1256 isolates of *S. maltophilia* and found that 72.4% of the *S. maltophilia* isolates were susceptible to polymyxin B (MIC_{50} and MIC_{90} values, 1 and 8 mg/L, respectively) (Gales et al., 2006). Colistin (polymyxin E) appears to have a considerable *in vitro* activity against *S. maltophilia* (83–88%) (Falagas and Kasiakou, 2005). In addition, synergy with rifampin, TMP/SMX, and doxycycline has been shown (Giamarellos-Bourboulis et al., 2002; Gülmez et al., 2010). Two studies by the SENTRY program in the year 2011 (globally) and during 2009–2012 (USA and Europe) that collected 362 and 494 isolates of *S. maltophilia*, respectively, reported very different rates of susceptibility to colistin (98.5%, Sader et al., 2013 and 38.7–49.7%, Sader et al., 2014a). In a recent study that collected 641 isolates of *S. maltophilia* in a university hospital in Argentina (Rodríguez et al., 2014), Rodríguez et al. showed that colistin resistance increased from 8% in 1996 to 45% in 2013 and found that the increase correlated with a marked increase (11.4-fold) in colistin consumption during the study period. Fosfomycin has been shown in several reports to be an inappropriate treatment option because of its poor activity and high MICs against *S. maltophilia* (Macleod et al., 2009; Khan et al., 2014; Rizek et al., 2015).

Combination Therapy

Owing to the impressive array of antimicrobial resistance mechanisms of *S. maltophilia*, various combinations of antimicrobial agents have been surveyed in order to overcome resistance or to attain synergism (**Table 5**). Combinations of two or three agents with good susceptibility results such as TMP/SMX, ceftazidime, ticarcillin/clavulanate, and aminoglycosides have demonstrated synergistic effects to different degrees in prior studies. In more recent studies, combinations of TMP/SMX or β-lactam/β-lactam inhibitors with new or old antibiotics such as tigecycline, fluoroquinolones, televancin (Hornsey et al., 2012), rifampin (Betts et al., 2014), and aerosolized colistin have been investigated; they demonstrated various extents of synergism and the ability to maintain effectivity in biofilm. TMP/SMX plus ceftazidime plus levofloxacin has been shown to be effective in treatment of meningitis (Correia et al., 2014) and intrabiliary infusion of colistin plus parenteral fosfomycin with tigecycline was reported to be effective at treating complicated biliary tract infection (Perez et al., 2014). Several combinations of novel agents are currently under investigation, including a β-lactam and dual β-lactamase inhibitor combination (Page et al., 2011) and MD3 (a novel synthetic inhibitor of peptidases) plus colistin (Personne et al., 2014). It is important to mention that *in vitro* synergy attained by combination should be further correlated with clinical outcomes.

Nebulized Antimicrobial Agents

Nebulization of antimicrobials results in high concentrations in the respiratory tract and is associated with low toxicity because this method of delivery results in limited systemic absorption (**Table 5**). These characteristics are especially important for patients with cystic fibrosis, who are prone to frequent infection and colonization of multidrug resistant pathogens including *S. maltophilia*. Wood et al. reported a case of recurrent ventilator-associated pneumonia successfully treated with aerosolized colistin and doxycycline (Wood et al., 2010). King et al. surveyed the *in vitro* pharmacodynamics of aerosolized levofloxacin and suggested that the high concentrations of levofloxacin achieved in the lung by aerosol delivery may be useful for the treatment of patients with cystic fibrosis (King et al., 2010). When *S.*

TABLE 5 | Combinations of antibiotics that demonstrate synergism and nebulized antimicrobial agents.

Year	Combinations	References
COMBINATIONS		
1974	TMP/SMX + colistin	Nord et al., 1974
1979	TMP/SMX + carbenicillin	Felegie et al., 1979
1980	Gentamicin + carbenicillin + rifampin TMP/SMX + carbenicillin + rifampin	Yu et al., 1980
1983	Gentamicin + carbenicillin + rifampicin TMP/SMX + carbenicillin + rifampicin	Berenbaum et al., 1983
1988	Ciprofloxacin + ceftazidime	Chow et al., 1988
1995	Ticarcillin/clavulanate + TMP/SMX Ticarcillin/clavulanate + ciprofloxacin	Poulos et al., 1995
1998	Levofloxacin + various beta-lactams	Visalli et al., 1998
2001	Ticarcillin/clavulanate + aztreonam	Krueger et al., 2001
2002	Azithromycin + TMP/SMX	Saiman et al., 2002
2009	Tigecycline + amikacin Tigecycline + TMP/SMX	Entenza and Moreillon, 2009
2010	TMP/SMX + ticarcillin/clavulanate TMP/SMX + ceftazidime	Gülmez et al., 2010
2012	Ticarcillin/clavulanate + aztreonam: most synergic combination Ticarcillin/clavulanate + colistin Ticarcillin/clavulanate + levofloxacin	Milne and Gould, 2012
2012	Telavancin + colistin: marked synergy	Hornsey et al., 2012
2013	Tigecycline + colistin: best result	Church et al., 2013
2013[a]	Ceftazidime + colistin^{200} Levofloxacin^{100} + ticarcillin/clavulanate Colistin^{200} + TMP/SMX	Wu et al., 2013
2013	TMP/SMX + ticarcillin/clavulanate: most synergistic combination	Chung et al., 2013
2014	Ceftazidime + TMP/SMX Ceftazidime + levofloxacin More effective than the combination of TMP/SMX + levofloxacin	Hu et al., 2014
2014	Colistin + rifampin: reliably bactericidal Colistin + tigecycline	Betts et al., 2014
NOVEL COMBINATIONS		
2011	BAL30376: β-lactam and dual β-lactamase inhibitor combination	Page et al., 2011
2014	MD3 + colistin MD3: a novel synthetic inhibitor of SPases (bacterial type I signal peptidases)	Personne et al., 2014
NEBULIZED ANTIMICROBIAL AGENTS		
2010	Doxycycline + aerosolized colistin	Wood et al., 2010; Harthan and Heger, 2013
2010	Levofloxacin	King et al., 2010
2015	Tobramycin inhalation powder	Ratjen et al., 2015

TMP/SMX, trimethoprim/sulfamethoxazole.

[a] The Top 3 effective combinations when S. maltophilia isolates grown as a biofilm. Colistin and levofloxacin were tested at high concentrations (200 and 100 mg/L, respectively), corresponding to the level achievable in sputum by aerosolization.

maltophilia isolates were grown as a biofilm, the top 3 most effective antibiotic combinations included high-dose levofloxacin or colistin delivered at doses achievable by aerosolization plus a β-lactam or TMP/SMX (**Table 5**) (Wu et al., 2013). The potentials of other antibiotics to be nebulized to achieve high drug levels in airway have been investigated in order to overcome the high MICs that cannot be conquered when the agents are administered systemically. A device (Podhaler device) that delivers new inhalational tobramycin (tobramycin inhalation powder, TIP) and attains high drug levels to the lung may be able to exceed current high MICs of tobramycin in *S. maltophilia* (Ratjen et al., 2015). Waters suggested a potential role of inhaled aztreonam lysine in the treatment of *S. maltophilia* pulmonary infection because of its resistance to the L1 β-lactamase produced by *S. maltophilia* and the ability to achieve high drug levels in respiratory secretions (approximately 1000-fold higher than the corresponding plasma concentration) (Waters, 2012). The antibacterial activity of a novel inhaled combination of fosfomycin and tobramycin (FTI) was investigated in patients with bronchiectasis. However, FTI demonstrated relatively poor activity against *S. maltophilia* (Macleod et al., 2009).

Conclusions

Worldwide, multi-institutional studies confirm that *S. maltophilia* is an emerging multi-drug resistant opportunistic pathogen in hospital and community settings, especially among immunocompromised hosts. TMP/SMX remains the most effective antimicrobial agent in the general population. Drugs with historically good susceptibility results include ceftazidime, ticarcillin-clavulanate, and fluoroquinolones; however, a number of studies show an alarming trend in resistance to those agents. Tetracyclines such as tigecycline, minocycline, and doxycycline are also effective agents and consistently display good activity against *S. maltophilia* in various geographic regions and across different time periods. Combination therapies, novel agents, and aerosolized forms of antimicrobial drugs are currently being tested for their ability to treat infections caused by this multi-drug resistant organism. In addition, recent advances in molecular methods have identified various new mechanisms contributing to drug resistance, which hopefully will lead to future breakthroughs in the treatment of infections due to *S. maltophilia*.

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The inactivation of RNase G reduces the *Stenotrophomonas maltophilia* susceptibility to quinolones by triggering the heat shock response

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Quinolone resistance is usually due to mutations in the genes encoding bacterial topoisomerases. However, different reports have shown that neither clinical quinolone resistant isolates nor *in vitro* obtained *Stenotrophomonas maltophilia* mutants present mutations in such genes. The mechanisms so far described consist on efflux pumps' overexpression. Our objective is to get information on novel mechanisms of *S. maltophilia* quinolone resistance. For this purpose, a transposon-insertion mutant library was obtained in *S. maltophilia* D457. One mutant presenting reduced susceptibility to nalidixic acid was selected. Inverse PCR showed that the inactivated gene encodes RNase G. Complementation of the mutant with wild-type RNase G allele restored the susceptibility to quinolones. Transcriptomic and real-time RT-PCR analyses showed that several genes encoding heat-shock response proteins were expressed at higher levels in the RNase defective mutant than in the wild-type strain. In agreement with this situation, heat-shock reduces the *S. maltophilia* susceptibility to quinolone. We can then conclude that the inactivation of the RNase G reduces the susceptibility of *S. maltophilia* to quinolones, most likely by regulating the expression of heat-shock response genes. Heat-shock induces a transient phenotype of quinolone resistance in *S. maltophilia*.

Keywords: ***Stenotrophomonas maltophilia*, quinolone resistance, antibiotic resistance, RNase G, heat shock**

Introduction

Stenotrophomonas maltophilia is a nosocomial opportunistic pathogen that is considered a prototype of intrinsically resistant bacterium. (Brooke, 2012) The characteristic low-susceptibility of this organism to different antibiotics mainly relies in the presence in its genome of genes encoding several intrinsic resistance elements that include antibiotic-inactivating enzymes, multidrug efflux pumps and a quinolone resistance protein; SmQnr. (Walsh et al., 1997; Lambert et al., 1999; Alonso and Martinez, 2000; Avison et al., 2002; Okazaki and Avison, 2007; Crossman et al., 2008; Sanchez et al., 2008; Shimizu et al., 2008; Al-Hamad et al., 2009; Sanchez and Martinez, 2010; Garcia-Leon et al., 2014b) Quinolones are synthetic antimicrobials which targets are the bacterial topoisomerases. Given their synthetic origin, it was expected that quinolone resistance genes should be absent in natural ecosystems and the only mechanism of resistance would be

mutations in the genes encoding bacterial topoisomerases. Further work demonstrated this not to be true; overexpression of efflux pumps can confer quinolone resistance, and the acquisition of genes encoding target-protecting proteins (quinolone resistance protein, Qnr) renders resistance to these antimicrobials as well (Courvalin, 1990; Hernandez et al., 2011).

Despite these findings, mutations at the genes encoding topoisomerases still remain as the most important mechanism conferring high-level quinolone resistance in clinically relevant bacteria. The only exception is *S. maltophilia*. Different studies have shown that neither clinical isolates presenting high-level quinolone resistance nor *in vitro* selected quinolone resistant mutants present mutations in genes encoding *S. maltophilia* topoisomerases (Ribera et al., 2002; Valdezate et al., 2002; Garcia-Leon et al., 2014b). The best studied mechanisms of quinolone resistance in this bacterial species consist on the overexpression of the multidrug efflux pumps SmeDEF or SmeVWX (Alonso and Martinez, 2001; Gould and Avison, 2006; Garcia-Leon et al., 2014b). Nevertheless, some quinolone resistant clinical isolates neither overproduce any of the already described *S. maltophilia* multidrug efflux pumps not present mutations on the genes encoding bacterial topoisomerases (Garcia-Leon et al., 2014b, 2015). This indicates that there are still mechanisms of quinolone resistance, including overexpression of other efflux pumps as SmrA, which is known to extrude quinolones (Al-Hamad et al., 2009) that remain to be unveiled in *S. maltophilia*. Consequently with this situation, we decided to screen a transposon-tagged insertion library in the aim of finding novel mechanisms of quinolone resistance in *S. maltophilia*. A recent article has reviewed the different definitions of resistance: based on clinically relevant breakpoints, on ecologically relevant breakpoints or in the comparison of two isogenic strains, the resistant one presenting a higher MIC than its parental (Martinez et al., 2015). Along this work we will make use of this third definition of resistance, which is the best suited for determining novel mechanisms of resistance irrespectively, on the level of MIC increase achieved. (Martinez et al., 2015) Using this criterion, we found that the inactivation of RNase G increases the quinolone MICs of *S. maltophilia*. The inactivation of RNases has been associated in few cases with changes in the susceptibility to antibiotics; however, in most published articles and opposite to our findings, such inactivation increases the susceptibility to the analyzed antimicrobials (Saramago et al., 2014).

The role of RNase G on *S. maltophilia* should be indirect by regulating the level of expression of the genes actually conferring resistance. Because of this, we analyzed the effect of inactivating this enzyme on *S. maltophilia* transcriptome and tracked the cause of resistance to the heat-shock response. It has been shown that antibiotics can trigger different stress responses and that such responses can in occasions produce a transient reduction in the susceptibility to antibiotics of bacterial pathogens (Utaida et al., 2003; Cardoso et al., 2010; Kindrachuk et al., 2011). Our data are consistent with these findings and support that mutations producing a de-repressed constitutive expression of the heat-shock response can reduce the susceptibility of *S. maltophilia* to quinolones.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains used were the *S. maltophilia* clinical strain D457 (Alonso and Martinez, 1997), the *S. maltophilia* D457 insertion mutant ALB001 and their isogenic derivatives ALB002 [D457(pVLT33)], ALB003 [D457(pVLT33-*rng*)], ALB004 [ALB001(pVLT33)], and ALB005 [ALB001(pVLT33-*rng*)]. *Escherichia coli* CC118λpir, S17-1 with pUT-miniTn5::Tc and *E. coli* 1047/pRK2013 strains were used for conjugation (de Lorenzo and Timmis, 1994). All strains were grown in LB medium at 37°C, unless indicated.

Generation of Transposon Insertion Mutant Libraries of *S. maltophilia* D457

Random transposon insertion mutant libraries were generated in the *S. maltophilia* D457 strain (Alonso and Martinez, 1997) as described using the minitransposon mini-Tn*5*-*Tc* (de Lorenzo et al., 1990). The transposon was transferred to *S. maltophilia* D457 by conjugation (de Lorenzo and Timmis, 1994) using the donor strain *E. coli* S17-1 λ*pir* (pUT mini-Tn*5*-*Tc*) (de Lorenzo et al., 1990) and the helper strain *E. coli* 1047/pRK2013 (Figurski and Helinski, 1979). One ml aliquots of overnight cultures of each strain were centrifuged 3 min at 5900x *g*. The pellets were suspended in 1 ml of 10 mM $MgSO_4$. For the mating, suspensions were mixed at a 1:10:10 (receptor:donor:helper) ratio, incubated for 10 min at 42°C and then 5 min at 4°C. Cell mixtures were filtered through 0.45 μm filters (Millipore). The filters were placed onto LB agar (LBA) plates and incubated at 30°C during 8 h.

Cells grown on the filter surface were suspended in 5 ml of M9 medium and 200 μl of each suspension were spread in LBA plates containing 10 mg/L tetracycline and 20 mg/L imipenem. The number of colonies was recorded after 24 h of incubation. The colonies were recovered in 1 ml of M9 medium, centrifuged and recovered in PBS with 20% glycerol to store at −80°C.

Antibiotic Susceptibility Assays

The MICs of the different antibiotics were determined on Mueller Hinton agar plates by MIC Test Strip (Liofilchem). The antibiotics used were ciprofloxacin, gatifloxacin, levofloxacin, norfloxacin, nalidixic acid, erythromycin, tigecycline, cotrimoxazole, and ceftazidime. The results were recorded after 24 h of incubation at 37°C.

Inverse-PCR

Inverse-PCR was performed, mainly as described (Fajardo et al., 2008), to identify the gene interrupted by the transposon in the mutant strain ALB001. Chromosomal DNA was obtained using the GNOME DNA kit (MP Biomedicals). One micro gram of genomic DNA was cut using 20 units of the restriction enzyme PstI (Biolabs Inc.) at 37°C for 2 h. One hundred ng of the digested DNA were auto-ligated using 200 units of T4 DNA ligase (BioLabs Inc.) at 16°C overnight in 200 μl of ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 mg/ml bovine serum albumin, pH 7.5). After ligation, DNA was precipitated by adding 20 μl

of 3 M sodium acetate, pH 5.2 and 500 μl of ethanol 100%. The mixture was incubated 20 min at 4°C, and the DNA was pelleted by centrifugation. DNA was washed with ethanol 70%, dried at 37°C, resuspended in water and used for inverse-PCR of the circularized fragments using the primers Tn5A2 and Tn5A3 (**Table 1**). The PCR reaction was made in 50 μl reaction mixture containing 5 μl of PCR buffer 1 10X from the Expand Long Template PCR System (Roche), each deoxynucleotide at a concentration of 350 μM, 300 nM of each primer, 2.5 units of Expand Long Template Enzyme mix (Roche) and the template DNA obtained as described above. The mixture was heated at 94°C for 2 min, followed by 30 cycles of 10 s at 94°C, 30 s at 55°C and 6 min at 68°C, and finally one 7 min extension step at 68°C. The PCR product was run on 1% agarose gel (Sambrook and Russell, 2001) and purified using the QIAquick PCR purification kit (Qiagen). The amplicon was sequenced with the same primers used for the PCR in the Parque Científico of Madrid.

Complementation of *S. maltophilia* ALB001 Strain with the *rng* gene of *S. maltophilia* D457

The *rng* gene of *S. maltophilia* D457 was amplified using the primers RNaseF and RNaseR (**Table 1**). The reaction was performed using the Expand Long Template PCR system (Roche), 500 ng of genomic DNA of *S. maltophilia* D457 as template, 350 μM of each deoxynucleotide, and 0.5 μM of each primer. The reaction had one denaturation step at 94°C for 5 min, followed by 30 amplification cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1.5 min, with a final extension step of 68°C for 7 min. The PCR product was cloned into the pGEM-T plasmid (Promega), generating the pGEM-T-*rng* plasmid. The inserted fragment was sequenced by Macrogen[1] to ensure that no mutations were introduced during PCR. The pGEM-T-*rng* plasmid was *Eco*RI-*Hin*dIII digested, and the fragment containing the *rng* gene was purified and cloned into pVLT33 (de Lorenzo et al., 1993), in the same enzyme restriction sites. The plasmid containing the wild-type allele of *rng* was dubbed pBA01. This plasmid as well as the cloning vector pVLT33 were introduced into *E. coli* CC118λpir and mobilized into *S. maltophilia* D457 and ALB001, using the *E. coli* 1047/pRK2013 strain as a helper, by triple conjugation (de Lorenzo and Timmis, 1994) at a rate of 4:1:2 (receptor-donor-helper) and using M9 (Atlas, 1993) to recover the mating mixture from the filter. The exconjugants were selected on LB plus 300 mg/L kanamycin and 20 mg/L imipenem and the presence of the pVLT33 and pBA01 plasmid was checked by extracting the plasmid by QIAprep Spin Miniprep Kit (Qiagen) and pBA01 was further analyzed by *Eco*RI-*Hin*dIII digestion and electrophoresis on a 0.8% agarose-ethidium bromide gel.

[1]http://www.macrogen.com

TABLE 1 | Primers used along this work for DNA amplification.

Primers	5′-3′ sequence	Description
Tn5 A3	gccttgatgttacccgagagc	Used for inverse-PCR
Tn5 A2	aaaatctagcgagggctttac	Used for inverse-PCR
RNaseF	cggaattccgatgtctgaggaa	To amplify and clone *rng* (*Eco*RI target underlined)
RNaseR	cccaagcttggatcagagcagaa	To amplify and clone *rng* (*Hin*dIII target underlined)
Sme27	tgccagcgacagtgcaaagggtc	To amplify a *Stenotrophomonas maltophilia* specific region (Sanchez et al., 2002)
Sme48	ccgtgttcatggaagcaggc	To amplify a *S. maltophilia* specific region (Sanchez et al., 2002)

RNA Preparation and Real-time RT-PCR

Flasks containing 25 ml LB were inoculated with either *S. maltophilia* D457 or ALB001 overnight cultures to 0.01 OD_{600nm} and were grown at 37°C until an OD_{600nm} of 0.6 was reached; in order to synchronize growing cells, new flasks containing 25 ml LB were inoculated with the aforementioned cultures to 0.01 OD_{600nm} and grown at 37°C until an OD_{600nm} of 0.6 was reached. Ten milliliters of cultures were mixed with 10 ml of RNAprotect Bacteria Reagent (Qiagen) during 10 min at room temperature. Afterward, cells were spun down at 6,000× g for 10 min at 4°C and immediately frozen on dry ice and stored at −80°C. Total RNA extraction, DNA elimination, RNA integrity verification, DNA absence confirmation, cDNA generation, and real-time PCR were performed as described previously (Olivares et al., 2012). Total RNA was extracted from cell pellets by RNeasy mini- kit (Qiagen) according to the manufacturer's instructions. To further eliminate any remaining DNA, Turbo DNA-free (Ambion) was used. RNA integrity was verified on a 1% agarose gel, and the absence of DNA was verified by PCR using primers Sme27 and Sme48 (**Table 1**). cDNA was obtained from 5 μg RNA by a High Capacity cDNA reverse transcription (RT) kit (AB Applied Biosystems). Real time PCR mixture was obtained using the Power SYBR green kit (Applied Biosystems) as indicated by the manufacturer and the reaction was performed as follows; a first denaturation step, 95°C for 10 min, was followed by 40 temperature cycles (95°C for 15 s, 60°C for 1 min). Differences in the relative amounts of mRNA for the different genes were determined according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). In all cases, the mean values for relative mRNA expression obtained in three independent experiments, each one with three technical replicates were considered. Expression of the reference gene *rpoD* was used for the normalization of the results (Garcia-Leon et al., 2014a). The primers used for the real-time PCR are indicated in **Table 2**.

Transcriptomic Analysis

To assess the transcriptome of *S. maltophilia* D457 and ALB001 strains, RNA was obtained from three independent cultures of each strain. The triplicates RNAs from each strain were then pooled to reduce biological variability. After elimination of rRNA with RiboZero, cDNAs were synthesized and sequenced using the Illumina technology in a 75 bp-single-end format at the Parque Científico of Madrid. The data of RNA-sequencing were normalized

TABLE 2 | Primers used along this work for real time RT-PCR.

Primers	5′-3′ sequence	Final concentration (nM)
dnaK 1	gcgtcatcgagtacctggtt	400
dnaK 2	caggttcacttcggtctgct	400
emrA 1	atgagccagacccaagacac	200
emrA 2	ggccgaacatgaagtaccac	200
emrB 1	agcacatctcggcctatcag	200
emrB 2	gatgtcgttgaagcccatct	200
groEL 1	aagaaggtgcaggtctccaa	200
groEL 2	tcgtaatccgaggaggtgtc	200
groES 1	ccaaggaaaagtccaccaag	200
groES 2	cgtactggccgtagatgacc	200
grpE 1	caagttcgccaacgagaag	200
grpE 2	tgcttgtaggtcagctccag	200
hslU 1	agaccgaccacatcctgttc	600
hslU 2	cacgaaatcgttcttgctca	800
htpG 1	catcaccatcgaagacaacg	600
htpG 2	agctgcgaatccttcttctg	800
clpB 3	acttcaagctggtgcaggac	400
clpB 4	gttgtgcagcacttcttcca	400
dnaJ 3	aagcctacgaagtgctgtcc	400
dnaJ 4	ccaaaaatgttgccgaagat	400
hslV 3	ggtggctcgtatgcactgtc	600
hslV 4	acgttgcggttggtgtagat	800
rnasaG 1	gaggacatcgcctacctgtc	200
rnasaG 2	accttcaccttgtccacgtc	200
rpoD1	ggtgcacatgatcgaaacga	500
rpoD2	gccgtactgctggagcatct	500

as reads per kilobase per million mapped reads (RPKM; Mortazavi et al., 2008) analyzed, and visualized using Fiesta 1.1[2].

Determination of mRNAs Half Lives

A rifampicin run-out experiment was conducted by adding rifampicin 200 mg/L to synchronized cultures (OD_{600nm} 0.6) and taking samples every 5 min after transcription inhibition by rifampicin. RNA extraction and real-time RT-PCR were carried out as described above. The values of half-lives were estimated from the mean decay rate for each mRNA.

Killing Curves under Heat Shock

The killing curves of *S. maltophilia* D457 and ALB001 with or without plasmid pBA01 were established, under antibiotic pressure and at different temperatures. The different strains were inoculated from overnight cultures and grown at 37°C until they reached an OD_{600nm} of 0.6. Then, cultures were diluted 1:10 on LB medium containing nalidixic acid 48 mg/L and grown either at 37°C or at 42°C for 60 min. Ten minutes after heat shock, RNA was extracted from the different cultures and used for real-time RT-PCR as described above. At different times after inoculation, samples were taken and serial dilutions of such samples were plated in LBA Petri dishes. Colony-forming-units (cfu) were recorded after 24 h of incubation at 37°C.

[2]http://bioinfogp.cnb.csic.es/tools/FIESTA

Results and Discussion

Generation of a *S. maltophilia* Transposon-insertion Library and Selection of a Quinolone Resistant Mutant

Four independent conjugations were performed as described in methods to obtain different libraries. Each library contained between 1600 and 2000 mutants. The whole library then contains around 7000 independent mutants. A screening was performed by plating the library on Mueller–Hinton agar Petri dishes containing nalidixic acid 128 mg/L. Selected mutants were grown in medium without antibiotic (two passages) to assure that the observed phenotype was not transient and the susceptibility to quinolones was tested by disk diffusion (not shown). One mutant presenting a decreased susceptibility to different quinolones was chosen for further studies and dubbed ALB001.

To determine the gene in which the transposon had been inserted, an inverse PCR reaction was performed as described in methods and the amplified DNA was sequenced using Sanger technology. Comparison of the sequence with the *S. maltophilia* D457 genome (Lira et al., 2012) showed that the transposon was inserted inside the SMD_3054 gene, which encodes an ortholog of RNase G. To further confirm the position of the transposon, a PCR was performed using primers flanking the gene encoding RNase G. Confirming the results of the inverse PCR, the size of the amplicon from ALB001 fits with that predicted for the presence of the miniTn5 inside *rng* (not shown).

To further confirm the role of *rng* inactivation in the observed phenotype, the wild-type allele of *rng* was cloned and expressed in the wild-type *S. maltophilia* strain D457 and the *rng* defective mutant ALB001 as described in Methods. The strains were also transformed with the pVLT33 vector and the resultant strains used as controls. The susceptibility of the different strains to different quinolones was then analyzed by using MIC Test Strip. As shown in **Table 3**, the *in-trans* expression of the wild-type allele of *rng* in ALB001 reduced the susceptibility of the mutant to levels close to those of the wild-type strain. To address whether or not the inactivation of *rng* also alters *S. maltophilia* susceptibility to other non-quinolone antibiotics, the MICs of erythromycin, tigecyclin, cotrimoxazole, and ceftazidime were measured. We found that the inactivation of *rng* did not produce any effect on *S. maltophilia* susceptibility to these non-quinolone antimicrobials. Altogether, these results further support that the inactivation of RNase G reduces the susceptibility to quinolones of *S. maltophilia*.

The Inactivation of RNase G alters the Transcriptome of *S. maltophilia*

Since RNases are involved in RNA processing and the targets of the quinolones are the bacterial topoisomerases, a direct effect of the inactivation of RNase G on quinolone susceptibility is unlikely. Indeed, it has been reported that in *E. coli* RNase G is involved in the regulation of the central metabolism (Lee et al., 2002; Sakai et al., 2007). The observed resistance might be due to another determinant(s) the expression of which is regulated by RNase G. To have further insights of these putative elements,

TABLE 3 | Susceptibility to quinolones of the strains used in this work.

Strains	MIC (mg/L)								
	Nalidixic acid	Norfloxacin	Levofloxacin	Ciprofloxacin	Gatifloxacin	Erythromycin	Tigecycline	Cotrimoxazole	Ceftazidime
D457	6	12	0.5	1.5	0.5	128	1	0.125	2
D457 (pVLT33)	4	12	0.5	1.5	0.38	128	1.5	0.125	1.5
D457 (pBA001)	4	12	0.5	1.5	0.38	128	1	0.125	1.5
ALB001	64	32	1.5	3	0.75	128	1.5	0.125	1.5
ALB001 (pVLT33)	128	48	3	6	1	128	1.5	0.125	1.5
ALB001 (pBA001)	8	12	0.75	2	0.5	128	1.5	0.094	1.5

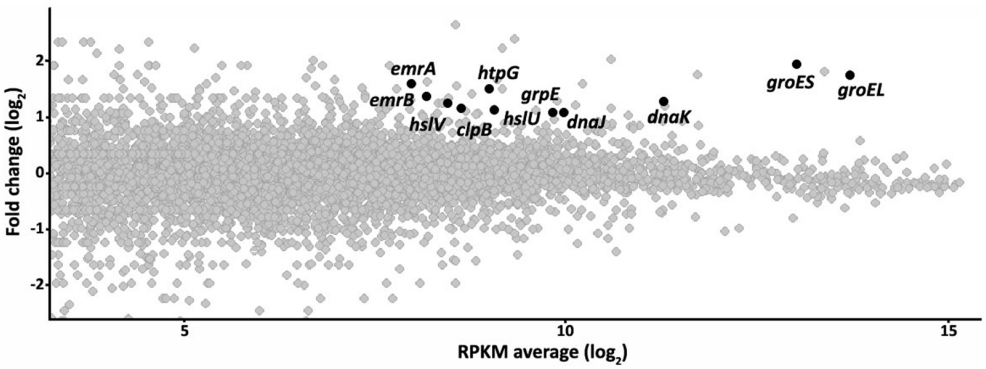

FIGURE 1 | The inactivation of the RNase G alters the transcriptome of *Stenotrophomonas maltophilia*. The effect of inactivating the RNase G in the transcriptome of *S. maltophilia* was studied by RNA sequencing as described in the text. The figure represents the fold change in the expression level of RNAs from the RNase G-defective mutant ALB001 in comparison with the wild-type strain D457, as a function of the average of the RPKM values gene in both strains. The Figure is represented in a $\log_2$ scale. Genes, presenting higher expression in the RNase G defective mutant and selected for further studies are highlighted.

the transcriptome of the ALB001 RNase G defective mutant was analyzed by Illumina sequencing in comparison with that of the wild-type strain D457. As shown in **Figure 1** and Supplementary Table S1, several genes were expressed at a higher level in the mutant ALB001 than in the wild-type strain D457.

The Inactivation of RNase G Increases the Expression of Genes Involved in *S. maltophilia* Heat Shock Response

Among the genes that were overexpressed in the ALB001 mutant when compared with the isogenic parental strain D457, some of them encoded proteins forming part of the heat shock response. The genes *emrA* and *emrB*, which encode a multidrug efflux pump were also expressed at a higher level in ALB001 than in D457. RpoH is an alternative sigma factor involved in the heat-shock response (Grossman et al., 1984). Our transcriptomic study shows that *rpoH* expression in slightly higher (1.4-fold change) in the *rng* mutant as compared with the wild-type strain. Whether or not this slight increase in the expression of this transcriptional activator is enough for triggering the heat-shock response remains to be elucidated.

To further confirm the transcriptomic studies, the expression of this set of selected genes was measured by real-time RT-PCR as described in Methods. As shown in **Figure 2**, for all the selected genes, namely *clpB*, *dnaJ*, *dnaK*, *groEL*, *groES*, *grpE*, *hslU*, *hslV*, *htpG*, *emrA*, and *emrB*, expression was higher in the RNase G defective mutant than in the wild-type strain. This result further confirms that RNase G down-regulates *emrA, emrB* and different genes encoding proteins involved in *S. maltophilia* heat-shock

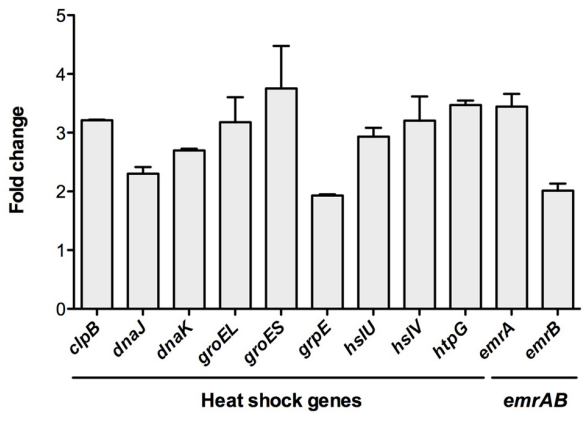

FIGURE 2 | The inactivation of RNase gene increase the expression of heat-shock response genes. To confirm the transcriptomic assays, the expression of the genes *clpB*, *dnaJ*, *dnaK*, *groEL*, *groES*, *grpE*, *hslU*, *hslV*, *htpG*, *emrA,* and *emrB* was determined by real-time RT-PCR in the wild-type strain and the RNase defective mutant ALB001. As shown, all genes were expressed at a higher level in the RNase G defective mutant, confirming the results of the RNA-seq analysis.

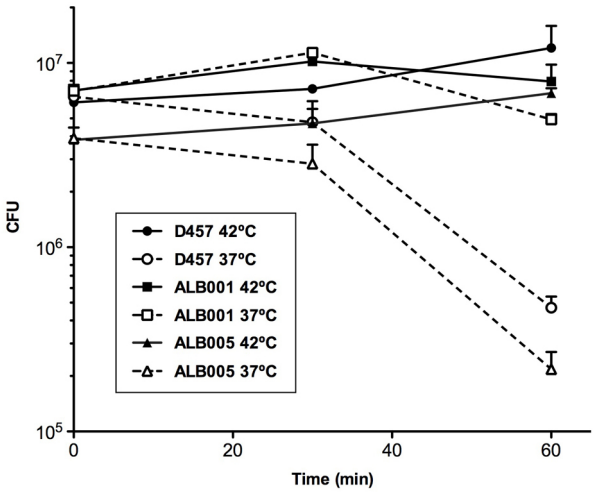

FIGURE 3 | Heat-shock reduces the susceptibility of *S. maltophilia* to nalidixic acid. The wild-type strain *S. maltophilia* D457 and its isogenic, RNase G defective mutant ALB001, were either grown at 37°C or subjected to a 42°C heat-shock in the presence of 48 mg/L of nalidixic acid (NA) and the killing curves of each of the cultures were determined. As shown, the wild-type strain D457 was killed by the quinolone when growing at 37°C, but it was not affected under heat-shock conditions. The RNase defective mutant ALB001 was not affected by quinolones under any of the tested conditions. The means and standard deviations of three independent experiments are shown.

TABLE 4 | Effect of RNase G on the stability of *groES* and *groEL* mRNAs.

Gene	mRNA half-life (min)		Increase (%)
	D457	ALB001	
groES (SMD_3814)	3,65	4,01	10
groEL (SMD_3813)	3,69	4,41	20

response. It would be possible that these genes are targets of RNase G, in which case the half lives or their messenger RNAs will be longer. For addressing this possibility a rifampicin run-out experiment was conducted and half-lives of *groEL* and *groES* mRNAs were calculated in the wild-type strain and in the RNase G defective mutant. As shown in **Table 4**, inactivation of *rng* led to a minor increase in the half lives of *groEL* and *groES* mRNAs (10 and 20%), suggesting these mRNAs are not direct targets of RNase G.

Overexpression of multidrug efflux pumps is involved in the acquisition of resistance to quinolones in *S. maltophilia* (Chang et al., 2004; Sanchez et al., 2004; Gould and Avison, 2006; Garcia-Leon et al., 2014b). Indeed, it has been shown that EmrAB overexpression protects *E. coli* from the antibiotics nalidixic acid and thiolactomycin in *E. coli* (Lomovskaya et al., 1995). It is then possible that the responsible for the resistance of the ALB001 mutant would be the overexpression of the EmrAB efflux pump. (Lomovskaya et al., 1995).

In addition, it has been shown in *E. coli* that the inhibition of the heat shock chaperon DnaK (Credito et al., 2009) as well as mutations in the heat-shock-response genes *dnaK*, *groEL,* and *lon*, increase the susceptibility to quinolones (Yamaguchi et al., 2003). If the heat-shock response is involved in the intrinsic resistance to quinolones, it might be speculated that triggering the heat-shock response might induce resistance. In this regard, it is worth mentioning that the inhibition of the DNA gyrase, the target of quinolones, induces the heat shock response in *E. coli* (Kaneko et al., 1996).

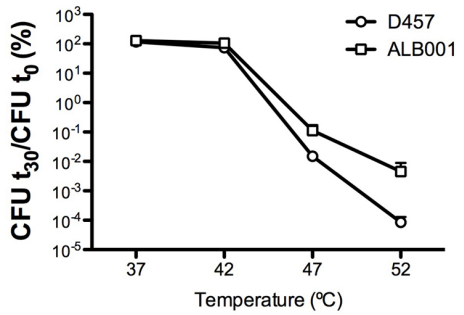

FIGURE 4 | The lack of RNase G increases the survival of *S. maltophilia* at high temperatures. The wild-type strain *S. maltophilia* D457 and its isogenic, RNase G defective mutant ALB001, were grown at either 37, 42, 47, or 52°C. Colony forming units (CFUs) were counted after 30 min of incubation. As shown, the RNase defective mutant ALB001 survived better than the wild-type strain when growing at 47 and 52°C. The means and standard deviations of three independent experiments are shown.

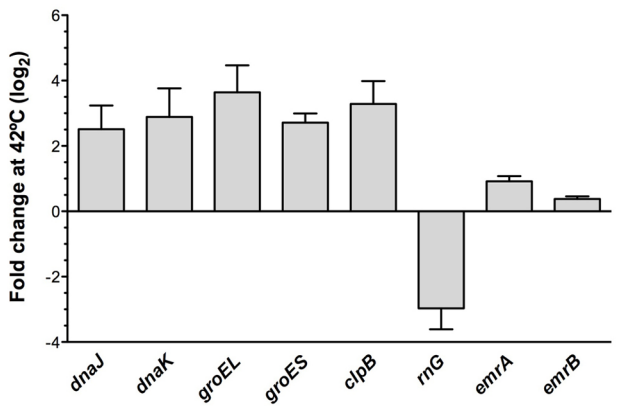

FIGURE 5 | Expression of heat-shock genes, *rng*, *emrA,* and *emrB* under heat shock conditions. The wild-type strain *S. maltophilia* D457 was grown either at 37 or under heat-shock conditions at 42°C. RNA was isolated after 10 min of incubation and the expression of *clpB*, *dnaJ*, *dnaK*, *groEL*, *groES*, *rng*, *emrA,* and *emrB* was measured by real time RT-PCR. Gene expression at 42°C relative to 37°C. As shown, all the analyzed heat shock genes are over-expressed under heat shock conditions. While, opposite to this situation, *rng* expression is down-regulated after a heat shock. Notably, expression of *emrA* and *emrB* is not significantly affected by the heat shock.

The Heat-shock Response Reduces the Susceptibility to Quinolones of *S. maltophilia* without Increasing *emrA*, *emrB* Expression

To determine whether or not a heat-shock might reduce the susceptibility to quinolones of *S. maltophilia*, the kinetic of death in the presence of the quinolone nalidixic acid of the different

strains was measured for bacteria growing at 37°C and upon a 42°C heat-shock (**Figure 3**). As shown, more than 90% of the population of the wild-type strain D457 was killed after 60 min of incubation at 37°C in the presence of nalidixic acid, whereas the quinolone did not exert any effect over the heat-shocked D457 population. Consistent with the role of the inactivation of RNase G on quinolone resistance, growth of the ALB001 mutant was inhibited neither at 37°C nor upon heat-shock. Notably, *in-trans* expression of the wild-type allele of *rng* restored the quinolones susceptibility of the ALB001 mutant to the levels of the wild-type strain D457 when growing at 37°C (**Figure 3**). Further supporting a role of RNase G on *S. maltophilia* heat shock response and its survival at high temperatures is the finding that the ALB001 mutant survives better than the wild-type strain upon incubation at high temperatures (47 or 52°C; **Figure 4**).

Since RNase G downregulates expression of heat-shock genes at permissive temperature, it is possible that the induction of the expression of such genes under heat shock is due to the down-regulation of the expression of *rng* under such conditions. To address this possibility, the expression of the heat shock genes *dnaJ*, *dnaK*, *groEL*, *groES*, *clpB* as well as *rng, emrA,* and *emrB* was measured at 37°C and upon heat-shock conditions. As shown in **Figure 5**, *rng* is expressed at lower levels under the same heat-shock conditions where the genes of the heat-shock response are overexpressed. This finding is consistent with the proposed role of RNase G in regulating the *S. maltophilia* heat-shock response. Notably, expression of *emrA* and *emrB* is not increased upon heat-shock conditions, strongly suggesting they are not involved in the heat-shock-mediated quinolone resistance of *S. maltophilia*.

Conclusion

Differing to other organisms in which high-level quinolone resistance is usually due to mutations at the genes encoding the bacterial topoisomerases, this type of quinolone-resistance mutations have never been described neither in *S. maltophilia* clinical isolates nor in the case of *in vitro* selected quinolone resistant mutants of this bacterial species (Ribera et al., 2002; Valdezate et al., 2002; Garcia-Leon et al., 2014b). While in some isolates quinolone resistance is associated to overexpression of multidrug efflux pumps (Alonso and Martinez, 2001; Sanchez et al., 2004; Garcia-Leon et al., 2015), some other mechanisms of resistance remain to be explored in this pathogen. Herein, we show that heat-shock induces a phenotype of quinolone resistance in *S. maltophilia*. In addition, RNase G regulates the expression of heat-shock responding genes, hence modulating the susceptibility to quinolones of *S. maltophilia*. Our work strongly supports that heat-shock response, a well conserved system in different bacterial species, triggers quinolone resistance (at least in *S. maltophilia*). In this way, heat-shock response proteins might be good targets for the development of new antimicrobials to be used together with quinolones.

Some works have shown that nearly 3% of the bacterial genome contributes to the characteristic phenotype of resistance of a given bacterial species. (Fajardo et al., 2008) Among those genes which inactivation modifies the susceptibility to antibiotics, several of them belong to basic categories of bacterial physiology (in principle not directly linked with antibiotics), including general metabolism, transport, and regulation among others. The finding that the lack of RNase G renders quinolone resistance, by triggering *S. maltophilia* the heat-shock response, fits with this situation and indicates that novel, non-classical mechanisms, might be involved in the acquisition of antibiotic resistance by this pathogen.

Transcriptomic Data Accession Number

The results of the transcriptomic analysis described in this article were deposited in the SRA (Sequence Read Archive) database of NCBI (accession number SRR2128156).

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01068

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In vitro interaction of *Stenotrophomonas maltophilia* with human monocyte-derived dendritic cells

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Stenotrophomonas maltophilia is increasingly identified as an opportunistic pathogen in immunocompromised, cancer and cystic fibrosis (CF) patients. Knowledge on innate immune responses to *S. maltophilia* and its potential modulation is poor. The present work investigated the ability of 12 clinical *S. maltophilia* strains (five from CF patients, seven from non-CF patients) and one environmental strain to survive inside human monocyte-derived dendritic cells (DCs). The effects of the bacteria on maturation of and cytokine secretion by DCs were also measured. *S. maltophilia* strains presented a high degree of heterogeneity in internalization and intracellular replication efficiencies as well as in the ability of *S. maltophilia* to interfere with normal DCs maturation. By contrast, all *S. maltophilia* strains were able to activate DCs, as measured by increase in the expression of surface maturation markers and proinflammatory cytokines secretion.

Keywords: DC maturation markers, cytokines secretion, cystic fibrosis, opportunistic pathogen, innate immune response

Introduction

The environmental Gram-negative bacillus *Stenotrophomonas maltophilia* is increasingly identified as an opportunistic nosocomial pathogen for susceptible individuals, including immunocompromised, cancer, and cystic fibrosis (CF) patients (Brooke, 2012; Waters et al., 2013). Cases of community-acquired *S. maltophilia* infections have also been reported (Falagas et al., 2009b; Chang et al., 2014). Molecular analyses have revealed considerable heterogeneity among *S. maltophilia* isolates, including those coming from just one hospital (Roscetto et al., 2008; Kaiser et al., 2009; Rocco et al., 2009).

The intrinsic resistance to a wide spectrum of antibiotics (Crossman et al., 2008; Brooke, 2014), the ability to adhere and form biofilm on medical devices surfaces and airway epithelial cells (Pompilio et al., 2010), make *S. maltophilia* responsable of persistent nosocomial infections. *S. maltophilia* can cause various serious infections but it is most commonly associated with pneumonia (Weber et al., 2007; Chang et al., 2014; Chawla et al., 2014). *S. maltophilia* pneumonia may be complicated by persistent bacteremia associated with a significant increased mortality (Elsner et al., 1997; Falagas et al., 2009a).

Little is known about pathogenesis of *S. maltophilia*, and the genomic diversity exhibited by clinical isolates makes difficult the identification of pathogenic traits. Factors likely involved in

TABLE 1 | *Stenotrophomonas maltophilia* strains used in DC infection assay.

Strain name	Source	Swimming motility	Twiching motility	Biofilm production
CF1545	Sputum (P)	++	+	++
CF781	Sputum (G)	++	+	++
CF635	Sputum (P)	–	–	–
CF2315	Sputum (G)	+	+++	++
CF1445	Pharyngeal aspirate (P)	+	++	++
NCF879	Sputum (ICU)	+++	+++	+++
NCF1489	Sputum (H)	+++	+++	+++
NCF1732	Bronchial aspirate (ICU)	+++	+++	+++
NCF1376	Bronchial aspirate (ICU)	++	++	+
NCF2035	Bronchial aspirate (ICU)	++	+++	+++
TFE66	Foot wound swab (D)	+++	++	+++
K279a	Blood infection	++	+	++
LMG11104	Tuberous roots	++	++	++

P, Pediatrics; G, Geriatrics; ICU, Intensive Care Unit; H, Hematology; D, Diabetology; LMG-labeled strain is from the Laboratorium voor Microbiologie Gent Culture Collection, Belgium. (+, weak; ++, moderate; +++, strong).

the pathogenesis of *S. maltophilia* have been described (Chhibber et al., 2008; Pompilio et al., 2011; Roscetto et al., 2012). The ability to adhere and penetrate inside airway epithelial cell lines (Pompilio et al., 2010), and stimulate robust cytokine production in macrophages and monocytes are key steps in *S. maltophilia* infections (Waters et al., 2007; Härtel et al., 2013). Little is known on the interactions between *S. maltophilia* and dendritic cells (DCs). Immature DCs (iDCs) are sentinel cells found in skin, respiratory and gastrointestinal mucosae constantly recruited from blood to peripheral tissues, even in the absence of inflammatory processes (Guermonprez et al., 2002). Upon capture of soluble antigens or bacteria, iDCs undergo maturation, a profound change in gene expression transforming them from antigen-capturing to mature antigen-processing and -presenting cells (Guermonprez et al., 2002; Villadangos and Health, 2005).

Mature DCs migrate to secondary lymphoid organs to prime naïve T cells differentiation into effector T cells (Reis e Sousa, 2006). DCs produce interleukin-12p70 (IL-12p70), which activates the differentiation of T helper 1 (Hsieh et al., 1993) and cytotoxic T cells, and IL-1 beta, IL-6, and IL-23, required for differentiation of Th17 cells (Acosta-Rodriguez et al., 2007). Pathogenic bacteria have developed strategies which enable them to escape phagocytosis and killing by DCs, or affect DC maturation (Bueno et al., 2012).

The aim of this study was to compare the ability of *S. maltophilia* strains from CF and non-CF patients to survive in human monocyte-derived DCs, and evaluate whether isolates from CF patients have unique properties. We also examined the effect of *S. maltophilia* strains on the maturation of DCs, monitoring the expression of specific maturation markers by flow cytometry.

Materials and Methods

Bacterial Strains and Culture Conditions

Thirteen non-clonal clinical isolates of *S. maltophilia* were included in the study. Strains were isolated from diverse clinical settings, including CF and non-CF respiratory specimens, as well as non-respiratory specimens, collected at the Laboratory of Clinical Microbiology, University Federico II of Naples. The sequenced strain K279a was kindly provided by M. B. Avison (University of Bristol, Bristol, UK). The environmental strain LMG11104 was obtained from the BCCM (Belgium Coordinated Collections of Microorganisms/Laboratorium) voor Microbiologie Ghent, University of Ghent, Belgium. Details of the bacterial strains used in this study are given in **Table 1**. Bacteria were identified as *S. maltophilia* by biochemical characteristics and antibiotic resistance analysis, using the VITEK II system (bioMerieux) and confirmed by MALDI TOF MS (Bruker). Bacteria were grown overnight at 37°C for 24 h in Brain-Hearth Infusion broth (BHI; Becton Dickinson), and aliquots were frozen in BHI-glycerol at −80°C until use.

Motility Assays

Swimming motility was evaluated on plates 1% tryptone, 0.5% NaCl containing 0.3% agar. A colony of bacteria was inoculated with a sterile toothpick placed directly into the center of the agar. After incubation for 48 h at 30°C, the diameters of the swimming motility zones were measured. Twitching motility was evaluated on plates 1% tryptone, 0.5% yeast extract, 0.5% NaCl containing 1% agar. A colony of bacteria was inoculated deep into the agar with a sterile toothpick at the bottom of twitch plates. After incubation at 37°C for 24 h, the zone of motility at the agar-petri dish interface was measured by staining with 1% crystal violet.

Biofilm Assay

Biofilm formation was tested in 96-well microtiter plates according to Stepanovic et al. (2000). Briefly, one colony of the overnight cultures of bacterial strains was diluted in trypticase soy broth (TSB) in order to adjust the turbidity of the bacterial suspension to 0.5 McFarland standard, approximately 10^8 CFU (colony forming units)/mL. Bacterial suspension was diluted 1:100 in TSB and 200 μL of the diluted bacteria was added to each well. Negative control wells contained broth only. The plates were incubated aerobically for 48 h at 37°C. Thereafter, the content of each well was aspirated and the wells washed three times with 300 μl of sterile phosphate buffered saline (PBS). Biofilm was fixed incubating microtiter plates for 1 h at 60°C. The plates were stained with 30 μl per well of 1% crystal violet for 15 min and washed with 150 μl of sterile PBS. The dye bound to the

adherent cells was resolubilized with 150 μl of 95% ethanol per well. The optical density of each well was measured at 630 nm using a microplate reader (Biorad). Based on the optical densities of bacterial biofilms, all strains were classified into the following categories: no biofilm producers (−), weak (+), moderate (+), or strong (+++) biofilm producers, as previously described (Stepanovic et al., 2000).

In vitro Generation and Culture of Human DCs

Immature dendritic cells were generated from peripheral blood mononuclear cells, as described (Sallusto and Lanzavecchia, 1994). Briefly, peripheral blood mononuclear cells were obtained from 30 ml of leukocyte-enriched buffy coat from healthy donors by centrifugation on a Ficoll-Hypaque plus (GE Healthcare) through density gradient. Monocytes were purified by positive selection using anti-CD14 mAb-conjugated magnetic microbeads (Miltenyi Biotec). $CD14^+$ cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (Invitrogen), and 2 mM glutamine (Invitrogen) containing 50 ng/ml GM-CSF (granulocytes monocytes-colony stimulating factor) and 250 ng/ml IL-4 (Immunotools). Cells were cultured for 5–7 days in 5% CO_2 atmosphere to obtain a population of iDCs. Purity of generated DCs was checked by flow cytometry, using Phycoerythrin (PE) conjugated anti-CD1a antibody (Becton Dickinson) on a FACScalibur flow cytometer (Becton Dickinson). DC populations were used when CD1a expression was $>$ 95%. Written informed consent was obtained from each donor at the time of venous peripheral blood donation, in accordance with the Declaration of Helsinki, as approved by Azienda Ospedaliera Universitaria Federico II. All the experiments done by using blood donations were performed and analyzed anonymously, without any biographical reference to donors.

DC Infection Assays

iDCs were seeded at a concentration of 10^6 cells per well into 24-wells culture plates, infected with 2×10^7 CFU/ml of mid-log growth phase *S. maltophilia* cells to yield bacterium-to-dendritic cell ratio of 20:1 and incubated for 1 h at 37°C in 5% CO_2.

To assess the number of intracellular bacteria, the culture medium was gently removed, cells were washed twice with sterile PBS and further incubated for 1 h in fresh medium containing 600 μg/ml gentamicin or 1 mg/ml ceftazidime to kill extracellular bacteria. DCs were subsequently washed and incubated in 10 mM EDTA, 0.25% Triton X-100 (Sigma) for 5 min to release internalized bacteria. Lysates were serially diluted 1:10 and plated on TSA (Becton Dickinson) to quantify the number of viable intracellular bacteria. Entry Index (EI), i.e., the percentage (mean) of the inoculated CFUs that were internalized by iDCs after 1 h of infection, was determined as follows: EI = (CFU ml^{-1} recovered from DCs after 1 h of infection/CFU ml^{-1} applied to DCs) × 100.

To measure intracellular replication, after infection of DCs with *S. maltophilia* and antibiotic treatment cells were extensively washed and fresh medium was added. Incubation was continued for additional 16 h. After washing three times with PBS, intracellular bacteria were released by addition of 10 mM EDTA, 0.25% Triton X-100 and the number of CFU was determined by plating 10-fold dilutions on TSA. Intracellular Replication Index (RI), i.e., the percentage (mean) of the internalized CFUs that survived by iDCs after 18 h of infection, was determined as follows: RI = (CFU ml^{-1} recovered from DCs after 18 h of infection/CFU ml^{-1} recovered from DCs after 1 h of infection) × 100.

The bactericidal activity of gentamicin or ceftazidime at the used concentrations had been previously assessed. The effectiveness of drugs during infection assays was tested by supernatant sampling before DCs lysis and plating on TSA (data not shown).

DCs Maturation Analysis

Dendritic cells were seeded in 24-well plates at a concentration of 10^6 cells/ml and then exposed to RPMI 1640 only, 1 mg/ml LPS or live bacteria at an MOI of 20:1. After 18 h of incubation at 37°C and 5% CO_2, DCs were harvested, washed, re-suspended in FACS buffer and DC maturation was then analyzed measuring CD86 and CD80 expression by flow cytometry, using FITC-conjugated anti-CD80 antibody and a PE-conjugated anti-CD86 antibody (Becton Dickinson).

For TNFα expression, cells were cultured for 2 h with the bacterial strains in the presence of 5 mg/ml Brefeldin A (Sigma) and then stained with anti-TNFα antibody (Becton Dickinson) using Cytofix/Cytoperm method (Becton Dickinson), according to manufacturer's instructions. IL -12p70 production by DCs was measured from supernatants using OptEIA ELISA kits (Becton Dickinson), according to manufacturer's instructions.

Statistical Analysis

All assays were performed in three independent experiments. Paired Student's *t*-test was used to evaluate the statistical differences between uninfected-DCs and *S. maltophilia*-infected DCs and between CFUs recovered from DCs after 1 h and after 17 h of incubation for each analyzed strain. Statistical differences between multiple groups of *S. maltophilia* strains were assessed using a Mann-Whitney non-parametric test. Values of $p < 0.05$ were taken as statistically significant differences.

Results

Internalization and Intracellular Replication of *S. maltophilia* in Immature Monocytes-derived Dendritic Cells

We have selected 13 strains from diverse clinical setting (**Table 1**). Some were isolated from the respiratory tract of CF patients (group 1, CF strains), others from the respiratory tract of non-CF patients (group 2, NCF strains), the remaining from non-respiratory specimens (group 3, NR strains). Motility is crucial both to colonize an environment and to induce formation of structured surface-associated communities of bacteria called biofilm. For each strain, both motility and the ability to form biofilm in polystyrene microtiter plates were analyzed. All CF and NR strains exhibited reduced swimming and twiching motilities

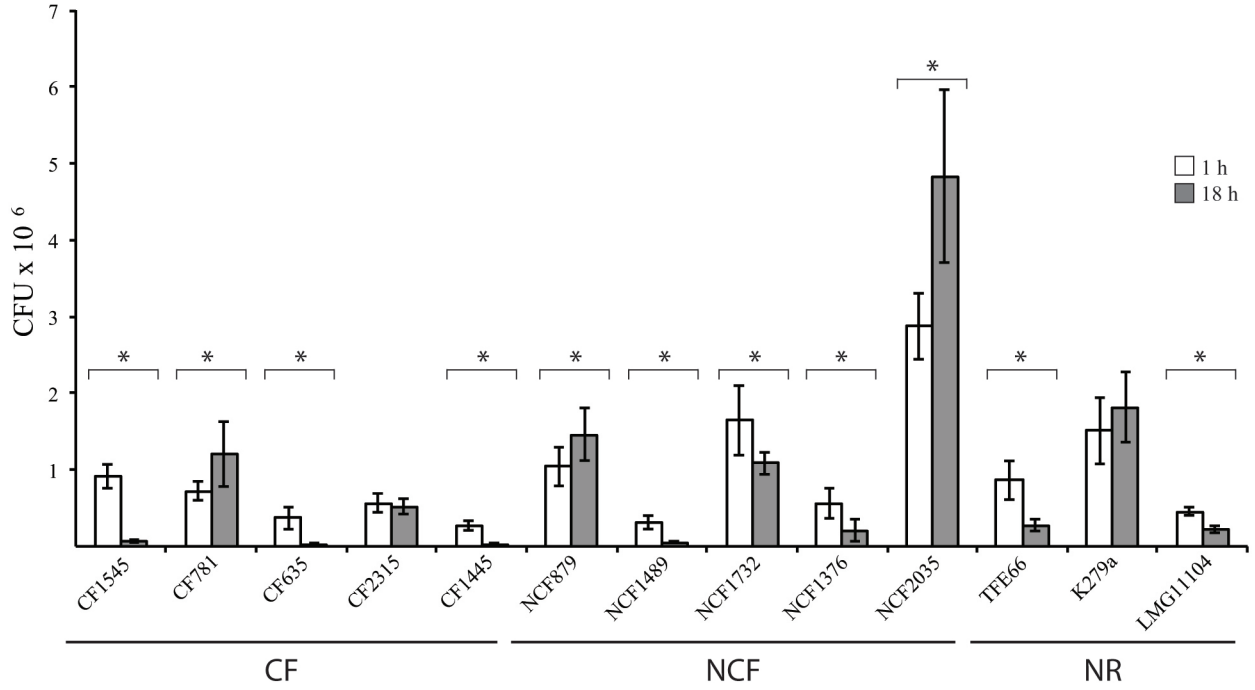

FIGURE 1 | Interactions of *S. maltophilia* strains with iDCs. White and gray bar graphs indicate CFUs recovered by iDCs after 1 h and 18 h of infection, respectively. *$p < 0.05$. Each bar indicates the mean value ± standard deviation of at least three independent experiments in triplicate.

compared to NCF strains (**Table 1**). Biofilm forming ability greatly varied among strains tested (OD_{630} range: 0.08–1.2). Most CF strains (80%) were moderate biofilm producers, whereas the majority of the NCF and NR strains (75%) were strong producers (**Table 1**). Strong biofilm producers exhibited increased swimming and twitching activity.

The uptake and intracellular replication were analyzed by infecting immature monocytes-derived DCs (iDCs) with tested *S. maltophilia* strains at a MOI of 20. All strains were internalized and were able to survive within iDCs, but internalization and replication significantly varied among strains of the same group, as among strains of different groups (**Figure 1**). The difference between CFUs at 1 h and 18 h of infection resulted statistically significant ($p < 0.05$) for 11/13 strains (**Figure 1**). Values of Entry Index (EI) of CF strains ranged from 1.35 for (CF1445 strain) to 4.6 (CF1545 strain; **Figure 2A**). Only the CF781 strain was able to grow intracellularly ($p < 0.05$) with Intracellular Replication Index (RI) of 166.7 (**Figure 2B**). EI values of NCF strains ranged from 1,6 (NCF 1489 strain) to 14.38 (NCF2035 strain; **Figure 2A**). Two NCF isolates (NCF879 and NCF2035 strains) were able to multiply intracellularly ($p < 0.05$) with RI mean values of 140.38 and 170.83 respectively (**Figure 2B**). Among NR strains, only the K279a strain could survive into iDCs, but was unable to replicate, because no significant differences ($p = 0{,}27$) between CFUs at 1 h and 18 h were found (**Figure 1**). In spite of the limited number of clones analyzed, no significative difference could be observed among CF, NCF, and NR strains. This weakens the hypothesis made by several groups suggesting that unique properties may be associated to CF strains (see Waters et al., 2007).

S. maltophilia Induces DCs Maturation

Changes induced in DCs by tested strains were assessed by measuring by flow cytometry the expression of CD86 and CD80, two costimulatory molecules upregulated during DC maturation. As revealed by the fold increase in mean fluorescence intensity (MFI) of CD80 and CD86 (**Figure 3**), most strains induced a consistent upregulation of both molecules. Only for the NCF2035 strain no statistically significative difference in the expression of both CD80 and CD86 in infected and uninfected cells could be observed.

Along with changes in the expression of surface maturation markers, DC secrete several cytokines in response to intracellular infection. All strains induce a statistically significative ($p < 0.001$) increase of TNFα and IL-12 cytokines (**Figure 4**). Also in DCs maturation, no significant differences ($p > 0.05$) was observed by comparing CF isolates to either NCF or NR strains.

Discussion

By sensing danger signals in peripheral tissues, iDCs activate both adaptive and innate immunity, leading to bacterial clearance. Several microbial pathogens are able to avoid or subvert DCs, thwarting DC differentiation, survival, maturation, antigen processing and presentation and the induction of T cell immunity (Rescigno, 2015). This is the first report of the interaction between human monocyte-derived DCs and *S. maltophilia*. All the analyzed strains were internalized by DCs (**Figure 1**), but the efficiency of internalization and intracellular survival differed highly (**Figure 2**). Six of thirteen strains exhibited low EI values,

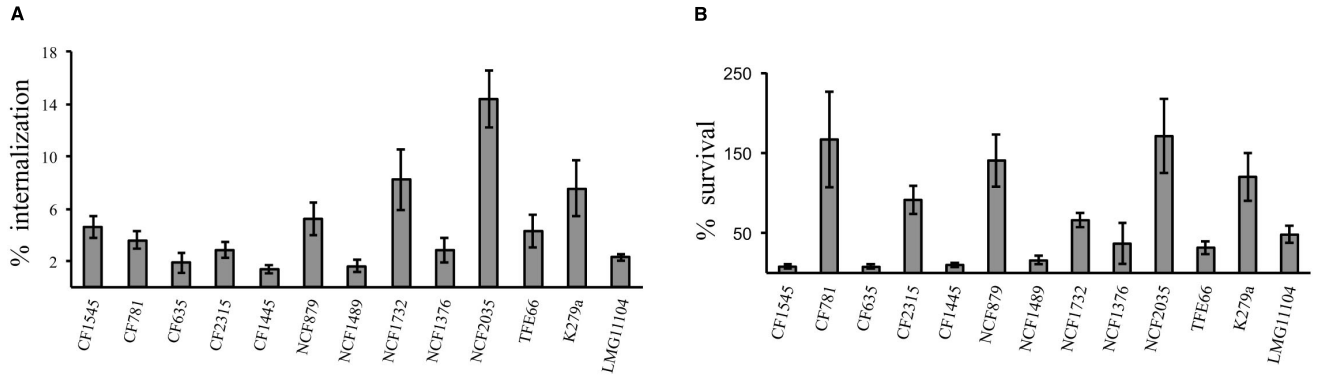

FIGURE 2 | DC infection assays. (A) Entry Index (EI). Bar graphs shown the percentage internalization of *S. maltophilia* strains after 1 h of infection with DCs. **(B)** Intracellular Replication Index (RI). Bar graphs shown the percentage survival of *S. maltophilia* strains after 18 h of infection within DCs. Each bar indicates the mean value ± standard deviation of at least three independent experiments in triplicate.

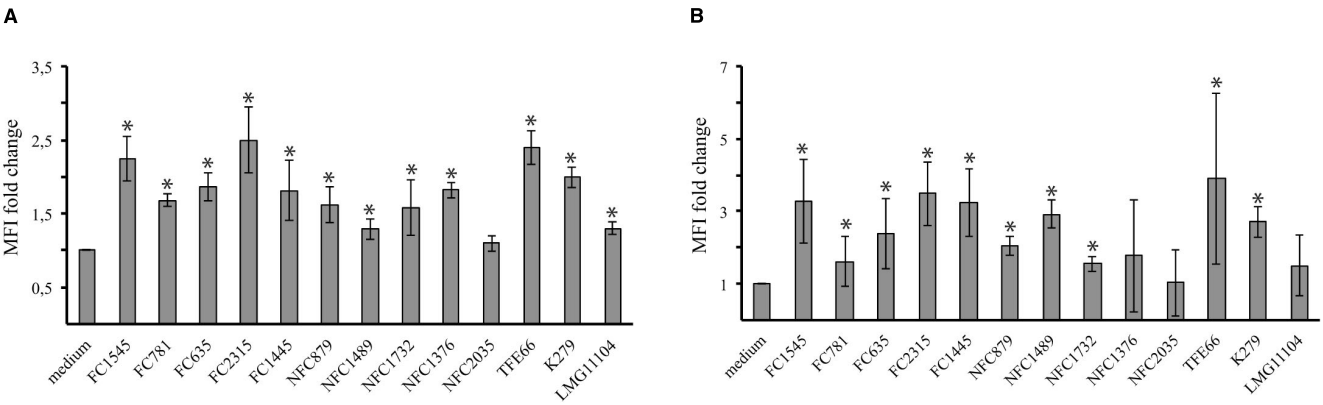

FIGURE 3 | *Stenotrophomonas maltophilia* strains induce dendritic cells maturation. Bar graphs shown the fold change in CD80 **(A)** and CD86 **(B)** expression after 18 h of incubation. CD86 and CD80 expression on DCs was measured by flow cytometry. The mean fluorescence intensity (MFI) reflects the upregulation of molecule on cell surface. Each bar indicates the mean ± standard deviation of three independent experiments. *$p < 0.05$. Medium: iDCs uninfected.

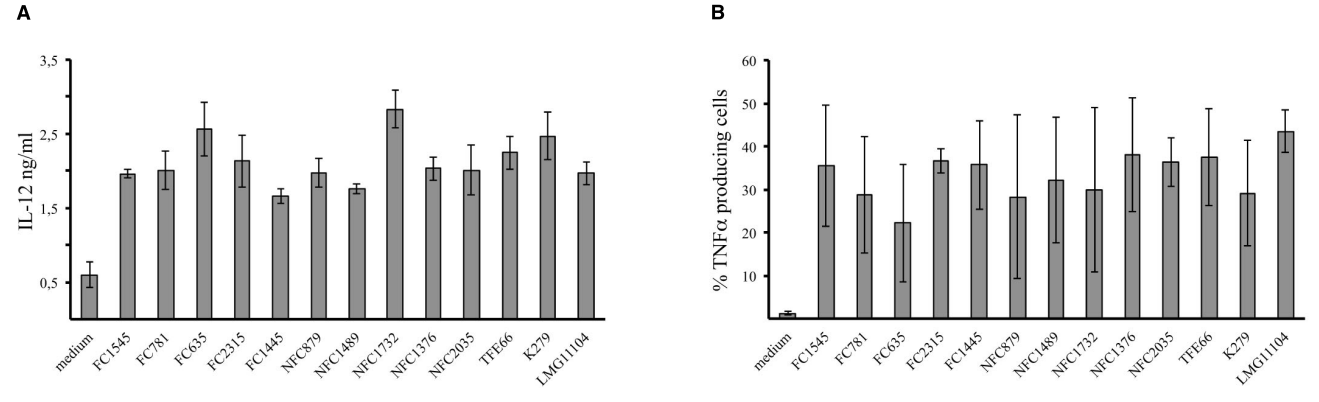

FIGURE 4 | *Stenotrophomonas maltophilia* strains induce cytokines production by dendritic cells. Bar graphs shown the production of IL-12 **(A)** and TNFα **(B)** by dendritic cells after incubation with the *S. maltophilia* strains. IL-12 and TNFα expression by DCs was measured by ELISA and flow cytometry, respectively. Each bar indicates mean ± standard deviation of at three independent experiments. All the bacterial strains induced the production of TNFα and IL-12 by DCs ($p < 0.001$). Medium: iDCs uninfected.

and were unable to persist inside host cells. Thus, these *S. maltophilia* isolates plausibly escape the effector cells of innate immunity by circumventing phagocytosis. Four strains showed high EI values, and two (NFC879 and NFC2035) also exhibited high RI values. NFC879- and NFC2035-like strains could safely be mobilized in the host evading antimicrobial therapy and immune response. The intracellular survival and replication could contribute to *S. maltophilia* virulence, and DCs may serve as dissemination vectors of bacteria outside of the infection site. Monitoring changes in gene expression occurred in bacteria recovered by DCs after 1 h and 18 h of infection could eventually help to clarify the intracellular survival strategy of *S. maltophilia* inside DCs.

Most of tested strains induced a significant increase in the expression of DC surface maturation markers (**Figure 3**). This suggests that DCs exposed to *S. maltophilia* can prime naïve T cells. Noteworthy, the NFC2035 strain, which exhibited the highest RI value, selectively failed to upregulate CD80 and CD86 costimulatory molecules. Pathogens as diverse as *Mycobacterium tuberculosis*, HIV-1, and *Leishmania* sp., downregulate the expression of costimulatory molecules in order to establish chronic infection. Impairment in the signaling events delivered by costimulatory molecules may be responsible for defective T-cell responses, enabling infective organisms to grow unhindered in the host cells (Khan et al., 2012).

All tested strains were able to induce the production of TNFα and IL-12 in DCs (**Figure 4**), and this is on line with data obtained by monitoring the response of macrophages to *S. maltophilia* (Waters et al., 2007). The production of both cytokines may play a crucial role in tissue inflammation, and contribute to a slow damage in CF lung. Clinical retrospective studies indeed showed deterioration of lung function after prolonged colonization with *S. maltophilia* in CF- as in hospital-acquired pneumonias (Karpati et al., 1994).

No significant difference was observed in the maturation of iDCs stimulated with *S. maltophilia* strains belonging to CF, NCF, and NR group. The data suggested that internalization, intracellular survival and immunostimulatory properties of *S. maltophilia* are strain-dependent. This is in accord with studies showing that different *S. maltophilia* strains replicate and persist in the murine lungs to significantly different degrees (Rouf et al., 2011). The observed heterogeneity is not surprising, in light of the high genetic diversity exhibited by *S. maltophilia* isolates (Roscetto et al., 2008; Kaiser et al., 2009; Rocco et al., 2009).

Further experiments will be needed to characterize the DC-driven T-cell responses, analyzing the ability of DCs infected with *S. maltophilia* to bias naive allogenic T-cell differentiation toward a TH_1 or TH_2 phenotype.

Author Contributions

All authors read and approved this manuscript. ER and MC conceived and designed the experiments. ER, LV, RM, and AS performed the experiments. ER, LV, and EDG analyzed the data. VI and AV provided strains for analysis. The manuscript was prepared by ER, EDG, and MC.

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Iron is a signal for *Stenotrophomonas maltophilia* biofilm formation, oxidative stress response, OMPs expression, and virulence

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Stenotrophomonas maltophilia is an emerging nosocomial pathogen. In many bacteria iron availability regulates, through the Fur system, not only iron homeostasis but also virulence. The aim of this work was to assess the role of iron on *S. maltophilia* biofilm formation, EPS production, oxidative stress response, OMPs regulation, quorum sensing (QS), and virulence. Studies were done on K279a and its isogenic *fur* mutant F60 cultured in the presence or absence of dipyridyl. This is the first report of spontaneous *fur* mutants obtained in *S. maltophilia*. F60 produced higher amounts of biofilms than K279a and CLSM analysis demonstrated improved adherence and biofilm organization. Under iron restricted conditions, K279a produced biofilms with more biomass and enhanced thickness. In addition, F60 produced higher amounts of EPS than K279a but with a similar composition, as revealed by ATR-FTIR spectroscopy. With respect to the oxidative stress response, MnSOD was the only SOD isoenzyme detected in K279a. F60 presented higher SOD activity than the wt strain in planktonic and biofilm cultures, and iron deprivation increased K279a SOD activity. Under iron starvation, SDS-PAGE profile from K279a presented two iron-repressed proteins. Mass spectrometry analysis revealed homology with FepA and another putative TonB-dependent siderophore receptor of K279a. *In silico* analysis allowed the detection of potential Fur boxes in the respective coding genes. K279a encodes the QS diffusible signal factor (DSF). Under iron restriction K279a produced higher amounts of DSF than under iron rich condition. Finally, F60 was more virulent than K279a in the *Galleria mellonella* killing assay. These results put in evidence that iron levels regulate, likely through the Fur system, *S. maltophilia* biofilm formation, oxidative stress response, OMPs expression, DSF production and virulence.

Keywords: *Stenotrophomonas maltophilia*, iron, Fur, biofilms, oxidative stress response, IROMPs, virulence, DSF

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Introduction

Stenotrophomonas maltophilia is a widespread environmental, multidrug resistant bacterium. It has become a nosocomial pathogen of increasing importance; in fact, it is the third most common nosocomial non-fermenting Gram-negative bacterium. Infection occurs principally in immunocompromised subjects, and in patients exposed to invasive devices and/or broad spectrum antibiotics (Looney et al., 2009; Brooke, 2012). *S. maltophilia* has also emerged as one of the most

common isolated bacteria from the airway of cystic fibrosis (CF) patients (Pompilio et al., 2011; Vidigal et al., 2014).

Despite the broad spectrum of clinical syndromes associated with *S. maltophilia* infections, little is known about its virulence factors (Adamek et al., 2011). Factors that could be involved in the virulence of *S. maltophilia* include Smf1-fimbrial operon (de Oliveira-Garcia et al., 2003), protease StmPr1 (Windhorst et al., 2002; Nicoletti et al., 2011), exopolysaccharides and lipopolysaccharides (Huang et al., 2006), and siderophores (Garcia et al., 2012). Another important virulence factor of *S. maltophilia* is its capacity to form biofilms, communities of microbial cells that grow on biotic or abiotic surfaces embedded within extracellular polymeric substances (EPS) (Huang et al., 2006; Passerini de Rossi et al., 2007; Pompilio et al., 2008). *S. maltophilia* biofilms exhibit phenotypic characteristics that are distinct from those of planktonic organisms, including increased resistance to antimicrobial compounds (Di Bonaventura et al., 2004; Passerini de Rossi et al., 2009, 2012; Pompilio et al., 2010).

The genome of *S. maltophilia* K279a encodes a diffusible signal factor (DSF) dependent quorum sensing (QS) system that was first identified in *Xanthomonas campestris pv. campestris* (Xcc) (Fouhy et al., 2007; Huang and Wong, 2007). DSF synthesis is completely dependent on *rpfF*, which is part of the *rpf* operon (for regulation of pathogenicity factors) (Barber et al., 1997). Fouhy et al. (2007) demonstrated that the disruption of DSF signaling has pleiotropic effects in *S. maltophilia* K279a. The *rpfF* mutant had severely reduced motility, reduced levels of extracellular protease and altered LPS profiles. Their results showed that DSF controls aggregative and biofilm behavior and virulence in a nematode model. A recent study demonstrated that the significance of the *rpf*/DSF QS system is not confined to the virulence caused by *S. maltophilia* but also used by the plant-associated biocontrol agent *S. maltophilia* R551-3 (Alavi et al., 2013). Furthermore, the *S. maltophilia* QS signal is involved in interspecies signaling between different bacterial species within the CF lung and also has cross-kingdom antagonistic activity on *Candida albicans* (Ryan et al., 2008; Passerini De Rossi et al., 2014).

Besides QS signals, iron availability is a regulatory signal not only for the acquisition and utilization of this metal but also for the production of virulence factors in many pathogenic bacteria (Carpenter et al., 2009). Bacteria find iron limiting conditions in mammalian hosts, where free iron is limited and it is normally bound to sequestering proteins such as transferrin and lactoferrin. Thus, siderophores are considered important virulence factors for many pathogens allowing the microorganism to survive in the host. However, an excess of iron is toxic because of its ability to catalyze Fenton reactions and the formation of reactive oxygen species (ROS). In consequence, iron uptake has to be carefully regulated to maintain the intracellular concentration of the metal between desirable limits (Escolar et al., 1999). Iron-dependent gene regulation is mediated, in many bacterial species by Fur (ferric uptake regulator). Fur regulates the expression of iron uptake genes and is also involved in virulence and protection against oxidative stress (Carpenter et al., 2009). Fur is a global regulator that can act as either a repressor or an activator. Iron limitation induces or inhibits biofilm formation depending on the species (Wu and Outten, 2009).

Previous studies reported a relationship between Fur and the QS system. Fur positively regulates acyl homoserine lactone (AHL) production by *Pseudomonas syringae* pv. tabaci 11528 (Cha et al., 2008). In *Vibrio vulnificus* the gene *vvpE*, encoding the virulence factor elastase, is repressed under iron-rich conditions, and the repression was due to a Fur-dependent repression of *smcR*, a gene encoding a QS master regulator with similarity to *luxR* in *Vibrio harveyi* (Kim et al., 2013). Recently, evidence that iron limitation enhances AHL production was reported in *A. baumannii* (Modarresi et al., 2015).

S. maltophilia is an aerobic bacterium which generates ROS during metabolism. Superoxide dismutase catalyzes the dismutation of toxic superoxide radicals into molecular oxygen and hydrogen peroxide. Three SOD isoenzymes have been discovered, all prokaryotic organisms contain Mn-SOD or Fe-SOD while Cu/Zn-SOD is absent except for a few cases (Fridovich, 1978). Currently very little is known about the oxidative stress response of *S. maltophilia*.

The aim of this work was to assess the role of iron on *S. maltophilia* biofilm formation, EPS production, oxidative stress response, OMPs regulation, QS and virulence.

Materials and Methods

Bacterial Strains and Culture Conditions

The reference strain *Stenotrophomonas maltophilia* K279a, which genome is fully sequenced (GenBank: AM743169.1) was used in this study (Crossman et al., 2008). The spontaneous *fur* mutant, F60, derivative from the wild-type (wt) strain *S. maltophilia* K279a, was isolated in this study. *Xanthomonas campestris* pv. *campestris* (Xcc) 8004 and Xcc 8523 (*rpfF* mutant) were used in the DSF bioassay described by Barber et al. (1997). Strains were kept frozen at −20°C in 15% glycerol. Before use, bacteria were cultured on tryptone soya agar (TSA; Oxoid Ltd, Basingstoke, Hampshire, UK) for 24 h at 35°C. Unless otherwise stated, all cultures were grown in tryptone soya broth (TSB, Oxoid Ltd) in the presence or absence of 200 μM 2,2′-dipyridyl (Dip; Sigma-Aldrich), and incubated for 48 h at 35°C. When required, the cultures were vigorously aerated on a gyratory water bath shaker (Model G75, New Brunswick Scientific Co. Edison NJ, USA) at 200 r.p.m.

Isolation of *fur* Mutants

The manganese mutagenesis technique (Hantke, 1987) with few modifications was used to isolate spontaneous *fur* mutants of *S. maltophilia*. Briefly, an overnight culture of K279a in LB broth was plated on LB agar containing 20 mM $MnSO_4$ and 200 μM Dip. After 72 h of incubation, the robustly growing manganese-resistant colonies were tested on chrome azurol S (CAS) agar plates prepared with the modifications described previously by our group (Garcia et al., 2012) and supplemented with 20 mM $FeCl_3$, to identify *fur* mutants by constitutive siderophore production. In order to confirm the mutations, the full-length *fur* gene and 100 bp upstream from the start codon were PCR amplified using primers designed in this

study (For 5′-GGCGGTTGGGGAAATCAAAC-3′ and Rev 5′-CAACGAAAAACCCCGGGCA-3′) and sequenced. PCR was performed using *Taq* DNA polymerase (Promega Corporation) and chromosomal DNA from *S. maltophilia* putative *fur* mutants as template under the following conditions: 1 cycle of 96°C for 5 min; 30 cycles of 96°C for 1 min, 48°C for 30 s, and 72°C for 1 min, and a final cycle of 72°C for 7 min (Biometra, TPersonal48). DNA sequences were determined at Unidad de Genómica (Instituto de Biotecnología, Instituto Nacional de Tecnología Agropecuaria-INTA, Argentina). The nucleotide sequences were edited using the BioEdit Sequence Alignment Editor (Hall, 1999) and sequences were aligned with MEGA v4.0 (Tamura et al., 2007).

Biofilm Formation Assay

Biofilms were prepared using a static microtitre plate model as previously described (Passerini de Rossi et al., 2009). Briefly, overnight cultures of *S. maltophilia* strains were standardized to contain approximately 10^6 CFU/ml. For each test condition (presence or absence of 200 μM Dip in TSB medium), 8 wells of a sterile flat-bottom 96-well polystyrene microtiter plate (TPP, Trasandingen, Switzerland) were filled with 200 μl of the standardized inoculum. Uninoculated medium controls were included. After 48 h incubation, the final culture density was determined by measuring the OD_{546} using a Multiskan EX plate reader (Thermo electron corporation, Hudson, United States). Then, the culture medium was removed from each well and plates were washed three times with phosphate buffered saline (PBS) to remove non-adherent cells. Biofilms were stained with 0.01% crystal violet (CV; Mallinckrodt, Chemical Works, NY, USA) for 30 min. The plates were washed, and the dye bound to the biofilm was extracted with ethanol 95%. The total biomass (attached cells and extracellular matrix) was quantified by measuring the OD_{546} of dissolved CV. Results were expressed as the level of CV staining relative to the final culture density [CV (OD_{546})/Growth (OD_{546})], to avoid variations due to differences in bacterial growth generated by the chelator.

Confocal Laser Scanning Microscopy (CLSM)

For confocal microscopy biofilms were formed on Nunc Lab-Tek 8-well chamber slide (No. 155411) containing a borosilicate glass base 100 μm thick. Overnight cultures of K279a and F60 were standardized to contain approximately 10^6 CFU/ml and for each test condition (presence or absence of 200 μM Dip), chambers were filled with 400 μl of the standardized inoculum. After 48 h of incubation, the wells were rinsed with sterile physiological saline (0.9% NaCl) in order to eliminate any non-adherent bacteria. The wells were refilled with physiological saline containing 2.5 μM Syto9® (Molecular Probes, Grand Island, NY), a green fluorescent nucleic acid marker, and incubated in the dark for 20 min. Images were acquired with a confocal laser-scanning microscope Carl Zeiss LSM510-Axiovert 100 M by sequentially scanning with a 488 nm argon laser using a 40X water immersion objective lens, at the Instituto de Investigaciones Biomédicas (Pontificia Universidad Católica Argentina-CONICET, Argentina). Emitted fluorescence was recorded within the 505–530 nm range to visualize Syto9 fluorescence, and Z-stacks were captured every10 μm at different areas in the well. Images were analyzed using the ZEN 2009 Light Edition (Carl Zeiss) and three-dimensional projections of the biofilms' structure were reconstructed using the ImageJ program (ImageJ. Available online: http://rsbweb.nih.gov/ij/).

Extracellular Polymeric Substances (EPS) Production Assays

The EPS production was measured by ethanol precipitation as previously described (Boon et al., 2008) with some modifications. *S. maltophilia* strains were cultured in 100 ml of LB supplemented with 0.1% glucose (LB-glc) in the presence or absence of 200 μM Dip. After 48 h of incubation with shaking, the supernatants were collected by centrifugation at 14,000 r.p.m for 20 min. The pellets were dried overnight at 56°C before determination of dry weight of the biomass. After filter sterilization, supernatants were mixed with 2 volumes of absolute ethanol and incubated at −20°C overnight. The precipitated EPS were centrifuged and dried overnight at 56°C. Results were expressed as the amount of EPS (μg) relative to the dry weight of the biomass [EPS (μg)/Biomass (mg)]. The carbohydrate content in EPS was quantified by the phenol-sulfuric acid method (Dubois et al., 1956) with glucose as the standard, and the protein content was measured by the Bradford method (Bradford, 1976) using bovine serum albumin (Sigma-Aldrich) as standard.

Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopy

ATR-FTIR spectroscopy was performed with *S. maltophilia* ethanol-precipitated EPS samples. Infrared spectra were recorded using a Nicolet 380 FT-IR (Thermo Scientific Electron Corporation) with an ATR accessory equipped with a 45° single-reflection ZnSe prism. Thin particle films were prepared by placing a suspension of dried EPS in Milli-Q water (7.5 mg of EPS in 150 μl of water) on the ZnSe prism and drying for 60 min. Absorption spectra were recorded between 4000 and 900 cm^{-1} with a resolution of 4 cm^{-1} and co-addition of 32 scans. Recording of spectra, data storage and data processing were performed using the OMNIC software version 7.2 (Thermo Scientific Electron Corporation).

Microscopy Analysis of Matrix Exopolysaccharides

The presence of exopolysaccharides in the matrix of *S. maltophilia* biofilms was detected as previously described (Passerini de Rossi et al., 2009). Sterile microscope borosilicate coverslips were aseptically placed into Petri plates along with 15 ml TSB inoculated with *S. maltophilia* strains (10^6 CFU/ml) and incubated for 48 h. The coverslips were removed, rinsed with distilled water and stained in the dark with 0.1% calcofluor white dye (Fluorescent brightener 28, Sigma-Aldrich) for 10 min. Then, coverslips were rinsed, mounted on the microscope slides and examined with an Olympus BX50-DP73 microscope (Olympus, New York, USA). Images were obtained with a 40X lens objective. Calcofluor emissions were detected using a DAPI filter (excitation/emission wavelengths: 330–385/420 nm). The polysaccharide matrix fluoresces blue under the DAPI light filter.

Determination of SOD Isoenzymes

The determination of the SOD isoenzymes in *S.maltophilia* was performed using inhibition methods according to Dunlap and Steinman (1986). First, crude extracts were prepared from planktonic cultures. *S. maltophilia* cells from TSB or TSB-Dip 48 h-cultures were harvested by centrifugation, washed and suspended in 7 ml of PBS with 0.25 mM PMSF (Fluka Biochemika). Suspensions were sonicated using two 3 min pulses (Vibra cell, Sonics & Materials Inc. Danbury, Connecticut, USA) and centrifuged at 10,000 r.p.m. for 10 min at 4°C. The supernatant protein concentration was determined by the Bradford assay. Proteins were separated by using a 10% non-denaturing PAGE in the presence of 50 mM Tris, 300 mM glycine, and 1.8 mM EDTA at 120 V. Gel lanes were loaded with 20 μg total protein and purified MnSOD from *Escherichia coli* (Sigma-Aldrich) was used as standard. The metal present in the active site of SOD molecule was determined using inhibition methods. Gels were treated with 10 mM NaCN or 3.7 mM H_2O_2 to inactivate Cu/ZnSOD or FeSOD, respectively. Resistance to both cyanide and H_2O_2 is characteristic of MnSODs. SOD activity was visualized by staining with NBT as described by Beauchamp and Fridovich (1971). Briefly, after inhibition assays gels were incubated with shaking for 30 min in the dark in a solution of 500 μM NBT (Sigma Aldrich), 50 mM potassium phosphate buffer pH 7.8, 1 mM EDTA, 20 mM TEMED (Invitrogen, Carlsbad, CA, USA) and 30 μM riboflavin (Sigma-Aldrich). The gels were illuminated until achromatic zones indicating SOD activity were visible in a uniformly blue background.

SOD Activity Assay

The SOD activity was determined by the riboflavin/methionine system (Beauchamp and Fridovich, 1971) in crude extracts from planktonic (see Determination of the SOD isoenzymes) and biofilm cultures of *S. maltophilia* strains under iron replete (TSB) or iron restricted conditions (TSB-Dip). Biofilm crude extracts were obtained by using 12-well microtiter plates. Wells were filled with 3 ml of the standardized inoculum (ca. 10^6 CFU/ml). After 48 h of incubation, wells were aspirated, washed and then filled with 1 ml of PBS. Adherent cells were detached using a sterile cell scraper and the resulting cell suspension from 9 wells was centrifuged. The pellet was suspended in 1 ml of PBS with 0.25 mM PMSF. Then, crude extracts were obtained and their protein concentration was determined as described. For SOD activity determination, aliquots of 100 μl from each extract were treated with 300 μl of 13 mM methionine (Sigma-Aldrich), 100 μl of 1 mg/ml NBT, 300 μl of 100 nM EDTA, and 300 μl of 0.5 mM riboflavin in the presence of light. After 15 min, OD_{560} was determined. A unit of SOD was defined as the quantity of enzyme required to produce a 50% inhibition of NBT reduction. Activity was expressed as units of SOD per mg of protein [SOD (U)/Total protein (mg)].

Nitro Blue Tetrazolium (NBT) Assay

The NBT assay was performed as described by Aiassa et al. (2010) with the following modifications. *S. maltophilia* biofilms formed in 96-well microtiter plates under iron replete or iron restricted conditions, as described above, were washed two times with PBS, then 100 μl NBT (1 mg/ml) were added to each well. After incubation for 30 min at 37°C in the dark the reaction was stopped with 20 μl of 0.1 N HCl. Each well was treated with 50 μl of DMSO (Calbiochem, La Jolla, CA, USA) to extract the reduced NBT. Reduced NBT was measured as formazan blue at 540 nm. Results were expressed as reduced NBT relative to the biomass of biofilm normalized to cell density [NBT (OD_{546})/Biomass (CV/OD)].

SDS-PAGE of OMP-enriched Fractions

S. maltophilia cells from 48 h-cultures in TSB or TSB-Dip were harvested by centrifugation (15 min, 4°C, 10,000 r.p.m) and used for preparation of Sarkosyl-insoluble OMP-enriched fractions as described previously (Passerini De Rossi et al., 2003). SDS gel electrophoresis was carried out according to the Tris/tricine method (Schagger and Von Jagow, 1987). Gradient gels of 4–20% (1 mm thick) were used. Gels were stained with Coomassie brilliant blue R-250 (Sigma-Aldrich). A high molecular weight standard mixture for SDS gel electrophoresis (SDS-6H, Sigma-Aldrich) was used as molecular size standards.

Mass Spectrometric Analysis of Proteins

Selected bands were cut from the SDS-polyacrylamide gel stained with Coomassie blue and the proteins were subjected to enzymatic hydrolysis with 25 ng/μl trypsin. The samples were analyzed at the Laboratorio Nacional de Investigación y Servicios en Péptidos y Proteínas (LANAIS–PRO, CONICET-UBA). The sizes of the peptide fragments generated were determined by mass spectrometry on a LCQ Duo ESI/TRAP (Thermo Fisher) after separation by HPLC with a column Vydac RP-C18 (1.0 × 200 mm).The data were used to interrogate the National Center for Biotechnology Information nonredundant protein data bases by using the MASCOT MSMS program (Matrix Science) available at www.matrixscience.com.

DSF Production

The bioassay described by Barber et al. (1997) was generally followed. DSF production was assayed by measuring the restoration of endoglucanase activity to the *rpfF* mutant Xcc 8523 by extracts from culture supernatants. To obtain the extracts, *S. maltophilia* strains were grown in Stainer-Scholte (SS) minimal medium plus 0.1% casamino acids (Difco laboratories, Detroit, MI, USA) (SSC, iron-limited condition) and in SSC supplemented with 100 μM $FeCl_3$ (iron rich condition). After 48 h of incubation the final culture density was adjusted to an OD_{540} of 1.00 to avoid variations due to differences in bacterial growth generated by iron supplementation. Then, the cells were removed by centrifugation, the supernatants were acidified to pH 3.0 with chlorhydric acid and loaded onto 30 mg Oasis MAX columns (Waters Oasis® Milford, USA). The column was washed with 5 mM sodium acetate:methanol (95:5) and elution was performed with methanol followed by 2% acetic acid in methanol. The extracts were evaporated and suspended in 500 μl sterile water for the quantitative assay for the DSF. Diameters of zones of carboxymethyl cellulose (CMC; Sigma-Aldrich) hydrolysis, produced by 50 μl of extract, were measured and converted to relative endoglucanase units with a standard

curve constructed using dilutions of a standard of cellulase I (Sigma Aldrich). One unit of endoglucanase was defined as the amount which gave a hydrolysis zone of 12 mm diameter.

Galleria Mellonella Killing Assay

The virulence of *S. maltophilia* strains was evaluated by infecting larvae of the wax moth *G. mellonella* as described by Seed and Dennis (2008) with some modifications. First, in order to establish the optimal inoculum of *S. maltophilia* required to kill *G. mellonella* over 24–96 h, caterpillars of 250–350 mg in weight with a cream-colored cuticle, were inoculated with 10 μl of K279a suspensions containing 10^4, 10^5, and 10^6 CFU. Bacterial suspensions were obtained from overnight TSA cultures. The cells were washed twice with physiological saline and diluted to obtain the different inocula. The concentration of each inoculum was confirmed by colony counting on TSA plates. Bacteria were injected into the hemocoels of the caterpillars via a left proleg using 20 μl Hamilton syringes. Caterpillars were incubated in Petri dishes lined with filter paper at 30°C for 96 h and scored for survival daily. Insects were considered dead when they displayed increased melanization and failed to respond to touch. Second, *G. mellonella* killing assays were performed on K279a and F60 using the determined optimal inoculum. In all experiments, 12 caterpillars were used for each condition, including a control group of caterpillars inoculated with physiological saline to monitor for killing due to physical trauma. Experiments that had more than one dead caterpillar in the control group were discarded and repeated. Survival curves were plotted using the Kaplan-Meier method, and differences in survival were calculated by using the log-rank test (Graph Pad Prism version 5.0, Software Inc., La Jolla, CA).

Statistical Analysis

All experiments were performed at least in triplicate and repeated on three different occasions. Statistical analysis was performed using GraphPad InStat version 3.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Results were analyzed by One-Way ANOVA with Dunnett's post-test, and differences were considered significant at $p < 0.05$.

Results and Discussion

In order to assess the role of iron on the production of biofilms and factors potentially involved in biofilm formation and virulence of *S. maltophilia* the following studies were performed under iron-limiting and iron-replete conditions. Iron-dependent gene regulation in bacteria is generally mediated by the Fur system (Escolar et al., 1999). To our knowledge, *S. maltophilia fur* mutants have not yet been obtained. Thus, we carried out the manganese-induced mutagenesis of the wt strain K279a to select *fur* mutants and test the possible role of Fur in iron dependent regulation of these factors.

Isolation of *S. maltophilia fur* mutants

The genome of *S. maltophilia* K279a (GenBank: AM743169.1) revealed a gene SMLT_RS09600 (old locus tag Smlt1986, putative *fur* gene) encoding a 135 amino acid Fur family transcriptional regulator.

Sequence similarity searches of the available nucleotide databases were performed with the BLASTN program (http://www.ncbi.nlm.nih.gov/blast). The putative *fur* gene is highly conserved in *S. maltophilia*, SMLT_RS09600 from K279a is 99–96% identical to putative *fur* genes of *S. maltophilia* strain 13637 (Accession: CP008838, region: 2056865–2057272), *S. maltophilia* JV3 (Accession: CP002986, region: 1789094–1789500), and *S. maltophilia* R551-3 (Accession: CP001111, region: 1790286–1790693). Furthermore, K279a putative *fur* gene is 86% identical to Xcc ATCC 33913 *fur* (XCC1470) and is 76% identical to *Pseudomonas aeruginosa* PAO1 *fur* (PA4764).

These data prompted us to carry out the manganese selection technique (Hantke, 1987) to select spontaneous *fur* mutants of *S. maltophilia*. This method has been successfully used to isolate *fur* mutants in other Gram-negative bacteria (Hantke, 1987; Passerini De Rossi et al., 2003). A total of 51 independent clones from K279a were obtained on manganese LB agar. A clone, named F60, showed constitutive siderophore production. To discard reversion back to the wild type phenotype, the mutant was tested for the deregulated phenotype on CAS agar plates supplemented with iron several additional times with consistent results.

With the aim of confirming the presence of mutations in F60, a pair of primers were designed to PCR amplify the full-length *fur* gene and 100 bp upstream from the start codon. Sequenced PCR products revealed a point mutation (T→C) 65 bp upstream of the ATG initiation codon, in the promoter region located within the −10 sequence.

Iron as a Signal for Biofilm Formation

In order to assess the role of iron on *S. maltophilia* biofilm formation, K279a and its *fur* mutant were grown for 48 h in TSB in the presence or absence of Dip in polystyrene microtiter plates. The growth yield of F60 in these media was similar to that of K279a (data not shown). Biofilm formation was quantified by crystal violet (CV) staining. **Figure 1A** shows that K279a was significantly more efficient in producing biofilms in the presence of Dip than in TSB ($p < 0.05$). On the other hand, under iron-replete conditions the amount of biofilm produced by the *fur* mutant was higher than that of the wt strain ($p < 0.05$), and the addition of the iron chelator did not affect its biofilm production.

To further investigate the role of iron on biofilm formation, CLSM was used to analyze the architecture of the 48 h-biofilms stained with Syto9. **Figure 2A** shows confocal images acquired from K279a and F60 biofilms. K279a biofilm grown in TSB presented a confluent growth with microcolonies scattered on the surface (x–y plane) and the *z*-projection of the *x*–*y* stacks revealed variable thickness. In the presence of Dip, K279a produced a much more compact biofilm with enhanced thickness. On the other hand, F60 biofilms grown under both iron conditions showed larger microcolonies and were thicker than biofilms from K279a grown in TSB. **Figure 2B** shows three-dimensional reconstructions obtained from confocal stack images using the ImageJ program. The architecture of biofilms consisted of peaks interlaced with water channels. K279a biofilm presented peaks with a range of 20–30 μm in height while iron restriction resulted in the formation of taller peaks up to 50 μm. Irrespective of

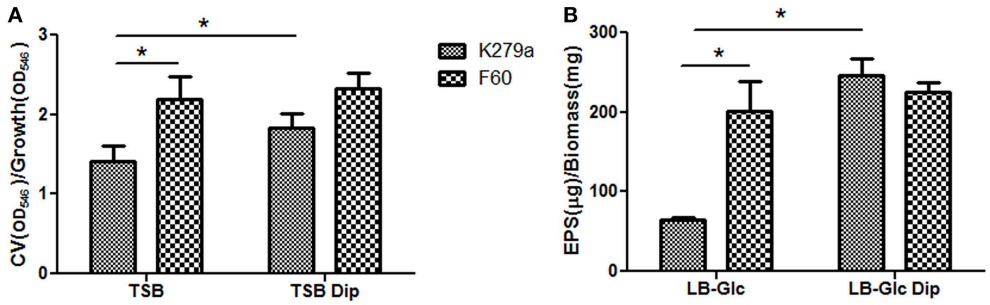

FIGURE 1 | Biofilm formation and production of EPS by *S. maltophilia* K279a and its Fur mutant. (A) Biofilm formation assessed by microplate colorimetric assay. Biofilms were grown in TSB with or without 200 µM Dip for 48 h in static 96-well polystyrene plates. The biomass of biofilms was quantified by CV staining and expressed relative to the final culture density. **(B)** Production of EPS. *S. maltophilia* K279a and F60 were cultured in LB supplemented with 0.1% glucose (LB-glc) in the presence or absence of 200 µM Dip for 48 h at 35°C. Supernatants were mixed with 2 volumes of absolute ethanol and the precipitated EPS were dried overnight at 56°C before determination of dry weights. Results were expressed as the amount of EPS relative to the dry weight of the biomass. Results represent the mean ± standard deviation of one representative experiment. Asterisks indicate significant difference (*$p < 0.01$).

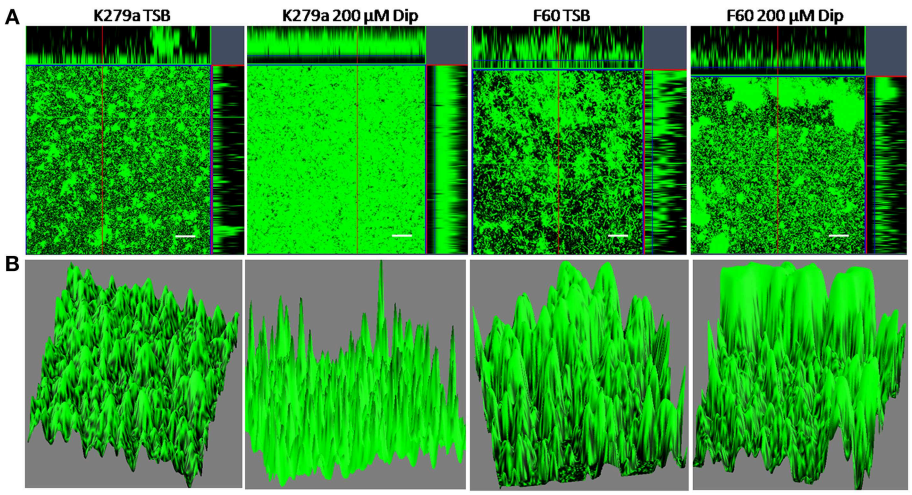

FIGURE 2 | CLSM analysis of biofilms from *S. maltophilia* K279a and its Fur mutant. Biofilms were grown in glass LabTek chambers, in TSB and TSB supplemented with 200 µM Dip for 48 h. Then, biofilms were stained with Syto9 and analyzed by CSLM, using a 40X water immersion objective lens. **(A)** Central panels represent the x–y plane, and the top and right side panels represent the x–z and y–z planes, respectively. Scale bars represent 20 µm. **(B)** Three-dimensional projections of biofilm structure were reconstructed using the Image J program. The images are representative of those obtained on three independent occasions.

culture conditions F60 formed biofilms with more and taller peaks of up to 90 µm in height.

Since the growth yield of F60 was similar to that of K279a, the phenotypic differences observed between these strains were not due to a growth defect. Our results put in evidence that iron levels regulate, possible through the Fur system, *S. maltophilia* biofilm formation and architecture.

Iron limitation induces or inhibits biofilm formation depending on the species (Wu and Outten, 2009). The presented data show that in *S. maltophilia* iron restriction induces biofilm formation, a similar behavior to that previously reported for *Legionella pneumophila*, *Acinetobacter baumannii* and *Staphylococcus aureus* (Tomaras et al., 2003; Johnson et al., 2005; Hindre et al., 2008; Modarresi et al., 2015).

Effect of Iron on Extracellular Polymeric Substances Production and Chemical Composition

The production of extracellular polymeric substances (EPS) in planktonic cultures grown under iron-limiting and iron-replete conditions was quantified by ethanol precipitation. Results showed that F60 produces 3-fold higher amounts of EPS than K279a, and the presence of Dip improved the EPS production only in the wt strain (**Figure 1B**). Hence, iron limitation, likely through Fur, increased EPS production in *S. maltophilia*. This is in accordance with the enhanced alginate production detected in *P. aeruginosa* under iron limitation (Wiens et al., 2014).

The EPS of the majority of bacterial biofilms including *P. aeruginosa* consists mainly of polysaccharides, proteins,

TABLE 1 | Macromolecular composition of EPS extracted from *S. maltophilia* strains.

Strains	EPS (μg EPS/mg Biomass)	Chemical analysis of EPS*		
		Carbohydrates (mg glc/mg EPS)	Proteins (mg proteins/mg EPS)	Carbohydrate/Protein (mg glc/mg protein)
K279a	64.9 ± 3.3	0.25 ± 0.02	0.49 ± 0.03	0.51
F60	201.1 ± 38.0	0.26 ± 0.05	0.52 ± 0.05	0.50

*Results represent the mean ± standard deviation of one representative experiment. *Carbohydrates and proteins were determined by the phenol-sulfuric acid method (Dubois et al., 1956) and the Bradford method (Bradford, 1976), respectively.*

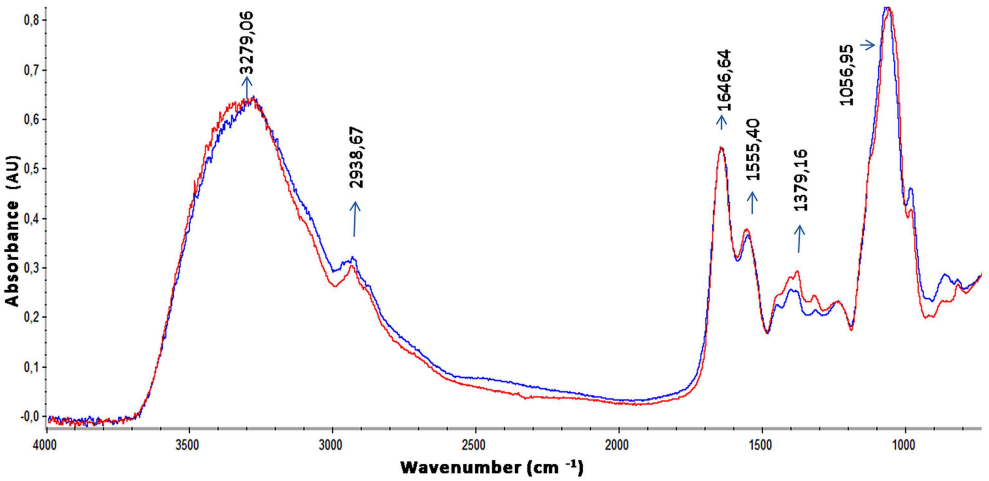

FIGURE 3 | ATR-FTIR spectra of EPS from *S. maltophilia* strains. Spectra of ethanol precipitated EPS from K279a and F60 are shown in blue and in red, respectively. Bands associated with lipids (3100–2800 cm^{-1}), proteins (1700–1500 cm^{-1}) mainly identified by the amide I (1646,64 cm^{-1}) and amide II bands (1555,40 cm^{-1}), and polysaccharides and nucleic acids (1300–900 cm^{-1}) are indicated in the spectra.

and nucleic acids (Laverty et al., 2014). The chemical composition of the *S. maltophilia* EPS fractions was assessed by measuring the total carbohydrate and protein contents (**Table 1**). Interestingly, EPS from K279a and F60 had a similar carbohydrate/protein ratio (∼0.50). To gain more insight, at the biochemical composition level, the EPS of both strains were also compared using ATR-FTIR spectroscopy. **Figure 3** shows the bands associated with lipids (3100–2800 cm^{-1}), proteins (1700–1500 cm^{-1}), and polysaccharides and nucleic acids (1300–900 cm^{-1}) (Jiao et al., 2010). The FTIR spectra revealed no significant variations in relative abundance of the components between the two EPS fractions. These results put in evidence that F60 produces a higher amount of EPS than K279a in the absence of Dip, but with a similar biochemical composition.

The EPS matrix is important in forming the biofilm architecture and in protecting bacteria from antimicrobials and host defense mechanisms. Exopolysaccharides, fundamental components of EPS, are recognized as virulence factors (Cescutti et al., 2011; Laverty et al., 2014). In order to detect the presence of exopolysaccharides in the matrix of *S. maltophilia* biofilms samples stained with calcofluor white were examined with epifluorescence microscopy. **Figure 4** shows micrographs from K279a and F60 biofilms formed in TSB and TSB-Dip. Samples stained with calcofluor white showed that the cells and microcolonies of both strains attached to borosilicate were embedded within a blue fluorescent material. According to the higher amounts of EPS produced by F60, the mutant formed more compact structures, resembling cotton wool, than K279a. Again, under iron-restricted conditions, the biofilm from K279a showed the presence of EPS as an almost continuous sheet.

We have previously reported that binding of calcofluor white indicates that β-linked polysaccharides, such as cellulose and chitin, are part of the matrix of *S. maltophilia* biofilms (Passerini de Rossi et al., 2007). This is in accordance with the presence of many β-1-4 unions in the primary structure of *S. maltophilia* exopolysaccharides characterized by Cescutti et al. (2011) from planktonic cultures of two mucoid clinical isolates obtained from two CF patients. This is a novel structure among bacterial polysaccharides: It has three uronic acid residues on a total of four sugars in the repeating unit and bears an additional negative charge due to the d-lactate substituent. The authors suggested that the abundance of negative charges, a common feature of the exopolysaccharides produced by two other bacteria infecting CF patients (*P. aeruginosa* and *Inquilinus limosus*), is a characteristic which somehow constitutes an advantage for the microbes in the lung environment.

Xiao et al. (2012) reported that exopolysaccharides modulate the development and spatial distribution of microcolonies in *Streptococcus mutans* biofilms. These authors suggested that individual microcolonies encased in polysaccharides may serve

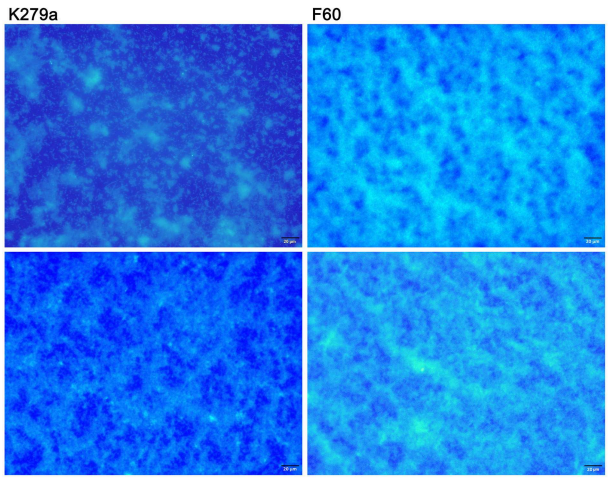

FIGURE 4 | Microscopy detection of matrix exopolysaccharides in *S. maltophilia* biofilms. K279a and F60 biofilms formed on borosilicate coverslips in TSB (upper panel) or TSB-Dip (lower panel) for 48 h were stained with calcofluor white and examined by epifluorescence microscopy. The polysaccharide matrix fluoresces blue under the DAPI light filter. Calcofluor stains cells as discrete points while EPS is visible as a continuous sheet. Biofilms were viewed at 400X magnification.

as architectural units that become connected during biofilm construction, forming compartmentalized networks that confer highly heterogeneous yet cohesive environments within the 3D architecture. CLSM analysis demonstrated that, under iron restriction, K279a produces a much more compact biofilm with enhanced thickness and 3D organization, similar to that of F60 biofilms. These results could be in part due to an increment in EPS production.

Role of Iron on the Oxidative Stress Response of *S. maltophilia*

S. maltophilia is an aerobic bacterium which generates reactive oxygen species (ROS) during metabolism. Aerobic bacteria prevent the oxidative stress by producing antioxidant enzymes including superoxide dismutases (SODs). Superoxide dismutases is a family of three metalloenzymes containing manganese (MnSOD) or iron (FeSOD) or both copper and zinc (Cu/ZnSOD) cofactors. FeSOD and MnSOD are generally evidenced in prokaryotes (Fridovich, 1978).

First, we decided to determine the type of SOD isoenzymes produced by *S. maltophilia* K279a using inhibition methods (Dunlap and Steinman, 1986). Crude extracts of planktonic cells of *S. maltophilia* K279a cultured in TSB or TSB-Dip were separated by using a 10% non-denaturing PAGE. A single band was seen under both growth conditions and inhibition experiments showed that MnSOD is the only SOD isoenzyme present since this band was inhibited neither by H_2O_2 nor by NaCN (data not shown). The mobility of the K279a MnSOD differs from that of the MnSOD purified from *E. coli* used as a reference; this fact could reflect some structural differences between them. Analysis of the *S. maltophilia* K279a genome (GenBank: AM743169.1) showed the presence of five *sod* genes, two of them, SMLT_RS13450 and SMLT_RS15415 coding for putative MnSODs. This was the only isoenzyme detected under the experimental conditions used in this study. Our result is in accordance with the report of the expression of only MnSOD by the quinclorac-degrading strain *S. maltophilia* WZ2 (Lü et al., 2009). This fact allowed us to study the role of iron in SOD regulation by determining the total SOD activity by the riboflavin/methionine system (Beauchamp and Fridovich, 1971).

Comparative studies of SOD activity were conducted on crude extracts obtained from biofilms and planktonic cultures of K279a and F60 in the presence or absence of Dip (**Figure 5A**). The use of 12-well microtitre plates instead of 96-well plates for biofilm formation allowed the recovering of larger amounts of biomass needed for the assay. The crude extracts obtained from the biofilm of K279a in the absence of Dip showed the lowest total SOD activity (12.06 ± 0.5 U/mg of protein), and iron deprivation produced a significant increase in SOD activity (19.6 ± 1.4 U/mg of protein). On the other hand, the SOD activity in crude extracts of F60 biofilms obtained in the presence or absence of Dip (22.06 ± 0.7 and 18.20 ± 0.5 U/mg of protein, respectively) was higher than that of K279a cultured in the absence of Dip. The SOD activity from planktonic cultures of K279a and F60 under both conditions showed the same behavior. In conclusion, these results demonstrate that SOD activity in *S. maltophilia* is negatively regulated by iron, likely through Fur. In many organisms Fur regulates the expression of the *sodA* gene which encodes MnSOD. Our results are in agreement with those of Hassett et al. (1996) who reported that, the addition of Dip to the wt *P. aeruginosa* PAO1 produced an increase in MnSOD activity, similar to the *fur* mutant phenotype. Interestingly, the SOD activity of biofilms was lower than that of the respective planktonic counterparts, a fact that could be due to the lower metabolic activity of biofilms.

Finally, the production of ROS by *S. maltophilia* in biofilms grown under iron replete or iron restricted conditions was evaluated by the NBT assay (Aiassa et al., 2010). **Figure 5B** shows that the biofilm of K279a cultured in the presence of Dip had 2.5-fold lower levels of ROS than biofilms cultured in TSB. The biofilms of F60 formed under both conditions showed levels of ROS similar to that of K279a cultured in TSB-Dip. These results are in concordance with the respective levels of SOD activity in the biofilm (**Figure 5A**). Thus, the formation of biofilms under low iron conditions led to low ROS generation. A similar result was obtained when the production of ROS was determined in supernatants of planktonic cultures (data not shown). One limitation of these results is that the methodology used for evaluating ROS production is not quite specific. Further studies with specific fluorescent probes for detection of superoxide radicals or hydrogen peroxide, as well as electron spin resonance (ESR) spectrometry, are needed to quantify and identify these radicals.

Identification of Iron Regulated OMPs by Mass Spectrometry

Under iron-limiting growth conditions many bacteria express high affinity systems to scavenge this metal from different sources. In Gram-negative bacteria specific outer membrane

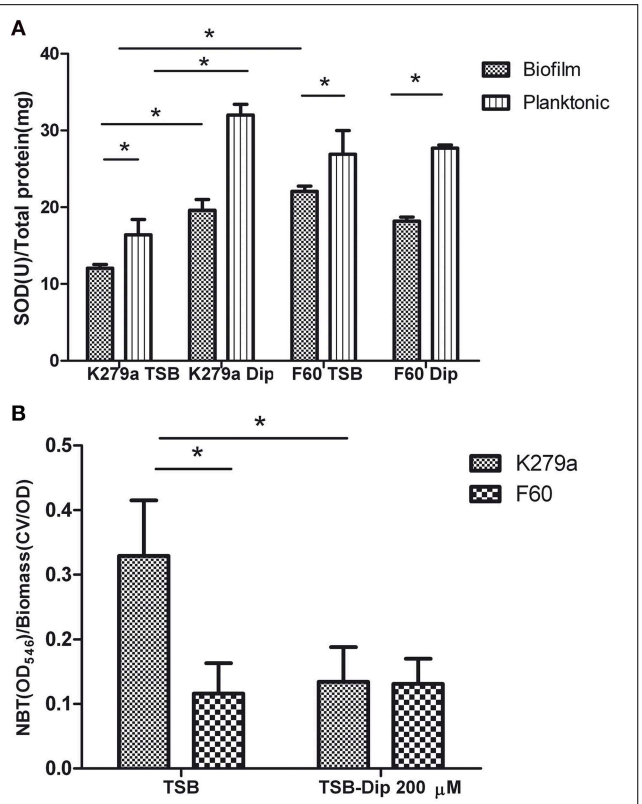

FIGURE 5 | SOD activity and ROS levels in *S. maltophilia* K279a and F60. Bacterial cultures were performed in TSB and TSB supplemented with 200 μM Dip for 48 h **(A)** SOD activity of crude extracts from plaktonic and biofilms cells determined by the riboflavin/methionine system, as described in the text. Activity was expressed as units of SOD per mg of protein. **(B)** Levels of ROS in biofilms of *S. maltophilia*. The biofilms formed in microtiter plates were treated with NBT. Reduced NBT was measured as formazan blue at 540 nm and expressed relative to the biomass of biofilm. Results represent the mean ± standard deviation of one representative experiment. Asterisks indicate significant difference (*$p < 0.01$).

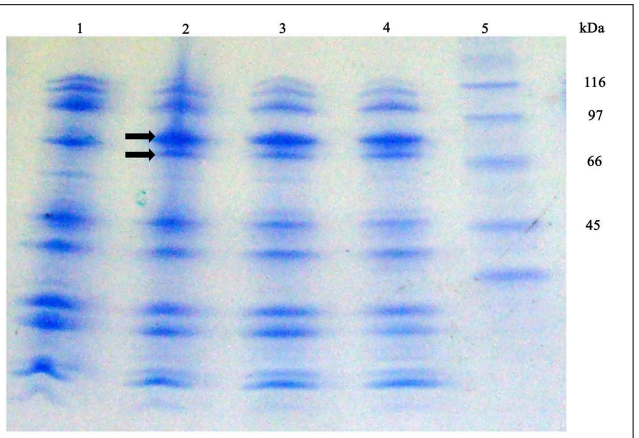

FIGURE 6 | SDS-PAGE profiles of Sarkosyl-insoluble OMP-enriched fractions from *S. maltophilia* K279a and F60. Bacterial cultures were performed in TSB and TSB supplemented with 200 μM Dip for 48 h. Outer membrane proteins were extracted as described in the text. Proteins from Sarkosyl-insoluble OMP-enriched fractions (10 μg per lane) were separated by SDS gel electrophoresis according to the Tris/tricine method (Schagger and Von Jagow, 1987) using gradient gels of 4–20%. Gels were stained with Coomassie blue. Lane 1, K279a (TSB); 2, K279a (TSB-Dip); 3, F60 (TSB); 4, F60 (TSB-Dip), and 5: molecular mass markers in kDa. Arrows show two bands, in the range of 70–90 kDa, regulated by iron: Band 1 (higher MW) and Band 2.

receptors bind FeIII-siderophore complexes, which are generally internalized into the periplasm across the outer membrane with energy transduced by the TonB system (TonB/ExbB/ExbD complex) from the cytoplasmic membrane (Braun, 1995). Furthermore, many of these genes are repressed by Fur (Crosa, 1997).

With the purpose of detecting *S. maltophilia* iron-repressed outer membrane proteins (IROMP) Sarkosyl-insoluble OMP-enriched fractions from K279a and F60, grown in TSB and TSB-Dip, were compared by SDS-PAGE (**Figure 6**) Under iron starvation, the SDS-PAGE profile from K279a presented a pattern of two iron-repressed proteins in the range of 70–90 kDa. Band 1 (higher MW) appeared to be stronger under low-iron conditions while Band 2 was only seen when K279a was grown with Dip. The SDS-PAGE profiles from F60, cultured in both conditions, were similar to that of K279a grown with Dip.

Subsequently, Band 1 and Band 2 from K279a were analyzed by mass spectrometry and identified based on the analysis of their mass spectra by Mascot (www.matrixscience.com). Band 1 was matched to the outer membrane receptor FepA from *S. maltophilia* K279a (gi|190573428, YP_001971273, score: 283, protein sequence coverage: 57%). Reference sequence YP_001971273 has been replaced by WP_012479564. The sequence YP_001971273 is 100% identical to WP_012479564 over its full length (749 aa). SMLT_RS06850 (gi|190572091:1472622-1474871) codifies WP_012479564, a protein with a calculated mass of 80.67 KDa.

Band 2 closest match was a colicin I receptor from *S. maltophilia* K279a (gi|190575965, YP_001973810, score: 419, protein sequence coverage: 60%). The old locus tag Smlt4135 (gi|190010013:4245161-4247299) codifies for the putative precursor of colicin I (also known as CirA, YP_001973810), a protein of 712 aa. This locus have been replaced by SMLT_RS19685 (gi|190572091:4245254-4247299) which codifies for the TonB-dependent receptor WP_044570913, a protein of 681 aa with a calculated mass of 73.95 KDa. The sequence YP_001973810 is 100% identical to WP_044570913 over the shared 681 aa.

A BLAST search indicated that the *S. maltophilia* WP_012479564 protein displays closest homology to the outer membrane receptor FepA of *Xanthomonas citri* (Accession: WP_011052402, 66% identity), *P. aeruginosa* (Accession WP_003111874, 50% identity) and *E. coli* (Accession: ADB98042, 47% identity). On the other hand, WP_044570913 showed significant homology to a TonB-dependent receptor of *Pseudomonas putida* (Accession: WP_043199708, 55% identity), a putative colicin I receptor of *Acinetobacter* sp. WC-323 (Accession: EKU56436, 48% identity), a colicin I receptor of *Acinetobacter guillouiae* (Accession: BAP36722, 47% identity), and a colicin IA outer membrane receptor and translocator, ferric

iron-catecholate transporter of *E. coli* str. K-12 substr.MG1655 (Accession: NP_416660, 34% identity).

Therefore, by using SDS-PAGE followed by mass spectrometry we identified two IROMPs, WP_012479564, and WP_044570913, which are TonB-dependent receptors. *E. coli* K-12 possesses at least five TonB-dependent receptors for the uptake of different siderophores, including FepA and Cir (also termed CirA). All of them consist of a 22-β-strand barrel formed by ca. 600 C-terminal residues, while ca. 150 N-terminal residues fold inside the barrel to form a hatch, cork or plug domain. The plug domain acts as the channel gate, blocking the pore until the channel is bound by ligand. At this point it undergoes conformational changes which opens the channel (Miethke and Marahiel, 2007). Consequently, we decided to search the protein accession numbers obtained through MASCOT in the conserved domain databases (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The domain hits for WP_012479564 were PRK13524 (multi-domain, Accession PRK13524), outer membrane receptor FepA, for the interval of 28-749 aa (*E*-value 0e+00), and the ligand_gated_channel (Accession cd01347) corresponding to TonB dependent/Ligand-Gated channels in the interval of 52-749 aa (*E*-value 6.06e–122). Furthermore, in the N-terminal plug of ligand_gated_channel (residues 52–171) 54 of 54 of the residues that compose this conserved feature have been mapped to the query sequence. With respect to WP_044570913, the domain hits were FepA (multi-domain, Accession COG4771), outer membrane receptor for ferrienterochelin and colicins, for the interval of 45–681 aa (*E*-value 0e+00), and the ligand_gated_channel (Accession cd01347) in the interval of 66–681aa (*E*-value 2.07e–115). Besides, in the N-terminal plug of ligand_gated_channel (residues 60–172) 54 of 54 of the residues that compose this conserved feature have been mapped to the query sequence. Another hit was PRK13483 (Accession PRK13483) corresponding to enterobactin receptor protein in the interval of 43–681 aa (*E*-value 2.74e–142).

In addition, the SignalP 4.0 program (http://www.cbs.dtu.dk/services/SignalP-4.0) predicts a cleavable N-terminal signal sequence for both *S. maltophilia* proteins, with a potential cleavage site at amino acid 29 for WP_012479564 and at amino acid 35 for WP_044570913.

Since several OMPs which are TonB-dependent receptors are encoded by *fur*-repressed genes (Andrews et al., 2003), the presence of putative Fur-binding sites was searched in the upstream regions of SMLT_RS06850 and SMLT_RS19685, coding for WP_012479564 and WP_044570913, respectively. Firstly, −10 and −35 elements similar to the *E. coli* consensus promoter sequences were identified in the upstream regions (−200 to +21 relative to the start codon) of both genes by using BPROM (Softberry, Inc.) (**Figure 7A**). Secondly, for the detection of potential Fur boxes in *S. maltophilia* genes a scoring matrix was defined from the 43 Fur binding sites characterized in other bacteria (Zaini et al., 2008) using the MEME tool (http://meme-suite.org). Then, the *S. maltophilia* K279a genome (GenBank: AM743169.1) was analyzed with the resulting scoring matrix using the MAST tool (http://meme-suite.org/tools/mast). Twenty putative *S. maltophilia* Fur boxes ($p < 10e^{-4}$) were detected with MAST tool. **Figure 7A** shows

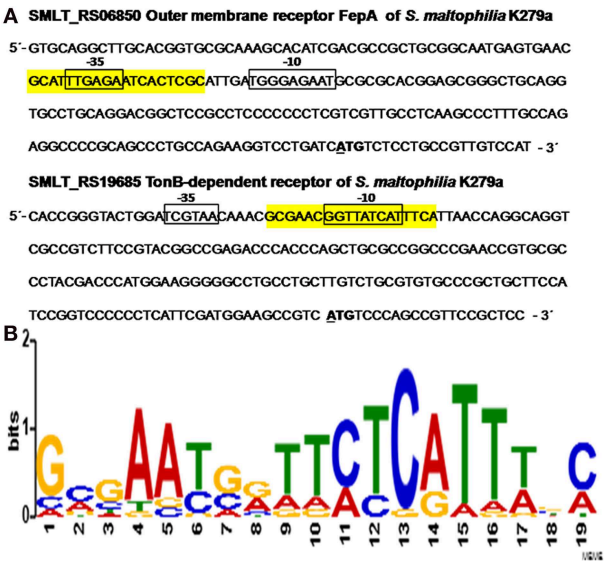

FIGURE 7 | *In silico* analysis of putative Fur boxes of *S. maltophilia* K279a iron regulated genes. (A) Upstream regions (−200 to +21) of SMLT_RS06850 and SMLT_RS19685, coding for the outer membrane receptor FepA and a TonB-dependent receptor, respectively. The -35 and -10 sites of the predicted promoters are shown in boxes and the +1 transcription start sites are in bold and underlined. The putative Fur boxes of SMLT_RS06850 (−144 to −125) and SMLT_RS19685 (−175 to −156) are highlighted in yellow. **(B)** Sequence logo of the putative Fur box of *S. maltophilia*. Detection of potential Fur boxes present in the genome of *S. maltophilia* (GenBank: AM743169.1; (Crossman et al., 2008) was performed with 43 Fur boxes previously characterized (Zaini et al., 2008) using MEME tool. A consensus was built from 20 putative *S. maltophilia* Fur boxes detected with MAST tool.

the putative Fur boxes of SMLT_RS06850 (−144 to −125, 5′ GCATTTGAGAATCACTCGC 3′) and SMLT_RS19685 (−175 to −156, 5′ GCGAACGGTTATCATTTCA 3′) identified in a region comprising the −35 element and the −10 element of the respective promoters. In *E. coli,* the iron-bound Fur binds to a well-conserved consensus sequence, known as the Fur box, located in the promoter region of genes directly repressed by Fur (Escolar et al., 1999). Therefore, our results suggest that Fur could regulate the expression of the studied *S. maltophilia* genes in response to iron availability. However, the two identified sequences do not appear to be particularly well conserved since they are identical in 11 over 19 bases to the Fur-box consensus sequence 5′ GATAATGATAATCATTATC 3′ of *E. coli* (de Lorenzo et al., 1987). Escolar et al. (1999) proposed that the intracellular iron concentration and the variability and extension of the sequences targeted by Fur could cause a wide range of responses in each specific case. Some genes undergo mild regulation or coregulation by iron, while others are subjected to a strong repression/induction switch. **Figure 7B** shows a consensus *S. maltophilia* FUR motif built from 20 putative Fur boxes detected within the 200 bp region upstream a start codon with MAST tool.

With respect to protein functions, in *E. coli* K-12 FepA and CirA are the catecholate siderophore OM receptors for

the uptake of Fe-enterobactin and linear Fe-enterobactin degradation products such as dihydroxybenzoyl serine, respectively (Buchanan et al., 2007; Miethke and Marahiel, 2007). We have previously reported the optimization of the chrome azurol S agar assay, based on the addition of Casamino acids and Dip to the CAS medium, for the detection of siderophores in *S. maltophilia*. Moreover, K279a and all local nosocomial isolates studied produced only catechol-type siderophores (Garcia et al., 2012). Interestingly, Ryan et al. (2009) suggested that *S. maltophilia* K279a and R551-3 produce the catechol-type compound enterobactin based on their sequenced genomes. In the genome of *S. maltophilia* K279a, SMLT_RS13395, SMLT_RS13400, SMLT_RS13415, and SMLT_RS13420 encode putative enterobactin synthasa components. According to these data, our results put in evidence the presence of WP_012479564 and WP_044570913, which are TonB-dependent receptors homologs to those related to the uptake of Fe-enterobactin. We are currently performing studies to structurally characterize *S. maltophilia* siderophores.

Little is known about *S. maltophilia* iron uptake systems. Huang and Wong (2007) identified a homolog of the ferric citrate receptor FecA in *S. maltophilia* WR-C, which was induced in the iron-depleted medium supplemented with a low concentration of ferric citrate. Interestingly, their results suggest that this TonB-dependent receptor is regulated by the *rpf*/DSF cell-cell communication system. Consequently, we are going to investigate whether the two iron regulated TonB-dependent receptors described in this study are also regulated by *S. maltophilia* quorum sensing.

Effect of Iron on DSF Production

The effect of iron on DSF production was evaluated through the bioassay described by Barber et al. (1997). *S. maltophilia* strains were grown in SSC (iron-limited condition) and in SSC supplemented with $FeCl_3$, and DSF present in the supernatants was extracted with Oasis MAX columns. The iron-limited condition cannot be achieved by the addition of Dip, since the chelator present in the extracts inhibits the growth of the reporter strain. **Figure 8** shows that the wt strain under the iron-limited condition produced significantly higher amounts of DSF measured as endoglucanase units (18.7 ± 0.6 U) than under the iron rich condition (16.2 ± 0.4 U). On the other hand, the mutant F60 under both conditions, produced higher amounts of DSF (17.9 ± 0.5 and 18.1 ± 0.5 U, respectively) than K279a cultured with 100 μM $FeCl_3$. In conclusion, iron, probably through the Fur system, negatively regulates DSF production.

Previous studies, mentioned in the introduction, reported a relationship between Fur and the QS system. In some Gram negative bacteria iron limitation enhances AHL production. This is the first report of the regulation of DSF, an unusual QS signal, by iron, probably through the Fur system.

Virulence of *S. maltophilia* K279a and F60 in the *Galleria mellonella* Infection model

G. mellonella has been utilized to study host-pathogen interactions in bacteria, including *Burkholderia cepacia* and *S. maltophilia* (Seed and Dennis, 2008; McCarthy et al., 2011; Nicoletti et al., 2011). *G. mellonella* caterpillars have a humoral immune response which involves melanization and production of antimicrobial peptides, and a cellular response which includes phagocytosis (Hoffmann, 1995). *G. mellonella* is an attractive alternative infection model since its innate immune system shares a high degree of structural and functional homology to that of mammals.

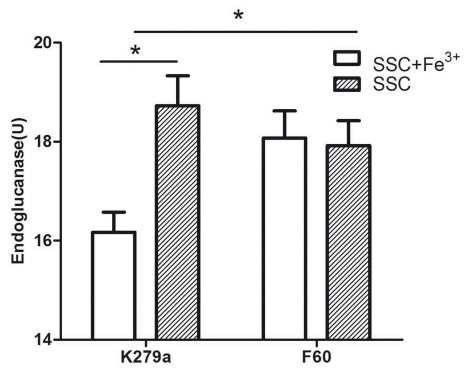

FIGURE 8 | DSF production by *S. maltophilia* strains grown under iron-limited (SSC) and iron rich conditions (SSC-100 μM $FeCl_3$). DSF production was assayed by measuring the restoration of endoglucanase activity to the Xcc *rpfF* mutant strain 8523 by culture supernatant extracts obtained using Oasis MAX columns. The results were expressed as units of endoglucanase. Results represent the mean ± standard deviation of one representative experiment. Asterisks indicate significant difference (*$p < 0.01$).

The influence of the Fur system in *S. maltophilia* virulence was evaluated by infecting larvae of *G. mellonella*. First, we established the optimal inoculum size of *S. maltophilia* required to kill *G. mellonella* over 24 to 96 h by injecting larvae with 10 μl of K279a suspensions containing 10^4, 10^5, and 10^6 CFU. The killing was significantly dependent on the number of *S. maltophilia* cells injected. Inoculation of larvae with 10^4 CFU of K279a did not produce the killing of any of them after 96 h of infection, whereas the 10^6 CFU/larva inoculum resulted in the rapid killing of more than 90% of caterpillars within 24 h. On the other hand, the dose of 10^5 CFU/larva which produced a progressive death of caterpillars over the incubation time was chosen as the optimal inoculum (data not shown). Then, *G. mellonella* killing assays were performed with K279a and F60. **Figure 9** shows the corresponding survival curves of a single representative trial. Infection of caterpillars with K279a resulted in 20% of death after 48 h of inoculation, and 43% of death after 96 h, a response significantly different from that obtained with the mutant F60 ($p < 0.001$) which was able to kill 62% of caterpillars after 48 h of inoculation and reached 81% of death after 96 h. No dead caterpillars were detected in the control group. Therefore, the *fur* mutation led to increased virulence to *G. mellonella* larvae, compared to that of its isogenic parental strain, which could be due to the pleitropic effects of this mutation observed in the present study, including increased biofilm formation, and EPS and SOD production.

Very little is known about the pathogenic mechanisms of *S. maltophilia. One* study using the *G. mellonella* infection model suggests that the major extracellular protease StmPr1 may be a

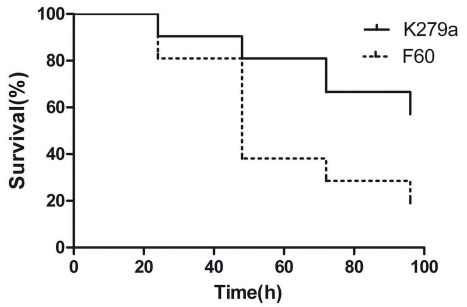

FIGURE 9 | Virulence of *S. maltophilia* K279a and F60 in the *Galleria mellonella* infection model. Kaplan-Meier survival curves of *G. mellonella* larvae after 96 h from injection with 1×10^5 UFC/larva of K279a and F60 are shown. Each data set corresponds to a single representative trial with the specified strain ($n = 12$). Differences in survival were calculated using the log-rank test for multiple comparisons and were considered statistically significant at $p < 0.05$. No more than one control larvae injected with sterile physiological saline died in any given trial (not shown).

relevant virulence factor of *S. maltophilia* (Nicoletti et al., 2011). Another study (McCarthy et al., 2011) using this model reported that Ax21 protein is a cell-cell signal that regulates virulence in *S. maltophilia*. These results and the herein presented show that the *G. mellonella* infection model is a useful tool for future research on *S. maltophilia* virulence.

Conclusion

The studies described herein are the first to provide evidence about the important role of iron as a signal, likely through the Fur system, for *S. maltophilia* biofilm formation and virulence. For these studies, a spontaneous *fur* mutant was obtained for the first time in *S. maltophilia*. Iron limitation improved biofilm formation and organization, as well as EPS production and SOD activity. Furthermore, MnSOD was responsible for the oxidative stress response of *S. maltophilia*. The *G. mellonella* infection model was useful to evaluate the virulence of the strains used in this work. F60 was more virulent than K279a in the killing assay, in accordance with the described role of iron in the regulation of potential virulence/survival factors. These observations are significant since *S. maltophilia* would be exposed to iron-limiting conditions either in the host or the nosocomial environment.

Moreover, we report the presence of two IROMPs which showed homology with FepA and another putative TonB-dependent siderophore receptor of K279a. *In silico* analysis allowed the detection of potential Fur boxes in the respective coding genes. Additionally, several promoters containing consensus Fur boxes were detected in the genome of K279a, and a consensus *S. maltophilia* FUR motif was built.

This is the first report of the regulation of DSF, an unusual QS signal, by iron, probably through the Fur system. Some authors suggest that iron and QS converge to regulate the expression of some virulence factors while an alternative interpretation could be that QS-regulated traits are likely to be a component of the Fur regulon (Cha et al., 2008). Future additional studies are required to fully define the role of the *S. maltophilia fur* homolog and to characterize the interaction between the QS system and Fur.

Finally, it has been proposed that interference with iron signaling processes could provide an interesting approach to aid the treatment of bacterial infections (Thompson et al., 2012). However, our results as well as those of Wiens et al. (2014) raise concerns about the use of iron chelators in the treatment of CF infections.

Author Contributions

Conceived and designed the study: BP and MF. Performed the experiments and analyzed the data: BP, CG, and EA. Coordinated the study and wrote the manuscript: BP. All authors read and approved the final manuscript.

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Decoding the genetic and functional diversity of the DSF quorum-sensing system in *Stenotrophomonas maltophilia*

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Stenotrophomonas maltophilia uses the Diffusible Signal Factor (DSF) quorum sensing (QS) system to mediate intra- and inter-specific signaling and regulate virulence-related processes. The components of this system are encoded by the *rpf* cluster, with genes *rpfF* and *rpfC* encoding for the DSF synthase RpfF and sensor RpfC, respectively. Recently, we have shown that there exist two variants of the *rpf* cluster (*rpf*-1 and *rpf*-2), distinguishing two groups of *S. maltophilia* strains. Surprisingly, only *rpf*-1 strains produce detectable DSF, correlating with their ability to control biofilm formation, swarming motility and virulence. The evolutive advantage of acquiring two different *rpf* clusters, the phylogenetic time point and mechanism of this acquisition and the conditions that activate DSF production in *rpf*-2 strains, are however not known. Examination of this cluster in various species suggests that its variability originated most probably by genetic exchange between rhizosphere bacteria. We propose that *rpf*-2 variant strains make use of a strategy recently termed as "social cheating." Analysis of cellular and extracellular fatty acids (FAs) of strains E77 (*rpf*-1) and M30 (*rpf*-2) suggests that their RpfFs have also a thioesterase activity that facilitates the release of unspecific FAs to the medium in addition to DSF. Production of DSF in *rpf*-1 strains appears in fact to be modulated by some of these extracellular FAs in addition to other factors such as temperature and nutrients, while in *rpf-2* strains DSF biosynthesis is derepressed only upon detection of DSF itself, suggesting that they require cohabitation with DSF-producer bacteria to activate their DSF regulatory machinery. Finally, we show that the mixed *rpf*-1/*rpf*-2 population presents synergism in DSF production and virulence capacity in an *in vivo* infection model. Recovery and quantification of DSF from co-infected animals correlates with the observed mortality rate.

Keywords: *rpf* cluster, virulence in zebrafish, fatty acids, DSF bioassay, social cheating, bacterial cross-talk

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Introduction

Quorum Sensing (QS) refers to bacterial communication processes that allow populations to synchronize gene expression when reaching a critical cellular density. These communication systems rely on the production and detection of signal molecules by which bacteria can coordinate a global response and promptly adapt to environmental fluctuations. The QS system described for the nosocomial pathogen *Stenotrophomonas maltophilia* is based on the fatty acid (FA) signal DSF (*cis*-11-methyl-2-dodecenoic acid) (Fouhy et al., 2007; Huang and Lee Wong, 2007; Huedo et al., 2014b). This cell-cell communication system was described for the first time in the phytopathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) as a novel regulation mechanism of virulence-factor synthesis (Barber et al., 1997). Its components are arrayed in the *rpf* (Regulation of Pathogenicity Factors) cluster, which includes the genes *rpfB* (fatty acyl-CoA ligase), *rpfF* (DSF synthase), *rpfC* (hybrid histidine-kinase receptor and effector), and *rpfG* (cytoplasmic regulator element) (Barber et al., 1997; Slater et al., 2000; Cheng et al., 2010).

In the past few years it has been shown that DSF-family signals are widespread (He and Zhang, 2008). This QS system has been well studied in *Xanthomonas* spp. (Barber et al., 1997; Slater et al., 2000; Dow et al., 2003; He et al., 2006; Ryan et al., 2006; He and Zhang, 2008; Cheng et al., 2010), *Xylella fastidiosa* (Chatterjee et al., 2008; Beaulieu et al., 2013; Ionescu et al., 2013) and *Burkholderia* sp. (Deng et al., 2009, 2010, 2013; McCarthy et al., 2010). In *S. maltophilia*, it has been reported that DSF-QS regulates bacterial motility (Fouhy et al., 2007; Huang and Lee Wong, 2007; Huedo et al., 2014b), biofilm development (Fouhy et al., 2007; Huedo et al., 2014b), antibiotic resistance (Fouhy et al., 2007), and virulence (Fouhy et al., 2007; Huedo et al., 2014b). Nonetheless, little is known about the mechanisms participating in the synthesis and perception of DSF molecules by *S. maltophilia*.

In a recent work we have reported that, although the DSF-QS system is found in all *S. maltophilia* strains analyzed, two populations can be clearly distinguished based on the *rpf* cluster they harbor—which we have named *rpf*-1 and *rpf*-2 (Huedo et al., 2014b)—, entangling the understanding of the QS system in this bacterium. We have shown that the two *rpf* clusters differ mainly in the region encoding the N-terminus of both the synthase RpfF and the sensor RpfC. Moreover, there exists a full association between the RpfF and RpfC variants, so that in the studied population we always observed the same RpfF/RpfC combination (called RpfF-1/RpfC-1 for the *rpf*-1 cluster and RpfF-2/RpfC-2 for the *rpf*-2 cluster). Strains with the RpfF-1/RpfC-1 combination are more commonly isolated, representing nearly 60% of the tested population (Huedo et al., 2014b). Interestingly, only those strains harboring the *rpf*-1 variant produce detectable DSF levels under the assayed conditions. The conditions that may lead to DSF production and perception in the *rpf*-2 variant group, which also includes clinically relevant strains, are not yet known.

In *Xcc*, RpfF activity is regulated by RpfC, whose REC domain (CheY-like receiver domain) interacts with the RpfF substrate-binding domain blocking DSF production (Cheng et al., 2010). Therefore, dissociation of the RpfF-RpfC complex is necessary to liberate free-active RpfF and produce DSF. The high homologies of the corresponding components in *S. maltophilia*, as well as our previous results (Huedo et al., 2014b), strongly suggest that a similar mechanism regulates DSF production in *S. maltophilia*.

In this study, we have investigated further the environmental conditions that may modulate DSF production in model strains belonging to each *rpf* variant group, in particular, temperature, medium composition and presence of other FAs. These factors, notably the presence of 13-methyl-tetradecanoic acid (*iso*-15:0), affect DSF synthesis only in the *rpf*-1 variant strain. We have also characterized cellular and extracellular FAs in wild-type and Δ*rpfF* mutant strains of each variant. The dependence of some of these FAs on the expression of the *rpfF* gene suggests that both RpfF variants have an unspecific thioesterase activity, as previously found for the *Burkholderia cenocepacia* homolog, cleaving a variety of acyl-ACP (acyl-acyl carrier protein) substrates and releasing FAs that are then secreted to the medium. Finally, we also show that in *S. maltophilia* DSF is produced in a positive-feedback manner and that the two *rpf*-variant groups act synergistically by enhancing the DSF production and virulence potential of a mixed *rpf*-variant population during *in vivo* infection.

Materials and Methods

rpf Cluster Comparison and Phylogenetic Tests

Sequences of the *rpf* cluster and corresponding genes from selected Xanthomonadales strains were retrieved from their respective NCBI genome sequences [http://www.ncbi.nlm.nih.gov/genome/]. The recently described genome sequence of *S. maltophilia* M30 (Huedo et al., 2014a) was used as a reference for the *rpf*-2 variant. To manually confirm some annotations, orthology relations were defined as reciprocal best matches by blastn and blastx. Concatenated nucleotide sequences for the *rpfF* and *rpfC* genes were aligned using ClustalW as implemented in MEGA 6 (Tamura et al., 2013) with default parameters. *rpfH* was also included in the analysis, if present. Neighbour-joining trees were generated and displayed using MEGA 6. The GARD program (Kosakovsky Pond et al., 2006) was used to search for putative recombination breakpoints in sequence alignments. Detailed information about the sequences analyzed in recombination tests is found in Supplementary Material (**File S1**).

Bacterial Strains and Growth Conditions

All bacterial strains used in this study are listed in **Table 1**. *S. maltophilia* strains E77 (*rpf*-1) and M30 (*rpf*-2) (Ferrer-Navarro et al., 2013) and their respective Δ*rpfF* mutants (Huedo et al., 2014b) were taken as model strains to investigate the molecular mechanisms underlying DSF production and perception in each *rpf* cluster variant. *X. campestris* pv. *campestris* (*Xcc*) strain 8523 pL6engGUS was used as a reporter strain to detect DSF activity and was provided by the authors (Slater et al., 2000). *S. maltophilia* strains were routinely grown at 30°C in Luria-Bertani (LB) medium on a rotary shaker. When needed, Δ*rpfF* mutants were grown in LB supplemented with

TABLE 1 | Strains used in this study.

Strains	Relevant characteristics	References
S. maltophilia		
E77	Wild type, *rpf*-1 cluster variant	Ferrer-Navarro et al., 2013
M30	Wild type, *rpf*-2 cluster variant	Ferrer-Navarro et al., 2013
E77 Δ*rpfF*	E77 Δ*rpfF* mutant, Erm^r	Huedo et al., 2014b
M30 Δ*rpfF*	M30 Δ*rpfF* mutant, Erm^r	Huedo et al., 2014b
E77 Δ*rpfF* p*rpfF*E77	E77 Δ*rpfF* mutant harboring pBBR1MCS1-Cm-*rpfF* from E77, Cm^r Erm^r	Huedo et al., 2014b
M30 Δ*rpfF* p*rpfF*M30	M30 Δ*rpfF* mutant harboring pBBR1MCS1-Cm-*rpfF* from M30, Cm^r Erm^r	Huedo et al., 2014b
X. campestris* pv. *campestris		
Xcc 8523	Δ*rpfF* mutant. DSF reporter strain. carring plasmid pL6engGUS, Tc^r. Kan^r	Slater et al., 2000

erythromycin (Erm) at 500 μg/mL. The *Xcc* reporter strain was routinely grown at 28°C in NYG medium (0.5% peptone. 0.3% yeast extract and 2% glycerol) supplemented with Tc 10 μg/mL. To investigate the role of diverse FAs in DSF production by *S. maltophilia*, strains E77 and M30 were grown in 150 mL LB liquid cultures to an optical density of 0.1 at 600 nm. Subsequently, cultures were supplemented with diverse FAs, including lauric acid (12:0), myristic acid (14:0), 13-methyl-tetradecanoic acid (*iso*-15:0), palmitic acid (16:0), and stearic acid (18:0) (Sigma-Aldrich), at 5 μM concentration and incubated at 30°C for 24 h. Extraction and quantification of DSF molecules was done as described below.

Colony-based DSF Bioassay

DSF determination was performed using strain *Xcc* 8523 pL6engGUS (DSF-reporter strain) as previously described (Slater et al., 2000), with few modifications. Briefly, the DSF-reporter strain was grown in 10 mL of NYG medium supplemented with Tc (10 μg/mL) to an optical density of 0.7 at 600 nm. Cells were harvested and reconstituted with 1 mL of fresh NYG and added to 100 mL of temperate NYG medium, giving a final OD600nm of 0.07, containing 1% of BD Difco Agar Noble (NYGA) and supplemented with 80 μg/mL X-Glu (5-Bromo-4-chloro-3-indolyl β-D-glucopyranoside) (Sigma) and then plated into petri plates upon solidification. Candidate strains were pin inoculated onto NYGA plates containing 80 μg/mL X-Glu and the DSF-reporter strain and incubated for 24 h at 28°C. Presence of a blue halo around the colony indicated DSF activity.

DSF Extraction from Culture Supernatants

For liquid and supernatant-based DSF bioassay, bacterial cultures were grown in 250 mL of LB for 48 h at 30°C (OD600 nm about 4). Supernatants were extracted with the ethyl acetate method (Barber et al., 1997) and residues were dissolved in 200 μL of 30% methanol.

Supernatant-based DSF Bioassay

Three microliters of each sample were deposited into hand-generated wells (3 mm in diameter) in 5.5 cm petri plates containing NYGA supplemented with 80 μg/mL of X-Glu and seeded with the DSF-reporter strain previously prepared to a final optical density of 0.07 at 600 nm. Plates were incubated for 24 h at 28°C. DSF activity was determined by the presence of a blue halo around the well.

Microtitter DSF Bioassay

Two-hundred microliters of a DSF reporter solution consisting of a suspension of the *Xcc* 8523 pL6engGUS strain (adjusted to an optical density of 0.07 at 600 nm) in NYG medium and supplemented with X-Glu (80 μg/mL), were deposited into wells of sterile 96-well flat-bottomed microtitter plates (BrandTech 781662). As a standard curve, synthetic DSF was added in duplicate to separate wells at different increasing concentrations (from 0.05 to 1.5 mM) and incubated at 28°C for 24 h. After incubation, the presence of DSF molecules turned the reaction color to blue with intensity (620 nm) proportional to the initial concentration of DSF (see Supplementary Material, **Figure S1**). Test samples were dissolved in methanol and then tested in this bioassay. Serial dilutions of test samples were added into wells and after incubation the absorbance was read at 620 nm, as done for the calibration curve. Finally, DSF quantification was done using the standard calibration curve.

Determination of DSF Production in Mixed and DSF-supplemented *S. maltophilia* Liquid Cultures

Overnight cultures of *S. maltophilia* E77 and M30 grown in LB at 30°C were combined in a single shake flask containing 150 mL of fresh LB medium to a final OD of 0.1 each at 600 nm, to obtain a mixed initial culture (E77:M30, 1:1). The mixed culture was incubated at 30°C for 24 h and prior to DSF extraction the CFUs of each strain were evaluated by serial dilution and colony counting (note that the colony morphology of strains E77 and M30 can be distinguished). Direct specific PCR on colonies were done if necessary to corroborate strain identity. At this time point both strains reached similar number of CFU/mL. Then, culture supernatants were extracted as described above and DSF quantification was done by microtitter DSF bioassay. To validate the synergic role of synthetic DSF in the production of DSF in strain M30, initial 150 mL LB cultures of M30 (optical density of 0.1 at 600 nm) were supplemented with 0.05 μM of synthetic DSF and incubated at 30°C for 24 h. Culture supernatants were extracted as described above and DSF quantification was done by microtitter DSF bioassay. The same volume of the media supplemented with 0.05 μM DSF was extracted and quantified by microtitter DSF bioassay as control.

Analysis of Fatty Acids

Analysis of total cellular FAs was carried out by the Identification Service of the DMSZ, Braunschweig, Germany, as follows. FA methyl esters were obtained from 40 mg cells scraped from Petri dishes by saponification, methylation and extraction using minor modifications of the method of Miller (Miller, 1982) and Kuykendall (Kuykendall et al., 1988). The FA methyl ester

mixtures were separated using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.), which consists of an Agilent 6890N gas chromatograph equipped with a 5% phenyl-methyl silicone capillary column (0.2 mm × 25 m), a flame ionization detector, an Agilent 7683A automatic sampler and a computer with the MIDI data base. Peaks were automatically integrated and FA names and percentages calculated by the MIS Standard Software (Microbial ID). The gas chromatographic parameters were as follows: carrier gas, ultra-high-purity hydrogen: column head pressure, 60 kPa: injection volume, 2 μL: column split ratio, 100:1: septum purge, 5 mL/min: column temperature. 170 to 270°C at 5°C/min: injection port temperature, 240°C: and detector temperature, 300°C. To analyze FAs present in the medium, bacterial cultures were grown in 2 L of LB for 48 h at 30°C with vigorous shaking (250 rpm). Cultures were centrifuged, and supernatants were extracted by the ethyl acetate method. Dry residues were dissolved in 3 mL of dichloromethane and esterified to generate FA methyl esters. Esterified extracellular FAs were identified by gas chromatography (GC) (Agilent Technologies 6890) with an Agilent 19091S-433 column coupled to a mass spectrometer (MS) detector (Hewlett-Packard 5973).

Determination of Virulence in the Adult Zebrafish Infection Model

Adult (9–12 months) wild-type zebrafish (*Danio rerio*) were kept in a 12 h light:12 h dark cycle at 28°C and fed twice daily with dry food. All fish used in infection experiments were transferred to an isolated system and acclimated for 3 days before infection. Adult zebrafish ($n = 16$ per condition) were infected intraperitoneally (Kinkel et al., 2010) with 20 μL of a bacterial suspension corresponding approximately to 50% of lethal dose (LD50). The LD50 for M30 and E77 was previously estimated in zebrafish by injecting 20 μL of bacterial suspensions at concentrations ranging from 10^8 to 10^{10} CFU/mL and proved to be very similar (1–5 × 10^8 CFU per animal). Four conditions were tested, including animals injected with a single dose corresponding to the LD50 of an axenic inoculum of E77, an axenic inoculum of M30, a mixed inoculum of E77 and M30 (1:1) and a mixed inoculum of mutants Δ*rpfF*-1:Δ*rpfF-2* (1:1). Each inoculum consisted in 20 μL of a bacterial suspension previously adjusted to approximately 6–7 × 10^9 CFU/mL in sterile PBS. Mixed inocula were prepared by mixing equal amounts of adjusted bacterial suspensions. *S. maltophilia* strains were previously grown at 28°C in Columbia blood agar plates (BioMérieux) for 20 h and collected directly from the plates with sterile phosphate buffered saline (PBS). Two control groups were injected with PBS and showed no mortality. Fishes were observed daily for signs of disease and mortality.

DSF Detection and Quantification from Infected Animal Tissues

Two fishes from each experimental group (E77; M30; E77:M30 and Δ*rpfF*-1:Δ*rpfF*-2, plus two PBS-injected animals) were randomly chosen and sacrificed by an overdose of anesthetic solution (MS-222, 220 ppm) 48 h post infection (hpi). Since in the mixed wild-type inoculum all fishes died within the first 48 h, this was chosen as the time point for evaluating DSF production inside the infected animals. Two dead fishes from each group were sacrificed and introduced into their corresponding falcon tube containing 15 mL of PBS and homogenized using a Politron Homogenizer (MARK). The homogenized solution was extracted twice with the same volume of ethyl acetate and subsequently dried. Dry residues were dissolved in 100 μL of 30% methanol and DSF activity was detected and quantified by microtitter and supernatant-based DSF bioassays.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism software version 5.00. Comparison of phenotypic data was performed by One-Way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-test or unpaired *t*-test with Welch correction for unequal variances. Survival curves of zebrafish infection experiments were analyzed using the Kaplan-Meier method and differences were evaluated using the log-rank test.

Ethics Statements

Zebrafish were handled in compliance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and with Decree 214/1997 of the Government of Catalonia, which regulates the use of animals for experimental and other scientific purposes. Experimental protocols have been reviewed and approved by the Animal and Human Experimentation Ethics Committee (CEEAH) of Universitat Autònoma de Barcelona (UAB), Spain (ref #CEEAH-1968).

Results

The Two *rpf* Cluster Variants in *S. maltophilia* May Have Originated by Horizontal Exchange

To infer the origin of the two *rpf* cluster variants in *S. maltophilia* the diversity of this cluster among Xanthomonadales species was studied. In addition to *S. maltophilia* strains K279a (*rpf*-1) and M30 (*rpf*-2), 16 different species with available genome information were included in the analysis (**Figure 1**). In all the species analyzed, *rpfC* and *rpfF* are part of two contiguous but convergent operons, both involved in DSF synthesis and perception. Among the genes in the two *rpf* clusters, *rpfC* and *rpfF* are the most variable and were therefore selected for phylogenetic analysis. A phylogenetic tree based on the *rpfC* and *rpfF* concatenated sequences (**Figure 1**) suggests that the origin of the two *rpf* variants in *S. maltophilia* may be explained by horizontal gene transfer. In this tree, *S. maltophilia* K279a clusters together with *Stenotrophomonas rhizophila*, while M30 occupies a distinct, more distant branch, together with *Pseudoxanthomonas* spp., *Arenimonas* spp. and *Lysobacter* spp. strains. The nucleotide sequence of the *rpfF* gene of strain M30 (*rpf*-2 variant) is more similar to that of *Pseudoxanthomonas* spp. (80% identity) than to that of the *S. maltophilia rpf*-1 variant strain K279a (71% identity). In addition, the *rpfC* gene of *S. maltophilia* K279a and *S. rhizophila* are similar in that both encode a protein with a sensor input domain with ten transmembrane regions (TMR). This

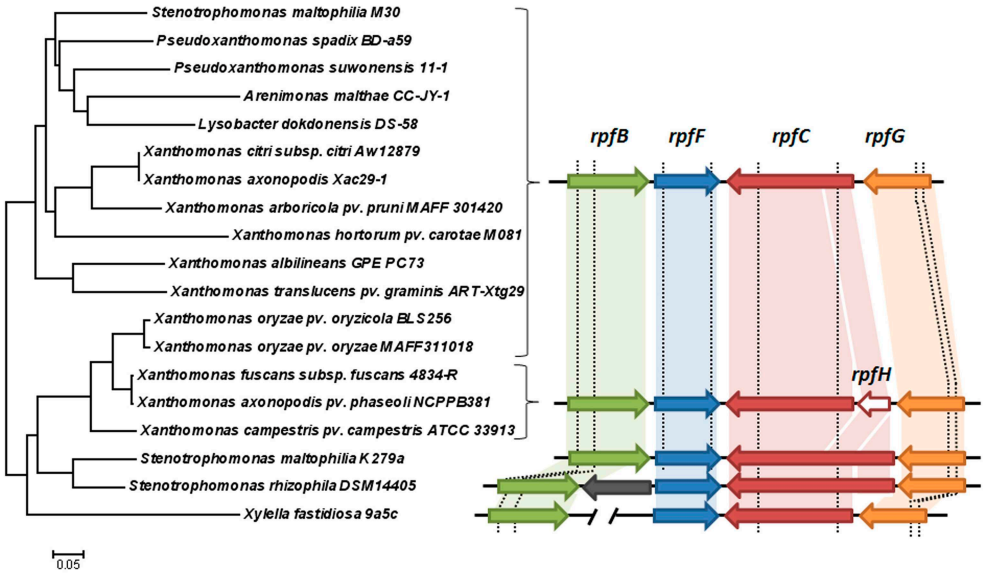

FIGURE 1 | Left: neighbor-joining tree illustrating the relationships across Xanthomonadales strains based on concatenated coding sequences (nucleotide alignment) of the *rpfF* and *rpfC* genes. **Right**: comparison of the cluster of genes involved in DSF synthesis and perception among the analyzed strains. Arrows with the same colors indicate orthologies. The gray arrow represents a gene with unknown function unique to *S. rhizophila* DSM14405 (Alavi et al., 2014). Note that the 5′ portions of the *rpfC* coding sequence in K279a and DSM14405 strains are similar to the *rpfH* gene (conserved regions are paired with a shaded block of the same color). In *X. fastidiosa*, *rpfB* is located elsewhere in the chromosome. The vertical dashed lines represent recombination breakpoint sites predicted with GARD (Kosakovsky Pond et al., 2006).

input domain is encoded by a repetitive region at the 5′ end of the gen, similar to the *rpfH* of *Xcc*. On the other hand, the *rpfC* gene of *S. maltophilia* M30 encodes a shorter protein with only five TMRs (Huedo et al., 2014b), as in most Xanthomonadales. The tree topology also suggests that a similar genetic event could have acted on the *rpf* cluster in the *Xanthomonas axonopodis* clade, as it has been already suggested (Lu et al., 2008). The presence of putative recombination breakpoints within *rpf* genes was confirmed using a genetic algorithm for recombination detection (see **Figure 1** and **File S1**).

Characterization of Cellular and Extracellular Fatty Acids for the Two *rpf* Variant Strains

Analysis of total cellular FAs revealed that the *rpf*-1 and *rpf*-2 variant strains display a very heterogeneous but similar FA profile, with near 20 different FAs (**Table 2**). In both cases, the most abundant FA was found to be 13-methyl-tetradecanoic acid (*iso*-15:0). The presence of *iso*-15:0 as a major FA is characteristic of the genus *Stenotrophomonas* (Kim et al., 2010). Such high representation (ca. 33%) indicates that this branched FA is part of cell-membrane phospholipids (resulting from FA methyl ester extraction). Total cellular FAs were also evaluated for the Δ*rpfF*-1 and Δ*rpfF*-2 mutants and no differences were observed compared to the FA profile of their respective parental strain (**Table S1**).

Extracellular FAs present in the supernatants of the two variant strains were also identified by GC/MS (**Figure 2** and **Figure S2**). As for the cellular FAs, the FAs identified in the medium supernatants of the two strains were mostly the same, except for the signal DSF, which was not detected in M30 (*rpf*-2) supernatants (Huedo et al., 2014b). These included *iso*-15:0, 16:0, and 18:0. Deletion of *rpfF*-1 (strain E77) led to a decrease in *iso*-15:0 release, with levels restored (in excess) by *in trans* complementation (**Figure 2A**). The same effect was

TABLE 2 | Total cellular fatty acids in *S. maltophilia* strains E77 (*rpf*-1) and M30 (*rpf*-2).

E77 (*rpf*-1)		M30 (*rpf*-2)	
Fatty acid	**Percentage**	**Fatty acid**	**Percentage**
iso-15:0	32.3	*iso*-15:0	33.29
iso-15:0 2OH or 16:1 w7c	12.6	*iso*-15:0 2OH or 16:1 w7c	11.94
anteiso-15:0	11.9	*anteiso*-15:0	10.35
16:0	5.95	16:0	9.23
iso-17:1 w9c	4.22	*iso*-11:0	4.22
16:1 w9c	3.86	*iso*-17:1 w9c	3.95
14:0	3.77	*iso*-17:0	3.88
12:0 3OH	3.29	*iso*-12:0 3OH	3.28
iso-13:0 3OH	3.27	*iso*-13:0 3OH	3.08
iso-11:0	2.9	16:1 w9c	2.96
iso-17:0	2.49	14:0	2.9
iso-11:0 3OH	1.66	18:1 w9c	1.85
18:1 w9c	1.47	*iso*-11:0 3OH	1.82
iso-16:0	1.46	Unknown	1.53
Unknown	1.23	*iso*-16:0	1.51
14:0 ISO	1.06	18:1 w7c	1.13

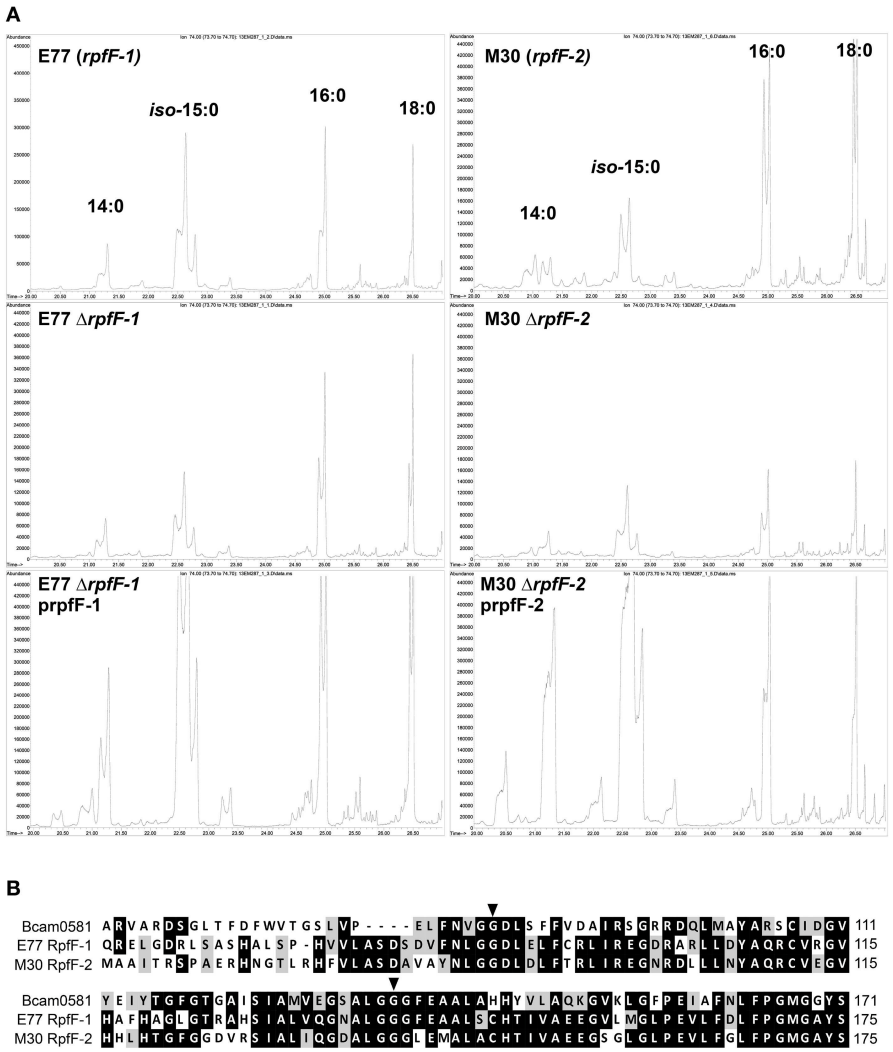

FIGURE 2 | (A) Gas Chromatography analysis of FAs present in the supernatants of *S. maltophilia* E77 and M30, their respective Δ*rpfF* mutants and complemented strains. **(B)** Alignment of RpfF (partial sequence) in *B. cenocepacia* (Bcam0581) and *S. maltophilia* E77 and M30. Marked aminoacids indicate the residues responsible for thioesterase activity (Bi et al., 2012).

observed in the M30 strain, although in this case deletion of *rpfF*-2 led also to a significant decrease in the release of 16:0 and 18:0. These results suggest that, as described for the RpfF homolog Bcam0581 of *B. cenocepacia* (Bi et al., 2012), both *S. maltophilia* RpfF variants could have, apart from a dehydratase activity, an unspecific thioesterase activity that cleaves acyl-ACP bonds generating free FAs that are then released to the extracellular environment. Protein alignment (**Figure 2B**) shows that both RpfF variants conserve the amino-acids G87 and G139 responsible for the thioesterase activity in the *B. cenocepacia* ortholog (Bi et al., 2012). Overall, these results suggest that RpfF is not involved in the main synthetic pathway of cellular FAs (their composition not being affected by RpfF deletion), but participates in fatty-acid release and, therefore, determines to some extent the extracellular fatty-acid composition.

Effect of the Temperature, the Medium and the Presence of Extracellular FAs on DSF Production by *S. maltophilia*

Little is known about the growth conditions that modulate DSF production in *S. maltophilia*. In a recent report, we have shown that although only *rpf*-1 strains produce detectable levels of DSF under standard laboratory growth conditions, both synthase variants RpfF-1 and RpfF-2 are functional on DSF synthesis (Huedo et al., 2014b). Here, we aimed at investigating the conditions that may modulate such production, especially in *rpf*-2 strains. To this end, we developed a new, simple and efficient DSF-detection methodology based on a microtitter-plate bioassay (see Materials and Methods and **Figure S1**), which allowed us to accurately quantify DSF produced by *S. maltophilia* strains grown in different media and incubation temperatures. Using this method, we first quantified the DSF

TABLE 3 | Effect of temperature, medium composition and presence of extracellular fatty acids on DSF production by *S. maltophilia* strains E77 (*rpf*-1) and M30 (*rpf*-2).

Experiment	Growth conditions		DSF production (μg/mL)	
	Medium	Temperature°C	E77	M30
Temperature effect (LB)	LB	20	ND	ND
	LB	25	0.6 ± 0.07	ND
	LB	30	1.4 ± 0.2*	ND
	LB	37	0.25 ± 0.03	ND
	LB	42	ND	ND
Medium composition effect (30°C)	M9	30	0.3 ± 0.03	ND
	BM2	30	0.4 ± 0.03	ND
	NYG	30	1.1 ± 0.15	ND
	LB	30	1.4 ± 0.2*	ND
Fatty acid supplementation effect (5 μM)	LB + 12:0	30	1.6 ± 0.2	ND
	LB + 14:0	30	1.8 ± 0.3	ND
	LB + *iso*-15:0	30	2.4 ± 0.4*	ND
	LB + 16:0	30	1.35 ± 0.1	ND
	LB + 18:0	30	ND	ND

*ND, Not detected. *p < 0.05 (One-Way ANOVA and post-test).*

levels in culture supernatants from bacteria grown in LB medium at different temperatures (20, 25, 30, 37, and 42°C) for 48 h (**Table 3**). DSF production in strain E77 (*rpf*-1 variant) was temperature dependent and statistically significant only at 30°C ($p < 0.05$). Production was however detected also at 25 and 37°C, and not at 20 or 42°C. On the other hand, DSF was not detected in the supernatants of M30 (*rpf*-2 variant) grown in LB at any of the tested temperatures (**Table 3**).

The effect of medium composition at the optimal temperature (30°C) was then evaluated using a second rich medium (NYG) and two minimal media (BM2 and modified M9-salts). DSF production by E77 showed to be clearly higher in rich media (LB and NYG), while none of these conditions induced DSF production by the M30 strain (**Table 3**).

The finding that *S. maltophilia* RpfFs may have, just like *B. cenocepacia* Bcam0581 (Bi et al., 2012) and *Xcc* RpfF (Bi et al., 2014), a thiosterase activity that contributes to the accumulation of diverse FAs in the extracellular environment, led us to speculate that these FAs could contribute to modulate DSF production. To test this hypothesis, we supplemented E77 and M30 cultures with the FAs that were identified as predominant in their supernatants (12:0, 14:0, *iso*-15:0, 16:0, and 18:0, at 5 μM each). After incubation, DSF was quantified using the microtitter DSF bioassay. As negative control, the same volume of non-supplemented medium was in each case extracted and tested with the bioassay, resulting in no DSF activity (data not shown). Results showed that *iso*-15:0 significantly stimulates DSF production in E77 (**Table 3**). Interestingly, while 12:0, 14:0, and 16:0 have no significant effect on DSF synthesis in this strain, 18:0 appears to have an inhibitory effect. As expected, none of the FAs promoted DSF production in the M30 strain.

S. maltophilia rpf-1 and *rpf*-2 Variant Strains Cross Talk Producing DSF in a Positive-feedback Manner

Since we had previously observed that the RpfF-2 variant is functional when the RpfF-2:RpfC-2 stoichiometric ratio is favorable to the RpfF-2 protein (RpfF-2 > RpfC-2) (Huedo et al., 2014b), we now aimed to determine the conditions under which the RpfF-2/RpfC-2 complex could dissociate and synthesize DSF in a wild-type background. We have observed *in vitro* that, when colonies of *S. maltophilia* E77 and M30 are grown at a distance at which the halo of DSF production from E77 invades the growing zone of M30, the latter begins to produce DSF (**Figure 3**). Additionally, it seems that this synergism is reciprocal, since both strains produce higher levels of DSF when are seeded closely than when are seeded separately (**Figures 3A,C**). Also, when E77 is grown close to a Δ*rpfF*-1 or Δ*rpfF*-2 mutant colony, no increment in DSF production is observed (**Figure 3C**), which corroborates that under wild-type conditions the observed synergism in DSF production depends only on the DSF molecule (**Figure 3C**). Furthermore, mixed cultures of E77 and M30 grown in LB broth at 30°C for 48 h displayed higher DSF production than E77 axenic cultures (**Figure 3B**). Finally, supplementation of M30 cultures in LB with 0.05 μM DSF significantly triggers DSF production ($p < 0.05$) after 48 h of growth (**Figure 3B**). Together, these results confirm that RpfC-2 is able to sense DSF, that DSF is produced in a positive feedback-manner and that the variant groups *rpf*-1 and *rpf*-2 produce DSF synergistically if they grow in close contact.

S. maltophilia rpf-1 and *rpf*-2 Variant Strains Act Synergistically to Promote Virulence in a Zebrafish Infection Model

The observation that the two populations produced DSF synergistically led us to investigate whether these strains would also cooperate to develop virulence *in vivo*. To this end, we performed infection experiments in a recently developed adult zebrafish model using groups of 16 fishes. Each group was inoculated with either: an axenic inoculum of E77, an axenic inoculum of M30, a mixed inoculum of E77 and M30 wild type (1:1) and a mixed inoculum of Δ*rpfF* mutant strains Δ*rpfF*-1:Δ*rpfF*-2 (1:1), all of them with the same total bacterial load ($\sim 5 \times 10^8$ CFU). Control animals were injected with PBS.

The infection experiments showed that when animals were infected with the mixed wild-type inoculum all fishes ($n = 16$) died within the first 48 h, whereas the fishes infected with the axenic cultures E77 and M30 showed a survival between 20 and 50% at 120 hpi (**Figure 4A**). Moreover, when fishes were infected with a mixed inoculum consisting of E77 Δ*rpfF*-1 and M30 Δ*rpfF*-2 the survival ratio was close to 40% at 120 hpi, demonstrating that RpfF is an essential trait for the virulence capacity of the *S. maltophilia* population (**Figure 4A**). Symptoms of disease as well as fish behavior were evaluated during the days of infection. Interestingly, fishes infected with the mixed wild-type inoculum showed a drastic change in behavior (spasms, compromised swimming and sudden death), especially between 24 and 48 h (data not shown).

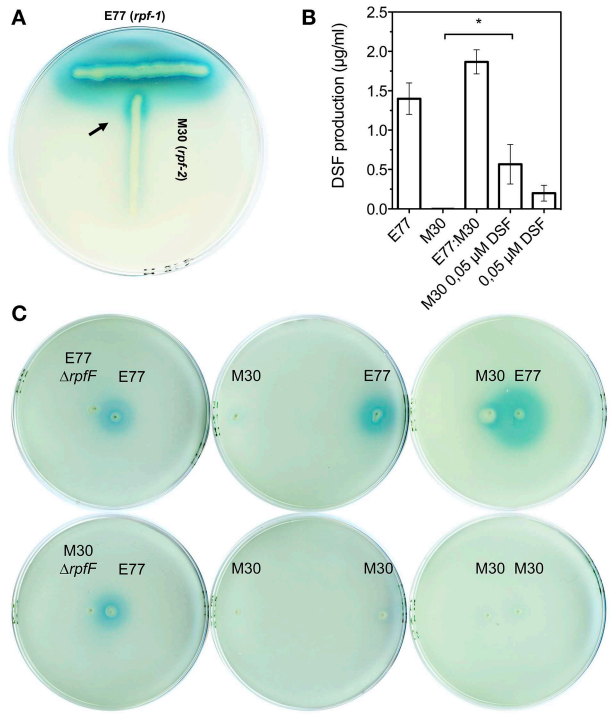

FIGURE 3 | (A) T-seeded colony-based DSF bioassay of strains E77 and M30. Arrow indicates DSF production of M30 (*rpf*-2) strain upon detection of DSF molecules produced by E77 (*rpf*-1). **(B)** DSF quantification of supernatants from axenic cultures of E77 and M30, a mixed culture E77:M30 (1:1), an axenic culture of M30 supplemented with synthetic DSF at 0.05 µM final concentration and a corresponding control (equal volume of LB broth containing 0.05 µM DSF), using the microtitter DSF bioassay; $*p < 0.05$ by One-Way ANOVA and post-test. **(C)** Colony-based DSF bioassay of E77, M30 and their respective Δ*rpfF* mutants seeded at different distances on the same agar plate.

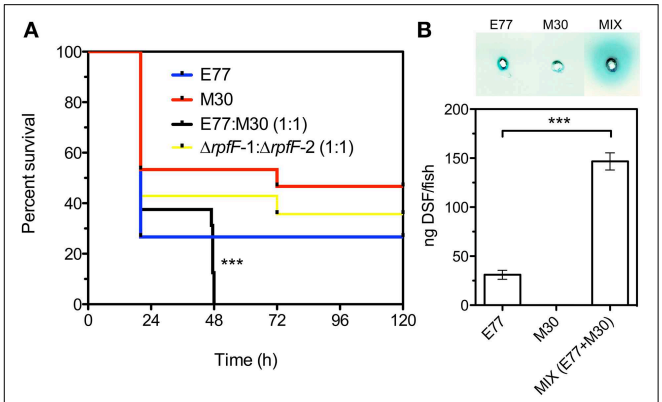

FIGURE 4 | (A) Virulence determination of E77, M30, a mixed inoculum of E77 and M30 (1:1) and a mixed inoculum of E77 Δ*rpfF-1* and M30 Δ*rpfF-2* (1:1), in the adult zebrafish model; $***p < 0.001$ (log-rank test). **(B)** Supernatant-based DSF bioassay of tissue extracted from sacrificed fishes 48 h after infection with E77, M30 and a mixed inoculum (E77:M30, 1:1) (top) and quantification of DSF in the samples using the microtitter bioassay (bottom); $***p < 0.001$ by One-Way ANOVA and post-test.

To assess DSF production during the *in vivo* infection and investigate the eventual correlation with the lethal capacity of the inocula, two fishes from each condition were sacrificed at 48 hpi and the DSF inside the infected animals was quantified. The condition that leads to 100% mortality (mixed inoculum of E77 and M30 wt) showed significant increment ($p < 0.001$) in DSF content relative to the axenic inoculum with E77 (**Figure 4B**), showing a clear correlation between *in vivo* DSF production and virulence capacity. No DSF was detected in PBS-injected animals.

Discussion

Although the DSF-QS system has been deeply studied in Xanthomonadales species (Deng et al., 2011), the biosynthetic pathway of DSF, its regulation and the way in which the sensor RpfC perceives DSF signals remain unclear. This is especially true for the opportunistic human pathogen *S. maltophilia*, in which DSF-QS regulation appears to be particularly complex. In a recent work, our group demonstrated that, in contrast to other species featuring the DSF-QS system, in *S. maltophilia* there exist two populations—designated *rpf*-1 and *rpf*-2—that differentially regulate DSF production and detection (Huedo et al., 2014b). In *rpf*-1 variant strains DSF-QS regulation seems to be reasonably similar to that described in *Xcc*. Phylogenetic analysis of the *rpf* cluster among Xanthomonadales further confirms the similarity between the *S. maltophilia rpf*-1 cluster variant and the *Xcc rpf* cluster, both in terms of sequence identity and genomic organization (**Figure 1**). On the other hand, the *rpf*-2 cluster organization is more similar to that of the *rpf* clusters of *Pseudoxanthomonas* spp., *Arenimonas* spp. and *Lysobacter* spp. (**Figure 1**), bacteria commonly isolated from the rhizosphere. The high competition and enhanced horizontal gene transfer rates characteristic of this competitive ecological niche (Berg et al., 2005) might have contributed to the genetic and metabolic variability observed in *S. maltophilia*, also reflected in DSF-QS regulation. The aim of this study was to get further insight into the complexity of the DSF-QS system of *S. maltophilia*.

The elevated proportion of *iso*-15:0 found in the analysis of total cellular FAs indicates that the identified methyl ester derives from membrane phospholipids. In line with this result, *iso*-15:0 has been also reported as the most abundant FA in several *Xanthomonas* species, including *Xcc, Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *X. axonopodis* pv. *citri* (*Xac*), among others (Vauterin et al., 1996). Surprisingly, *iso*-15:0 has been also found in similar proportion in total phospholipids of Gram-positive bacteria such as *Bacillus* spp. PS3 (34%) (Kagawa et al., 1978) and is actually considered a biomarker phospholipid FA for the Gram-positive group (Kaur et al., 2005). In this context, it is remarkable that among the Gram-negative group *iso*-15:0 appears to be present only, and with similar relative abundances, in DSF-producer bacteria such as *S. maltophilia* (**Table 2**) and *Xanthomonas* spp. (Vauterin et al., 1996; Bi et al., 2014). By similarity to protein Bcam0581 from *B. cenocepacia* (which produces *cis*-2-dodecenoic acid or BDSF), RpfF has been postulated to have a double acyl-ACP dehydratase and thioesterase activity, which would catalyze the conversion of (*R*)-3-hydroxy-11-methyl-dodecanoyl-ACP to DSF in two steps

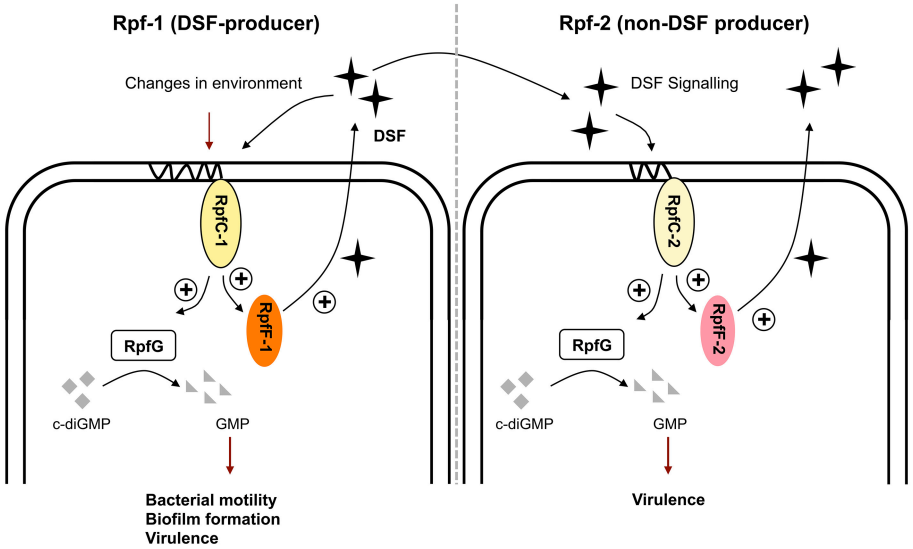

FIGURE 5 | Schematic model of the DSF-QS proposed for *S. maltophilia rpf*-1 (DSF-producer) and *rpf*-2 (non-DSF producer) variant strains. Rpf-1: RpfC-1 (containing 10 TMR) allows basal activation of RpfF-1, with subsequent DSF production, when reaching high cellular density. Changes in the environment including temperature, nutrients and presence of extracellular fatty acids modulate DSF production. The DSF-QS system controls bacterial motility, biofilm dispersion, and virulence. Rpf-2: RpfC-2 (containing 5 TMR) does not allow basal DSF production. None of the mentioned factors stimulate DSF production in these variant strains. Activation of RpfF-2 and subsequent DSF production happens only upon detection of DSF itself, likely coming from neighbor DSF-producer strains (e.g., *Xcc* or *S. maltophilia rpf*-1). In this situation, *rpf*-2 strains regulate synergistically virulence capacity and DSF production in co-infection with *rpf*-1 variant strains.

(Bi et al., 2012). Furthermore, the thioesterase activity of both Bcam0581 and the RpfF from *Xcc* has been observed to be unspecific, cleaving a variety of acyl-ACP substrates and thus generating free medium- and long-length FAs that are then released to the medium (Bi et al., 2012, 2014). Our analysis of extracellular FAs suggests that the two RpfF variants of *S. maltophilia* do also have a thioesterase activity that is not specific for the DSF precursor but may also cleave, at least, the acyl-ACP form of *iso*-15:0 (**Figure 2A**). Interestingly, the putative DSF precursor (*R*)-3-hydroxy-11-methyl-dodecanoyl-ACP is also a precursor of *iso*-15:0 in the fatty-acid synthesis cycle (Heath et al., 2002) (note that methylation of *iso*-branched FAs originates from the primer for biosynthesis, which derives from valine in odd-numbered chains and leucine in even-numbered chains). The potential connection between DSF and *iso*-15:0 biosynthesis leads us to speculate on a corresponding potential connection between DSF production and membrane synthesis.

In this context, we have observed that, besides temperature and medium composition, the presence of RpfF-dependent FAs in the medium can modulate DSF production in variant 1 (**Table 3**). Indeed, it seems that the presence of exogenous *iso*-15:0 has a stimulatory effect on DSF synthesis in this variant strain. Since RpfC has both a sensor and a RpfF-repression function (Cheng et al., 2010; Huedo et al., 2014b), we hypothesize that RpfC-1 may be able to detect *iso*-15:0—and possibly other FAs—leading to the liberation of RpfF-1 and subsequent synthesis of DSF. On the contrary, RpfC-2—with a shorter sensor input domain containing only five TMRs (Huedo et al., 2014b)—would be incompetent for promiscuous perception, resulting in a permanent repression of the RpfF-2 synthase despite the presence of *iso*-15:0 and other FAs in the medium. Indeed, it appears that the RpfF-2/RpfC-2 complex is able to dissociate, and thus produce DSF, only upon detection of DSF-itself (**Figure 3**), indicating that, contrary to *rpf*-1, the sensor complex in *rpf*-2 is more specific. Recent findings on *X. fastidiosa* (RpfC-2-like variant with 5 TMR) show that in this species RpfF is required for DSF detection (Ionescu et al., 2013). It seems thus clear that some initial DSF production must therefore occur in order to trigger DSF synthesis by *rpf*-2 strains. The specific conditions that would facilitate this initial DSF production in axenic cultures have not yet been elucidated. However, it is well established that in the natural environments of *S. maltophilia* there exists extensive intra- and inter-population competence and communication (Wang et al., 2004), even between organisms from different domains (Boon et al., 2008). Since bacterial species sharing DSF-QS are almost ubiquitous and frequently share ecological niches, it is likely that *rpf*-2 variant strains will often be in contact with DSF-producer bacteria (e.g., *Xcc* or *S. maltophilia rpf*-1 variant). In this situation, a DSF-producer strain would act as a starter strain, triggering the reciprocal DSF-communication by synthesizing the initial DSF molecules (**Figure 5**). Interestingly, we have observed that, besides showing *in vitro* synergism in DSF production (**Figure 3**), the two variant-groups communicate enhancing the virulence capacity of a mixed *S. maltophilia* population during *in vivo* infection (**Figure 4**). It seems that *rpf*-2 strains have evolved as a receptor group in this DSF communication, showing a lethargic DSF-deficient phenotype in axenic conditions that would save energy and allow a finer regulation of processes related to DSF communication. Recent studies have evidenced that within bacterial communities there

exist individual cells that turn off their QS communication—by accumulation of diverse mutations—and take advantage of public goods, thus saving energy. This recently discovered behavior is termed "social cheating" and has raised much interest. It has been observed that several *P. aeruginosa* isolates from cystic fibrosis patients accumulate mutations in the gene encoding for the QS regulator LasR (Smith et al., 2006; Tingpej et al., 2007; Hoffman et al., 2009). Although *lasR* mutants are not able to trigger the QS response (Haas, 2006), they frequently coexist with wild-type isolates and take advantages from their intact QS-regulation (Sandoz et al., 2007). Inspired by this phenomenon, we speculate that *S. maltophilia rpf*-2 variant strains may constitute a conserved, therefore successful, population of "social cheaters." The specific advantages (besides energy saving) and disadvantages (in the absence of DSF-producer bacteria) of this behavior are yet to be elucidated.

Interspecies communication mediated by DSF-like signal molecules appears to be common in Proteobacteria, not only among Xanthomonadales (Deng et al., 2011). For example, it has been shown that DSF produced by *S. maltophilia* modulates the behavior of *P. aeruginosa*, including biofilm formation and antibiotic resistance (Ryan et al., 2008) and virulence and persistence in lungs of CF patients (Twomey et al., 2012). *Xcc* can also regulate certain virulence factors in response to BDSF produced by *B. cenocepacia* (Deng et al., 2010). It has been also reported that the DSF-related signal *cis*-DA (*cis*-decenoic acid) produced by *P. aeruginosa* (Davies and Marques, 2009; Amari et al., 2013) induces biofilm dispersion of several Gram-negative and Gram-positive bacteria (Davies and Marques, 2009). Recently, our group has reported that *S. maltophilia* can also respond to acyl homoserine lactone (AHL) signals produced by *P. aeruginosa* and regulate swarming motility (Martínez et al., 2015). Additionally, the morphological transition of *C. albicans* is inhibited in the presence of various DSF-like molecules (Wang et al., 2004; Boon et al., 2008; Deng et al., 2010) including SDSF (*trans*-2-decenoic acid), a FA produced by *Streptococcus mutans* (Vílchez et al., 2010). Clearly, FA-mediated communication is largely distributed in bacteria and has a very relevant interspecies component. Combining these data with our results, it becomes obvious that complex population processes, such as QS, are not well addressed when they are studied only in axenic conditions.

Acknowledgments

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00761

File S1 | Detection of putative recombination breakpoints in the *rpf* cluster.

Figure S1 | DSF microtitter bioassay. (Top) Wells containing DSF-reporter solution (see Materials and Methods) inoculated with increasing concentrations (0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mM) of synthetic DSF (Sigma). **(Bottom)** Calibration curve ($rl = 0.9787$) generated by reading the absorbance of the wells at 620 nm after incubation at 28°C for 24 h.

Figure S2 | Mass spectra of the Gas Chromatography peaks corresponding to the fatty acids 14:0 (21.2 min), *iso*-15:0 (22.5 min), 16:0 (25.0 min), and 18:0 (26.5 min).

Table S1 | Total cellular fatty acids in *S. maltophilia* mutants E77 Δ*rpfF*-1 and M30 Δ*rpfF*-2.

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Cooperative pathogenicity in cystic fibrosis: *Stenotrophomonas maltophilia* modulates *Pseudomonas aeruginosa* virulence in mixed biofilm

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The present study was undertaken in order to understand more about the interaction occurring between *S. maltophilia* and *P. aeruginosa*, which are frequently co-isolated from CF airways. For this purpose, *S. maltophilia* RR7 and *P. aeruginosa* RR8 strains, co-isolated from the lung of a chronically infected CF patient during a pulmonary exacerbation episode, were evaluated for reciprocal effect during planktonic growth, adhesion and biofilm formation onto both polystyrene and CF bronchial cell monolayer, motility, as well as for gene expression in mixed biofilms. *P. aeruginosa* significantly affected *S. maltophilia* growth in both planktonic and biofilm cultures, due to an inhibitory activity probably requiring direct contact. Conversely, no effect was observed on *P. aeruginosa* by *S. maltophilia*. Compared with monocultures, the adhesiveness of *P. aeruginosa* on CFBE41o- cells was significantly reduced by *S. maltophilia*, which probably acts by reducing *P. aeruginosa's* swimming motility. An opposite trend was observed for biofilm formation, confirming the findings obtained using polystyrene. When grown in mixed biofilm with *S. maltophilia*, *P. aeruginosa* significantly over-expressed *aprA*, and *algD*—codifying for protease and alginate, respectively—while the quorum sensing related *rhlR* and *lasI* genes were down-regulated. The induced alginate expression by *P. aeruginosa* might be responsible for the protection of *S. maltophilia* against tobramycin activity we observed in mixed biofilms. Taken together, our results suggest that the existence of reciprocal interference of *S. maltophilia* and *P. aeruginosa* in CF lung is plausible. In particular, *S. maltophilia* might confer some selective "fitness advantage" to *P. aeruginosa* under the specific conditions of chronic infection or, alternatively, increase the virulence of *P. aeruginosa* thus leading to pulmonary exacerbation.

Keywords: cystic fibrosis, lung infections, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, microbial interactions

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Introduction

Pulmonary disease is the leading cause of morbidity and mortality in cystic fibrosis (CF) patients, in whom defective mucociliary clearance and impaired innate immunity lead to chronic pulmonary infections (Lyczak et al., 2002). During CF airway disease periods of stability are punctuated by acute pulmonary exacerbations (PEs) in which overt immunological responses are the main causes of irreversible lung damage (Amadori et al., 2009; Sanders et al., 2011). Recurrent PEs are associated with a shortened survival (Emerson et al., 2002; Marshall, 2004).

The pathophysiology of PEs is not yet completely understood. Aside from acute viral infections which, especially in children, have been associated with up to one-third of PEs (Armstrong et al., 1998; Clifton et al., 2008), *Pseudomonas aeruginosa* has been considered the primary cause of PEs and related long-term decline in lung function (Goss and Burns, 2007; Sanders et al., 2010).

The pathogenesis of *P. aeruginosa* infection depends on several cell-associated and extracellular virulence factors, including proteases and toxins, whose expression is mainly regulated by hierarchically organized inter-bacterial communication LasRI and RhIRI quorum sensing (QS) systems, which monitor population size using various diffusible N-acylhomoserine lactones as signal molecules (Goodman and Lory, 2004).

However, this view is not convincingly supported by either the clinical or the microbiological evidence available.

Several studies have reported that in adult CF patients the anti-pseudomonal antibiotic therapy frequently did not reduce *P. aeruginosa* load and airways inflammation (Wolter et al., 1999; Reid et al., 2004), and that about 25% of patients experiencing a PE did not recover their baseline lung function after treatment, leading to a progressive deterioration in their clinical status over time (Sanders et al., 2010, 2011). Furthermore, in other studies no increase in *P. aeruginosa* concentration was observed immediately prior to, or at the time of, PE (Stressmann et al., 2011; Reid et al., 2013). Recent epidemiological data indicate that coinfections involving different species of bacteria are common, and probably represent the norm, in CF lung (Harrison, 2007; Bittar et al., 2008; Sibley et al., 2008a; Rogers et al., 2010a). *P. aeruginosa* is the most common species found in CF airways, but other species are frequently co-isolated in CF lung (Harrison, 2007).

The pathophysiology of PEs in CF could be, therefore, directly related to changes in microbial behavior and/or to the dynamics of the interactions between the constituents of the complex microbial communities present. Several studies have recently highlighted the potential role of interspecies interactions in influencing infection status, clinical outcomes or response to therapy in CF patients (Harrison, 2007; Ryan et al., 2008; Sibley et al., 2008b, 2009; Shank and Kolter, 2009; Rogers et al., 2010b). Taken together, these findings suggest that the role of microbial species other than *P. aeruginosa* needs to be considered.

Stenotrophomonas maltophilia is one of the most common emerging multi-drug resistant organisms found in the lungs of people with CF where its prevalence is increasing (Ciofu et al., 2013). Nevertheless, the role of *S. maltophilia* in the pathogenesis of CF lung disease is not yet clear because of conflicting results from clinical studies which focused on the correlation between the presence of this microorganism and lung damage (Karpati et al., 1994; Goss et al., 2002). In a series of studies, we found evidence highly suggestive of the pathogenic role of *S. maltophilia* in CF patients (Di Bonaventura et al., 2004, 2007a,b, 2010; Pompilio et al., 2008, 2010, 2011). This microorganism can grow as biofilms—sessile communities inherently resistant to antibiotics and host immune response—not only on abiotic surfaces (Di Bonaventura et al., 2004, 2007a,b; Pompilio et al., 2008), but also on CF-derived epithelial monolayer (Pompilio et al., 2010), probably because of a selective adaptation to CF airways (Pompilio et al., 2011). Furthermore, in a murine model of acute respiratory infection we observed that *S. maltophilia* significantly contributes to the inflammatory process resulting in compromised respiratory function and death (Di Bonaventura et al., 2010).

S. maltophilia is often co-isolated with *P. aeruginosa*, with a frequency ranging from 10 to 60% of CF patients (Spicuzza et al., 2009; Blau et al., 2014; Zemanick et al., 2015); however, no data are available on this at the onset of PE. It is, therefore, plausible to hypothesize that these species interact and that this could potentially affect the virulence and persistence of *P. aeruginosa*. While several studies have focused on the interaction of *P. aeruginosa* with other bacterial species (Qin et al., 2009; Pihl et al., 2010; Baldan et al., 2014), very little has been published on the interaction between *P. aeruginosa* and *S. maltophilia*. In this regard, Ryan et al. (2008) found that the presence of *S. maltophilia* increases *P. aeruginosa* resistance to polymyxin by a diffusible signal factor, while Kataoka et al. (2003) observed that β-lactamases leaking from *S. maltophilia* can encourage the growth of *P. aeruginosa* in the presence of imipenem or ceftazidime.

Recently, we observed that the pre-incubation of the CF bronchial epithelial IB3-1 cells with *S. maltophilia* decreases the adherence of *P. aeruginosa*, while previous *P. aeruginosa* infection may increase the likelihood of *S. maltophilia* colonizing a damaged CF pulmonary mucosa (Pompilio et al., 2010).

Our hypothesis, based on these clinical and experimental findings, was that in CF lung *S. maltophilia* may indirectly contribute to disease development by providing a favorable growth environment for *P. aeruginosa* and/or by modulating some virulence traits exhibited by *P. aeruginosa*. In order to test this hypothesis, *S. maltophilia* RR7 and *P. aeruginosa* RR8—two strains co-isolated at the same time from the lung of a CF patient during a PE episode—were evaluated, both in combined and monomicrobial cultures, with respect to planktonic growth, adhesion and biofilm formation onto both polystyrene and CF bronchial cell monolayer, and motility. The effect of

Abbreviations: CF, cystic fibrosis; PE, pulmonary exacerbation; QS, quorum sensing; MHA, Mueller-Hinton agar; TSB, tryptone soya broth; CAMHB, cation-adjusted Mueller-Hinton broth; PBS, phosphate buffered saline; OD, optical density; EDTA, ethylenediaminetetraacetic acid; MOI, multiplicity of infection; RT-PCR, real-time PCR; CI, competitive index; RIR, relative increase ratio; EPS, extracellular polymeric substance.

S. maltophilia RR7 on the expression of selected *P. aeruginosa* RR8 virulence genes was also assessed in mixed biofilms.

We show that *S. maltophilia* has the potential to significantly affect *P. aeruginosa* virulence, which suggests that interspecies interactions are operative in promoting bacterial pathogenicity in CF lung infections.

Materials and Methods

Bacterial Strains and Growth Conditions

P. aeruginosa RR8 and *S. maltophilia* RR7 strains were co-isolated from the same sputum sample collected, during a PE, from a 15-year-old CF patient (named "RR") attending Children Hospital "Bambino Gesù" of Rome. The patient was selected because he was chronically infected by both *P. aeruginosa* and *S. maltophilia*, according to the definition proposed by the EuroCareCF Working Group (Pressler et al., 2011). The strains were identified by the API 20-NE system (bioMérieux, Marcy-L'Etoile, France) and stored at −80°C until use. Fresh bacterial stocks were thawed and grown twice on Mueller-Hinton agar (MHA; Oxoid S.p.A., Garbagnate M.se, Italy) to check for purity and to regain the original phenotype: (i) *P. aeruginosa* RR8 strain was mucoid and resistant to amikacin and gentamicin only; (ii) *S. maltophilia* RR7 strain, not mucoid, showed resistance to ceftazidime, piperacillin/tazobactam, amikacin, gentamicin, ciprofloxacin, and levofloxacin.

All assays were carried out by using a standardized bacterial inoculum. Briefly, an overnight culture in tryptone soya broth (TSB), grew under agitation (130 rpm) at 37°C, was adjusted with sterile broth to an OD_{550} corresponding to $1–3 \times 10^8$ CFU/ml for both strains, then diluted 1:10 in cation-adjusted Mueller-Hinton broth (CAMHB; Becton, Dickinson and Company; Milan, Italy) (pH 7.2–7.4).

Mono- and Co-culture Planktonic Growth

Each well of a 96-well polystyrene, flat bottom, tissue culture-treated microtiter (BD Falcon, Milan, Italy) was seeded with 100 μl of each standardized inoculum (dual cultures) or 100 μl of standardized inoculum +100 μl sterile medium (single cultures). Microtiters were then statically incubated at 37°C, under aerobic atmosphere, for 24 h. At different time points (2, 4, 6, 8, 10, and 24 h) samples were taken, serially diluted in sterile phosphate buffered saline (PBS; Sigma-Aldrich S.p.A.; Milan, Italy) (pH 7.2), then plated onto MHA + imipenem at 32 μg/ml or *Pseudomonas* cetrimide agar (Oxoid) to discriminate *S. maltophilia*, and *P. aeruginosa* growth, respectively. The agar plates were incubated at 37°C for 24 h when the CFU number was recorded.

Kinetics of Adhesion and Biofilm Formation by Single and Mixed Cultures

Each well of a 96-well polystyrene, flat bottom, tissue culture-treated microtiter was seeded as described above, and statically incubated at 37°C, under aerobic atmosphere, for 3 h (adhesion) or up to 7 days (biofilm formation). During biofilm formation assay, CAMHB was replaced daily with fresh broth. At each time-point considered (3 h, and 1–7 days), samples were washed twice with sterile PBS, and then adhesion or biofilm were measured for both biomass and viability. Biomass was measured in terms of optical density read at 492 nm (OD_{492}) following crystal violet staining, as previously described (Pompilio et al., 2008), considering a low cut-off of ODc + 3 × SDs, where ODc is OD_{492} of control wells (containing medium alone without bacteria). For viability assessment, samples were exposed to trypsin-ethylenediaminetetraacetic acid (EDTA) 0.25% (Sigma-Aldrich) to allow detachment from the polystyrene, and then the viable count was carried out as described above.

Preparation of Cell-free Culture Supernatant

For each strain, some colonies from an overnight MHA growth were suspended in 20 ml of TSB and incubated overnight at 37°C, under agitation (220 rpm). Supernatants were obtained after centrifugation at 12,000 × g, for 10 min at 4°C, followed by sterile filtration using a 0.20 μm-pore-size filter (Corning; Tewksbury, MA, USA), and storage at −80°C until use.

Evaluation of Antibacterial Activity by *S. maltophilia* RR7 and *P. aeruginosa* RR8

The antibacterial activity of each strain was evaluated by the drop test assay. Briefly, 100 μl of the standardized inoculum containing the indicator strain were streaked onto an MHA surface using a cotton swab, and then the plate was dried at 30°C for 30 min. The test strain was then evaluated for antimicrobial activity, both as cell suspension and supernatant. Drops containing 10 μl of the supernatant or standardized suspension, and a control, were placed on the agar surface and the inhibition zone diameter was measured after incubation at 37°C for 24 h.

To evaluate whether the inhibitory activity of *P. aeruginosa* RR8 on *S. maltophilia* RR7 required direct cell-cell interactions, a transwell-based assay was performed. Briefly, *P. aeruginosa* RR8 was inoculated at $1–5 \times 10^8$ CFU/ml in the upper insert (Cell Culture Insert; BD Falcon), while *S. maltophilia* RR7 was inoculated at the same concentration in the bottom of the culture well. Following 24 h-incubation at 37°C, suspensions from both compartments underwent a viable count. A sample where the upper insert contained TSB without *P. aeruginosa* RR8 was used as control.

Adhesion to, and Biofilm Formation on CF Bronchial Cells

CFBE41o- bronchial cells, derived from a CF patient homozygous for the F508del CFTR mutation, were obtained from Dr. Dieter C. Gruenert (University of California, San Francisco, USA). Briefly, confluent CFBEo- cells were grown in tissue polystyrene culture flasks (BD Falcon) in Minimum Essential Medium (GIBCO, Life-technologies; Monza, Italy) supplemented with 10% fetal bovine serum (GIBCO), 50 U/ml penicillin, 50 μg/ml streptomycin (both from Sigma-Aldrich), and 2 mM L-glutamine (GIBCO). Monolayers were simultaneously infected with *S. maltophilia* RR7 and *P. aeruginosa* RR8 strains (ratio 1:1, each at 10^6 CFU/ml; multiplicity of infection, MOI: 10), then incubated at 37°C for 3 h (adhesion assay) or 24 h (biofilm formation assay). CFBE41o- cell monolayers infected with a single strain (at 10^6 CFU/ml) were prepared as control. At the end of incubation, infected

monolayers were washed twice with PBS, then detached by 0.25% trypsin/EDTA, and finally plated for colony count.

Tobramycin Activity against Planktonic and Biofilm Cells

MICs of tobramycin (Sigma-Aldrich) for *S. maltophilia* RR7 and *P. aeruginosa* RR8 were respectively 128 and 4 μg/ml, as assessed by microdilution technique in accordance with the M100-S20 protocol [Clinical and Laboratory Standards Institute, 2010]. Tobramycin was also tested against preformed single species and mixed biofilms. Briefly, biofilms were allowed to form at 37°C for 24 h as described in "Kinetics of adhesion and biofilm formation by single and mixed cultures." Samples were washed once with sterile CAMHB, and then exposed to 200 μl of tobramycin at 128 μg/ml. After incubation at 37°C for 24 h, non-adherent bacteria were removed by washing twice with sterile PBS, and biofilm cells were scraped with a pipette tip following 5 min-exposure to 100 μl trypsin-EDTA 0.25%. Cell suspension was vortexed at high speed for 1 min to break up the clumps, and then bacterial counts were assessed by plating serial 10-fold dilutions of the biofilm cell suspension onto selective media MHA plates.

Motility Assays

Swimming, swarming, and twitching motilities were evaluated using dedicated agar media, as previously described (Pompilio et al., 2008). The relative motility of two strains was assessed by using agar prepared with and without 1:2 diluted culture supernatant of the other strain.

Gene Expression Assay in Mixed Biofilm

The effect of *S. maltophilia* RR7 on the transcription levels of several virulence factors (**Table 1**) of *P. aeruginosa* RR8 was assessed in mixed biofilms by real-time PCR (RT-PCR). *P. aeruginosa* RR8 was cultured as biofilm, both alone and with *S. maltophilia* RR7, in 24-well polystyrene, flat bottom, tissue culture-treated microtiter (BD Falcon), as described above. Following 24 h-incubation at 37°C, biofilms were washed, and then harvested by scraping in Qiazol (Qiagen; Milan, Italy). RNA was then extracted by the phenol-chloroform technique (Kang et al., 2009), treated with DNase I (Applied Biosystems Italia; Monza, Italy), and checked for purity by NanoDrop-2000 spectrophotometer (Thermo Scientific Italia; Milan, Italy). First strand cDNA was synthesized using a High Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. Gene expression was evaluated using a SYBR green (Applied Biosystems) RT-PCR assay. The primers' specificity was assessed both *in silico* with BLAST and by PCR endpoint under the same RT-PCR conditions. Each amplification assay was also tested for *S. maltophilia* RR7 as an additional negative control in each RT-PCR. The ΔΔCt method was used to determine the relative gene expression of each gene in co-culture vs. monoculture biofilms normalized to the expression of the housekeeping gene *proC*.

Interpretative Criteria and Statistical Analysis

Each experiment was performed in triplicate and repeated on two different occasions. All statistical analyses were performed by GraphPad Prism software (ver. 4.0; GraphPad Inc, San

TABLE 1 | List of primer sequences used in RT-PCR for expression analysis of virulence genes by *P. aeruginosa* RR8 grown as biofilm, both in solo and co-cultured with *S. maltophilia* RR7.

Primers, 5′–3′	Primer ID	Target gene	Gene function	References
CTCAGGATGATGGCGATTTC	rhlR-F	*rhlR*	Quorum sensing	Pérez-Osorio et al., 2010
AATTTGCTCAGCGTGCTTTC	rhlR-R	–	–	–
GCGTGCTGCACTACTCCATG	toxA-F	*toxA*	Exotoxin A	Davinic et al., 2009
GTTACCGGCGTTCAGTTCGT	toxA-R	–	–	–
GCTGCGAAACGCTGTTCTTC	vfr-F	*vfr*	Virulence regulator las system	Davinic et al., 2009
GCTGCCGAGGGTGTAGAGG	vfr-R	–	–	–
GCGACCTGGACCTGGGCT	algD-F	*algD*	Enhancer for alginate synthesis	Joly et al., 2005
TCCTCGATCAGCGGGATC	algD-R	–	–	–
GCTTCTGCACGGCAAGGA	lasI-F	*lasI*	Quorum sensing	Joly et al., 2005
ATGGCGAAACGGCTGAGTT	lasI-R	–	–	–
GGCGGATGCGGAAAAGTAC	exoS-F	*exoS*	Type III system effector	Joly et al., 2005
CTGACGCAGAGCGCGATT	exoS-R	–	–	–
TACTCGCTGGGCAAGTTCAGGCG	aprA-F	*aprA*	Alkaline protease	Vettoretti et al., 2009
GTAGCTCATCACCGAATAGGCG	aprA-R	–	–	–
GTACCGGCGTCATGC AGGGTTC	mexC-F	*mexC*	Efflux pump	Savli et al., 2003
TTACTGTTGCGGCGCAGGTGACT	mexC-R	–	–	–
CCAGGACCAGCACGAACT TCT TGC	mexE-F	*mexE*	Efflux pump	Vettoretti et al., 2009
CGACAACGCCAAGGGCGAGTTCACC	mexE-R	–	–	–
CAGGCCGGGCAGTTGCTGTC	proC-F	*proC**	Proline biosynthesis	Savli et al., 2003
GGTCAGGCGCGAGGCTGTCT	proC-R	–	–	–

**proC was tested as housekeeping gene.*

Diego, USA), considering as statistically significant a *p*-value less than 0.05. Differences were assessed by ANOVA-test followed by Newman-Keuls multiple comparison post-test (kinetics of planktonic growth, adhesion and biofilm formation onto both polystyrene and CFBE41o- cells, and tobramycin activity against preformed biofilms), chi-square test (adhesion and biofilm formation efficiency) or paired Student's *t*-test (motility and gene expression). The efficiency of adhesion and biofilm formation onto CFBE41o- cells was calculated for each strain, both in single and mixed infections, as a percentage referred to the initial inoculum: (A/B) × 100, where A is the number of adhered or biofilm bacteria, and B is the number of bacteria in the initial inoculum.

In mixed cultures, the Competitive Index (CI) was defined as the *S. maltophilia*/*P. aeruginosa* ratio within the output sample divided by the corresponding ratio in the inoculum (input): $CI = (S.\ maltophilia/P.\ aeruginosa)_{output}/(S.\ maltophilia/P.\ aeruginosa)_{input}$, where output and input samples were assessed after plating onto MHA serial dilutions of the sample taken at fixed times or the inoculum ($t = 0$), respectively (Macho et al., 2007). For statistical analyses, CI values were first subjected to a Log transformation for normal distribution, then interpreted as follows: a CI value equal to 0 indicates equal competition of the two species; a positive CI value indicates a competitive advantage for *S. maltophilia*; a negative CI value indicates a competitive advantage for *P. aeruginosa*.

Similarly to CI, the Relative Increase Ratio (RIR) was calculated based on the growth results obtained from monocultures of each strain (Macho et al., 2007). Each CI and RIR was analyzed using the Student's *t*-test and the null hypothesis: the mean index was not significantly different from 1.0. When appropriate, CI and RIR from a given experiment were compared using unpaired Student's *t*-test, and significant differences are suggestive of a meaningful competition between the species (Macho et al., 2007).

Results

P. aeruginosa Significantly Affects *S. maltophilia* Growth in Planktonic Culture

The growth curve kinetics of *P. aeruginosa* RR8 and *S. maltophilia* RR7 strains, tested as alone or in mixed culture, were assessed by colony count over 24 h, and the results are shown in **Figure 1A**. When grown in mixed cultures, the growth kinetics of the two strains were comparable. However, compared with single cultures, the growth of both *S. maltophilia* RR7 and *P. aeruginosa* RR8 in mixed cultures was negatively affected during both log and stationary phases.

To assess further the meaning of the differences between the variations observed in single vs. mixed cultures, CI and RIR were calculated and compared, as shown in **Figure 1B**. The CI of *S. maltophilia* RR7 vs. *P. aeruginosa* RR8 was significantly different from the respective RIR values between 6 h- and 24 h-incubation, suggesting that *P. aeruginosa* RR8 exerts an inhibitory effect on *S. maltophilia* RR8 growth during both log and stationary growth phases.

P. aeruginosa Exhibits Antimicrobial Activity against *S. maltophilia* in a Contact-dependent Manner

The agar spot assay results showed that *P. aeruginosa* RR8 is active against *S. maltophilia* RR7, although only when tested as cell suspension (diameter of inhibition zone, mean ± SD: 12.5 ± 1.4 mm), and not as supernatant (**Figure 1C**). However, the inhibition halo showed a partial regrowth, suggesting that a partial inhibition occurred. Conversely, *S. maltophilia* RR7 showed no activity against *P. aeruginosa* RR8, as supernatant or suspension (**Figure 1C**).

The inhibitory effect of *P. aeruginosa* RR8 on *S. maltophilia* RR7 was also assessed using a transwell-based assay, and the results are shown in **Figure 1D**. *S. maltophilia* growth was not significantly affected by the presence of *P. aeruginosa* ($9.6 \pm 3.4 \times 10^8$ CFU/ml vs. $5.9 \pm 3.9 \times 10^8$ CFU/ml for unexposed and exposed *S. maltophilia*, respectively; $p > 0.05$), which suggests that *P. aeruginosa* inhibits *S. maltophilia* growth in a contact-dependent manner.

Kinetics of *S. maltophilia* and *P. aeruginosa* Interaction during Adhesion and Biofilm Formation onto Polystyrene

The interaction during adhesion phase between *S. maltophilia* RR7 and *P. aeruginosa* RR8 strains was assessed, tested as single and mixed cultures, after 3 h-incubation, both by crystal violet and the viable count assays; the results are reported in **Figure 2**.

The crystal violet assay showed that *P. aeruginosa* RR8, when cultured alone, exhibited a significantly higher adhesiveness than *S. maltophilia* RR7 (OD_{492}, mean ± SD: 0.781 ± 0.095 vs. 0.079 ± 0.026, respectively; $p < 0.001$) (**Figure 2A**). No differences, however, were found between the strains in terms of cell viability (**Figure 2B**), which suggests that extracellular polymeric substance (EPS) contributes substantially to *P. aeruginosa* RR8 adhesion.

In mixed infections, the numbers of viable cells of each strain that adhered to polystyrene were not affected by the presence of the other (**Figure 2B**), as was confirmed by comparable CI and RIR values (**Figure 2C**). However, in mixed culture the biomass adhesion level of *S. maltophilia* RR7 was significantly lower than that of *P. aeruginosa* RR8 (OD_{492}, mean ± SD: 0.645 ± 0.051 vs. 0.781 ± 0.095, respectively; $p < 0.01$), which indicates that the presence of *S. maltophilia* RR7 leads to a reduction in *P. aeruginosa* RR8 EPS.

The interaction in biofilm growth between *S. maltophilia* RR7 and *P. aeruginosa* RR8, tested as single and mixed cultures, was monitored over 7 days, by both crystal violet and viable count assays; results are shown in **Figure 3**.

The kinetics of biofilm formation, as assessed by crystal violet staining, are summarized in **Figure 3A**. In monoculture, from day 1 until day 5 of incubation *P. aeruginosa* RR8 formed significantly higher biofilm biomass amount than *S. maltophilia* RR7. The kinetics of biofilm formation by *P. aeruginosa* RR8 and RR7+RR8 mixed infection

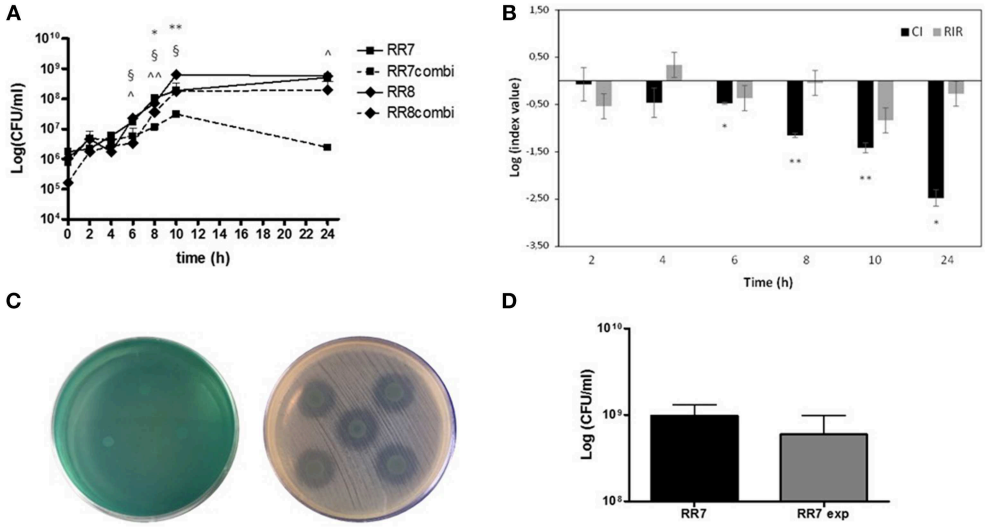

FIGURE 1 | Kinetics of planktonic growth exhibited by *S. maltophilia* RR7 and *P. aeruginosa* RR8, considered both as alone and in combination. *S. maltophilia* RR7 and *P. aeruginosa* RR8 were grown for 24 h in CAMHB in single culture and in co-culture after inoculation at equal ratio from mid-exponential phase pure cultures. Growth rate was monitored by colony count after plating on selective media for both species. **(A)** Growth curves of *S. maltophilia* RR7 and *P. aeruginosa* RR8 strains in pure culture (RR7, RR8) and in co-culture (RR7combi, RR8combi). The results are shown as mean + SD ($n = 6$). *$p < 0.05$, **$p < 0.01$, RR7 vs. RR8; §$p < 0.05$, RR8 vs. RR8combi; ^$p < 0.05$, ^^$p < 0.01$, RR7 vs. RR7combi; ANOVA + Newman-Keuls post-test. **(B)** Competitive index (CI; black bars) and Relative Increase Ratio (RIR; gray bars) calculated from single and dual planktonic cultures of *S. maltophilia* RR7 and *P. aeruginosa* RR8 strains. The results are shown as mean ± SD ($n = 6$). *$p < 0.05$, **$p < 0.01$, CI vs. RIR, unpaired *t*-test. **(C)** Evaluation of antibacterial activity using agar spot assay. *S. maltophilia* RR7 vs. *P. aeruginosa* RR8 (left): no antibacterial activity. *P. aeruginosa* RR8 vs. *S. maltophilia* RR7 (right): partial antibacterial activity, as suggested by the regrowth observed within the inhibition zone. **(D)** Transwell assay: *S. maltophilia* RR7 growth without or with (exp) *P. aeruginosa* RR8. The results are expressed as mean + SD ($n = 6$).

followed a comparable trend throughout the 7 day period. From day 2 until day 5 of incubation, the mean biofilm biomass amount formed by both *P. aeruginosa* RR8 and mixed infection was significantly higher than when *S. maltophilia* RR7 was cultured alone. On day 6, all conditions tested produced comparable mean biofilm biomass values.

The biofilm formation kinetics evaluated by the viable count are shown in **Figure 3B**. In monoculture, the viability of biofilm formed by *S. maltophilia* RR7 was higher than that of *P. aeruginosa* RR8 over the entire period (**Figure 3B**). Given the different endpoints measured by the crystal violet (biofilm biomass, consisting of both EPS and cells) and the viable count (biofilm viability) assays, our results indicate that only in *P. aeruginosa* does the amount of EPS increase over time during biofilm formation, as is also confirmed by the mucoid appearance of the biofilm samples observed during macroscopic analysis of the 96-well plate.

When *S. maltophilia* RR7 was co-cultured with *P. aeruginosa* RR8 in mixed biofilms, its viability was significantly decreased throughout the period. Conversely, the biofilm viability of *P. aeruginosa* RR8 was not affected by the presence of *S. maltophilia* RR7.

The CI values of *S. maltophilia* vs. *P. aeruginosa* were always significantly different from the RIR values ($p < 0.001$) throughout the 7 days of incubation, which suggests that *P. aeruginosa* outcompetes *S. maltophilia* affecting its growth in mixed biofilms as well as in planktonic cultures (**Figure 3C**).

Adhesion to and Biofilm Formation on CFBE41o- cells

P. aeruginosa RR8 and *S. maltophilia* RR7 were evaluated, both alone and in mixed culture, for adhesion to, and biofilm formation onto, CFBE41o- CF cell monolayer. The results of the viable count assay are summarized in **Figure 4**.

When monolayers were separately infected, the adhesiveness of *P. aeruginosa* RR8 to CFBE41o- cells was significantly higher than that of *S. maltophilia* RR7 ($1.3 \pm 0.3 \times 10^7$ vs. $2.8 \pm 0.7 \times 10^5$ CFU/well, respectively; $p < 0.01$) (**Figure 4A**). Conversely, when CFBE41o- cell monolayer was concomitantly challenged by both strains their degrees of adhesiveness were comparable. However, the adhesiveness of *P. aeruginosa* RR8 was significantly less than that observed in monoculture ($1.1 \pm 0.7 \times 10^6$ vs. $1.3 \pm 0.3 \times 10^7$ CFU/well, respectively; $p < 0.05$), unlike *S. maltophilia* RR7, whose adhesiveness was not affected by the presence of *P. aeruginosa* RR8. The comparative analysis of the CI and the RIR values showed no statistically significant difference (**Figure 4C**), although a comparison of efficiency values, from single and mixed cultures, showed a significant decrease for *P. aeruginosa* RR8 (from 12.9 to 4.9%, respectively; $p < 0.05$), whereas a significant increase was found for *S. maltophilia* RR7 (from 0.08 to 0.3%, respectively; $p < 0.001$).

Both *S. maltophilia* RR7 and *P. aeruginosa* RR8 showed comparable biofilm viability when tested in monoculture (**Figure 4B**). However, when CFBE41o- cell monolayer was simultaneously infected by both strains, the concentration of

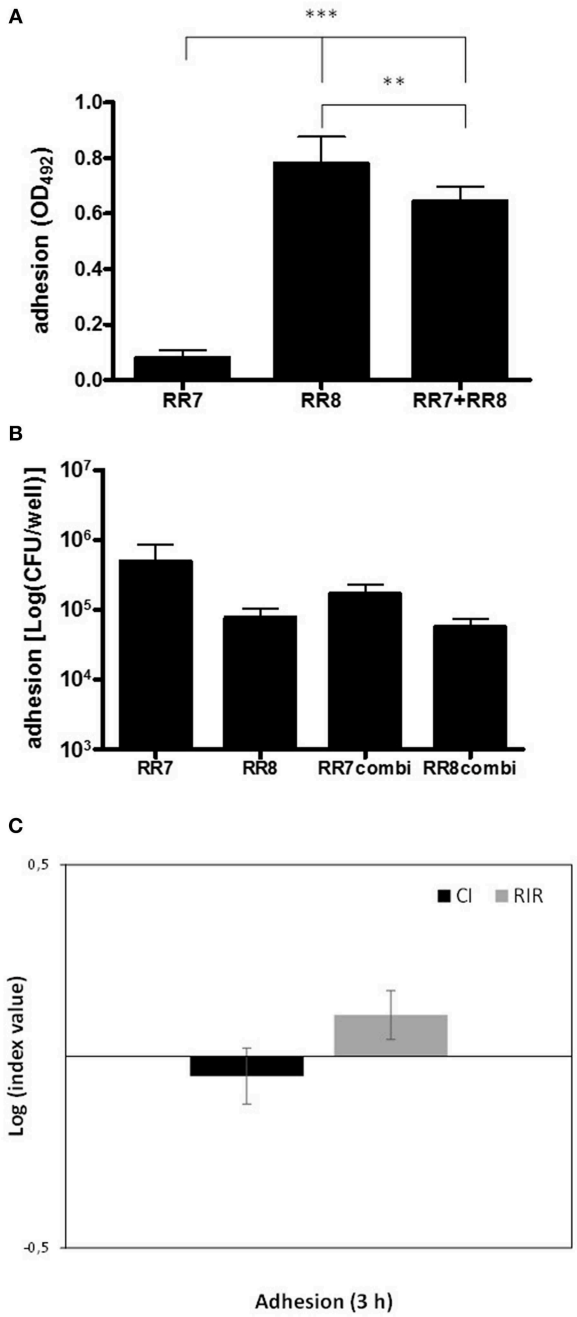

FIGURE 2 | Kinetics of adhesion onto polystyrene by *S. maltophilia* RR7 and *P. aeruginosa* RR8, considered both as alone and in combination. Adhesion was assessed, after 3 h of incubation at 37°C, both by crystal violet and viable count assays. **(A)** Crystal violet assay. *S. maltophilia* RR7, and *P. aeruginosa* RR8 were tested both alone (RR7, RR8), and in mixed infection (RR7 + RR8), and the results are shown as mean + SD ($n = 6$). $^{**}p < 0.01$, $^{***}p < 0.001$, ANOVA + Newman-Keuls post-test. **(B)** Viable count assay. *S. maltophilia* RR7 and *P. aeruginosa* RR8 strains were tested both alone (RR7, RR8), and in mixed infection (RR7combi, RR8combi), and the results are shown as mean + SD ($n = 6$). No statistically significant differences were found among groups by ANOVA + Newman-Keuls post-test. **(C)** Competitive index (CI; black bars) and Relative Increase Ratio (RIR; gray bars), calculated from single and dual cultures of *S. maltophilia* RR7 and *P. aeruginosa* RR8. The results are shown as mean ± SD ($n = 6$). CI vs. RIR, no statistically significant difference, unpaired *t*-test.

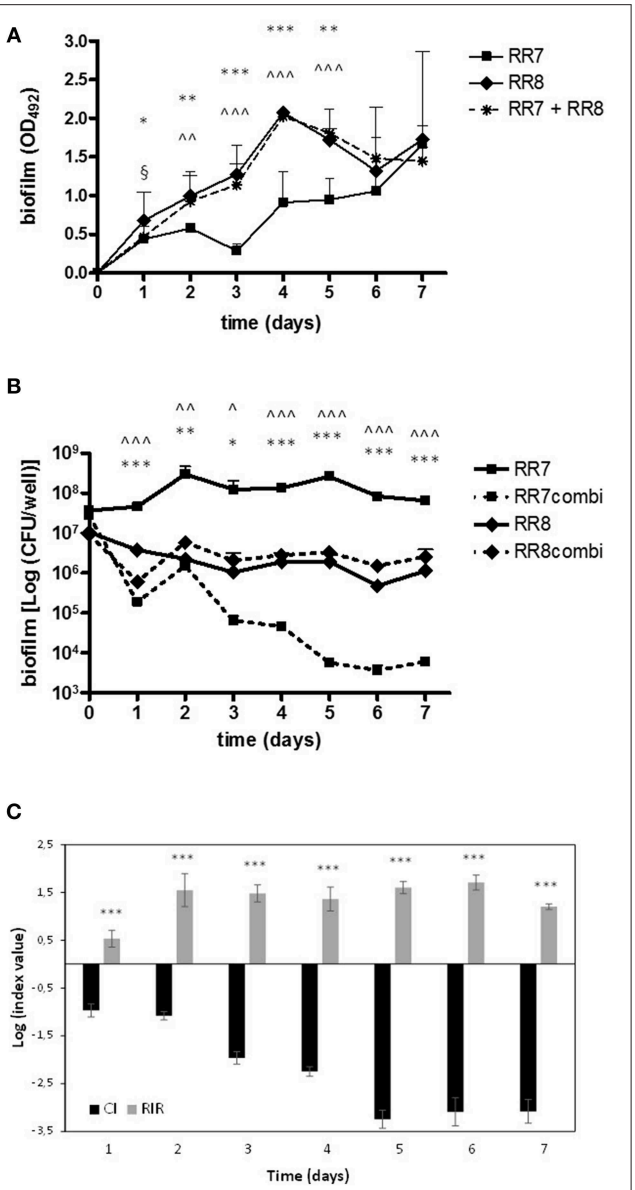

FIGURE 3 | Kinetics of biofilm formation onto polystyrene by *S. maltophilia* RR7 and *P. aeruginosa* RR8, both considered as alone and in combination. Biofilm formation was assessed, over 7 days, both by **(A)** crystal violet and **(B)** viable count assays. *S. maltophilia* RR7 and *P. aeruginosa* RR8 were tested both alone (RR7, RR8), and in mixed infection (RR7 + RR8), and the results are shown as mean + SD ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, RR7 vs. RR8; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, RR7 vs. RR7combi; $^{\S}p < 0.05$, RR8 vs. RR8combi; ANOVA + Newman-Keuls post-test. **(C)** Competitive index (CI; black bars) and Relative Increase Ratio (RIR; gray bars) calculated from single and dual biofilm cultures of *S. maltophilia* RR7 and *P. aeruginosa* RR8. The results are shown as mean ± SD ($n = 6$). $^{***}p < 0.001$, CI vs. RIR, unpaired *t*-test.

P. aeruginosa RR8 in mixed biofilm was significantly higher than that of *S. maltophilia* RR7 ($2.2 \pm 1.5 \times 10^7$ vs. $3.2 \pm 2.9 \times 10^5$ CFU/well, respectively; $p < 0.05$), whose concentration was significantly lower than that observed in monomicrobial biofilm ($2.3 \pm 0.5 \times 10^7$ CFU/well; $p < 0.05$) (**Figure 4B**).

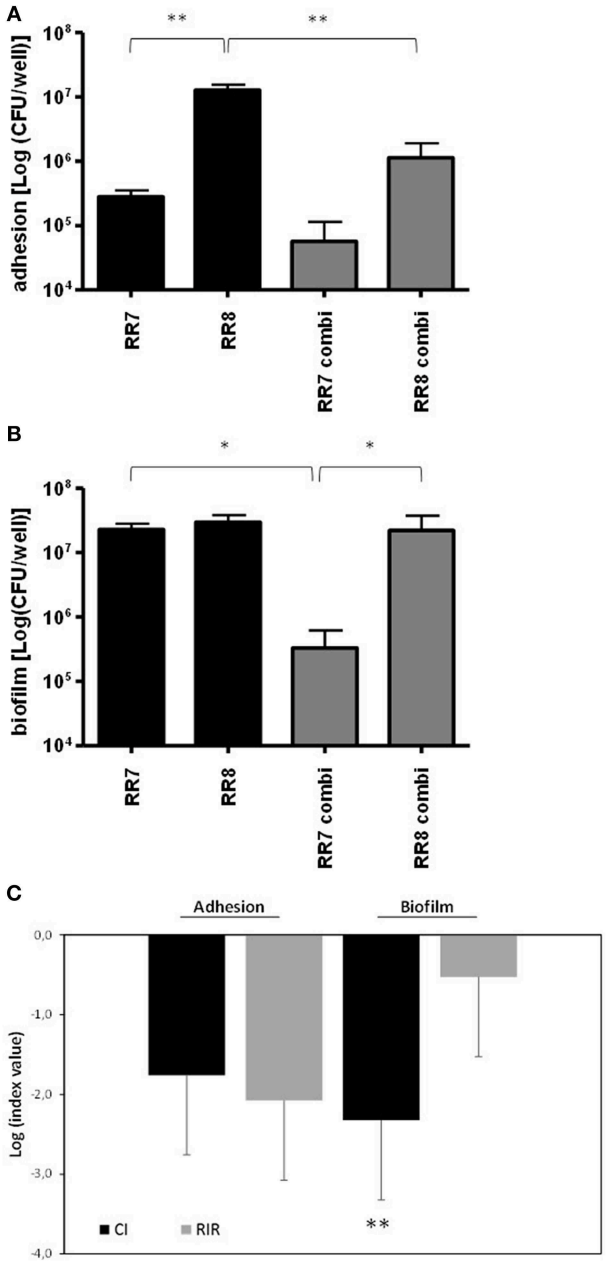

FIGURE 4 | Adhesion and biofilm formation by *S. maltophilia* RR7 and *P. aeruginosa* RR8 onto CFBE41o- CF bronchial cells. CFBE41o- cell monolayers were exposed for **(A)** 3 h (adhesion assay) or **(B)** 24 h (biofilm formation assay) to *S. maltophilia* RR7 and *P. aeruginosa* RR8 (each at 10^6 CFU/ml; MOI: 10), tested as alone (RR7, RR8) or in mixed cultures (RR7combi, RR8combi). *$p < 0.05$, **$p < 0.01$, ANOVA + Newman-Keuls post-test. **(C)** In mixed cultures, the Competitive Index (CI) and the Relative Increase Ratio (RIR) were calculated as described in Materials and Methods. The results are shown as mean + SD ($n = 6$). **$p < 0.01$; unpaired *t*-test.

The comparative analysis of the CI and the RIR values obtained for biofilm formation assay showed statistically different values (-2.3 ± 0.4 vs. -0.5 ± 0.4, respectively; $p < 0.01$) (**Figure 4C**). Relative to the biofilm cells/initial inoculum ratio calculated for each strain, the values obtained from mono- and mixed cultures were significantly elevated for *P. aeruginosa* RR8 (from 31.8 to 96.2%, respectively; $p < 0.001$), while a significant decrease was observed for *S. maltophilia* RR7 (from 6.9 to 0.5%, respectively; $p < 0.001$).

Taken together, these results suggest the existence of a reciprocal interference between these two species in the adhesion step. *S. maltophilia* RR7 negatively affects *P. aeruginosa* RR8 adhesiveness, whereas later, during biofilm formation onto CFBE41o- cells, *P. aeruginosa* RR8 outcompetes *S. maltophilia* RR7, probably by inhibiting its growth.

Exposure to *S. maltophilia* Culture Supernatant Significantly Reduces Swimming Motility in *P. aeruginosa*

The effect of the presence of one strain, tested as culture supernatant, on swimming, swarming and twitching motilities of the other strain was evaluated and the results are summarized in **Figures 5A,B**.

The swimming motility exhibited by *P. aeruginosa* RR8 was significantly reduced by exposure to *S. maltophilia* RR7 culture supernatant (mean ± SD: 18.6 ± 1.3 vs. 25.8 ± 0.8 mm, with or without supernatant, respectively; $p < 0.001$) (**Figure 5A**). No significant variations were observed between groups for swarming and twitching motilities. *P. aeruginosa* RR8 supernatant did not affect any of *S. maltophilia* RR7 motility types tested (**Figure 5B**).

S. maltophilia is Less Susceptible than *P. aeruginosa* to Tobramycin in Mixed Biofilms

The activity of tobramycin at 128 μg/ml against single and mixed preformed biofilm was assessed by the viable count and the results are summarized in **Figure 5C**. Exposure to tobramycin significantly reduced the viability of both *S. maltophilia* RR7 and *P. aeruginosa* RR8 monomicrobial biofilms (*S. maltophilia* RR7: $1.1 \pm 0.5 \times 10^7$ vs. $8.0 \pm 5.0 \times 10^5$ CFU/well; *P. aeruginosa* RR8: $2.2 \pm 2.5 \times 10^6$ vs. $2.2 \pm 2.4 \times 10^4$ CFU/well; unexposed and tobramycin-treated biofilms, respectively; $p < 0.001$). However, when mixed biofilms were tested, tobramycin resulted to be effective against *P. aeruginosa* RR8 only ($1.1 \pm 0.9 \times 10^7$ vs. $3.4 \pm 4.7 \times 10^4$ CFU/well; unexposed and tobramycin-treated biofilms, respectively; $p < 0.001$).

S. maltophilia Significantly Affects *P. aeruginosa* Virulence in Mixed Biofilm

The effect of exposure to *S. maltophilia* RR7 on the expression of nine selected virulence genes (*rhlR, lasI, aprA, vfr, exoS, toxA, algD, mexC, mexE*) of *P. aeruginosa* RR8 was assayed, by RT-PCR, both in mono- and co-cultured biofilms; the results are summarized in **Figure 6**. When grown in mixed biofilm with *S. maltophilia* RR7, *P. aeruginosa* RR8 significantly overexpressed *aprA*, and *algD* genes ($p < 0.05$), codifying for protease and alginate, respectively, whereas the QS-related *rhlR* and *lasI* genes were down-regulated ($p < 0.05$). The presence of *S. maltophilia* RR7 also caused a considerable increase of efflux pump systems-related *mexC* and *mexE* genes, as well as of *toxA*, codifying for exotoxin A, expression by *P. aeruginosa* RR8, although this trend

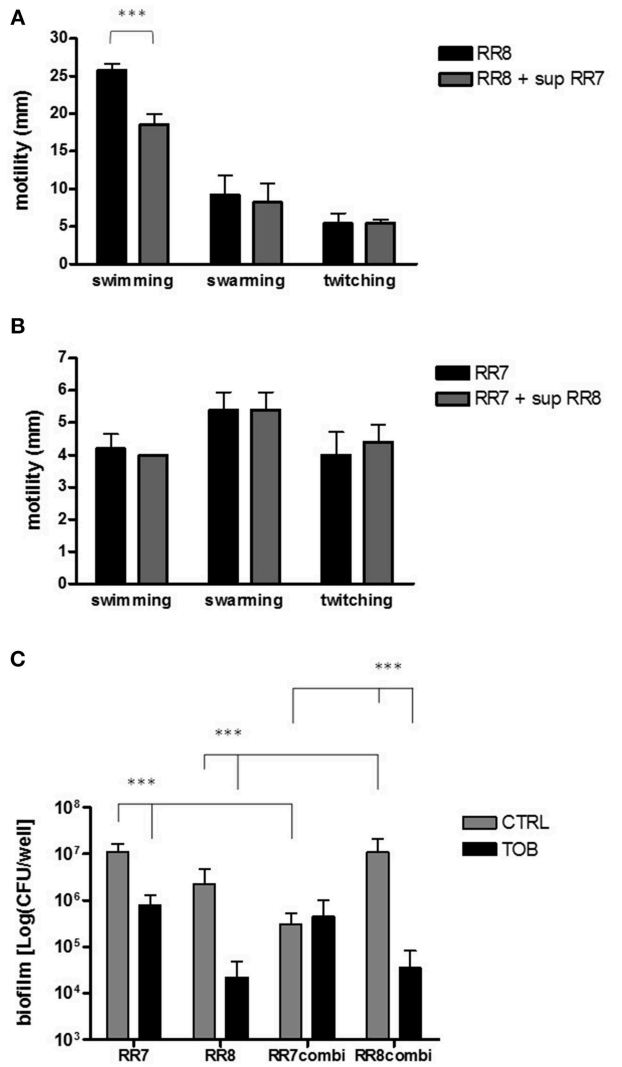

FIGURE 5 | (A,B) Effect of culture supernatants on motility. Swimming, swarming, and twitching motilities of each strain were assessed as previously described (Pompilio et al., 2008). **(A)** *P. aeruginosa* RR8 motility was evaluated both in the presence and absence of *S. maltophilia* RR7 culture supernatant (sup RR7). **(B)** *S. maltophilia* RR7 motility was evaluated both in the presence and absence of *P. aeruginosa* RR8 culture supernatant (sup RR8). The results are shown as mean + SD ($n = 6$). ***$p < 0.001$, paired-*t* test. **(C)** Activity of tobramycin against preformed biofilms. Monomicrobial and mixed biofilms were allowed to grow for 24 h, and then they were exposed to tobramycin 128 μg/ml (TOB) or CAMHB only (control, CTRL) for further 24 h. Biofilm's viability was then assessed by viable count. The results are shown as mean + SD ($n = 6$). ***$p < 0.001$, ANOVA + Newman-Keuls post-test.

resulted not to be statistically significant, probably due to high SD values.

Discussion

The originality of this study is two-fold. Firstly, it evaluates the nature of interactions occurring between *P. aeruginosa* and *S. maltophilia* under both planktonic and biofilm growth, and specifically whether these interactions offer enhanced fitness compared to single cultures. Secondly, it considers *S. maltophilia* and *P. aeruginosa* strains isolated at the same time from the same CF patient during a PE episode, whereas previous works were conducted using strains isolated from different, and non-CF, patients (Kataoka et al., 2003; Ryan et al., 2008; Varposhti et al., 2014).

Following examination of the interaction between *S. maltophilia* and *P. aeruginosa* under planktonic growth conditions, the CI and RIR values suggested that *P. aeruginosa* outcompetes *S. maltophilia*, during both exponential and stationary growth phases. This microbial antagonism could be due to competition for both nutrients and space or, more directly, to an antibacterial effect of one species toward the other (Harrison, 2007; Hibbing et al., 2010). Our findings from the agar spot assay in fact clearly showed, for the first time, that *P. aeruginosa* exerts antibacterial activity against *S. maltophilia*. As suggested by the transwell-based assay results, this effect is probably not mediated by exoproducts, but requires direct contact between cells, which suggests that *P. aeruginosa*, in order to achieve new niches, might kill *S. maltophilia* by injecting specific proteins via the type VI secretion system (Tashiro et al., 2013).

Despite the increasing interest in the crucial role played by biofilm in determining CF infections, interspecies interactions in mixed biofilms are still poorly understood. Using a polystyrene microtiter assay, the reciprocal interaction between *S. maltophilia* and *P. aeruginosa* was initially evaluated at the adhesion phase, the first step of biofilm formation. Our findings confirmed that EPS plays a crucial role during *P. aeruginosa* adhesion, and interestingly showed that the presence of *S. maltophilia* reduces the amount of EPS, probably secondary to loss or a reduced synthesis, therefore affecting *P. aeruginosa* adhesion to the substratum.

The kinetics of biofilm formation were then monitored throughout the 7 day-incubation period with daily medium replacement, in order to simulate a chronic infection. When both species were cultured alone, we found that in *P. aeruginosa*, unlike *S. maltophilia*, EPS amount increases over time during biofilm formation. When simultaneously cultured in mixed biofilms, we observed that *S. maltophilia* and *P. aeruginosa* cannot coexist in a dynamic equilibrium in mixed biofilm: *S. maltophilia* will always be outcompeted. The results from the viable count in fact indicated that the contribution of *S. maltophilia* to the biomass of mixed biofilm is negligible, being at least 100-fold lower than *P. aeruginosa* concentration. The comparative evaluation between single and dual specie biofilms showed that the capacity of *P. aeruginosa* to form biofilms is unaffected by the presence of *S. maltophilia,* while that of *S. maltophilia* is significantly reduced in the presence of *P. aeruginosa*. The antibacterial activity exhibited in planktonic cultures by *P. aeruginosa* is probably one of the mechanisms that inhibits *S. maltophilia* biofilm formation.

In contrast with our findings, Varposhti et al. (2014) found that *S. maltophilia*, in mixed cultures, induces *P. aeruginosa* biofilm biomass formation on Foley catheter.

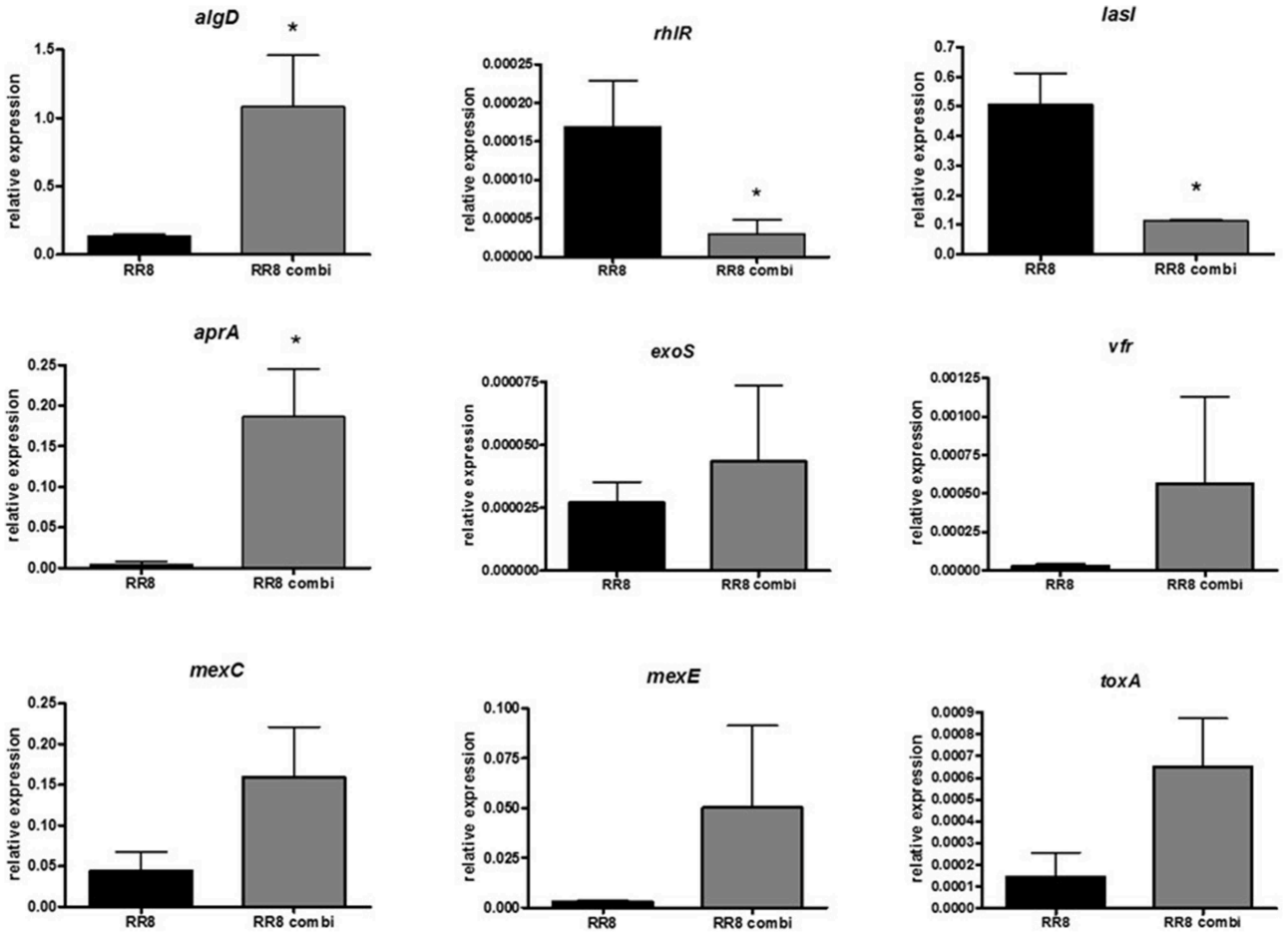

FIGURE 6 | Effect of *S. maltophilia* RR7 on *P. aeruginosa* RR8 virulence-related gene expression in mixed biofilm. *S. maltophilia* RR7 and *P. aeruginosa* RR8 were allowed to grow, as mixed biofilm, in a 24-well microtiter polystyrene plate at 37°C for 24 h (RR8 combi, gray bars). Relative expression of nine selected *P. aeruginosa* virulence genes (*algD, rhlR, lasI, aprA, exoS, vfr, mexC, mexE, toxA*) were then measured by RT-PCR. *P. aeruginosa* grown alone as biofilm was considered as control (RR8, black bars). The results are shown as mean + SD ($n = 6$). *$p < 0.05$, paired *t*-test.

The discrepancy could be explained by the fact that strains tested in combinations by Varposhti et al. were from different patients with polymicrobial lower respiratory infections. This might be particularly relevant in the case of CF chronic infections, where *P. aeruginosa* undergoes adaptation to the CF lung leading to patho-adaptative lineages genotypically and phenotypically different (Huse et al., 2010), which has been observed not only when comparing isolates from different CF patients, but also when comparing multiple isolates from the same CF respiratory specimen (Lee et al., 2005).

The polystyrene microtiter assay is classically used for biofilm studies due to its ease of use and ability for high-throughput testing. However, since not all variables can be controlled, the conditions still differ significantly from those observed during natural infections and, therefore, the results might be of limited clinical relevance. The interaction between *P. aeruginosa* and *S. maltophilia* during adhesion and biofilm formation was therefore also assessed using a CF-derived bronchial epithelial cell monolayer.

In single infections, *P. aeruginosa* was more adhesive than *S. maltophilia*, which disagrees with the polystyrene microtiter assay. However, when the cell monolayer was simultaneously challenged with mixed cultures, the adhesiveness of *P. aeruginosa* was significantly reduced relative to monoculture, while that of *S. maltophilia* remained unaffected. The results from motility assays suggested that *S. maltophilia* might reduce *P. aeruginosa* adhesiveness to bronchial cells by affecting its ability to reach the substratum via swimming motility, probably as a consequence of flagellar loss or a reduced expression of flagella-associated genes.

In accordance with the polystyrene microtiter assay, the viable count showed that *P. aeruginosa* outcompetes *S. maltophilia* in mixed biofilms formed onto CF bronchial cells. Accordingly, we also observed that *S. maltophilia* can not affect *P. aeruginosa* biofilm formation by modulating its swarming or twitching motility.

Recent studies on the behavior of multispecies communities have shown that the virulence and the gene expression of pathogens can be modulated by the presence of other

bacterial species (Duan et al., 2003). Therefore, we performed a comparative analysis of transcript levels of several *P. aeruginosa* virulence factors both in single- and dual-species biofilms. The quantitative PCR results suggested that *S. maltophilia* changes the physiology and, consequently, the virulence of *P. aeruginosa*. Specifically, both *aprA* and *algD* operon—codifying for the frank *P. aeruginosa* virulence factors alkaline protease and alginate, respectively—were up-regulated in the presence of *S. maltophilia*, whereas the QS-related *rhlR* and *lasI* genes were down-regulated. These differences may be the expression of a defensive reaction by *P. aeruginosa* to the presence of *S. maltophilia*. Alternatively, since both bacterial species exist ubiquitously in various environments, including the rhizosphere of plants (Berg et al., 2005), the lack of significant differences in the expression of other genes observed in co-culture may be highly suggestive of their relative acclimatization to each other.

P. aeruginosa causes severe tissue damage by the expression of the alkaline protease AprA, especially in CF patients where it has been associated with increased infectivity and virulence, suggesting a role in processes related to bacterial colonization and/or exacerbation in the CF lung (Burke et al., 1991; Kim et al., 2006). Our findings suggested that in mixed infections *S. maltophilia* might indirectly facilitate the onset of PEs in CF patients by inducing increased proteolytic activity in *P. aeruginosa*, probably via a Las/RhI independent pathway.

On the other hand, *S. maltophilia* might also promote *P. aeruginosa* adaptation to the airways of CF patients by favoring phenotypic traits acquired by *P. aeruginosa* only during chronic infections, which has the result of promoting long-term survival in CF lungs. Particularly, we observed in mixed biofilms the loss of LasR function and overproduction of alginate. Loss of QS regulation is generally considered a hallmark of chronic virulence and has been described for several *P. aeruginosa* CF isolates (Geisenberger et al., 2000; D'Argenio et al., 2007). Specifically, loss of LasR function has clinical implications for disease progression and antibiotic resistance since it produces an increase in β-lactamase activity, which augments tolerance to ceftazidime, a widely used β-lactam antibiotic in CF patients (D'Argenio et al., 2007). In addition, mucoid strains overproducing alginate have been found to be dominant in chronic lung infections, and therefore correlated with a poor prognosis (Yang et al., 2011). Furthermore, by increasing *algD* expression *S. maltophilia* might favor the protection of *P. aeruginosa* against oxygen radicals from activated PMNs (Govan and Deretic, 1996), and improve its ability to form more robust biofilms (Hoffmann et al., 2005).

Understanding the interspecies interactions is essential, not only because they can potentially modulate the virulence and the persistence of pathogens such as *P. aeruginosa*, but also because it might lead to the development of new, more effective, therapeutic strategies. We found that the exposure of a mixed biofilm to tobramycin reduced the viability of *P. aeruginosa* but not of *S. maltophilia*. It is, therefore, plausible that the *S. maltophilia*-induced alginate expression by *P. aeruginosa* might ensure added protection both to *S. maltophilia* against tobramycin, and to *P. aeruginosa* against other antimicrobial agents. This might explain why about 25% of patients do not recover their baseline lung function after treatment for a PE episode (Sanders et al., 2010, 2011), therefore suggesting that a suitable CF treatment should take into consideration the threat of such nosocomial indirect pathogens as *S. maltophilia*.

In conclusion, for the first time our findings show that a reciprocal interference between *S. maltophilia* and *P. aeruginosa* in CF lung is plausible. *P. aeruginosa* exhibits an aggressive lifestyle dominating *S. maltophilia* during competitive planktonic growth and biofilm development. However, in CF lung *S. maltophilia* cannot be considered as simply a "by-stander." In fact, growing in a biofilm together with *S. maltophilia* changes the physiology of *P. aeruginosa* and, therefore, modulates its virulence profile. This might confer some selective "fitness advantage" to *P. aeruginosa* under the specific conditions of chronic infection or, alternatively, cause its hypervirulentation thus leading to PE.

Our findings, however, need to be confirmed by *in vitro* and *in vivo* investigations focused on other CF *S. maltophilia/P. aeruginosa* combinations since, in infections where both species are present, the outcome over time could be highly influenced by the phenotype of the strains involved.

Author Contributions

AP performed analyses, statistically evaluated results and, together with GDB, drafted the manuscript and assisted in the study design. VC and SDN performed analyses. FV designed and supervised the analyses of gene expression. EF collected and processed clinical specimens, and provided clinical expertise for the discussion of the results. GDB supervised analyses, defined the study design, discussed the results and drafted the manuscript. All authors read, reviewed, and approved the final manuscript.

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Antibacterial Activity of *Stenotrophomonas maltophilia* Endolysin P28 against both Gram-Positive and Gram-Negative Bacteria

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Maltocin P28 is a phage-tail like bacteriocin produced by *Stenotrophomonas maltophilia* P28. The ORF8 of maltocin P28 gene cluster is predicted to encode an endolysin and we name it endolysin P28. Sequence analysis revealed that it contains the lysozyme_like superfamily conserved domain. Endolysin P28 has the four consensus motifs as that of *Escherichia coli* phage lambda gpR. In this study, endolysin P28 was expressed in *E. coli* BL21 (DE3) and purified with a C-terminal oligo-histidine tag. The antibacterial activity of endolysin P28 increased as the temperature rose from 25 to 45°C. Thermostability assays showed that endolysin P28 was stable up to 50°C, while its residual activity was reduced by 55% after treatment at 70°C for 30 min. Acidity and high salinity could enhance its antibacterial activity. Endolysin P28 exhibited a broad antibacterial activity against 14 out of 16 tested Gram-positive and Gram-negative bacteria besides *S. maltophilia*. Moreover, it could effectively lyse intact Gram-negative bacteria in the absence of ethylenediaminetetraacetic acid as an outer membrane permeabilizer. Therefore, the characteristics of endolysin P28 make it a potential therapeutic agent against multi-drug-resistant pathogens.

Keywords: endolysin, *Stenotrophomonas maltophilia*, antibacterial activity, maltocin, EDTA

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INTRODUCTION

Stenotrophomonas maltophilia is a Gram-negative bacillus and increasingly being recognized as an important nosocomial pathogen (Looney et al., 2009; Brooke, 2012). It can cause serious infections such as bacteremia, pneumonia, endocarditis, meningitis, urinary tract infections, skin and soft tissue infections in immunocompromised patients (Falagas et al., 2009; Sood et al., 2013; Hotta et al., 2014; Trignano et al., 2014; Guzoglu et al., 2015). The available therapeutic option for invasive *S. maltophilia* infection is limited, as this pathogen shows high levels of resistance to commonly used antibiotics (Sánchez, 2015). *S. maltophilia* not only exhibits intrinsic multidrug resistance but also can acquire antibiotic resistance during therapy like other pathogenic strains (Tan et al., 2008). Therefore, the novel treatment strategies and the effective antimicrobial agents are needed urgently to date for treatment of *S. maltophilia* infections.

Endolysins are enzymes encoded by phages at the end of their replication cycle to degrade the peptidoglycan of the host cell wall for cell lysis and release of the mature progeny phage particles (Loessner, 2005). Due to the absence of an outer membrane in Gram-positive bacteria, endolysins can easily access the peptidoglycan of cell wall and destroy these organisms when applied externally (Fischetti, 2010; Gilmer et al., 2013). When the endolysins work with Gram-negative bacteria, the chelating agents, like ethylenediaminetetraacetic acid (EDTA), are often used to increase the bacterial outer membrane permeability (Briers et al., 2011; Walmagh et al., 2013). Endolysins have been reported to be applied in medicine, control and detection of food-borne pathogens (Fischetti, 2008; Grandgirard et al., 2008; Coffey et al., 2010; Schmelcher et al., 2012). Endolysins can also be expressed by transgenic plants to prevent infection by phytopathogenic bacteria (Oey et al., 2009). Expanding databases of predicted proteins from the increasing number of sequenced and annotated bacterial genomes present a growing number of potential endolysins (Schmitz et al., 2010; Farris and Steinberg, 2014). As multidrug-resistant strains are becoming more prevalent, endolysins may be employed as novel alternatives to antibiotics (Bragg et al., 2014; Yang et al., 2014).

In the previous work, we have identified the maltocin P28 from *S. maltophilia* P28. The gene cluster of maltocin P28 is presumed to have 23 open reading frames (ORFs), and ORF8 is predicted to encode an endolysin (Liu et al., 2013). Here we designate this putative endolysin as endolysin P28. Sequence analysis revealed that the endolysin P28 contained the lysozyme-like superfamily conserved domain and its amino acid sequence had high identity with that of lambda phage gpR. In this study, we cloned the ORF8 into pET-26b(+) to express this endolysin gene in *Escherichia coli*. The recombinant endolysin P28 was purified and characterized. And the antibacterial activity of endolysin P28 against various Gram-negative and Gram-positive bacteria was also detected.

TABLE 1 | Antimicrobial activity of endolysin P28 against various species.

Strain	Species	Growth temperature (°C)	Antibacterial rate (%)
Gram-positive bacteria			
Bacillus cereus	CCTCC[a] AB200055	30	>99
Bacillus subtilis	CCTCC AB93017	30	>99
Listeria monocytogenes	CCTCC AB209106	30	90.4
Staphylococcus aureus	CCTCC AB91053	37	>99
Gram-negative bacteria			
Aeromonas hydrophila	XS91-4-1	30	47.1
Aeromonas salmonicida	CCTCC AB98041	22	19.2
Enterobacter aerogenes	CCTCC AB91102	37	58.3
Escherichia coli	ATCC47076	37	60.3
Klebsiella pneumoniae ssp. *pneumoniae*	CCTCC AB2010163	37	>99
Proteus vulgaris	CCTCC AB91103	30	61.2
Pseudomonas aeruginosa	ATCC 15692	30	70.6
Pseudomonas fluorescens	CCTCC AB92001	30	0
Pseudomonas putida	ATCC12633	30	34.7
Salmonella typhimurium	CCTCC AB204062	37	0
Shigella flexneri	CCTCC AB200061	37	>99
Xanthomonas citri ssp. *malvacearum*	CCTCC AB96030	30	>99

[a]*CCTCC, China Centre for Type Culture Collection.*

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Stenotrophomonas maltophilia P28 and *E. coli* BL21(DE3) were routinely cultivated in LB broth at 30°C. One hundred microgram per milliliter of ampicillin or 50 μg/mL kanamycin was added when necessary. Strains used for antimicrobial spectrum determination were all cultured in LB broth. The growth temperature was listed in **Table 1**. Most tested strains were taken from China Center for Type Culture Collection (CCTCC), the others were purchased from the American Type Culture Collection (ATCC) and *Aeromonas hydrophila* strain XS91-4-1 was kindly provided by Professor Aihua Li at Institute of Hydrobiology, Chinese Academy of Sciences.

Plasmid Construction and Transformation

DNA manipulations were performed according to standard protocols (Sambrook, 1989). Genomic DNA of *S. maltophilia* P28 was extracted using the genomic DNA extraction kit (TIANGEN, China) and used as the template to amplify the ORF8 of maltocin P28 gene cluster. Plasmid DNA was obtained with TIANprep Mini Plasmid kit (TIANGEN, China).

Cloning, Expression, and Purification of Endolysin P28

The ORF8 (putative endolysin gene) was amplified from the genomic DNA of *S. maltophilia* P28 by polymerase chain reaction (PCR). The PCR primers were endo-F (5′-CATATGACCGCCGC TGCAGCCAG-3′) and endo-R (5′-CTCGAGCTGCAGGGCTC CGCC-3′). Amplification was performed in PTC100 (BioRad, USA) with the following condition**:** 95°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and the final extension with 72°C for 10 min. The PCR product was purified with Cycle-pure-Kit (OMEGA) and cloned into pDM19-T vector to confirm the sequence. Then the recombinant plasmid pDM19-T-ORF8 was digested with restriction enzymes *Nde*I and *Xho*I. The obtained 492-bp fragment was purified and inserted into pET-26b(+) by *Nde*I and *Xho*I, designating the recombinant plasmid pET-26b-ORF8. The *E. coli* BL21 (DE3) was transformed with plasmid pET-26b-ORF8. Transformants were cultivated in LB broth containing 50 μg/mL kanamycin at 37°C. Protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD_{600} 0.5–0.8, followed by incubation for 6 h at 30°C. Bacterial cells were harvested at

4°C, suspended in binding buffer (0.5 M sodium chloride, 50 mM Tris-HCl, pH 7.5), and disrupted by sonication on ice. The lysates were centrifugated at 13,000 *g* for 10 min. After centrifugation, the supernatant was passed through a Ni-NTA column (Novagen). Purification of endolysin P28 was performed according to the manufacturer's instructions. The identity and purity of the protein were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained by Coomassie brilliant blue R-250 to visualize the bands. The purified endolysin P28 was stored at −20°C after the buffer was changed to the storage buffer (20 mM Tris-HCl, pH 7.0) by ultrafiltration.

Determination of the Lytic Activity

The antibacterial activity of endolysin P28 was assessed by CFU reduction analysis as described previously (Rodriguez et al., 2011). Exponentially growing cells were centrifuged to discard the supernatant, and then the cell pellet was washed twice, resuspended in 20 mM Tris-HCl buffer (pH 7.0) and adjusted the OD_{600} to 0.5 ± 0.05. Purified endolysin (100 μL) was added to 300 μL cell suspension. The volume of reaction system was 400 μL. Generally the concentration of the endolysin added was adjusted to 2 mg/mL and the working concentration was 0.5 mg/mL. When the appropriate working concentration was tested, the concentration of the purified endolysin was changed while the reaction volumes of purified endolysin (100 μL) and cell suspension (300 μL) remained unchanged. After incubation for 1 h at 30°C, the mixture was serially diluted by 10-fold. To determine the survival rate, 100 μL 10-fold serial dilutions were plated on LB agar in triplicate, then colony forming units (CFUs) were counted after incubation at 30°C for 24 h. The number of CFUs between 30 and 300 on the spread plate was effectively counted. The control was performed by adding 100 μL 20 mM Tris-HCl buffer (pH 7.0) instead of endolysin P28. The antibacterial activity was expressed as the bacterial counts decrease. This value was calculated as the dead percentage referred to an untreated control. In this study, all the data were statistically analyzed by using the Origin8.1 program. Difference was investigated by the *T*-test at the 5% level. All experiments were repeated at least three times. The error bars represent the standard deviations.

In order to analyze the effect of EDTA, the above washed *S. maltophilia* culture (OD_{600} 0.5 ± 0.05) was incubated in 20 mM Tris-HCl buffer (pH 7.0) containing 0, 1 mM and 5 mM EDTA for 30 min. After centrifugation to remove the EDTA, the cell pellet was resuspended in 20 mM Tris-HCl buffer (pH 7.0). Then the purified endolysin was added and its antibacterial activity was determined as described above.

To evaluate the effect of pH on endolysin lytic activity, the endolysin (100 μL, 2.0 mg/mL) was added to 300 μL *S. maltophilia* cells suspended with a variety of buffers: 20 mM NaAc for pH 4.0 and pH 5.0, 20 mM Na_2HPO_4 -NaH_2PO_4 for pH 6.0, 20 mM Tris-HCl for pH 7.0, pH 8.0 and pH 9.0, Glycine-NaOH for pH 10.0–12.0. The control was carried out by adding 100 μL respective buffer instead of endolysin P28. After incubation for 1 h at 30°C, the CFUs of the mixture were determined by 10-fold dilution and the antibacterial activity was calculated as described above.

The influence of saline concentration on the lytic activity of endolysin was tested with NaCl concentration from 50 to 400 mM to cell suspension. One hundred microliter purified endolysin (2.5 mg/mL) was added to 300 μL exponentially growing cell (OD_{600} 0.5 ± 0.05), which was washed and resuspended in 20 mM Tris-HCl buffer (pH 7.0). And then 100 μL NaCl solution of different concentration was added to the mixture. The control was performed by adding 100 μL 20 mM Tris-HCl buffer (pH 7.0) instead of endolysin P28. After incubation for 1 h at 30°C, the CFUs were determined by 10-fold dilution and the antibacterial activity was calculated as described above.

In order to examine the effect of different temperatures on the enzymatic activity of endolysin P28, 100 μL purified endolysin (2.0 mg/mL) was added to 300 μL exponentially growing cell (OD_{600} 0.5 ± 0.05), which was washed and resuspended in 20 mM Tris-HCl buffer (pH 7.0). The control was performed by adding 100 μL 20 mM Tris-HCl buffer (pH 7.0) instead of endolysin P28. After incubation for 1 h at different temperatures (25–45°C), the CFUs were determined by 10-fold dilution and the antibacterial activity was calculated as described above.

Thermostability was tested by incubating endolysin P28 (2.0 mg/mL) in 30, 40, 50, 60, 70 for 30 min, and then 100 μL treated endolysin P28 was mixed with the above washed *S. maltophilia* cells (OD_{600} 0.5 ± 0.05). The control was performed by adding 100 μL 20 mM Tris-HCl buffer (pH 7.0) instead of endolysin P28. After incubation for 1 h at 30°C, the CFUs of the mixture were determined by 10-fold dilution and the antibacterial activity was calculated as described above.

RESULTS

Sequence Analysis

Maltocin P28 is a novel phage tail-like bacteriocin produced by *S. maltophilia* P28 and has been characterized previously (Liu et al., 2013). The ORF8 of maltocin P28 gene cluster was predicted to encode an endolysin designated as endolysin P28. The deduced amino acid of endolysin P28 showed 48.48% identity with that of phage lambda gpR, which is the lambda lysozyme and function as a lytic transglycosylase. NCBI Blastp revealed that endolysin P28 included a conserved domain known as bacteriophage_lambda_lysozyme family. This family of lysozymes contains four consensus motifs (Blackburn and Clarke, 2001). Endolysin P28 has the same motifs, with the essential catalytic residue (Glu25) in motif I as lambda lysozyme gpR (Jespers et al., 1992) (**Figure 1A**). Compared with lambda gpR, endolysin P28 contained an N-terminal hydrophobic region (**Figure 1B**).

Expression and Purification of Endolysin P28

Endolysin P28 was overproduced as a C-terminal 6× His-tagged fusion protein which allowed purification by immobilized metal chelate affinity chromatography. The elution fractions

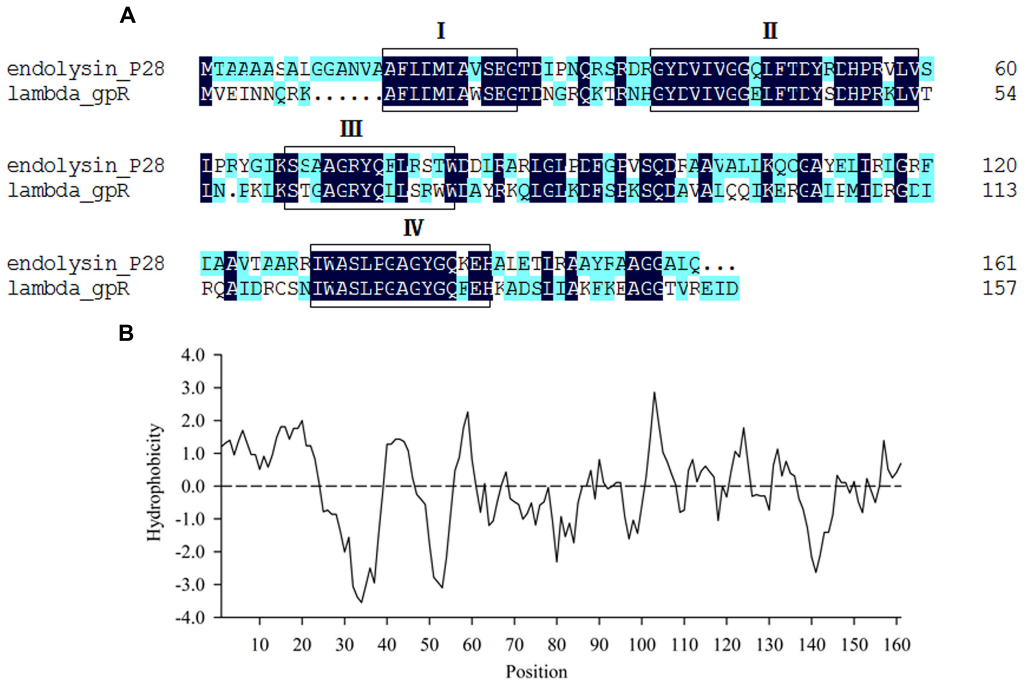

FIGURE 1 | Sequence analysis of endolysin P28. (A) Comparison of amino acid sequences between endolysin P28 and lambda gpR, the boxes indicate the four consensus motifs. **(B)** Hydrophobicity analysis of endolysin P28.

were pooled and analyzed by 15% SDS-PAGE. A major protein band was observed which correlated well with the calculated mass of the endolysin P28 fusion protein (18.1 kDa; **Figure 2**). The concentration of purified endolysin P28 was determined according to the Bradford assay.

Lytic Activity of Endolysin P28

For antibacterial activity measurement, *S. maltophilia* P28 was used as substrate for the purified endolysin. The lytic activity of endolysin P28 was conducted by CFU reduction assay. The addition of 125 μg/mL endolysin did not change the viable numbers of *S. maltophilia*. When the endolysin concentration rose more than 125 μg/mL, its antibacterial activity enhanced as the concentration increased (**Figure 3**). When the endolysin concentration was 500 μg/mL, the CFU number reduced about a half. Thus the endolysin concentration of 500 μg/mL was used as the standard to perform the following antibacterial assays.

Gram-negative bacteria are more sensitive to endolysins after they are treated with EDTA to enhance the permeability of their outer membrane (Briers et al., 2011). To determine the effect of EDTA on the antimicrobial activity of endolysin P28, both *S. maltophilia* P28 and *Pseudomonas aeruginosa* ATCC15692 were treated with the different concentrations of EDTA for 30 min. The addition of EDTA led the cell lysis of *P. aeruginosa* ATCC15692 to significantly increase compared with buffer alone. The viable cell number of *P. aeruginosa* ATCC15692 was reduced more than 2 logs by addition of 1 mM EDTA, while the sensitivity of *S. maltophilia* P28 to endolysin was not affected by addition of EDTA (**Figure 4**). Thus, we did not add EDTA in the following experiments.

Influence of Temperature, Salinity, and pH on Endolysin P28 Antibacterial Activity

Standard CFU reduction analysis was performed to analyze the effects of temperature, salinity, and pH on endolysin P28 antibacterial activity. The results showed that the antibacterial activity increased with the increase of temperature from 25 to 45°C (**Figure 5A**). And as the salt concentration increased from 100 mM to 200 mM, the antibacterial activity increased sharply. While the salt concentration up to 400 mM did not enhance the antibacterial activity further (**Figure 5C**). As for the effect of pH on endolysin P28, the result revealed that it had a relatively high lytic activity at a broad pH range of 5.0–8.0 and was ineffective when pH rose to 11.0 (**Figure 5B**). The thermal stability of endolysin P28 was characterized by challenges over a range of temperatures (30, 40, 50, 60, and 70°C). Residual activity of endolysin P28 was reduced 55% by following treatment at 70°C for 30 min (**Figure 5D**).

Antibacterial Spectrum

To test the antibacterial spectrum of endolysin P28, we used four Gram-positive and twelve Gram-negative bacteria as substrates. Maltocin P28 only lyses *S. maltophilia* (Liu et al.,

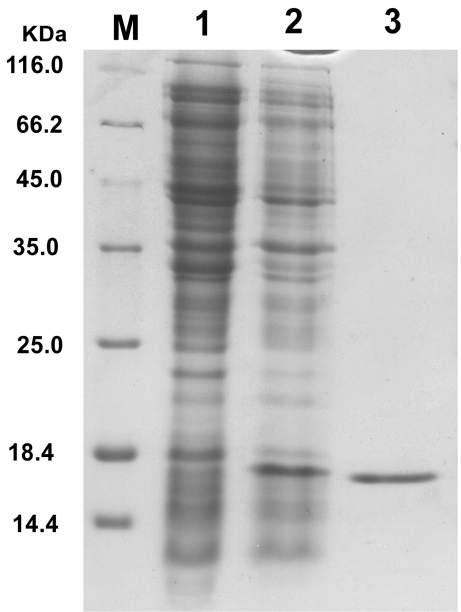

FIGURE 2 | Overproduction of endolysin P28 in *Escherichia coli* BL21 (DE3) cells. Shown is SDS-PAGE (15%) of the cellular proteins from 80 μL cultures of bacteria. The gel was stained by Coomassie brilliant blue R-250 to visualize the bands. *E. coli* BL21 (DE3; pET-26b-ORF8) before induction (lane 1) and 6 h after induction (1 mM IPTG; lane 2). The overexpressed oligohistidine-tagged endolysin P28 was purified (lane 3). M, molecular masses of reference proteins (Thermo Scientific; in kilodaltons).

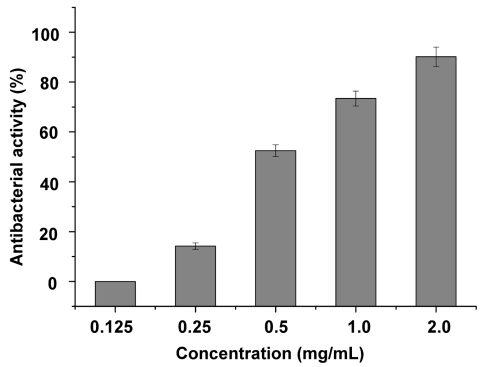

FIGURE 3 | Lytic activity of different concentration endolysin P28 against *Stenotrophomonas maltophilia* P28. One hundred microliter purified endolysin P28 was mixed with 300 μL cell suspension (OD_{600} 0.5 ± 0.05). The concentration of the purified endolysin was changed while the reaction volumes of purified endolysin (100 μL) and cell suspension (300 μL) remained unchanged. After incubation for 1 h at 30°C, the CFUs of the mixture was determined and the antibacterial activity was calculated as described in the section "Materials and Methods". The data were statistically analyzed by using the Origin8.1 program. All experiments were repeated at least three times. Mean values (±SD) are shown.

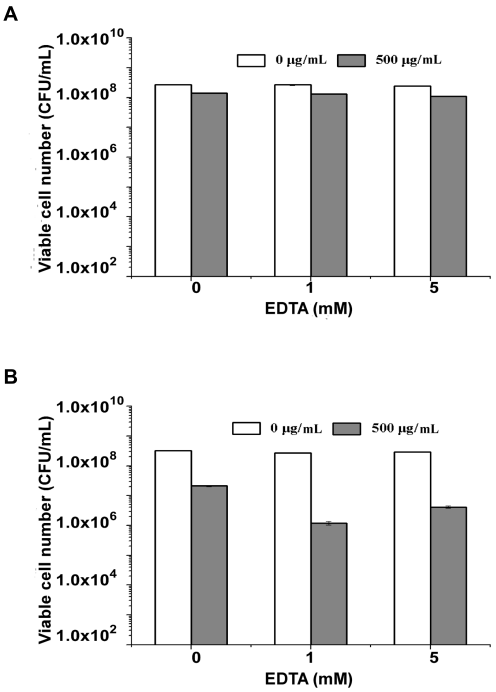

FIGURE 4 | Lytic activity of endolysin P28 over a range of ethylenediaminetetraacetic acid (EDTA). The viable cell numbers (CFUs) of *S. maltophilia* P28 **(A)** and *P. aeruginosa* ATCC 15692 **(B)** after the addition of 500 μg endolysin P28 were examined. The cells were treated with different concentrations of EDTA (0, 1, 5 mM) for 30 min before the endolysin P28 was added. After incubation for 1 h at 30°C, the CFUs of the mixture was determined and the antibacterial activity was calculated as described in the section "Materials and Methods". Error bars are the means ± standard deviation of three independent assays.

2013). Apparently endolysin P28 showed a broader antibacterial spectrum than maltocin P28 (**Table 1**). Endolysin P28 was able to lyse all of the tested Gram-positive bacteria and exhibited high lytic activity against three Gram-negative bacteria, *Klebsiella pneumoniae* ssp. *pneumoniae*, *Shigella flexneri*, *Xanthomonas citri* ssp. *malvacearum* (**Figure 6**). However, no activity was detected against *P. fluorescens* and *Salmonella typhimurium*.

DISCUSSION

The last decade has witnessed an increasing emergence of drug-resistant pathogens. Thus it is important to develop new alternatives to antibiotics. Phage endolysins have been reported to have good potential as a therapeutic agent for bacterial infections (Schmelcher et al., 2012). When added externally, they can cause a hole in the cell wall of the target bacterium through peptidoglycan digestion exerting a lethal effect (Fischetti, 2010). In general, exogenous endolysin is highly active against many Gram-positive species but ineffective against Gram-negative bacteria. It is probably due to the outer membrane existing in Gram-negative bacteria, which is impermeable to macromolecules and makes a physical barrier for endolysin to access the peptidoglycan layer (During et al., 1999; Fischetti, 2010).

Most endolysins from Gram-negative bacteriophage reported have only a catalytic domain. Some of them have a modular

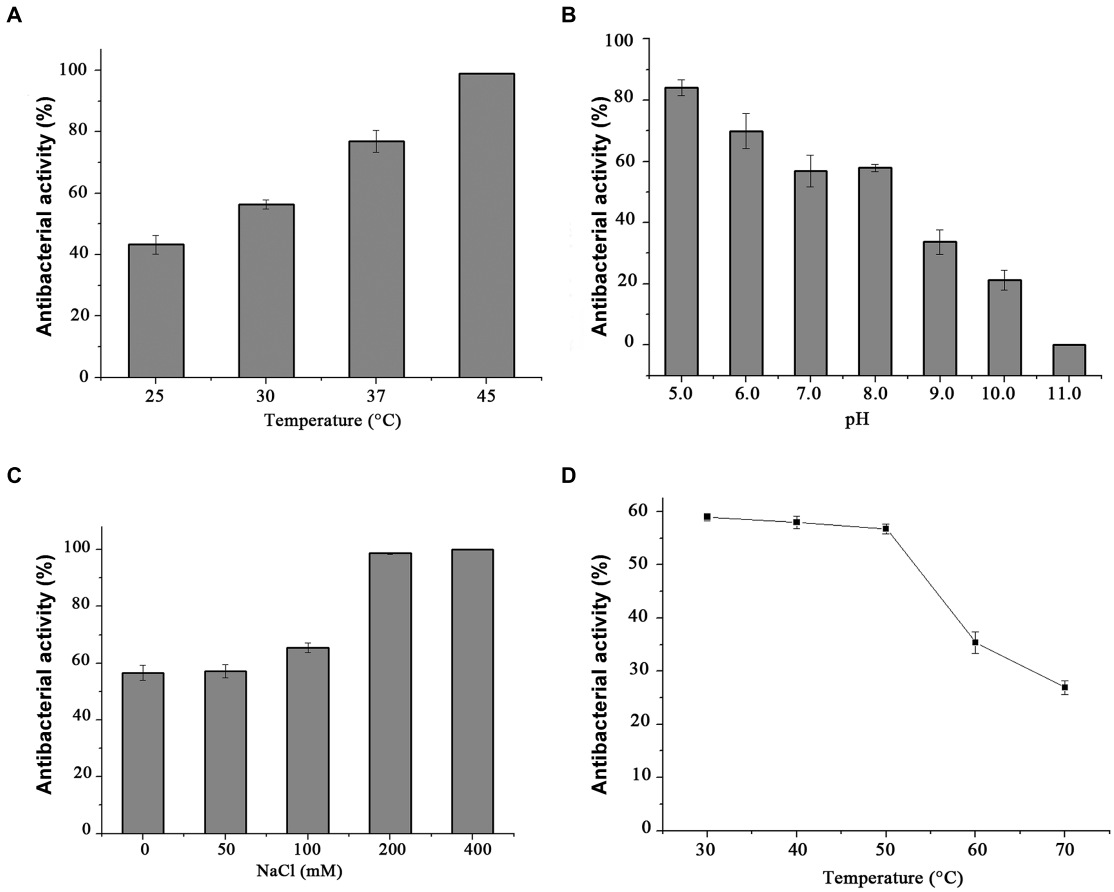

FIGURE 5 | Conditions for the enzymatic activity of the endolysin P28. The antimicrobial activity was performed at different temperatures for 1 h in 20 mM Tris-HCl buffer, pH 7.0 **(A)**, and in buffers with different pH values for 1 h at 30°C **(B)**. The influence of saline concentration on the lytic activity of endolysin was tested with different NaCl concentration to cell suspension for 1 h in 20 mM Tris-HCl buffer, pH 7.0 at 30°C **(C)**. Thermostability of endolysin P28 was conducted by incubating endolysin P28 in different temperatures for 30 min, and then the residual activity was calculated as described in the CFU reduction assay **(D)**. Error bars are the means ± standard deviation of three independent assays.

structure composed of a C-terminal catalytic domain and N-terminal cell wall binding domain (Briers et al., 2007; Fokine et al., 2008; Fischetti, 2010; Callewaert et al., 2011; Walmagh et al., 2013). Sequence analysis of endolysin P28 found no N-terminal cell wall binding domain or specific catalytic domain, rather it contained an N-terminal hydrophobic region (**Figure 1B**), which formed a putative helix domain. Some endolysins from phages of Gram-negative bacteria can destroy bacterial cells by means of a mechanism completely independent of their enzymatic activity (During et al., 1999; Orito et al., 2004). In these cases, it was found that helix-forming amphipathic peptides containing basic amino acid residues seem to interact with negatively charged membrane elements, such as lipopolysaccharide in Gram-negative bacteria (During et al., 1999; Lim et al., 2014). Therefore, we proposed that the N-terminal hydrophobic region of endolysin P28 should have the same function. Endolysin P28 showed a hydrolysis activity against peptidoglycan of *E. coli* and *Bacillus subtilis* through the turbidity reduction assay (data not shown). In addition to the four consensus motifs between endolysin P28 and lambda gpR, the catalytic residue Glu19 in lambda gpR playing an crucial role in the interaction of the enzyme with the GlcNAc of the peptidoglycan backbone is conserved in endolysin P28 (Glu25). So, further studies regarding the functions of N-terminal helix domain and Glu25 of endolysin P28 should be performed in the future.

Endolysin P28 had a good thermal stability, and showed a broad lytic spectrum against 14 out of 16 tested bacteria including medically important genera *Aeromonas*, *Bacillus*, *Escherichia*, *Klebsiella*, *Listeria*, *Pseudomonas, Proteus*, *Salmonella*, *Shigella*, *Staphylococcus*, and *Xanthomonas*. Since all the Gram-negative strains tested in this study are Gammaproteobacteria, further investigation into the effect of endolysin P28 on other bacterial species outside the two clusters of Gram-positive bacteria and Gammaproteobacteria is being carried out. Endolysin P28 could not lyse *S. typhimurium* and *P. fluorescens*. Gram-negative pathogens were protected by the presence of an outer membrane, preventing the entry of endolysins into the cell and reach the peptidoglycan (Nikaido, 2003). EDTA is a well-known used outer membrane permeabilizer that acts as a chelator by removing stabilizing cations from the outer

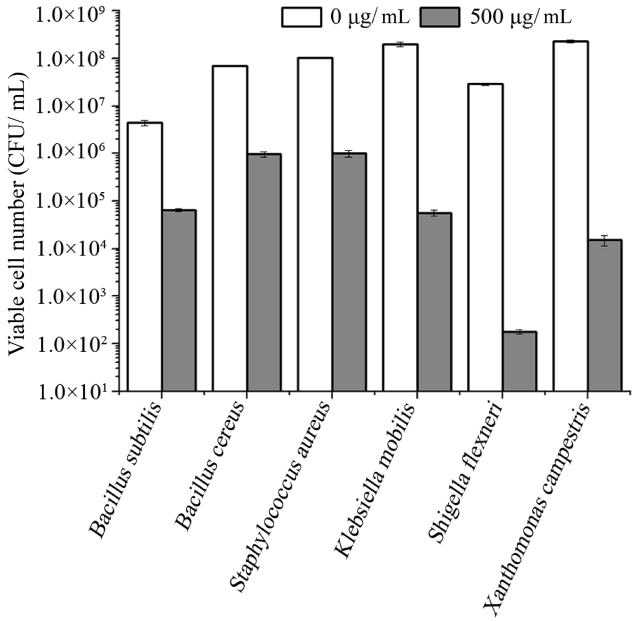

FIGURE 6 | Lytic activity of endolysin P28 against various Gram-positive and Gram-negative strains. Various exponentially growing bacteria were washed twice and resuspended in 20 mM Tris-HCl buffer (pH 7.0). Three hundred microliter bacterial suspension (OD_{600} 0.5 ± 0.05) was mixed with 100 μL purified endolysin P28. After incubation for 1 h at 30°C, the CFUs of the mixture was determined and the antibacterial activity was calculated as described in the section "Materials and Methods". Mean values (±SD) are shown.

membrane (Oliveira et al., 2014). In this study, we also found that EDTA could enhance the activity of endolysin P28 to *P. aeruginosa*. However, the tested strains were not treated by any outer membrane permeabilizer agent when the spectrum of endolysin P28 was detected. Thus, the reason for endolysin P28 ineffective against some strains may be the endolysin P28 could not pass through outer member and reach their peptidoglycans. Several *Salmonella* phage endolysins, such as Lys68, SPN1S, and SPN9CC, have been characterized (Lim et al., 2012, 2014; Oliveira et al., 2014). They have a wide spectrum of antibacterial activity against Gram-negative bacteria but no effect on the Gram-positive bacteria tested. More strains should be detected to ascertain how the antibacterial spectrum of endolysin is associated with the bacterial characteristics.

As *S. maltophilia* P28 is EDTA resistant, we have no wonder that EDTA had no effect on the sensitivity of *S. maltophilia* P28 to endolysin P28. However, it is important to note that endolysin P28 has antibacterial activity against other Gram-negative bacteria without EDTA treatment. To our knowledge, few endolysins and engineered phage lytic enzymes have been reported to have this property (Lai et al., 2011; Lukacik et al., 2012, 2013; Schmelcher et al., 2012). The antibacterial rate of endolysin P28 was more than 99% against six tested strains, which were three Gram-positive and three Gram-negative strains. The viable cell numbers of three Gram-positive strains were reduced about 2 logs, while the viable cell numbers of three Gram-negative strains were reduced more than 3 logs, especially the viable cell number of *S. flexneri* was reduced more than 5 logs (**Figure 6**). These results suggested that endolysin P28 is a potential therapeutic agent against both Gram-positive and Gram-negative pathogenic strains.

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