DOCTORAL THESIS

Fatty Acid-Mediated Quorum Sensing Systems in *Stenotrophomonas maltophilia*

Pol Huedo Moreno

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Parc de Recerca UAB

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A thesis submitted by **Pol Huedo Moreno** in partial fulfilment of the requirements for the Doctor of Philosophy degree in **Biotechnology** in the Universitat Autònoma de Barcelona.

Approval of the directors,

Dr. Isidre Gibert Gonzàlez **Dr. Daniel Yero Corona**

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Als meus pares, a en Lluís i a la Txell.

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PRESENTATIONS AND PUBLICATIONS

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ABBREVIATIONS

2-DE	2-Dimensional Electrophoresis
aa	Amino Acid
Ap	Ampicillin
Ara	Arabinose
ATP	Adenosil Triphosphate
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BM2	Basal Medium 2
cdi-GMP	Cyclic Diguanosine Monophosphate
CF	Cystic Fibrosis
CFTR	CF transmembrane conductance regulator
cfu	Colony Formation Units
Cm	Chloramphenicol
CMC	Carboxymethylcellulose
COPD	Chronic Obstructive Pulmonary Disease
DSF	Diffusible Signal Factor
DSF-QS	DSF-Quorum Sensing
Erm	Erythromycin
g	grams
GC/MS	Gas Chromatography/Mass Spectrometry
GMP	Guanosine Monophosphate
h	Hours
HATP	Histidine ATPase

HisKA	Histidine Kinase
hpi	Hours Post Injection
HPT	Histidine Phosphotransferase
ICUs	Intensive Care Units
Kan	Kanamycin
kDa	Kilodaltons
LB	Luria-Bertani
M	Molar
M9	Medium 9
MDR	Multi Drug Resistant
MDRO	Multi Drug Resistant Organism
MIC	Minimum Inhibitory Concentration
min	Minutes
ml	Milliliters
mM	Milimolar
N-ter	Amino-Terminus
nm	Nanometers
NYG	Nutrient Broth, Yeast Extract, Glucose
OD	Optical Density
ORF	Open Reading Frame
PAA	Peracetic Acid
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PQS	*Pseudomonas* Quinolone System
PVD	Pyoverdine

QS	Quorum Sensing
RBS	Ribosome Binding Site
REC	Receiver Domain
Rf	Ratio to Front
rpf	Regulation of Pathogenicity Factors
rpm	Revolutions Per Minute
RR	Response Regulator
SDS	Sodium Dodecyl Sulfate
SMX	Sulfamethoxazole
Tc	Tetracycline
TEM	Transmission Electron Microscopy
TFP	Type IV Pili
TMP	Trimethoprim
TMP-SMX	Trimethoprim- Sulfamethoxazole
TMR	Trans Membrane Region
wt	Wild Type
YEB	Yeast Extract Broth
µL	Microliters
µM	Micromolar

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ABSTRACT

Fatty-acid mediated Quorum Sensing (QS) systems have aroused considerably interest in the last years since it has been reported that many important bacterial pathogens use these communication systems to regulate virulence-related functions. It is known that *Stenotrophomonas maltophilia* presents the DSF (Diffusible Signal Factor) QS system, which is controlled by components that are encoded in the *rpf* cluster (Regulation of Pathogenicity Factors). However, the mechanisms by which *S. maltophilia* synthesize and sense as well as the biological functions that are under control of DSF-QS remain unclear. Here, we have first demonstrated that two populations of *S. maltophilia* can be distinguished depending on the *rpf* cluster (*rpf-1* or *rpf-2*) they harbour. Each variant cluster differs basically in the genes that encode for the synthase RpfF and the sensor RpfC. Moreover, we have observed that there exist a full association between both components, existing the pair RpfF-1/RpfC-1 for the *rpf-1* variant and RpfF-2/RpfC-2 for the *rpf-2* variant. In addition, we have demonstrated that only strains harbouring the *rpf-1* variant produce detectable levels of DSF and it seems to regulate bacterial motility, biofilm development and virulence. On the other hand, strains harbouring the *rpf-2* variant need extra copies of *rpfF-2* or the absence of *rpfC-2* to achieve detectable levels of DSF. In this case, DSF-QS seems to control only some virulence-related phenotypes in very specific environments (e.g., zebrafish infection). We also have shown that DSF is produced in a positive feedback-manner in *S. maltophilia*, and also, that both *rpf*-variant groups act synergistically in the DSF production and virulence ability of the whole population. In addition, we have observed that while RpfC-1 is a promiscuous sensor that liberates free active-RpfF-1 -with the subsequent DSF synthesis- upon detection not only DSF, but also saturated medium-length fatty acids, the sensor RpfC-2 only allows activation of RpfF-2 upon detection of DSF-itself, indicating that this sensor component is much more specific.

Here, we further report that the *cis*-DA (*cis*-decenoic acid) QS system recently described in *Pseudomonas aeruginosa* is also present in *S. maltophilia,* and it regulates various virulence factors. In this line, we have preliminary characterized two important components in the biosynthesis of *cis*-DA, the enoyl-CoA hydratases (ECH) Smlt0266 and Smlt0267. We have observed that while the mutation in the putative synthase *smlt0266* lead to alteration basically in biofilm formation, the mutation of the

alternative ECH *smlt0267* results in a drastic effect in many virulence-related behaviours such as biofilm formation, bacterial motility, exopolysaccharide production, antibiotic resistance and virulence. Similar results have been obtained for the mutants in the orthologous *P. aeruginosa* genes Δ*dspI* and Δ*dspII*. These results further support the significance of these two ECH, in addition to DSF-QS system, in virulence regulation of *S. maltophilia* and provide new interesting targets for developing new antimicrobial therapies against this potential human pathogen.

RESUM

Els sistemes de comunicació bacteriana -coneguts com *quorum sensing* (QS)- a través de molècules senyalitzadores del tipus àcid gras han despertat molt d'interès en els darrers anys ja que s'ha vist que molts bacteris patògens els utilitzen per regular funcions relacionades amb la virulència. Es coneix que *Stenotrophomonas maltophilia* presenta el sistema de QS DSF (*Diffusible Signal Factor*) el qual és controlat pels gens que conformen el clúster *rpf* (*Regulation of Pathogenecity Factors*). No obstant, no està clar els mecanismes pels quals *S. maltophilia* sintetitza i sensa les molècules senyal així com quines funcions estan regulades per aquest sistema. En aquest treball hem demostrat que existeixen dues poblacions de *S. maltophilia* les quals es diferencien en base al clúster *rpf* (*rpf-1* o *rpf-2*) que presenten. Cada variant difereix bàsicament en els gens que codifiquen per la sintasa RpfF i el sensor RpfC. A més, hem observat que existeix una associació entre ambdós components, generant-se la parella RpfF-1/RpfC-1 per les soques *rpf-1* i RpfF-2/RpfC-2 per les soques *rpf-2*. Addicionalment, hem demostrat que només aquelles soques que presenten la variant *rpf-1* produeixen nivells detectables de DSF i aquest regula motilitat bacteriana, formació de biofilm i virulència. Per altra banda, les soques de la variant *rpf-2* necessiten més còpies de la sintasa RpfF-2 o l'absència del repressor RpfC-2 per produir DSF. En aquest cas, el sistema de QS DSF sembla només regular pocs fenotips relacionats amb virulència en situacions molt específiques. També hem mostrat que existeix un *feedback* positiu en la síntesi de DSF i que ambdós grups de soques actuen de manera sinèrgica en la producció de DSF i la virulència de tota la població. Addicionalment, hem observat que, mentre la variant RpfC-1 és un sensor promiscu el qual permet l'alliberació de la sintasa RpfF-1 tant punt detecta no només DSF sinó també àcids grassos de cadena mitja, el sensor RpfC-2 és molt més específic, alliberant RpfF-2 només quan detecta DSF.

A més a més, aquí també mostrem com el sistema de QS *cis*-DA (*cis*-decenoic) descrit recentment a *Pseudomonas aeruginosa* és també present a *S. maltophilia* i regula un alt nombre de factors de virulència. En aquesta línia, hem sigut capaços de caracteritzar preliminarment dos components importants en la biosíntesi de l'àcid gras *cis*-DA: les enoil coA hidratases (ECH) Smlt0266 i Smlt0267. Hem observat que, mentre la mutació de la hipotètica sintasa Smlt0266 només condueix a l'increment de la formació de biofilm, la mutació en el gen que codifica per la ECH alternativa Smlt0267 implica una

reducció dràstica en la formació de biofilm, la motilitat bacteriana, la producció d'exopolisacàrids, la resistència a antibiòtics i la virulència. Resultats similars s'han obtingut per els mutants dels gens ortòlegs a *P. aeruginosa*, la qual cosa recolza la importància d'aquestes dues ECHs, a més a més del sistema DSF, en la regulació de la virulència i aporta noves dianes interessants pel desenvolupament de teràpies antimicrobianes contra aquest potencial patogen humà.

INTRODUCTION

1. INTRODUCTION

1.1 Microbiology of *Stenotrophomonas maltophilia*

1.1.1 Historical aspects

Stenotrophomonas maltophilia was for the first time isolated in 1943. It was originally named as *Bacterium bookeri* and subsequently *Pseudomonas maltophilia* (1). Later, rRNA sequence analysis determined that it was more appropriately named *Xanthomonas maltophilia* (2–4). Although there is current debate about nomenclature, during the last decades, DNA and rRNA sequence analysis of several *Xanthomonas maltophilia* isolates, have resulted in the classification and naming of *X. maltophilia* as *S. maltophilia* (2, 5–7).

1.1.2 Physiology and metabolism

S. maltophilia is a flagellar non-fermentative gram-negative bacillus, belonging to the *Xanthomonadaceae* family. It is slightly smaller than other members of the genus and posses two polar flagella that confer it bacterial motility. *S. maltophilia* is catalase-positive, oxidase-negative and have a positive reaction for extracellular DNase. Although *S. maltophilia* is aerobic, it is able to grow using nitrate as a terminal electron acceptor in the absence of oxygen (8). It usually produce positive reactions for ONPG (o-nitrophenyl-B-D-galactosidease), lysine decarboxylase, esculin hydrolyisis, and gelatinase when cultured on most blood agar media (9).

1.1.3 Genome

Five years ago, the number of publicly sequenced genomes was only four. At the date, it has increased up to twenty, including incomplete whole genome sequence (WGS) projects. During this four-years period, we have also contributed to rise the number of *S. maltophilia* publicly genomes, by sequencing the genome of a decubitus ulcer isolate -strain M30 (10) (Annex 1) - which has been used in the genomic analysis of the *rpf* cluster presented in the section 4.1.

As for the most of related bacteria, *S. maltophilia* genome consists of one large dsDNA circular chromosome containing between 4,500,000 and 5,000,000 base pairs. Typically, its genomes present a G+C content of 66.7% (8). Furthermore, while no plasmids have been reported in the genome of *S. maltophilia*, it is thought to have gained its multi-drug resistance through horizontal gene transfer from neighbour

bacteria present in its natural niche or similar nosocomial pathogens, probably by homologous recombination (9).

1.1.4 Ecology

S. maltophilia is ubiquitous in aqueous environments, and it is frequently isolated from soil, water, animals, plant matter, and hospital equipment. *S. maltophilia* is often associated with plants and has been isolated from the rhizosphere of wheat, oat, cucumber, maize, oilseed rape, and potato (11–14). However, while it is mainly isolated from the rhizosphere, they also can be isolated from the vascular tissues of the root and stem of the aforementioned plants. During the last years, the rhizosphere has been considered an important reservoir for opportunistic human pathogenic bacteria (15). This environment -the zone around the roots- is a 'microbial hot spot', due to the presence of a high content of nutrients. Various bacterial genera have been isolated from this habitat, including *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Ochrobactrum*, *Pseudomonas*, *Ralstonia*, *Staphylococcus* and *Stenotrophomonas,* most of them being considered as multi-drug resistant organisms (MDRO). Due to the inter-specific and inter-kingdom competence, there is a high presence of diverse antibiotics in the rhizosphere. Thereby, it has enhanced horizontal gene transfer rates in this microenvironment, which have contributed to the high levels of natural resistances.

The secretion of extracellular enzymes and secondary metabolites important for plant colonization, as well as the production of pili for adhesion and biofilm formation enables *Stenotrophomonas maltophilia* to colonize and survive to these competitive environments. Contrary to other related genus such as *Xanthomonas* or *Xylella*, none *Stenotrophomonas* spp. have been shown to be phytopathogenic (16), otherwise, the environmental strain R551-3 has been shown to have a positive role in the germination and growth of oilseed rape (17).

As most of the bacterial isolated from this particular niche, *S. maltophilia* is an environmental MDRO. However, due to its intrinsic resistance, metabolic versatility and rapid adaptation, it has been also recovered from animals, invertebrates, water treatment and distribution systems, wastewater plants, sinkholes, lakes, rivers, aquifers, among others environments (18). Since an important particularity of this ubiquitous MDR bacterium is its ability to form biofilms, it has been frequently identified on abiotic surfaces of medical instruments, such as cannulas, prosthetic devices, dental unit

waterlines, and nebulizers (19–21), which have made *S. maltophilia* infections become an important medical problem during the last decades.

1.2. Clinical Significance of *Stenotrophomonas maltophilia*

1.2.1 Hospital and community-acquired infections

S. maltophilia is responsible for an increasing number of hospital-acquired (nosocomial) infections, specially in immunocompromised patients. Nevertheless, cases of community-acquired infections caused by *S. maltophilia* have also been reported (22, 23). It is thought that the transmission of *S. maltophilia* to susceptible individuals may occur through direct contact with the contaminated device. Although *S. maltophilia* display limited invasiveness and weak pathogenic capacity, it is capable of infect a wide range of tissues and organs. Most commonly infections associated with *S. maltophilia* include respiratory tract infections (pneumonia and acute exacerbations of chronic obstructive pulmonary disease [COPD]); bacteremia; biliary sepsis; infections of the bones and joints, urinary tract, and soft tissues; endophthalmitis; eye infections (keratitis, scleritis, and dacryocystitis); endocarditis; and meningitis (18). *S. maltophilia* has been also considered a significant pathogen in cancer patients (24, 25). The crude mortality rates caused by this opportunistic pathogen ranged from 14 to 69% in patients with bacteremia (26).

1.2.2 Cystic fibrosis

Cystic fibrosis (CF), is an autosomal recessive genetic disorder that affects most critically the lungs, but also the pancreas, liver and intestine. CF is caused by mutations in a gene on chromosome 7 that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) (27). It is characterized by irregular transport of chloride and sodium ions across the epithelium, leading to thick and viscous secretions. The inhaled material including bacteria is entrapped, enabling microorganisms to colonize and establish infections within the mucus (28). In the early stages of CF, intermittent colonizations occur, which can be treated with antibiotics (29). Chronic infections appear over time and are characterized by the formation of bacterial aggregates, called biofilms (30, 31).

S. maltophilia has been frequently isolated from lungs of CF patients. However, there is some controversy whether *S. maltophilia* is a colonizer or the causal agent of the infection (32). While some studies point a harmful effect of *S. maltophilia* in CF lungs

(33), some others report that the presence of *S. maltophilia* do not reduce the survival of CF patients (34).

1.3. Molecular Mechanisms Involved in Pathogenesis

1.3.1 Exoproducts

Secretion of hydrolytic enzymes provides an advantage for the survival, growth, and spread of bacterial pathogens. The genome of *S. maltophilia* strain K279a contains genes encoding for extracellular enzymes including lipases, proteases, esterase, RNase, DNase and fibrolysin (8). It has been reported that some clinical *S. maltophilia* isolates displayed proteolitic, lipolitic, hemolytic and cytotoxic activities (35). Serine proteases have been shown to contribute pathogen's ability to degrade connective tissues in nosocomial *S. maltophilia* infections (36). The gene encoding the extracellular protease StmPr1 was also found in isolates that have been recovered from patients with CF chronic infections (37).

Bacterial lipopolysaccharide (LPS) has been frequently linked to bacterial adhesion, antimicrobial resistance and virulence. *S. maltophilia* presents a typical LPS structure containing lipid A, core oligosaccharide, and O-antigen (38). It has been shown that alteration in LPS reduces biofilm formation of *S. maltophilia* (39, 40), but also modulate cell's susceptibility to particular antimicrobial compounds, including polymyxin B, polymyxin E, nalidixic acid, gentamicin, and vancomycin (41). In addition, deficit of LPS reduced *S. maltophilia* virulence in a rat lung model of infection (41).

1.3.2 Antibiotic resistance

One important and particular feature that makes *S. maltophilia* an important global opportunistic pathogen is its high intrinsic resistance to a broad array of antibiotics. This includes resistance to: trimethoprim-sulphametoxazole (TMP-SMX), β -lactam antibiotics, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, and polymyxins (42–44). *S. maltophilia* cell membrane exhibits low permeability, which contributes to resistance to β-lactams including cefepime, ticarcillin-clavulanate, ceftazidime, and piperacillin-tazobactam (45). The presence of chromosomally encoded multidrug resistance efflux pumps (43, 46, 47) and β-lactamases (48–50) contribute to the intrinsic antibiotic resistance of *S. maltophilia* (44). It is thought that the resistance of *S. maltophilia* as well as other

opportunistic gram-negative pathogens have been acquired in its natural niche (i.e., rhizosphere), by horizontal gene transfer, and potentiated due to the use of antimicrobials in medical devices and treatments (51).

Considering the high intrinsic resistance that *S. maltophilia* displays against a wide range of antibiotics, it is evident that new antimicrobial strategies are needed to control and treat *S. maltophilia* infections. There is a lively debate about the use of monotherapy versus combination therapy to treat *S. maltophilia* infections. Currently, synergistic treatment combining different antibiotic is the selected choice. Indeed, the combined agent TMP-SMX has been frequently used in the last years, however, increasing resistances have been recently reported (52–54). More recently, it has been reported that colistin represents a valuable antibiotic against MDR bacterial infections with acceptable nephrotoxicity and considerable effectiveness (55). In this study, it was reported colistin therapy for 258 patients who received intravenous colistin for various MDR gram-negative bacterial infections, including *S. maltophilia.* In total, 79.1% of patients were cured and nephrotoxicity was found in 10% of patients. Hence, colistin has emerged as a new promising antimicrobial agent to treat infections caused by *S. maltophilia* and other MDR gram-negative bacteria.

Nevertheless, design new strategies against *S. maltophilia* infections represent a challenging and crucial issue in order to avoid serious complications in patients infected with *S. maltophilia.*

1.3.3 Biofilm formation

Another important virulence-related trait that this bacterium exploits to persist and resist to antimicrobials and immune system is its strong ability to form biofilms. A biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix (Fig. i). The first step of the biofilm formation is the adhesion of the bacteria to the surface. Then, the non-motile adhered bacteria grow and produce exopolysaccharide (EPS). Once the biofilm is matured, cells start to detach reverting the planktonic cells and completing the cycle (Fig. i).

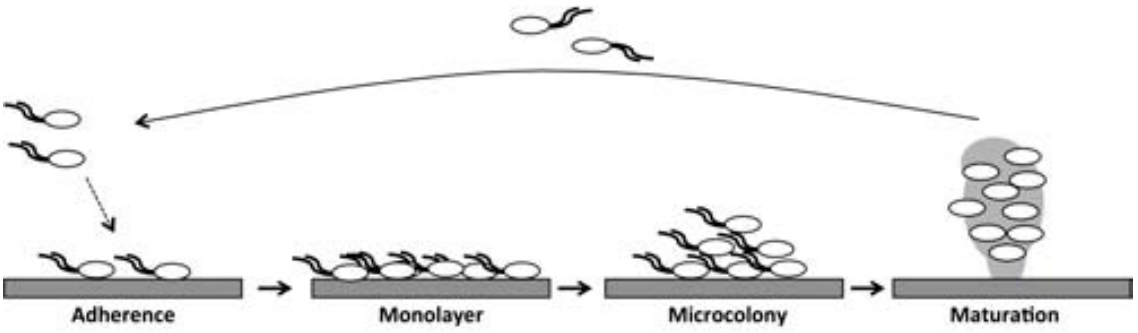

FIG i Schematic representation of a bacterial biofilm development.

Bacterial adherence and biofilm development depend on the physicochemical properties of the bacterial cell (presence of outer membrane proteins, lipopolysaccharides and cellular appendixes), the properties of the surfaces to be attached (biotic surface e.g., lungs, or abiotic surface such as hydrophilic glass or hydrophobic polyvinyl chloride) and the environmental conditions. Environmental factors that modulate the biofilms of *S. maltophilia* include phosphate and chloride concentration, pH, temperature, oxygen levels and the presence of copper and silver ions (18). Clinical *S. maltophilia* isolates have been observed to be more effective in biofilm formation at 32°C than at 37°C and 18°C (56). In addition, acid environments (pH 5.5) have been shown to reduce biofilm development compared to neutral o slightly basic conditions (pH 7.5-8.5) (18).

Flagellum is one of the most important appendixes of the cell that has been reported to mediate the initial steps of biofilm formation in bacteria. In the case of *S. maltophilia* flagella, it has been shown that these highly conserved immunogenic structures are crucial in attaching to mouse tracheal and lung mucus. Accordingly, flagellin pre-treatment of the mouse tracheal resulted in a reduction of *S. maltophilia* adhesion. Deflagellated bacteria also demonstrated a reduced adherence to mouse tracheal mucus (57).

An additional important appendix in biofilm formation is fimbriae or pili. *S. maltophilia* fimbriae 1 (SMF-1) has also been shown to be important for adherence to cultured HEp-2 monolayers and glass surface, specially in the early stages of the biofilm development (58).

Several treatments have been employed in order to remove biofilms formed by *S. maltophilia* in both abiotic and biotic surfaces. In a recent study, eight biocides (1% sodium dodecyl sulfate, 35% hydrogen peroxide, 5.25% sodium hypochlorite, 1% phenol, 4% Tween 20, 1% EDTA, 0.2% chlorhexidine gluconate, and 1% povidone-

iodine) were tested on *S. maltophilia* biofilms present in dental unit water lines (59). From these biocides, the most effective combination was the one composed for 5.25% sodium hypochlorite and 1% phenol, which was shown to remove almost the entire biofilm. The commonly used disinfecting agent peracetic acid (PAA) was found to inhibit the growth of a *S. maltophilia* monoculture biofilm and a dual-culture biofilm composed by *S. maltophilia* and *Candida parapsilosis* (60).

The use of antibiotics at their minimal inhibitory concentrations (MICs) and at concentrations below the MICs on *S. maltophilia* cell adherence and the subsequent biofilm formation have been studied (61, 62). It has been reported that some antibiotics at suboptimal MICs (e.g., moxifloxacin) are effective in preventing biofilm formation of *S. maltophilia* (62). On the other hand, It has been reported that the MDR isolates showed a higher level of biofilm formation than the non-MDR isolates. In addition, the biofilm ability have been correlated with resistance to ceftazidime, cefepime, ticarcillin-clavulanic acid, piperacillin-tazobactam, aztreonam, and gentamicin (63).

1.3.4 Bacterial motility

Three kinds of bacterial motilities have been described for *S. maltophilia*, and all of them have been related to pathogenesis. These include swimming, twitching and swarming.

Swimming motility is a mode of bacterial movement powered by rotating flagella that takes place as individual cells moving in liquid environments. It is usually tested into semi-liquid agar plates containing 0.25% agar. In *S. maltophilia*, deletion of *fliI* resulted in the suppression of swimming motility (64). It has been also reported that disruption of *rpfF* resulted in the loss of swimming motility in *S. maltophilia* strain K279a (65). Another study demonstrated that *xanB* gene -a lipopolysaccharide/exopolysaccharide biosynthetic gene- is also required for swimming motility in *S. maltophilia* strain WR-C (40). In this mentioned study, it is also reported that the other genes involved in the lipopolysaccharide/exopolysaccharide biosynthetic pathway *rmlA*, *rmlC*, and *xanB* were essential in twitching motility (40).

Twitching motility is a sort of surface motility powered by the extension and retraction of type IV pili, which results in a slow cell movement compared to other types of motility. Several clinical isolates have been studied to determine the possible correlation between twitching motility and biofilm formation, concluding that there is no correlation between these two phenotypes (37, 64).

Swarming motility is a rapid and coordinated translocation of a bacterial population across solid or semi-solid surfaces, which require cellular differentiation (hiperflagellation), self-production of biosurfactant and quorum sensing-mediated synchronization. Swarming motility is perhaps the most complex motility described in bacteria. It depends on several interconnected pathways that enable the whole population to colonize rapidly new environments. Thousands of papers have been recently published addressing this topic, the reader could easily address to extensive and detailed reviews about swarming motility in bacteria (66–71). However, swarming motility in *S. maltophilia* has been rarely reported. It is known that swarming motility requires very specific conditions to take place in the laboratory and many times these precise conditions are depending on the specie or even on the strain. In our laboratory, we have spent much time trying to optimize the conditions that facilitate this motility behaviour. Finally, although *S. maltophilia* do not display a fast swarming motility, we have observed that modified M9-salts and BM2 mediums are the most optimum conditions for the evaluation of swarming motility in this bacterium. Accordingly, we recently have reported swarming motility in *S. maltophilia* (72).

One important molecular mechanism involved in bacterial pathogenesis is quorum sensing (QS). During the last decasdes, QS has aroused much interest because it has been demonstrated that these cell–cell communication systems control many phenotypes, including virulence, in numerous bacterial pathogens.
Since QS is the main topic of this work, in the next section, I will address the issue of QS, with special emphasis in the one described in the nosocomial pathogen *S. maltophilia*, the DSF (Diffusible Signal Factor) quorum sensing system.

1.4 Quorum Sensing

1.4.1 Generalities

It is well established that bacterial cells can communicate with each other to facilitate their rapid adaptation to fluctuations in the environment. This cell-cell communication mechanism, known as quorum sensing (QS), relies on the production, detection, and response to diffusible signal molecules (also called autoinducers) in a cell-density-dependent manner (73–76). Throw this QS communication, numerous bacterial species regulate a variety of functions such as biofilm formation, toxin production,

exopolysaccharide synthesis, extracellular enzyme production, motility, and plasmid transfer (77–81). When the population density is low, each single cell produces basal levels of signalling molecules, which are accumulated in the extracellular space. Concentration of QS signals increase accordingly to the cell density and, once the concentration reaches a critical threshold, the population synchronize the gene expression and coordinate various biological activities.

The first QS system was described in the symbiotic gram-negative bacteria *Vibrio fishery*, during the 80's decade (82). In the present, it represents one of the best-characterized quorum sensing systems. The signalling molecule responsible for this communication is the AHL-signal (Acyl Homoserine Lactone), which is synthesized by the enzyme LuxI. When the signal concentration is the optimum, AHL interacts with its receptor LuxR and induces the transcriptional expression of the *lux* genes, which are responsible for the production of bioluminescence. Since this discovery, AHL-based QS have been described in numerous symbiotic and pathogenic bacteria (83). Hence, several clinically relevant gram-negative pathogens use quorum sensing systems to regulate processes associated with virulence (84, 85) (Fig. ii). Furthermore, QS systems are not restricted to gram-negative bacteria, since several gram-positive bacteria use peptide-based signalling molecules to regulate biological functions similarly to gram-negative bacteria (Fig. ii). Some interesting reviews could be consulted to obtain detailed information about quorum sensing in gram-positive bacteria (86–90).

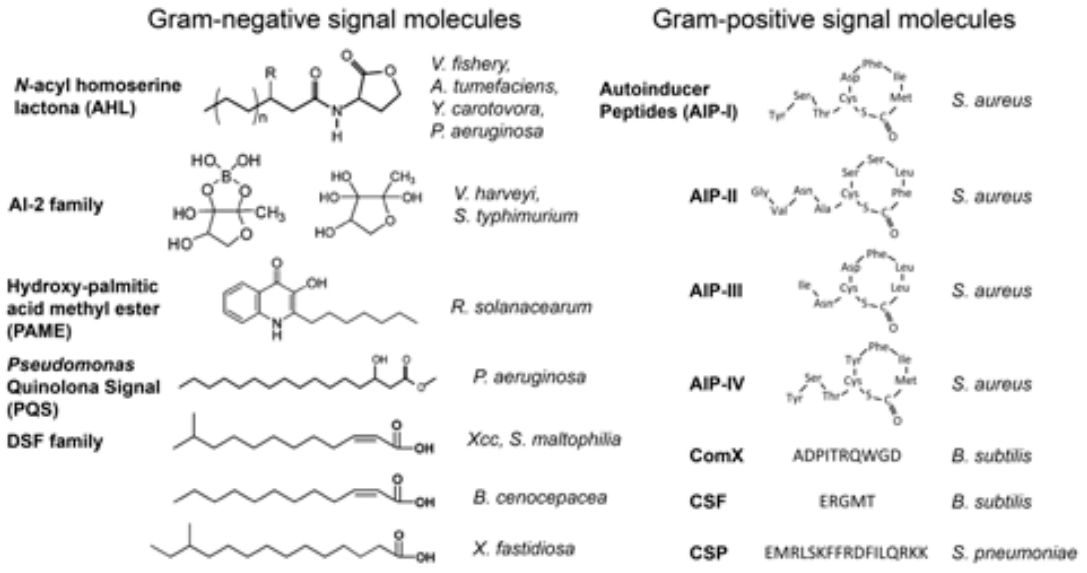

FIG ii. QS signalling molecules used in gram-negative (91) and gram-positive bacteria (89). Information has been extracted from cited reviews.

As observed in the figure ii, one important family of signalling molecules in gram-negative bacteria is DSF-family. These molecules are simple medium-length fatty acids that have been demonstrated to synchronize several biological processes in various gram-negative bacterial pathogens. These interesting features will be detailed in the following sections.

1.4.2 Generalities of DSF quorum sensing system

The diffusible signal factor (DSF) molecule represents an important type of QS signal found in many gram-negative bacterial pathogens. This small fatty acid molecule (*cis*-11-methyl-2-dodecenoic acid) was for the first time described in the phytopathogen *Xanthomonas campestris* pv. *campestris* (92, 93). In the past few years, it has been reported that DSF family signals are widespread (91). DSF-QS system has been described in various gram-negative human and plant pathogens, most of them belonging to the *Xanthomonadales,* including *X. campestris*, *X. axonopodis* (94, 95) and *X. oryzae* (96, 97); *Xylella fastidiosa* (98, 99), *Burkholderia cenocepacea* (100, 101) and *S. maltophilia* (65, 102). In addition, several DSF structural analogues have been identified in unrelated microorganisms including the fatty acid trans-2-decenoic acid (SDSF) produced by *Streptocuccus mutans* (103) and the farnesoic acid synthesized by the yeast *Candida albicans* (100).

Bacterial DSF activity has been primarily detected using different bacterial bioassays. The first-generation DSF detection methods are based on the protease and endoglucanase-restoration bioassay. Since it was demonstrated that in *Xanthomonas campestris* pv. *campestris* (*Xcc*) the production of exoproteases and endoglucanases depends on the DSF communication (92), the *Xcc* Δ*rpfF* mutant (DSF minus) is used as a reporter strain (92). Thus, the DSF-producer candidate strains are streaked onto plates containing either skimmed milk (for proteolysis-based bioassay) or carboxymethylcellulose (CMC) (for endoglucanase-based bioassay) closely to the *Xcc* reporter strain (Δ*rpfF* mutant) and the presence of a clear halo around the bacterial reporter colony indicates DSF activity (92).

Second-generation DSF-activity detection methods are based on engineered DSF biosensors strains. Classically, the promoter of the DSF-inducible operon *engXCA* (which encodes for an endoglucanase) is fused with a promoterless reporter *gusA* gene (which encodes for a ß-glucuronidase). The genic fusion is cloned into a replicative

vector and the resultant plasmid is mobilized to a DSF-minus *Xcc* mutant (*Xcc* Δ*rpfF*) generating the DSF reporter strain (104, 105). Then, a monolayer of the biosensor strain is seeded onto a nutrient broth plate containing the GusA substract X-Glu (5-Bromo-4-chloro-3-indolyl β-D-glucopyranoside) and the candidate strains are pin inoculated onto it. After an overnight incubation, the presence of a blue halo around the colony of candidate strains indicates DSF activity. These engineered biosensor strains have been widely used in detection of DSF-family signals in other bacterial species, including members of the *Burkholderia cepacia* complex and *Xanthomonas oryzae* pv. *oryzae* (100, 101). Another DSF biosensor, which was constructed using *engXCA* promoter fused to a promoterless *gfp* gene, has been employed to detect the DSF-family signals from *Xylella fastidiosa* and *Xanthomonas axonopodis* pv. *glycines* (95, 98, 106).

1.4.3 DSF-QS in *Xanthomonas campestris* pv. *campestris*

DSF communication was for the first time reported at the end of the nineties, analysing a *Xanthomomnas campestris* pv. *campestris* (*Xcc*) *rpf* (regulation of pathogenicity factors) cluster (92). The components involved in this cell-cell communication are organized in the *rpf* cluster (Fig. iii). It was observed that the *rpfF* mutant could restore the protease and endoglucanase activity when cultivated closeness to its wt strain. This led investigators to think that a diffusible signal factor (DSF) –produced by the wt *Xcc*- could be the responsible for the induction of protease and endoglucanase production in the *rpfF* mutant strain, thereby suggesting that the enoyl-CoA hydratase RpfF could be the DSF synthase. Then, *Xcc* has become a model organism to study DSF-QS and, at this time, extensive works have been published regarding the components involved in DSF production and detection, the chemistry of the biosynthetic pathway and the phenotypic implications of the components involved in this communication system not only in *Xcc,* but also among many related microorganisms.

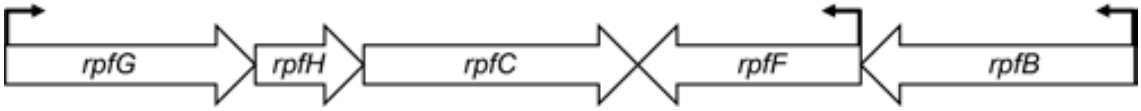

FIG iii. Gene organizations in the *rpf* cluster in *Xcc*. Arrows indicate the presence of a promoter.

1.4.3.1 DSF biosynthesis, RpfF and RpfB

It was found that mutation on *rpfF* or *rpfB* genes (Fig. iii) abolished DSF production, suggesting that these two enzymes are required for DSF synthesis in *Xcc* (92, 105) (Fig. iv). Further *in silico* analysis predicted that *rpfF* encode a putative enoyl-CoA hydratase and *rpfB* encode a putative long-chain fatty acyl CoA ligase (92, 105). More recently, it has been shown that RpfF belongs to the members of the crotonase superfamily (107). Two putative catalytic glutamate residues (Glu141 and Glu161) have been detected in RpfF, which are conserved in the enoyl-CoA hydratases. Accordingly, substitution of these two residues in RpfF resulted in the suppression of DSF synthesis, demonstrating their important role in DSF production (107). In addition, structural analysis of RpfF also revealed a hydrophobic pocket, which is comprised by several hydrophobic residues including Leu136, Gly137, Gly138, Gly85, Leu276, Met170, and Trp258 (107). It has been suggested that these amino acids compose the putative substrate-binding pocket for DSF biosynthesis (107). Accordingly, a single-point mutation in any of these residues resulted in a drastic fall or even abolition of DSF production (107).

1.4.3.2 DSF detection by RpfC

Another important component in the *rpf* cluster is the gene *rpfC* (Fig. iii), which encodes for a two-component hybrid sensor kinase (Fig. iv). It is composed by five transmembrane (TMR) domains, an histidine kinase (HisKA) domain, a receiver (REC) domain and a histidine phosphotransfer (HPT) domain. It has been reported that the exogenous addition of DSF could fully restore the *rpfF* but not *rpfC* mutant phenotype, strongly suggesting that RpfC is responsible of DSF detection (105, 108). It has been demonstrated that RpfC uses a conserved phosphorelay mechanism to transduce the DSF signal (Fig. iv). However, it still remains undetermined how the RpfC could detect DSF and trigger the phosphorelay process.

Curiously, it has been reported that –in addition to sense DSF- RpfC also acts negatively blocking the DSF biosynthesis (105) (Fig. iv). Based on classic DSF bioassay, it has been demonstrated that the *rpfC* mutant produced higher amount of DSF compared to the *Xcc* wt strain (105). It has been reported that the REC domain of RpfC directly interacts with the catalytic domain of the RpfF, blocking its DSF-synthesis activity (107) (Fig. iv).

Although these results provide evidences about the interaction between RpfC and RpfF, the mechanisms by which RpfC senses DSF and subsequently liberates free active-RpfF remain uncertain.

1.4.3.3 Signal transduction by RpfG

Since the genes *rpfG*, *rpfH*, and *rpfC* are organized into an operon in the genome of *Xcc*, it was suggested that these three genes could have related functions (105). Specifically, RpfG is a cytoplasmic regulator element that contains a typical receiver domain and a HD-GYP domain. It has been reported that mutation in the *rpfG* and *rpfC* genes lead to similar phenotypic disorders, related to insensitivity to DSF (105, 109). It has been demonstrated that the HD-GYP domain of RpfG plays a key role in DSF signal transduction (110). In particular, it has been revealed that this HD-GYP domain has a novel function related to cyclic diguanilate (c-diGMP) metabolism. It is known that c-diGMP is a global second messenger with important implications in the regulation of numerous biological functions in bacteria (111–113). It was postulated that this novel HD-GYP domain might be key component in bacterial signalling enabling adaptation to environmental changes by modulating the c-diGMP levels (114). It has been confirmed that the HD-GYP domain degraded c-di-GMP to generate two molecules of GMP (110) (Fig. iv). In concordance with the predicted function of this new phosphodiesterase domain, in trans expression of the HD-GYP domain in the *rpfG* mutant was sufficient to restore the mutant phenotypes (110).

Altogether, these findings reveal that the RpfG is a novel c-diGMP degradation enzyme, which is activated by the DSF signalling and regulates biological functions by the modulation of the intracellular concentration of this important second messenger (Fig. iv).

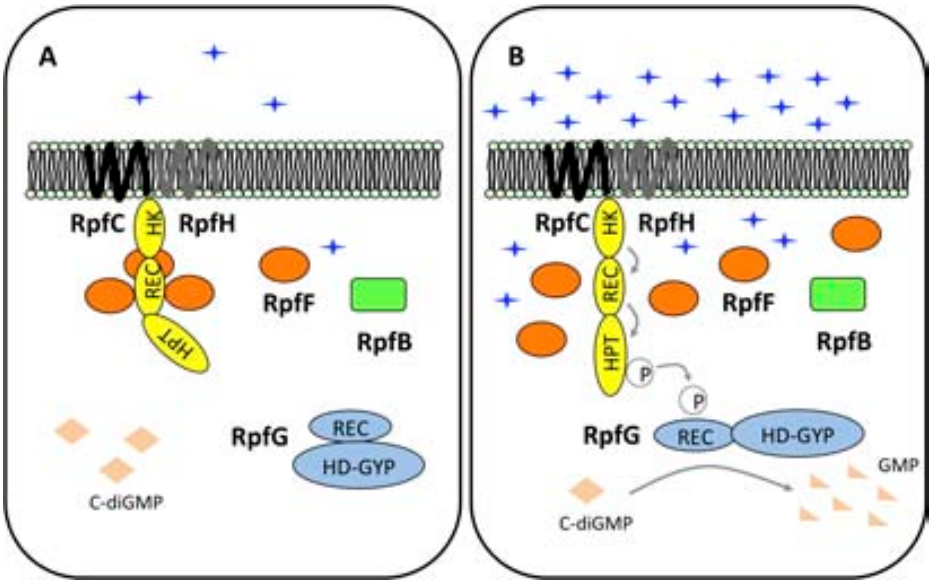

FIG iv. Schematic representation of the DSF-QS system in *Xcc*. (A) At low cell density, the DSF sensor RpfC blocks the DSF synthase RpfF through its receiver domain (REC), which restricts DSF biosynthesis at a basal level. The cytoplasmic regulator element RpfG remains inactive. (B) At high cell density, RpfC senses the accumulation of DSF molecules and triggers a phosphorylation cascade that ends in the activation of the phosphodiesterase RpfG. The increment of intracellular GMP triggers the expression of virulence genes.

1.4.4 DSF-QS in *Xylella fastidiosa*

X. fastidiosa is the causal agent of Pierce's disease, responsible for large economic losses in agriculture (115, 116). In the case of *X. fastidiosa*, the signalling communication is mediated by a DSF derivative, identified as 12-methyl-tetradecanoic acid and designated *xf*DSF (99). *In silico* analysis showed that this pathogen harbours a conserved *rpfGCF* gene cluster, displaying a high identity to the *Xcc rpf* (91). As expected, mutation of *rpfF* in *X. fastidiosa* also abolished *xf*DSF production and, contrary to that observed in *Xcc*, it enhanced virulence, impaired insect transmission, and disabled biofilm formation in host insects (106). Mutation of the *rpfC* also resulted in the overproduction of *xf*DSF, a deficiency in the virulence ability of *X. fastidiosa* (98). Interestingly, recent findings have evidenced that in the case of *X. fastidiosa*, the *xf*DSF synthase RpfF is also essential for *xf*DSF perception. This new findings have made reconsider the role of the RpfC not only in *X. fastidiosa*, but also in related bacteria sharing the DSF-QS (117).

Nevertheless, much work is needed to understand the complexity of *xf*DSF-QS system in this important bacterial pathogen.

1.4.5 DSF-QS in *Burkholderia spp.*

The *Burkholderia cepacia* complex is a group comprised of nine related species which have recently emerged as problematic opportunistic pathogens in patients with CF and immunocompromised individuals (118–121). A DSF derivative (*cis*-2-dodecenoic acid) has been recently identified in *B. cenocepacia* and designated BDSF (Fig. ii) (100). Although the synthase Bcam0581 shares near 40% of protein identity with the RpfF of *Xcc*, it has been demonstrated that Bcam0581 is essential for BDSF production and can functionally substitute the *rpfF* gene in *Xcc* Δ*rpfF* mutant (100). Nevertheless, contrary to the genetic organization observed for the other related bacteria sharing the DSF-QS, Bcam0581 is not encoded in the *rpf* gene cluster. Hence, genomes of *B. cepacea* strains do not contain neither *rpfC* nor *rpfG* homologues flanking *bcam0581* gene. This suggests that BDSF-QS system in *B. cenocepacia* could have a distinct evolutionary origin (100).

However, deletion of *bcam0581* resulted into alteration of similar processes as observed in the *rpfF* mutant in *Xcc*. These include delayed bacterial growth in minimal medium, reduced swarming motility, alteration in biofilm development and reduced virulence (101, 122–124). Interestingly, it has been suggested that certain biological functions such as motility, biofilm formation, and virulence, are coregulated by the BDSF signalling and the AHL-dependent QS system in *B. cenocepacia* (125). More recently, it has been identified Bcam0227 as a potential BDSF sensor, which shares 35.6% of identity with the RpfC of *Xcc* (124). This putative BDSF sensor contains four domains, including a histidine kinase phosphoacceptor domain, a histidine kinase domain, a CheY-like receiver domain, and a C-terminal histidine phosphotransfer domain (124). Deletion of *bcam0227* also resulted into attenuation of bacterial virulence (124).

Additionally, it has been shown that other members of the *B. cepacia* complex also produced different derivative DSF-QS signals (101), which complicates the understanding of the mechanism of the BDSF-QS in this important opportunistic bacterium.

1.4.6 DSF-QS in *Stenotrophomonas maltophila*

S. maltophilia harbours a *rpf* gene cluster highly conserved (≈65-80% peptide similarity) with the model strain *Xcc* (65). It has been demonstrated that disruption of

DSF signalling has a drastic effect on *S. maltophilia* K279a, since the *rpfF* mutant shows reduced swimming motility, reduced exoprotease production, altered LPS, reduced tolerance to a range of antibiotics and to heavy metals and reduced virulence in a *Caenorhabditis elegans* infection model (65). In addition, FecA, a ferric citrate receptor, has been shown to be positively regulated by the DSF-QS system (126). This receptor contributes to the internalization of iron, an essential element for the expression of virulence-related genes (126). In the *S. maltophilia* WR-C wild type strain and a flagella-defective *xanB* mutant, flagella-independent translocation was stimulated not only by the main DSF but also by its derivative 11-methyl-dodecanoic acid (127). Regarding the interaction of *S. maltophilia* with plants, DSF seems to be involved in oilseed germination, plant colonization and biofilm architecture in the environmental strain R551-3 (17). In a recent *S. maltophilia* population study, the authors detected *rpfF*+ genotypes in 61% of the 89 tested strains, suggesting that an important population of *S. maltophilia* lacks the *rpfF* gene (128).

With the rapid increase in the number of *S. maltophilia* sequenced genomes it is now possible to compare the *rpf* cluster among different strains. A preliminary analysis showed that all sequenced genomes contain the *rpf*F gene. In addition, at least two *rpf* cluster variants can be detected on the basis of sequence and genomic organization, with main differences found in the *rpfF* and *rpfC* genes. However, while numerous and extensive works have been published in other related bacteria sharing the DSF-QS, in the case of *S. maltophilia*, more efforts are needed to understand how this cell-cell communication system regulates virulence-related processes.

1.5 *Pseudomonas aeruginosa*, an overview

1.5.1 Microbiology of *P. aeruginosa*

P. aeruginosa is a gram-negative, aerobic, coccobacillus bacterium with unipolar motility. It belongs to γ-proteobacteria class and frequently it is responsible of cause disease in animals, including humans. *P. aeruginosa* contains a large genome, with one of the largest regulatory networks of any bacteria sequenced (129). Part of the significance of this large genome is that it provides the bacterium with a tremendous diversity of metabolic pathways and physiological responses, which allows the bacteria to acclimatize to many diverse environments.

Even though *P. aeruginosa* is most predominant in the environment, the organism is mainly studied in the context of lung infections in CF patients. It is the most prevalent cause of morbidity and mortality in CF patients, who are unable to clear the bacteria from their lungs. *P. aeruginosa* has also been isolated in samples taken from chronic sinusitis and burn patients, and has been implicated in urinary tract infections (130). The bacterium is a frequent culprit of hospital-acquired infections, and once *P. aeruginosa* infections have become established, they are characteristically difficult to treat.

1.5.2 Mechanisms involved in pathogenesis

P. aeruginosa displays three kinds of surface motility: swarming (131), twitching (132), and sliding (133). Twitching and swarming motilities are strongly linked with biofilm development and pathogenesis, and their mechanical components, the flagellum and type IV pili (TFP), have been demonstrated to be virulence factors (133, 134). TFP are essential for twitching motility, but they are generally not considered important for swarming behaviour. Little is known about sliding motility, but it doesn't seem to be dependent on flagella and TFP (133).

It is known that bacterial motility and adhesion are two important properties directly related to virulence. Biofilm development and swarming represent two contrasting lifestyles that are mutually complimentary. One opinion is that surface motility, including swarming, allows cells to colonize surfaces and then establish non-motile biofilms. It has been reported that surface attachment proteins are up-regulated in bacteria within the biofilm and predictably down-regulated in swarming cells (135). It has become evident that biofilm formation is a population strategy to protect the bacteria from desiccation, nutrient deficiency, antibiotics, and human immune response. Cell motility is necessary for the expansion and maturation of the biofilm, and the biofilm represents a 'safe-haven' community of sessile cells that have stopped moving. One important factor implied in the regulation of several virulence-related traits, including biofilm formation and bacterial motility, is quorum sensing.

1.5.3 Quorum sensing regulatory network in *P. aeruginosa*

The signalling network of *P. aeruginosa* is probably the most complex systems known and, to date, the most studied among bacteria. It consists of numerous interconnected signalling pathways that, together, regulate complex population behaviours. The best-studied communication system in *P. aeruginosa* is undoubtedly the AHL-mediated QS system, which is involved in the regulation of several virulence-related phenotypes, not

only in *P. aeruginosa* but also in most of the species sharing related QS systems (136–138). Nevertheless, some other well-studied QS systems based on different signalling molecules coexist in this bacterium. These include the *Pseudomonas* Quinolone System (PQS) -which is mainly based on the molecule 4-quinolone and has been shown to be essential for the binding of iron facilitating the work of the siderophores (139, 140) -, the pigmented signalling system, which includes pyoverdine (PVD), phenazine and pyocyanine which are responsible for the production of several virulence factors, including exotoxin A (ToxA) and PrpL endoprotease (141) - and the recently reported *cis*-DA system, based on the fatty acid molecule *cis*-decenoic (142, 143), which is indeed the subject of this study. *cis*-DA system has aroused attention recently, since this new small fatty acid produced by *P. aeruginosa* has been show to stimulate biofilm dispersion in several gram-negative and gram-positive bacteria (142). Although *P. aeruginosa* harbours 12 putative enoyl-CoA hydratases, it recently has been reported that the enoyl-CoA hydratase DspI (*PA0745*) is the responsible for the *cis*-DA synthesis (143). In addition, it has been recently reported that mutation of *PA0745* resulted into attenuation of virulence in the *C. elegans* model (144). Nevertheless, an alternative ECH is also present in the *PA0745* cluster, the ECH *PA0744*. Curiously, none of the mentioned studies reported information about this alternative ECH, which could be probably involved in the biosynthesis of the fatty acid signal *cis*-DA.

Interestingly, we have observed that in the *S. maltophilia* genome, there exists homologues of the *cis*-DA synthase PA0745 and the alternative ECH PA0744, which corresponded to Smlt0266 and Smlt0267, respectively. Since this fatty acid seems to represent a new fatty acid-mediated QS system in gram-negative bacteria and could present parallelisms to DSF-QS system, we aim to study this new communication system not only in *S. maltophilia* but also in *P. aeruginosa.*

OBJECTIVES

2. OBJECTIVES

The main objective of this thesis is to study the fatty acid-mediated quorum sensing systems that coexist in *Stenotrophomonas maltophilia* and the implications that they have in virulence regulation in this pathogen. More specifically, our aim is to get insights into the DSF-based and the recently reported *cis*-DA-based quorum sensing systems in *S. maltophilia.*

Specific objectives have been:

To analyse the molecular aspects that governs the DSF synthesis and perception

To investigate the environmental conditions that modulates DSF production

To identify and characterise the DSF molecules produced by *S. maltophilia.*

To evaluate the role of the DSF quorum sensing system in the regulation of biological functions

To investigate if the new *cis*-DA quorum sensing system is present in *S. maltophilia*

To evaluate the implication of the enoyl-CoA hydratases Smlt0266 and Smlt0267 in the regulation of *cis*-DA quorum sensing system in *S. maltophilia.*

To investigate the implication of the orthologous enoyl-CoA hydratases PA0745 and PA0744 in the regulation of the *cis*-DA quorum sensing in *Pseudomonas aeruginosa.*

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Bacterial strains and growth conditions.

3.1.1 *S. maltophilia* and other *Xanthomonads*

A panel of 78 *S. maltophilia* clinical isolates were collected from intensive care units (ICUs) of different European Hospitals. Name, geographic origin, hospital and isolation source per strain are given in Table 1. From this collection, E77 (RpfF-1-variant group) and M30 (RpfF-2-variant group) (145) were used as model strains to characterize Δ*rpfF* mutants (Sections 4.1 and 4.2). *S. maltophilia* strain M30 wt and the Δ *smlt0266*, Δ*smlt0267* and Δ*smlt0266-0267* mutants were also used to study the new *cis*-DA-QS system (Section 4.3). *S. maltophilia* strains were routinely grown at 37ºC in Luria-Bertani (LB) medium on a rotary shaker. When needed, LB was supplemented with the following antibiotics: tetracycline (Tc) 17 µg/mL, chloramphenicol (Cm) 32 µg/mL and erythromycin (Erm) 500 µg/mL. For phenotypic analysis in minimal media, strains were grown in BM2 medium (62 mM potassium phosphate buffer, pH 7, 2 mM $MgSO_4$, 10 µM $FeSO_4$, supplemented with glucose 0.4 %) (146) or a modified M9-salts medium (modified M9 salts [33.9g/L Na_2HPO_4 $7H_2O$, 15g/L KH_2PO_4, 2,5g/L NaCl without NH_4Cl], 0.5% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and supplemented with 0.2% glucose) (147).

Xanthomonas campestis pv. *campestris* (*Xcc*) 8523 pL6engGUS was obtained from the authors (148). *Xcc* 8523 pL6engGUS was used as a reporter strain for the detection of *S. maltophilia* DSF activity. *Xcc* was routinely grown at 28ºC in NYG medium (0.5% peptone, 0.3% yeast extract and 2% glycerol) supplemented with Tc 10 µg/mL.

3.1.2 *P. aeruginosa*

P. aeruginosa strain MPAO1 wt and the Δ*PA0745* (strain name PW2354) and Δ*PA0744* (strain name PW2353) mutants were obtained from the transposon mutant library from the University of Washington (149) and were used to study the new *cis*-DA-QS system (Section 4.3) in this bacterium. Bacteria were routinely grown at 37ºC in LB medium on a rotary shaker. When needed, LB was supplemented with Tc 17 µg/mL, Cm 32 µg/mL and Gm 40 µg/mL. For phenotypic analysis in minimal media, strains were grown in modified M9-salts medium (147).

TABLE 1. Clinical *S. maltophilia* strains used in the section 4.1 and 4.2.

Strain	RpfF Variant	Source	Hospital	Country
OL11	1	Oropharynx	Ljubljana Medical Center	SLO
PL12	1	Perineum	Ljubljana Medical Center	SLO
OL13	1	Oropharynx	Ljubljana Medical Center	SLO
OL16	2	Oropharynx	Ljubljana Medical Center	SLO
PL20	1	Perineum	Ljubljana Medical Center	SLO
OR41	2	Oropharynx	Hopital Raymond Poincare, Paris	FRA
PR45	2	Perineum	Hopital Raymond Poincare, Paris	FRA
PR59	2	Perineum	Hopital Raymond Poincare, Paris	FRA
OR79	1	Oropharynx	Hopital Raymond Poincare, Paris	FRA
OS87	2	Oropharynx	Hopital St Joseph, Paris	FRA
OS91	2	Oropharynx	Hopital St Joseph, Paris	FRA
PU100	1	Perineum	University of Antwerp	BEL
PU101	1	Perineum	University of Antwerp	BEL
PU102	1	Oropharynx	University of Antwerp	BEL
PU109	2	Perineum	University of Antwerp	BEL
OU110	2	Oropharynx	University of Antwerp	BEL
OU111	1	Oropharynx	University of Antwerp	BEL
PU140	2	Perineum	University of Antwerp	BEL
OU141	2	Oropharynx	University of Antwerp	BEL
OU148	1	Oropharynx	University of Antwerp	BEL
OU152	1	Oropharynx	University of Antwerp	BEL
PU153	2	Perineum	University of Antwerp	BEL
OG156	2	Oropharynx	University Clinic Golnik	SLO
PG157	1	Perineum	University Clinic Golnik	SLO
PC184	1	Perineum	ChariteClinic, Berlin	GER
PC185	1	Perineum	ChariteClinic, Berlin	GER
PC186	1	Perineum	ChariteClinic, Berlin	GER
PC187	1	Perineum	ChariteClinic, Berlin	GER
OC194	1	Oropharynx	ChariteClinic, Berlin	GER
OC195	1	Oropharynx	ChariteClinic, Berlin	GER
OC196	1	Oropharynx	ChariteClinic, Berlin	GER
OC197	1	Oropharynx	ChariteClinic, Berlin	GER
PC226	1	Perineum	ChariteClinic, Berlin	GER
PC227	1	Perineum	ChariteClinic, Berlin	GER
PC228	1	Perineum	ChariteClinic, Berlin	GER
PC229	1	Perineum	ChariteClinic, Berlin	GER
PC230	1	Perineum	ChariteClinic, Berlin	GER
PC231	1	Perineum	ChariteClinic, Berlin	GER
PC232	1	Perineum	ChariteClinic, Berlin	GER
PC237	2	Perineum	ChariteClinic, Berlin	GER
PC238	2	Perineum	ChariteClinic, Berlin	GER
PC239	2	Perineum	ChariteClinic, Berlin	GER
PC240	2	Perineum	ChariteClinic, Berlin	GER
PC245	1	Perineum	ChariteClinic, Berlin	GER

BEL: Belgium, ESP: Spain, FRA: France, GBR: Great Britain, GER: Germany, SLO: Slovenia, USA: United States of America.

TABLE 1. Clinical *S. maltophilia* strains used in the section 4.1 and 4.2 **(**Continuation).

Strain	RpfF Variant	Source	Hospital	Country
PC246	1	Perineum	CharitéClinic, Berlin	GER
PC254	2	Perineum	CharitéClinic, Berlin	GER
PC255	2	Perineum	CharitéClinic, Berlin	GER
PC256	2	Perineum	CharitéClinic, Berlin	GER
PC257	2	Perineum	CharitéClinic, Berlin	GER
PC271	1	Perineum	CharitéClinic, Berlin	GER
PC272	1	Perineum	CharitéClinic, Berlin	GER
PC273	1	Perineum	CharitéClinic, Berlin	GER
PC274	1	Perineum	CharitéClinic, Berlin	GER
PC312	2	Perineum	CharitéClinic, Berlin	GER
PC313	2	Perineum	CharitéClinic, Berlin	GER
PC314	2	Perineum	CharitéClinic, Berlin	GER
PC315	2	Perineum	CharitéClinic, Berlin	GER
OC322	2	Oropharynx	CharitéClinic, Berlin	GER
OC323	2	Oropharynx	CharitéClinic, Berlin	GER
OU343	1	Oropharynx	University of Antwerp	BEL
OU353	1	Oropharynx	University of Antwerp	BEL
ATCC13637	1	Collection	ATCC	USA
UV74	2	Vascular ulcer	Hospital Municipal de Badalona	ESP
L9.5R5	1	(150)	Hospital Clínic de Barcelona	ESP
4834-R	2	(151)	Hospital Clínic de Barcelona	ESP
D457	2	(152)	(152)	ESP
K279	1	Blood infection	(65)	GBR
E77	1	Sputum	Hospital Municipal Badalona	ESP
E50	1	Sputum	Hospital Municipal Badalona	ESP
M30	2	Decubitus ulcer	Hospital Municipal Badalona	ESP
S3149	1	Surgical wound	Hospital Trias i Pujol, Barcelona	ESP
P626	1	Oropharynx	Hospital Trias i Pujol, Barcelona	ESP
P815	1	Oropharynx	Hospital Trias i Pujol, Barcelona	ESP
E5520	2	Environmental control	Hospital Trias i Pujol, Barcelona	ESP
B5565	1	Bronchitis	Hospital Trias i Pujol, Barcelona	ESP
S5720	1	Sputum	Hospital Trias i Pujol, Barcelona	ESP
H5726	1	Hematologic neoplasia	Hospital Trias i Pujol, Barcelona	ESP
S289	2	Sputum	Hospital Trias i Pujol, Barcelona	ESP
B285	1	Bronchitis exudate	Hospital Trias i Pujol, Barcelona	ESP
B5742	1	Bronchitis exudate	Hospital Trias i Pujol, Barcelona	ESP

BEL: Belgium, ESP: Spain, FRA: France, GBR: Great Britain, GER: Germany, SLO: Slovenia, USA: United States of America.

3.1.3 *E. coli*

E. coli DH5α was obtained from our laboratory collection and was used for cloning purpose (Table 2). Bacteria were routinely grown at 37ºC in Luria-Bertani (LB) medium on a rotary shaker. When needed, bacterial strains were grown in LB

supplemented with ampicillin (Ap) 20 µg/mL, Tc 17 µg/mL, Cm 3.2 µg/mL, Gm 40 µg/mL and Erm 500 µg/mL. In order to avoid unnecessary information and tables, all *E. coli* DH5α cloning-derived strains have been excluded from this manuscript.

E. coli strain OP50 (Table 2) was provided by the "Caenorhabditis Genetic Center" (CGC) (University of Minnesota, USA) and used as food source for the maintenance of *Caenorhabditis elegans*. *E. coli* strain OP50 was routinely grown at 37ºC in Luria-Bertani (LB) medium on a rotary shaker.

TABLE 2. *E. coli* parental strains used in this study.

Strains	Relevant characteristics	Reference
DH5α	*fhuA2 lac(del)U169 phoA glnV44Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Lab. collection
OP50	Non-pathogenic strain, *C. elegans* maintenance	CGC

CGC: Caenorhabditis Genetics Center (*www.cbs.umn.edu/research/resources/cgc*).

3.1.4 Bacterial preservation

S. maltophilia, *Xcc* and *E. coli* bacterial strains were routinely conserved by cryopreservation. 1.5 mL from an overnight bacterial culture was mixed with 0.75 mL of 50% sterile glycerol (15% v/v final glycerol concentration), frozen in dry ice and then stored at -80ºC. For bacterial recovery, frozen culture surfaces were scrapped and seeded onto their respective medium plates.

P. aeruginosa strains were routinely preserved at DMSO 15% and then stored at -80ºC. For a short-term storage, *P. aeruginosa* strains were preserved in water. One single bacterial colony was suspended in 1.5 mL of sterile distilled water and stored at 4ºC. For bacterial recovery, 100 µL of the samples were seeded onto their respective medium plates.

3.2 Oligonucleotides and plasmid vectors

Primers for PCR experiments were designed according the following standard. The length of each primer was ranged from 18-25 nucleotides with a >50% of guanine/cytosine (GC) content greater than 50% in order to result in a melting temperature (Tm) from 50-65 ˚C. The software Oligos 7.0 was used to check and avoid the secondary structure, hairpin, primer dimmer and cross dimmer caused by designed primers. Common primers used in this study are listed in Table 3. All primer were synthesized and purchased at Sigma-Aldrich.

TABLE 3. Common primers used in this study.

Primer	Sequence 5'-3'	Restriction Site
Erm resistance gene		
P5Erm	GGATCCGAAACGTAAAAGAAGTTATG	*Bam*HI
P3Erm	GGATCCTACAAATTCCCCGTAGGC	*Bam*HI
P5Ermrev	GATACTGCACTATCAACACAC	-
P3Ermrev	CTTCCAAGGAGCTAAAGAGGT	-
Universal primers		
M13F	GTTTTCCCAGTCACGAC	-
M13R	CAGGAAACAGCTATGAC	-

Erm: Erythromycin.

Basic plasmid vectors used in this study are listed in Table 4 and include pGEM-T Easy Vector (Promega), pEX18Tc (153) pBBR1MCS plasmids (154) and pBAD18-Cm (155). From these, the non-replicative suicide vector pEX18Tc was used for mutant generation in *S. maltophilia* the broad-host-range plasmids pBBR1MCS were used for *in-trans* complementation of *S. maltophil*ia and *P. aeruginosa* mutants, and the arabinose inducible pBAD18-Cm was used for the overexpression of the putative *S. maltophilia cis*-DA synthases Smlt0266 and Smlt0267.

TABLE 4. Common basic plasmids used in this study.

Plasmids	Relevant Characteristics	Reference
pGEM-T Easy Vector	Cloning vector, Ap^r	Promega
pEX18Tc	Suicide allelic exchange vector; Tc^r	(153)
pBBR1MCS1Cm	Broad-host-range cloning vector, Cm^r	(154)
pBBR1MCS5Gm	Broad-host-range cloning vector, Gm^r	(154)
pBAD18-Cm	Arabinose-inducible plasmid Cm^r.	(155)

3.3 Chemical reagents

Synthetic fatty acids (FA) used in this study were all requested to Sigma-Aldrich and include DSF (11-*cis*-2-methyldodecenoic), 15:0 iso (13-methyltetradecanoic), 12:0 (lauric), 14:0 (myristic), 16:0 (palmitic) and 18:0 (stearic). X-Glu (5-Bromo-4-chloro-3-indolyl ß-D-glucuronide sodium salt) (Sigma-Aldrich) is the substrate used for the biosensor strain *Xcc* 8523 pL6engGUS to report DSF activity. Ethyl acetate (Panreac) was used for fatty acids (FA) extraction (92) and Thin-Layer Chromatography (TLC)

experiments, acetonitrile (Sigma-Aldrich) was used for FA separation using HPLC technique and hexane (Sigma-Aldrich) was also used in TLC experiments.

The antimicrobials tetracycline, minocycline, gentamicin, kanamycin, trimethoprim were purchased to Apollo Scientific Ltd. Sulfamethoxazole, norfloxacin, ciprofloxacin, erythromycin and levofloxacin were bought at Sigma-Aldrich and chloramphenicol was purchased to Roche Diagnostics.

The anaesthesia MS-222 (Sigma-Aldrich) was used in zebrafish experiments.

Crystal violet (Sigma-Aldrich) was used for biofilm staining; Congo Red (Sigma-Aldrich) was used for the evaluation of exopolysaccharide production and colony morphology and Coomassie Brilliant Blue (Sigma-Aldrich) was used to visualize protein spots in SDS-PAGE separation.

3.4 Molecular biology techniques

3.4.1 General guidelines

Molecular techniques have been performed following the standard guidelines according to Sambrook *et al.,* (1989) (156). Restriction enzymes, T4 DNA ligase and FastStart DNA Polymerase have been purchased to Roche. In all cases, protocols have been followed according to the manufacturer's instructions. Specific molecular techniques and protocols are detailed below, in its respective section.

3.4.2 Polymerase Chain Reaction (PCR) and quantitative reverse transcription-PCR (qRT-PCR).

For routinely PCR amplification, FastStart DNA Polymerase (Roche) has been used, following the manufacturer's instructions.

Primers for qRT-PCR were designed to have a length near 20 nt and a Tm circa 60˚C and were predicted to produce no secondary structure, hairpin, primer dimmer and cross dimmer, to amplify a desired 50-150 bp in size amplicon (Table 5). Total RNA was isolated from *S. maltophilia* cultures grown in the same conditions as for DSF extraction using GeneJet RNA Purification Kit (Thermo Scientific) and DNA was eliminated using TURBO DNase (Ambion, Life Technologies). 1 µg of RNA was used to synthetize cDNA using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real time PCR was performed using CFX96 real-time PCR system (Bio-Rad) and PCR amplification was detected using SsoAdvanced SYBR Green Supermix (Bio-Rad). PCR products between 80 and 110 bp were amplificated for *rpfC*, *rpfF* and *gyrA*, the latter

used as endogenous gene to normalize gene expression (157), using primers listed in Table 5. Differences in the relative amounts of mRNA for the four genes *rpfF-1, rpfC-1, rpfF-2* and *rpfC-2* were determined using the $2^{-\Delta\Delta Ct}$ method (158). RNA samples were extracted in three different experiments and results are given as mean values.

TABLE 5. Primers used in qRT-PCR analysis.

Primer	Sequence 5'-3'	Tm (ºC)
P1rpfC-RT	GTTCCGCACGCCGTTGAA	59
P2rpfC-RT	CGAGGCCTGGATGGTGTT	57
P1rpfF-RT	CTGAGCTGCCACACCATC	56
P2rpfF-RT	GAACAGCACCTCCGGCAG	58
P1gyrA-RT	GTCGACGGCCAGGGTAAC	58
P2gyrA-RT	GCCTCGGTGTATCGCATT	55

3.5 DNA Sequencing

All PCR amplifications and plasmid constructs have been routinely verified by sequencing (Macrogen Inc., Korea).

3.5.1 *rpfF* sequence determination and analysis

PCR products of 682 to 721 bp, containing the *rpfF* promoter and the region encoding the N-terminal fragment, were amplified from all 78 *S. maltophilia* strains using the primers PrpfFtypeUp and PrpfFTypeDw (Table 6) and directly sequenced (Macrogen Inc). Translation of partial ORFs to amino acids and sequence alignments were done using MEGA V5.2 (159) and BioEdit, respectively. A phylogenetic tree using neighbour-joining method (default parameters) was constructed with MEGA V5.2 based on a trimmed alignment to the 108 N-terminal residues of the RpfF from strain K279a. RpfC-variant determination was based on the RpfC sequences from the publicly available sequenced genomes (strains K279a, R551-3, D457 and JV3, with GenBank accession number AM743169.1, CP001111-1, HE798556.1 and CP002986.1, respectively) and our draft genome sequences (strains E77, M30 and UV74), using SMART software (160) for the identification and annotation of protein domains. The presence of two *rpfC* variants among the collection of *S. maltophilia* strains was verified by PCR, without sequencing, using primers PrpfCTypeUp and PrpfCTypeDw (Table 6). Lengths of the amplicons were used to discriminate *rpfC-1* (ca. 1015 bp) from *rpfC-2* (ca. 450 bp) variant strains.

All *rpfF*-amplified sequences from this *S. maltophilia* strain collection were deposited in GenBank under accession numbers KJ149475-KJ149552.

TABLE 6. Primers used in the determination of *S. maltophilia rpfF* and *rpfC* variants.

Primer	Sequence 5'-3'	Tm (ºC)
PrpfFtypeUp	GCAGAAGACCAACGTCGGCAAG	61
PrpfFtypeDw	CTTCCTAGGCGACGATGGTGTG	60
PrpfCTypeUp	TGTTCGGCTGGCTGCTGT	60
PrpfCTypeDw	GGCCAGCACTTCGGTCAT	57

3.5.2 Genome sequencing

Genomes of *S. maltophilia* strains M30, E77 and UV74 were sequenced at "Servei de Genòmica i Bioinformatica" (IBB, UAB, Barcelona, Spain) and a first draft constructed in collaboration with the group of Bioinformatics at IBB. From these, only the genome of the M30 was assembled and deposited in NCBI [http://www.ncbi.nlm.nih.gov/genome], at this time. Genomic DNA was extracted from the strain M30 using GenElute bacterial genomics DNA kit (Sigma-Aldrich), and whole-genome sequencing was performed using Illumina MiSeq technology. The low-quality reads were filtered, and the remaining reads were *de novo* assembled using VelvetOptimiser version 2.2.5 (161) relaying on Velvet version 1.2.10 (162) and improved with the IMAGE program from the PAGIT package version 1 (163). The assembly resulted in 193 contigs (G-C content, 66.3%), with an N50 contig size of 46,399 nucleotides, covering a total of 4,902,008 bp. The average length of the contigs was 25.4 kb and the biggest contig contained 142,025 bp. Genome annotation was performed by the NCBI Prokaryotic Genome Annotation Pipeline version 2.5 (rev. 434060), and 4,515 genes were predicted, of which 4,392 are coding sequences (CDSs), 43 are pseudogenes, 11 are rRNAs (5S, 16S, and 23S), 68 are tRNAs, and 1 is a noncoding RNA (ncRNA).

This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. JELS00000000. The version described here is JELS02000000.

3.6 Mutants generation and complementation

3.6.1 Generation and complementation of *S. maltophilia* Δ*rpfF* and Δ*rpfC* mutants

Primers, plasmids and strains used for the generation of *S. maltophilia* mutants are listed in Tables 7, 8 and 9, respectively. *S. maltophilia* E77 Δ*rpfF* and M30 Δ*rpfF* and Δ*rpfC* mutants were obtained by allelic-exchange recombination using erythromycin as antibiotic-resistance cassette. Briefly, *rpfF* upstream and downstream flanking regions were amplified by PCR (Table 7) and inserted, flanking the erythromycin cassette into pEX18Tc generating plasmids pEXE77rpfF and pEXM30rpfF for E77 and M30, respectively (Table 8). The erythromycin cassette was previously amplified from plasmid pGEM-Erm from our vector collection. Both strains were electroporated (164) with their respective suicide vector and transformants were selected in LB plates containing 500 μg/mL Erm and subsequently streaked onto LB plates containing 17 μg/mL Tc to discard single cross-over events. *rpfF* deletion was also verified by PCR using primers combination P1DemRpfFE77-P5Ermrev (for E77 upstream region), P2DemRpfFE77-P3Ermrev (for E77 downstream region), P1DemRpfFM30-P5Ermrev (for M30 upstream region) and P2DemRpfFM30-P3Ermrev (for M30 downstream region) (Table 7). The obtained fragments were subsequently sequenced.

To generate Δ*rpfC* mutant in the strain M30, the same strategy was followed. The upstream and downstream regions of *rpfC* from M30 were amplified using primers pairs P1M30rpfCUp-P2M30rpfCUp and P3M30rpfCDw-P4M30rpfCDw (Table 7), respectively. Both fragments were inserted, flanking the erythromycin cassette, into the pEX18Tc, generating pEXM30rpfC (Table 8). It was electroporated into *S. maltophilia* strain M30 and the mutant candidates were screened and verified as described above, with primer pairs P1DemRpfCM30-P5Ermrev (for upstream region) and P2DemRpfCM30-P3Ermrev (Table 7).

A fragment of ca. 1100 bp containing either the E77 or the M30 *rpfF* ORF and their predicted promoter was amplified by PCR using primers combination ProrpfFE77FUp-ProrpfFE77FDw (for E77) and ProrpfFM30FUp-ProrpfFM30FDw (for M30) (Table 7), ligated to pBBR1MCS1-Cm generating prpfFE77 (for E77) and prpfFM30 (for M30) (Table 8) and introduced into E77 and/or M30 for either homologous or heterologous *trans*-complementation of Δ*rpfF* (Table 9).

TABLE 7. Primers used in the generation and complementation of *S. maltophilia* Δ*rpfF* and Δ*rpfC* mutants.

Primer	Sequence 5'-3'	Restriction Site
E77		
P1E77rpfFUp	GGATCCAGTTCTCCGTGTGACCGTCC	*Hin*dIII
P2E77rpfFUp	AAGCTTCGAGGACGTCATCGCGATGAT	*Bam*HI
P3E77rpfFDw	GAATTCTGGGCATGGGCGATGGCTTC	*Bam*HI
P4E77rpfFDw	GGATCCTCGTCTGGCGGGTCAGGACG	*Eco*RI
P1DemRpfFE77	TCGACCGCAAGAAGGACATGA	-
P2DemRpfFE77	TCGGCGATGGCGTGCTCGATA	-
ProrpfFE77Up	AAGCTTCGTGAAGGTCGTGATCGTGAAGAA	*Hin*dIII
ProrpfFE77Dw	TCTAGAACGATCAGGCCGGGTCGCCAT	*Xba*I
P1RpfFE77Int	TGGTCGACATTCGTTGATACAC	-
P2RpfFE77Int	GCACGTCCTGTCACCGCAT	-
ProrpfGCE77Up	ATCAAGCTTGTGCTGCTGTTCCCGTACAT	*Hin*dIII
ProrpfGCE77Dw	CGTCTAGAGAGGTCAACGAGATCGCCAC	*Xba*I
M30		
P1M30rpfFUp	GCGGATCCAACGGTCCACACACGCGG	*Xba*I
P2M30rpfFUp	CATCTAGATGGTGTCCGGCTTCAACG	*Bam*HI
P3M30rpfFDw	CTGAATTCACTGGCTTCGCTGGGCAT	*Bam*HI
P4M30rpfFDw	CCGGATCCCAGCGCGCTTCACCATCA	*Eco*RI
P1DemRpfFM30	ATCCAGCGCTGTACTCAG	-
P2DemRpfFM30	CAGGTGATGAAGGGCTACT	-
ProrpfFM30Up	GCCAAGCTTGGGTTTGCGGCAATCTGGACAG	*Hin*dIII
ProrpfFM30Dw	GGTCTAGACGATGATGGTGAAGCGCGCTGA	*Xba*I
P1RpfFM30Int	GCCATGCGTCTGACAT	-
P2RpfFM30Int	AGACAATACTTGCTCAT	-
P1M30rpfCUp	AGGAATTCCCAGAATCGCTTCATCCAGGT	*Eco*RI
P2M30rpfCUp	GCGGATCCAGCTGCGACAACAGGCGTTTC	*Bam*HI
P3M30rpfCDw	ACGGATCCTGATGGTGAAGCGCGCTGA	*Bam*HI
P4M30rpfCDw	GCTCTAGACCCCATCTGGCCGAGAAGA	*Xba*I
P1DemRpfCM30	GAGGAAGAGCTGGCGATCAT	-
P2DemRpfCM30	GCGACCTCCTGCTGAACTAC	-
P1rpfCM30int	ATCGGTCTGATCCTGTTGCCA	-
P2rpfCM30int	TTCCAGATAGCCGATGTCACC	-
ProrpfGCM30Up	GTCAAGCTTGATCCGCGATGTACTGCTGT	*Hin*dIII
ProrpfGCM30Dw	TCGTCTAGAAGTCTCGACGCGGCCTGATT	*Xba*I

On the other hand, fragments of ca. 3000 bp containing the *rpfG* and *rpfC* genes with their predicted promoter were amplified from M30 and E77, using the primers pairs P1rpfGCM30-P2rpfGCM30 and P1rpfGCE77-P2rpfGCE77 (Table 7) to generate complementation vectors prpfGCM30 and prpfGCE77 (Table 8), respectively. Both fragments were digested with their respective restriction enzymes and ligated into pBBR1MCS1-Cm. Finally, prpfGCM30 and prpfGCE77 were introduced into E77,

M30 and M30 Δ *rpfC* mutant for either homologous or heterologous *trans*-complementation (Table 9).

TABLE 8. Plasmids used in the generation and complementation of *S. maltophilia* Δ*rpfF* and Δ*rpfC* mutants.

Plasmids	Relevant Characteristics	Reference
pGEM-Erm	pGEM-T carrying *Erm* resistance gene Erm^r, Tc^r	Lab. collection
pEXE77rpfF	pEX18Tc suicide vector, carrying E77 *rpfF* flanking regions interrupted with *Erm* gene, Tc^r, Erm^r	This work
prpfFE77	Complementation vector, pBBR1MCS1Cm carrying *rpfF* gene with its promoter from E77 strain, Cm^r	This work
prpfGCE77	Complementation vector, pBBR1MCS1Cm carrying *rpfGC* operon with its promoter from E77 strain, Cm^r	This work
pEXM30rpfF	pEX18Tc suicide vector, carrying M30 *rpfF* flanking regions interrupted with *Erm* gene, Tc^r, Erm^r	This work
prpfFM30	Complementation vector, pBBR1MCS1Cm carrying *rpfF* gene with its promoter from M30 strain, Cm^r	This work
pEXM30rpfC	pEX18Tc suicide vector, carrying M30 *rpfC* flanking regions interrupted with *Erm* gene, Tc^r, Erm^r	This work
prpfGCM30	Complementation vector, pBBR1MCS1Cm carrying *rpfGC* operon with its promoter from M30 strain, Cm^r	This work

TABLE 9. *S. maltophilia* Δ*rpfF* and Δ*rpfC* mutants and complemented strains.

Strains	Relevant characteristics	Reference
E77 Δ*rpfF*	E77 Δ*rpfF* mutant, Erm^r	This work
E77 prpfFE77	E77 wild type harbouring prpfFE77, Cm^r	This work
E77 pBBR1MCS1	E77 wild type harbouring pBBR1MCS1 Cm^r	This work
E77 Δ*rpfF* pBBR1MCS1	E77 Δ*rpfF* harbouring pBBR1MCS1 Erm^r Cm^r	This work
E77 Δ*rpfF* prpfFE77	E77 Δ*rpfF* harbouring prpfFE77 Erm^r Cm^r	This work
E77 Δ*rpfF* prpfFM30	E77 Δ*rpfF* harbouring prpfFM30 Erm^r Cm^r	This work
E77 prpfGCE77	E77 wild type harbouring prpfGCE77, Cm^r	This work
E77prpfGCM30	E77 wild type harbouring prpfGCM30, Cm^r	This work
M30 Δ*rpfF*	M30 Δ*rpfF* mutant, Erm^r	This work
M30 prpfFM30	M30 wild type harboring prpfFM30, Cm^r	This work
M30 pBBR1MCS1	M30 wild type harbouring pBBR1MCS1 Cm^r	This work
M30 Δ*rpfF* pBBR1MCS1	M30 Δ*rpfF* harbouring pBBR1MCS1 Erm^r Cm^r	This work
M30 Δ*rpfF* prpfM30	M30 Δ*rpfF* harbouring prpfFM30 Erm^r Cm^r	This work
M30 Δ*rpfF* prpfFE77	M30 Δ*rpfF* harbouring prpfFE77 Erm^r Cm^r	This work
M30 Δ*rpfC*	M30 Δ*rpfC* mutant, Erm^r	This work
M30 Δ*rpfC* prpGCM30	M30 Δ*rpfC* harbouring prpfGCM30 Erm^r Cm^r	This work
M30 Δ*rpfC* prpGCE77	M30 Δ*rpfC* harbouring prpfGCE77 Erm^r Cm^r	This work

3.6.2 Generation and complementation of *S. maltophilia* M30 Δ*smlt0266*, Δ*smlt0267* and Δ*smlt0266-0267* mutants

Primers and plasmids used for the cloning process are listed in Tables 10 and 11, respectively. Strains and mutants object of this study are listed in Table 12.

S. maltophilia Δ*smlt0266* and Δ*smlt0266-0267* mutants were obtained by allelic-exchange recombination using erythromycin as antibiotic-resistance cassette. *S. maltophilia* M30 Δ*sml0267* mutant was obtained by interrupting *smlt0267* gene with the whole suicide vector pEX0267 containing an intragenic region of *smlt0267* for homologous recombination.

Briefly, *smlt0266* and s*mlt0266-0267* upstream and downstream flanking regions were amplified by PCR using primer pairs P1BE0266-P2BE0266 and P3BD0266-P4BD0266 (for *smlt0266*) and P1BE0266-P2BE0266 and P3BD0267-P4BD0267 (for *smlt0267*) (Table 10) and inserted, flanking the erythromycin cassette, into the pEX18Tc vector, thus generating plasmids pEX0266 and pEX0266-0267 (Table 11). M30 was electroporated with these suicide vectors and transformants were selected in LB plates containing 500 μg/mL Erm and subsequently streaked onto LB plates containing 17 μg/mL Tc to discard single cross-over events. Δ*smlt0266* deleted mutant was also verified by PCR using primers pairs P1Dem0266-P3Ermrev (for upstream region) and P2Dem0266-P5Ermrev (for downstream region) (Table 10) and the amplified fragments were subsequently sequenced. The double mutant Δ*smlt0266-0267* was also verified by PCR using primers P1Dem02660267-P3Ermrev (for upstream region) and P2Dem02660267-P5Ermrev (for downstream region) and the amplified fragments were subsequently sequenced. To generate interrupted *smlt0267* mutant in the M30 strain, an intragenic fragment of ca. 500 bp, was amplified using primers P10267int-P20267int (Table 10) and cloned into pEX18Cm, generating pEX0267 (Table 11). M30 strain was electroporated and plated onto LB containing 3.2 μg/mL chloramphenicol. The mutant candidates were screened and verified by PCR and sequencing, using primers P1Dem0267-M13Fw (for upstream region) and P2Dem0267-M13Rv (for downstream region) (Table 10). In *S. maltophilia,* the genomic organization showed the same organization as *dsp* operon. However, in order to avoid interferences with the other components of the operon, *smlt0264, smlt0265 and smlt268* were excluded from the complementation vector. Then, a fragment of ca. 2000 bp corresponding to *smlt0266* (*dspI* homologue) and *smlt0267* (*dspII* homologue) was amplified and cloned into the non-replicative vector pBAD18-Cm, generating pBAD0266-0267 (Table 11). Then, a

fragment containing both genes plus the arabinose promoter was excised and subcloned into the replicative vector pBBR1MCS1-Cm, generating pBADSMdsp (Table 11). pBADSMdsp construction was confirmed by restriction and PCR using primers M13F and M13R and electroporated into the M30 wt and the M30 Δ*smlt0266*, Δ*smlt0266-0267* and Δ*sml0267* mutants for *in-trans* complementation.

TABLE 10. Primers used in the generation and complementation of *S. maltophilia* Δ*smlt0266,* Δ*sml0267* and Δ*smlt0266-0267* mutants.

Primer	Sequence 5'-3'	Restriction Site
P1BE0266	GAATTCGCGGTCTGTACATGGA	*Eco*RI
P2BE0266	GGATCCCGCAGTTCCTCTTCG	*Bam*HI
P1BD0266	AAGCTTCTGCAGCAGCGCATCGAACA	*Hin*dIII
P2BD0266	GGATCCATGAGCACCGACACCGTTGC	*Bam*HI
P1BD0267	GGATCCAGCCGCATTGCATTC	*Bam*HI
P2BD0267	AAGCTTCTGGTGATGAGCTGC	*Hin*dIII
P10267int	CTAGAATTCTGCTGCTGAAGCAGCTGAAT	*Eco*RI
P20267int	TCAGAATTCGTAGGTGTGGATGAGATGATC	*Eco*RI
P0266RBS	TCTAGACCCGTCACCTGCT	*Xba*I
P0267END	TCTAGATGCAATGCGGCTCAT	*Xba*I

3.6.3 Arabinose-induction of pBADSMdsp

In order to corroborate that the induction was functional in the hybrid vector, M30 was electroporated with this construct. A 25 mL culture of M30 transformed with pBADSMdsp was grown for an overnight at 37ºC in LB supplemented with 0.2% arabinose and 30 µg/mL Cm. 10 mL were centrifuged and the cell pellet was ressuspended twice in 10 mL of 250 mM sucrose. Finally, 1 mL aliquot was boiled for 10 minutes and total proteins were separated by SDS-PAGE and the gel was stained with coomasie brilliant blue.

3.6.4 Complementation of *P. aeruginosa* Δ*dspI* and Δ*dspII* mutants

P. aeruginosa Δ*dspI* (Δ*PA0745*, with strain name PW2354) and Δ*dspII* (Δ*PA0744*, with strain name PW2353) were obtained from the PAO1 transposon mutant library at the University of Washington (149).

In *P. aeruginosa, dspI* and *dspII* are expected to be co-transcribed in the *dsp* operon (*PA0747, PA0746, PA0745, PA0744, PA0743*). Consequently, a fragment of ca. 6750 bp containing the genes *PA0747, PA0746, PA0745, PA0744* and its predicted promoter was amplified by PCR using primers P1PAdsp (5'-CTGAAGCTTCGGCAGCATGCTGATGACCA-3') and P2PAdsp (5'-

CACTAGTACACTCCAGCCAGCCGCGTC-3') and cloned into the replicative vector pBBR1MCS1-Cm generating pPAdsp (Table 11), which was used for the *in-trans* complementation of *P. aeruginosa* Δ*dspI* and Δ*dspII* mutants (Table 12).

TABLE 11. Plasmids used in the generation and complementation of *S. maltophilia* Δ*smlt0266*, Δ*sml0267* and Δ *smlt0266-0267* and *P. aeruginosa* Δ*dspI* and Δ *dspII* mutants.

Plasmid	Relevant Characteristics	Reference
pEX0267	pEX18Cm carrying an internal fragment of *smlt0267* gene, Cm^r.	This work
pEX0266	pEX18Tc carrying the upstream and downstream regions of *smlt0266* interrupted by *Erm* gene,, Erm^r.	This work
pEX0266-0267	pEX18Tc carrying the upstream and downstream regions of *smlt0266* and *smlt0267* interrupted by *Erm* gene, Erm^r.	This work
pBAD18-Cm	Suicide vector in *S. maltophilia* and *P. aeruginosa* containing the MCS under the control of the arabinose promoter, Cm^r.	(155)
pBAD0266-0267	pBAD18-Cm carrying *smlt0266-0267* ORFs Cm^r.	This work
pBADSMdsp	Hybrid plasmid composed of the replication origin of pBBR1MCS1 and the genes *smlt0266* and *smlt0267* under the arabinose promoter control from pBAD18. Cm^r.	This work
pPAdsp	pBBR5MCS-Gm carrying the genes *PA0744, PA0745, PA0746* and *PA0747,* Gm^r.	This work

TABLE 12. Strains used in section 4.3.

Strains	Relevant characteristics	Reference
S. maltophilia		
M30	wild type	This work
M30 Δ*0266*	M30 Δ*smlt0266* mutant, Erm^r	This work
M30 Δ*0267*	M30 Δ*sml0267* mutant, Cm^r	This work
M30 Δ*0266-0267*	M30 Δ*smlt0266-0267* mutant, Erm^r	This work
M30 pBADSMdsp	M30 wild type harbouring pBADSMdsp Cm^r	This work
P. aeruginosa		
MPAO1	wild type	(149)
MPAO1 Δ*dspI*	MPAO1 Δ*PA0745* mutant, Kan^r	(149)
MPAO1 Δ*dspII*	MPAO1 Δ*PA0744* mutant, Kan^r	(149)
MPAO1 Δ*dspI* pPAdsp	MPAO1 Δ*PA0745* mutant harbouring pPAdsp, Gm^r, Kan^r	This work
MPAO1 Δ*dspII* pPAdsp	MPAO1 Δ*PA0744* mutant harbouring pPAdsp, Gm^r, Kan^r	This work

3.7 General analytical tools

3.7.1 SDS-PAGE

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as previously described (165) with modification (166) using a 12% polyacrylamide gel in a vertical slab gel apparatus (Bio-Rad).

3.7.2 Protein Identification by Mass Spectrometry

Protein bands of interest were excised from the gel using a cut tip. In-gel trypsin digestion was performed as described previously (167). MALDI-MS analysis of tryptic peptides was performed on an Ultraflex time-of-flight instrument (Bruker Daltonics). Samples were prepared using α -cyano-4-hydroxy-cinnamic acid. Calibration was performed in the external mode using a peptide calibration standard kit (Bruker Daltonics). The spectra were processed using Flex Analysis 2.2 software (Bruker Daltonics). Peak lists were generated using the signals in the 800–4000 mass:charge ratio (m/z) range, with a signal:noise threshold >3. The SNAP algorithm included in the software was used to select the monoisotopic peaks from the isotopic distributions observed. After removing m/z values corresponding to commonly observed matrix cluster ions, an internal statistical calibration was applied. Peaks corresponding to frequently seen keratin and trypsin autolysis peptides were then removed. The resulting final peak was used for the identification of the protein by peptide mass fingerprint. The Mascot 2.0 program (Matrix Science) was used to search the NCBI non-redundant database (http://ncbi.nlm.nih.gov), with no limitation on taxonomy. Search parameters were as follows: trypsin cleavages excluding N-terminal to P, one missed cleavage permission, carbamidomethylation and methionine oxidation as variable modification, mass tolerance <50 ppm, monoisotopic mass values.

3.7.3 Lipid extraction from culture supernatants

DSF extraction from culture supernatants was carried out following the ethyl acetate method (92). Briefly, overnight bacterial cultures grown on indicated media were harvested by centrifugation and the supernatant was extracted with the same volume of ethyl acetate. The organic phase was evaporated to dryness using a rotary evaporator and the residues were dissolved in 3 mL of methanol (for supernatant DSF bioassay and TLC analysis) or dichloromethane (for GC/MS analysis).

3.7.4 Lipid separation by Thin-Layer Chromatography (TLC)

For supernatant fatty acid separation using TLC technique, 5 µL aliquots of the lipid extractions dissolved in methanol were spotted onto a silica gel 60 TLC plate (20x20 cm, Merck) and separated with ethyl acetate:hexane (20:80, vol/vol) as running solvents.

3.7.5 Lipid identification from culture supernatants by GC/MS

This experiment was mainly performed at "Servei d'Ànalisi Química (SAQ)" in the "Universitat Autònoma de Barcelona (UAB)". Bacterial cultures were grown on 2 L of LB for 48 h at 30 ºC with vigorous shaking (250 rpm). Cultures were centrifuged and supernatants were extracted with the ethyl acetate method (92). Dry residues were dissolved in 3 mL of dichloromethane. Total fatty acids were methylated –generating Fatty Acids Methyl Esters (FAME)- and separated by Gas Chromatography (6890, Agilent Technologies) using an Agilent 19091S-433 column coupled to a Mass Spectrometry detector (Hewlett-Packard 5973).

3.7.6 Purification of fatty acids by High-Liquid Pressure Chromatography (HPLC)

Dry residues of concentrated supernatants were dissolved in 100 µL of 30% Methanol, and injected into the HPLC (Waters Alliance) and separated using a se C18 reverse-pha column (100 Å, pore size) (Phenomena) developed with a Methanol gradient 20-99% in 30 minutes at a flow rate of 1 ml/min and monitored at 210 nm. Fractions of interest were collected and used for further analysis. After each run, column was cleaned with 100% acetonitrile at the same flow rate to avoid cross contamination and interferences.

3.7.7 Extraction and analysis of total cellular fatty acids

Analysis of total cellular FA was carried out by the "Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures" (Braunschweig, Germany), as follows. Fatty acid 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 (1982) (168) and Kuykendall *et al*. (1988) (169). The fatty acid methyl esters mixtures are separated using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.) which consisted of an Agilent model 6890N gas chromatograph fitted with a 5% phenyl-methyl silicone capillary column (0.2 mm x 25 m), a flame ionization detector, Agilent model 7683A automatic sampler, and a HP-computer with MIDI data base (Hewlett-Packard Co., Palo Alto,

California, U.S.A.). Peaks are automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID). The gas chromatographic parameters are as follows: carrier gas, ultra-high-purity hydrogen; column head pressure 60kPa; 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.

3.8 DSF extraction, detection and quantification.

3.8.1 Colony and supernatant DSF bioassay

DSF determination was performed using strain *Xcc* 8523 pL6engGUS (DSF-reporter strain) as previously described (105), with few modifications. Briefly, the DSF-reporter strain was grown in 10 mLof 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 containing 1% of BD Difco Agar Noble (NYGA) and supplemented with 80 µg/mL X-Glu and then plated into petri plates upon solidification.

For colony-based DSF bioassay, candidate strains were pin inoculated onto NYGA plates containing 80 µg/mL X-Glu and the the DSF-reporter strain and incubated for 24 h at 28ºC. Presence of a blue halo around the colony indicates DSF activity.

For supernatant-based DSF bioassay, bacterial cultures were grown in 250 mL of LB for 48 h at 30ºC (OD600 nm about 4). Supernatants were extracted with the ethyl acetate method and residues were dissolved in 200 µL of methanol. 3 µL of each sample were deposited into hand-generated wells in 5.5-cm plates containing NYGA supplemented with 80 µg/mL of X-Glu and seeded with the DSF-reporter strain previously prepared to an estimated final optical density at 0.07 at 600 nm. Plates were incubated for 24 h at 30ºC. DSF activity was determined by the presence of a blue halo around the well.

3.8.2 TLC coupled to DSF bioassay

TLC analysis was used to separate DSF from total fatty acids present in the supernatant (See section 3.7.4). After separation, TLC plates were air-dried for at least 1 h and overlaid with 100 mL of cooled unsolidified NYGA containing 80 µg/mL of X-Glu and the DSF-reporter strain at an optical density of 0.07 at 600 nm. TLC plates were

incubated overnight at 28°C and DSF activity was identified by the presence of blue spots.

3.8.3 Liquid DSF bioassay

200 µL of DSF reporter solution consisting of *Xcc* 8523 pL6engGUS strain culture 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 in a sterile 96 microtitter well plate. Synthetic DSF was added to wells with an increasing concentration (from 0.05 to 1.5 mM) and incubated at 28ºC for 24 h. The presence of DSF molecules turned wells to blue with intensity proportional to the concentration of DSF molecules. The absorbance was read at 620 nm and a calibration curve was generated. Samples residues dissolved in methanol were then tested with increasing concentrations into wells and the lecture was done as described above. The quantification was measured using the formula generated by the calibration curve.

3.8.4 DSF detection and quantification from infected animal tissues

Two fishes from each tank (E77, M30, E77:M30 and Δ *rpfF*-1:Δ*rpfF*-2 plus two uninfected fishes) were randomly chosen and sacrificed by anaesthesia (MS-222, 220 ppm) after 48 hpi. Since in the mixed wild type inoculum all fishes died within the first 48 h, it was the criterion for evaluating DSF production from each culture in *in-vivo* infection. Sacrificed fishes were introduced into a falcon tube containing 15 mL of PBS and homogenized using a Politron Homogenizer (MARK). The homogenized solution was extracted 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 liquid and solid supernatant DSF bioassay.

3.9 Phenotypic analysis of mutant, complemented and wild type strains.

3.9.1 Biofilm formation

To analyze biofilm formation on polystyrene surface, 200 µL from an overnight bacterial cultures adjusted to an optical density of 0.1 at 600 nm in modified M9-salts or BM2 medium were inoculated into 96-well microtiter non-treated plates (BrandTech 781662) and incubated statically for 24 h at 30 ºC. Plates were then washed three times with water, fixed at 60 °C for 1 h and stained during 15 min with 200 µL of 0.1%

crystal violet. The dye was discarded and the plates were rinsed in standing water and allowed to dry for 30 min at 37°C. Crystal violet from attached bacteria was dissolved in 250 µL 95% ethanol for 15 min, and the OD of the extracted dye was measured at 550 nm.

Biofilm formation on glass surface was assayed by inoculating 2 mL from the same adjusted cultures as described above into glass tubes and incubated for 24 h at 30ºC with agitation (250 rpm). Biofilm formation was measured by crystal-violet method by staining the tubes with 2.5 mL of 0.1% crystal violet.

3.9.2 Swimming motility

The swimming motility was determined in TrA plates (1% tryptone, 0.5% NaCl, 0.25% agar) (40). Thus, 5 µL of adjusted overnight cultures were spotted on TrA plates and incubated at 30ºC for 24 h. Quantification was done by measuring the diameter of the colony.

3.9.3 Twitching motility

Twitching motility was assessed via subagar stab inoculations (stab assay) from overnight fresh plates as previously described (170). Plates were incubated at 30ºC for 24 h and the agar medium was extracted from the plate. Then, twitching zones were visualized by staining with 1% (wt/vol) crystal violet. The quantification was done by measuring the diameter of the blue halos.

3.9.4 Swarming motility

Swarm agar was made based on modified M9-salts medium without NH_4Cl, with 0.5% casamino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ supplemented with 0.4% glucose and solidified with 0.5% BD Difco Agar Noble (147). Plates containing 20 mL of fresh swarm medium were dried under laminar flow for 20 minutes before inoculation. The inoculation was performed using a sterile digralski spatula containing biomass from a fresh LB plate and softly deposited on the top of a semi-solid modified M9-salts medium plate. Inoculated swarm plates were sealed to keep the humidity and incubated at 28 ºC for 3-5 days.

3.9.5 Surfactant imaging

Strains of interest were seeded on modified M9-salts medium plates solidified with 0.7% Noble Agar (Difco) (to avoid swarming motility) and incubated at 28ºC for 5 days. Wetting agent images from isolated colonies were visualized using stereoscopic

microscope Olympus SZX16. Plates were illuminated using diffuse reflected light, and angled to capture the refractive quality of the layer.

3.9.6 TEM microscopy imaging

Samples for transmission electron microscopy (TEM) visualization were prepared as follows. Cells were picked from the edge of the tendril (for swarmer strains) and from the edge of the non-swarming colonies using a Kölle handle and ressuspended in 25 µL of distilled water. 5 µL of each sample were deposited on copper grids and contrasted with conventional 0.2% uranyl acetate solution. Observation was done using a Jeol 1400 (Jeol LTD, Tokyo, Japan) transmission electron microscope equipped with a CCD GATAN ES1000W Erlangshen camera.

3.9.7 Determination of antibiotic susceptibility

The susceptibility of *S. maltophilia* wt and mutant strains to the following antimicrobial agents was tested: tetracycline, minocycline, gentamicin, kanamycin, trimethoprim, sulfamethoxazole, norfloxacin, ciprofloxacin, erythromycin, levofloxacin or chloramphenicol. The MICs for selected antibiotics were determined by the resazurin microtitre assay (171) using 96-well plates by serial two-fold dilutions of each drug in 100 µL of LB. 100 µL of a bacterial suspension (final OD550nm = 0,005) was added to the antibiotic dilutions, mixed and incubated at 37°C for 16 h before developing with resazurin (30 µL 0.01%) [44]. The MIC was defined as the lowest drug concentration that prevented bacterial growth. The microdilution assay followed the Clinical and Laboratory Standards Institute (CLSI, www.clsi.org) guidelines for antimicrobial susceptibility testing.

3.9.8 Congo red binding assay

Congo red binding assays were performed as previously described (172). Briefly, overnight bacterial cultures grown in LB were diluted to OD600nm of 0.1 and 5 µL were spotted onto CR plates (10 g/l tryptone broth with 10 g/l agar, 40 µg/mL Congo red, and 20 µg/mL Coomassie brilliant blue). The plates were incubated for 72 h at room temperature to assess colony morphology.

3.9.9 Determination of virulence in the *Caenorhabditis elegans* model

Caenorhabditis elegans CF512 (fer-15(b26) II; fem-1(hc17) IV), a strain showing temperature-dependent sterility, was provided by CGC. Nematodes were routinely maintained in NGM plates (1.7% Agar, 50 mM NaCl, 0.25% peptone, 1 mM $CaCl_2$,

5 μg/mL cholesterol, 25 mM KH_2PO_4 , 1 mM $MgSO_4$) seeded with *E. coli* OP50, at 16 ºC.

Virulence determination of bacterial strains in the *C. elegans* CF512 infection model was based on the "Slow Killing" method (173). Bacterial strains were grown in BHI broth overnight at 30 °C and 100 μL of each strain culture were spread on a 5.5 cm diameter NGM agar plate and incubated at 30°C for 24 h. Each plate was then seeded with 15-20 adult hermaphrodite CF512 worms, incubated at 25°C (sterility conditions) and scored for live worms every 24 h. *E. coli* OP50 was used as a negative control. A worm was considered dead when it no longer responded to touch. Three replicates per strain were performed.

3.9.10 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 three days before infection. Adult zebrafish (n=12 per condition) were infected by intraperitoneal injection (174) with 20 μL of a 5×10^8 cfu/mL suspension of each *S. maltophilia* strain. The strains were previously grown at 28 °C in 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.

One fish from each tank was sacrificed at 72 h postinfection and divided into three sections (anterior, abdominal, and posterior regions) with a sterile surgical blade. All weights were annotated, and every section was homogenized in 3 ml of PBS. After serial dilution, bacteria were plated onto LB medium containing 20 μg/mL Ap (for WT E77), LB containing 500 μL Erm (for the E77 Δ*rpfF* mutant), or LB supplemented with Cm (for the complemented E77 Δ*rpfF* mutant). Finally, CFU were counted and divided per gram of tissue. All of the isolates obtained post-mortem from infected zebrafish were identified as *S. maltophilia* on the basis of cell and colony morphology, the analytical profile index, and the 16S rRNA gene sequence (data not shown).

3.10 Bioinformatic Tools

General bioinformatics tools used in this study include protein and nucleotide BLAST (175), ClustalW (176), Oligos 7.0 (177), CodonCode , Serial Cloner 5.2, MEGA 5.0

(159), Artemis and SMART (160). Software was run with default parameters unless otherwise stated.

3.11 Statistical Analysis

Statistical analyses were performed using the GraphPad Prism program version 5.00. Comparison of strain phenotypic data was performed by one-way analysis of variance (ANOVA) with a Bonferroni's multiple comparison post-test or unpaired t-test with Welch correction for unequal variances, as indicated in figure and table captions. Survival curves of zebrafish infection experiments were analyzed using the Kaplan-Meier method. Differences were evaluated using the log-rank test.

3.12 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 AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Molecular Basis of DSF-QS System in *S. maltophilia*

It is known that *S. maltophilia* presents the DSF-QS system like other members of the *Xanthomonadales* family (65, 127). Lot of literature is published regarding DSF-QS in *Xanthomonas* (91, 92, 105, 107–110), *Xylella* (98, 99, 117) and *Burkholderia* (101, 123–125) species, however, in the case of *S. maltophilia*, little is known about the mechanisms by which *S. maltophilia* synthesize and sense DSF molecules. In this chapter, we have focused on the molecular aspects of DSF-QS in *S. maltophilia*, more specifically, in the study of the molecular basis involved in DSF production.

4.1.1 Two variants of *rpf* cluster with main differences in *rpfF* and *rpfC* genes are distributed among *S. maltophilia* population.

A preliminary analysis comparing the few publicly available genomes (strains K279a, R551-3, D457, and JV3, with GenBank accession numbers AM743169.1, CP001111.1, HE798556.1, and CP002986.1, respectively) showed that all genomes contain the *rpfF* gene. Nevertheless, two different *rpfF* variants could be distinguished based on its N-terminus (Fig. 1). Taking the two model *S. maltophilia* strains D457 and K279a and comparing it to the well-studied DSF-producer strain *Xcc,* we could observe that two *rpf* clusters could be distinguished based on the *rpfF* but also on the *rpfC* gene (Fig. 1). Accordingly, we have named *rpf-1* (K279a) to the cluster variant displaying high similarity to the *Xcc rpf* cluster, and *rpf-2* (D457) to that with significant differences. Additionally, we have sequenced the genome of three more clinical isolates E77, UV74 and M30. From these, the genome of the strain M30 was submitted to the NCBI (with accession number JELS02000000) (10) and a first draft was constructed for the E77 and UV74 genomes. Analysis of the complete *rpf* cluster in these new genomes also led us to identify two *rpf* cluster variants among these strains. In order to conclude that two variants of the synthase RpfF and the sensor component RpfC were distributed among the population of *S. maltophilia*, the *rpfF* and *rpfC* genes were amplified for a collection of 78 clinical isolates (Table 1). Amplification of the corresponding DNA region demonstrated that all *S. maltophilia* strains contain the *rpfF* and *rpfC* genes. For the *rpfF* gene, slightly different fragment lengths were obtained due to the previously observed N-terminal region's variability. All of the amplified *rpfF* sequences were

deposited in the GenBank database and assigned accession numbers KJ149475 to KJ149552. In addition, alignment of the translated N-terminal regions and subsequent phylogenetic analysis confirmed that the RpfF of *S. maltophilia* might be distributed into two distinct variants, which we have named RpfF-1 and RpfF-2 (Fig. 1, 2).

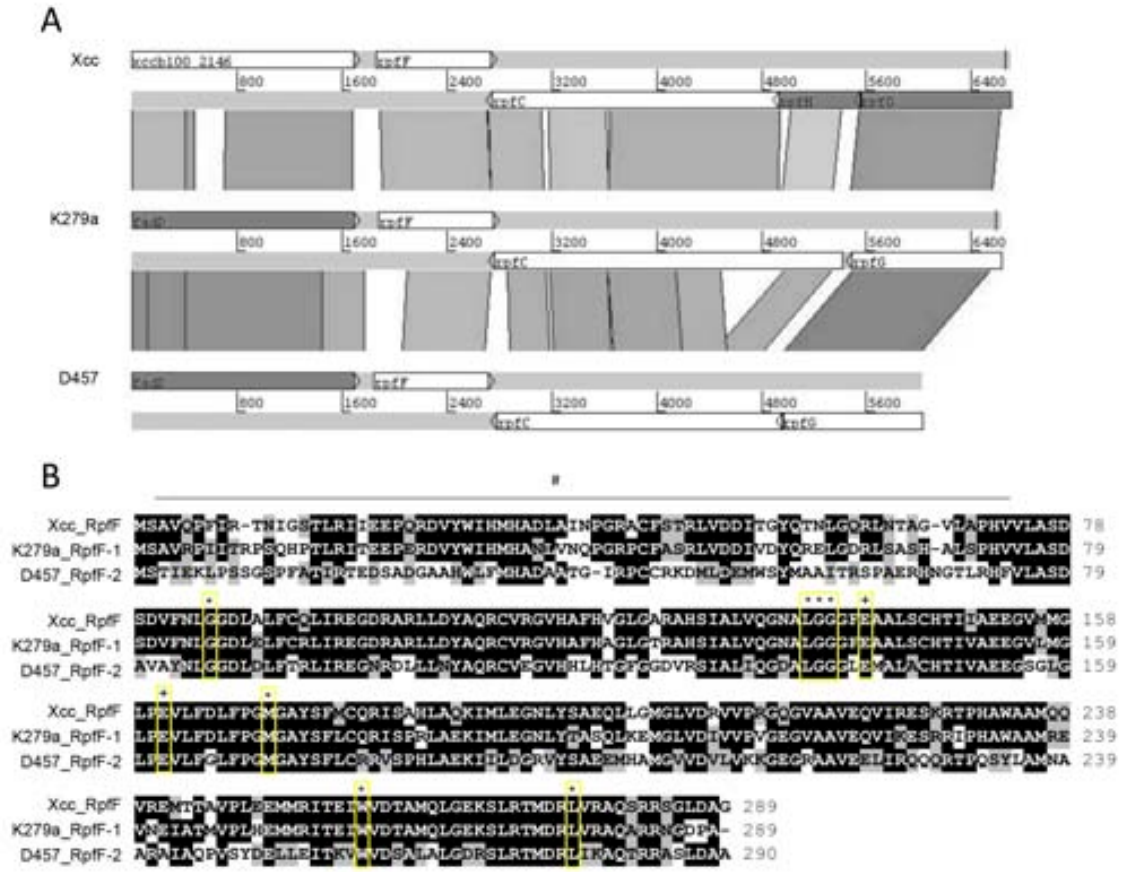

FIG 1 (A) Comparison of the *rpf* cluster in *Xanthomonas campestris* pv. *campestris* (*Xcc*) and two *Stenotrophomonas maltophilia* strains (K279a and D457). The alignment was performed using tblastx (percent identity cut-off = 45%) from the Blast suite and visualized with the Artemis Comparison Tool. Conserved protein regions are paired by shaded blocks where colour intensity is proportional to sequence identity. The scales are relative positions in base pairs. (B) Alignment of RpfF proteins from *Xcc* and *S. maltophilia* K279a (RpfF-1) and D457 (RpfF-2). Identical amino acid residues are shaded black with similar residues shaded grey. The hypervariable region is marked with "#", binding pocket residues are marked with "*" and Glutamate catalytic residues are marked with "+".

Continuing with the analysis of the *rpf* cluster in the *S. maltophilia* available genomes we observed that *rpfC* gene also differed significantly between the two *S. maltophilia*

variant groups defined by the *rpfF* gene (Fig. 1A). In order to demonstrate this association, we amplified the *rpfC* gene from all *S. maltophilia* strains in our collection. The obtained fragment lengths let us to discriminate and prove that two *rpfC* variants were also distributed among *S. maltophilia* population. It also verified that each RpfF-variant group carries an associated RpfC-variant. Hence, RpfF-1 variant strains have an associated RpfC-1 variant and RpfF-2 variant strains harbour an associated RpfC-2 variant.

Altogether, from all *S. maltophilia* strains in our collection, the RpfC/F-1 variant was present in 47 out of 78 strains (60.26%), whereas RpfC/F-2 was present in the remaining 31 strains (39.74%) (Fig. 2). Of the additional four complete genome sequences available at this time, K279a and R551-3 contain the RpfC/F-1 variant and D457 and JV3 contain the RpfC/F-2 variant.

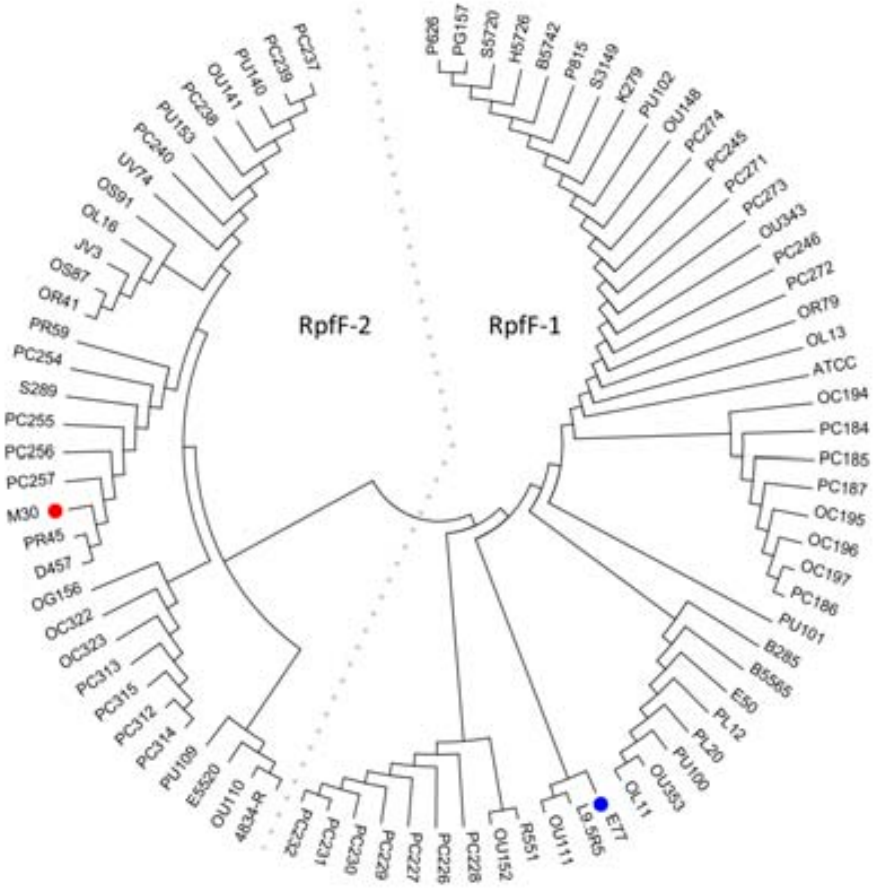

FIG 2 Phylogenetic analysis (Neighbourhood-Joining) of 82 *S. maltophilia* clinical strains based on the first 108 aminoacids of RpfF. E77 (blue) and M30 (red) were taken as model strains to study the implication of each variant *rpf-1* and *rpf-2* in DSF production and virulence regulation.

4.1.2 RpfC-1 but not RpfC-2 contains a transmembrane sensor input domain highly related to the *Xcc* RpfH-RpfC complex

RpfC-1 (belonging to RpfF-1 variant strains) and RpfC-2 (belonging to RpfF-2 variant strains) differ in their N-terminal regions, corresponding to the transmembrane (TMR) domain or sensor input domain (107) (Fig. 3). It has been postulated that in *Xcc* an additional integral membrane protein, RpfH, participates in DSF sensing (105). In *S. maltophilia* the RpfH protein appears to be fused to RpfC-1 generating a sensor input domain with ten TMR, as would happen in a putative *Xcc* RpfH-RpfC complex. However, the TMR domain of the RpfC-2 variant contains only five TMR. Interestingly, tblastx analysis revealed that the five TMR present in the RpfC-2 variant are highly related to *Xcc* RpfH, while the five regions that are absent would correspond to the *Xcc* RpfC TM domain (Fig. 3). This indicates that both RpfC-variant groups carry a putative RpfH but only the RpfC-1 variant contains its own five TMR in the sensor input domain.

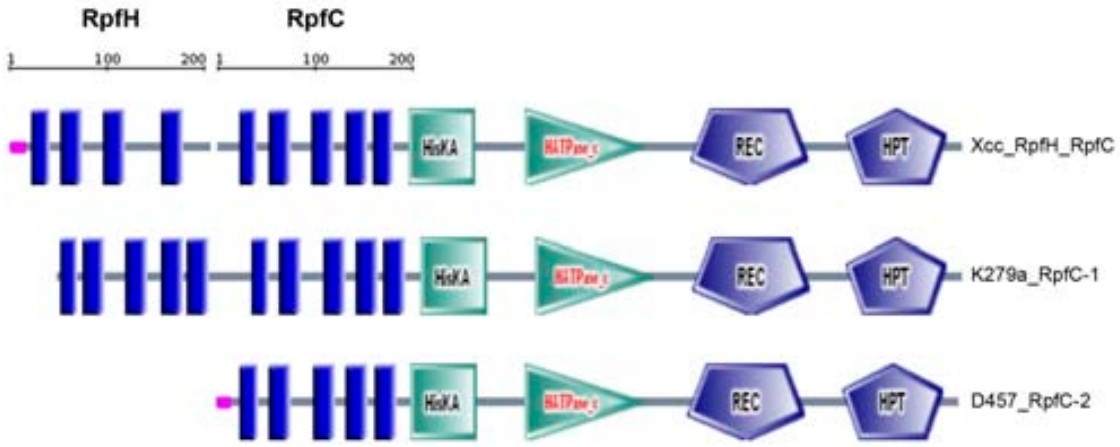

FIG 3 SMART-software analysis of RpfC and RpfH from *Xcc* and RpfC from *S. maltophilia* K279a and D457, where HisKA is a Histidine Kinase domain, HATPase_c a Histidine ATPase domain, REC a CheY-like receiver domain and HPT a Histidine phosphotransferase domain.

4.1.3 Only strains carrying RpfC/F-1 combination produce detectable levels of DSF.

We have tested all *S. maltophilia* strains in colony DSF bioassay. While all RpfC/F-1 variant strains showed DSF production; none strain carrying the RpfC/F-2 combination showed DSF activity at least under these conditions (Fig. 4).

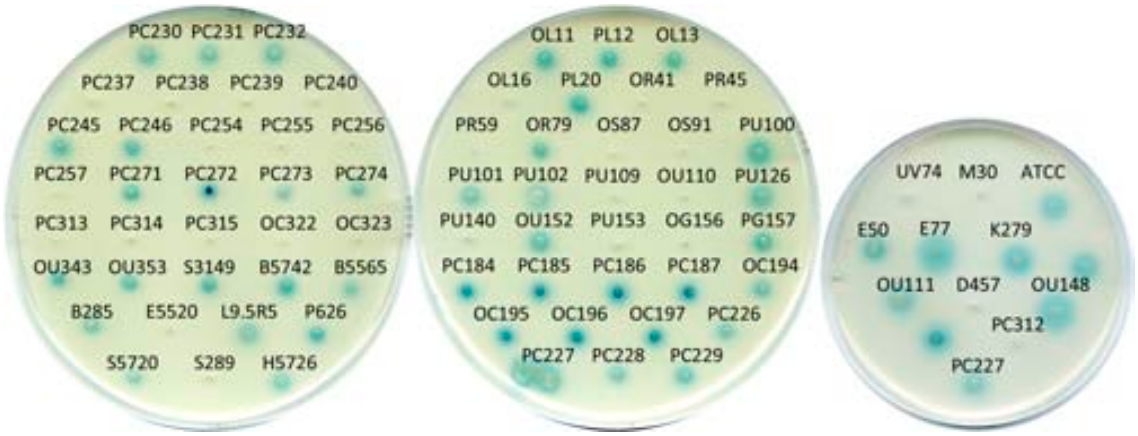

FIG 4 Colony DSF bioassay of 78 *S. maltophilia* strains using the *Xcc* 8523 pL6engGUS reporter strain. Single colonies were spotted on bioassay plates and incubated for 24 h at 28ºC. Blue halo around the colony indicates DSF activity and corresponded to the RpfF-1 variant strains. Colonies without DSF production corresponded to RpfF-2 variant strains.

M30 was taken as a RpfC/F-2 representative strain to corroborate the absence of DSF production in these *S. maltophilia* variant group. Thus, the culture supernatant of the strain M30 was analysed by supernatant DSF bioassay, TLC and GC/MS (see Materials and Methods, sections 3.8.1, 3.8.2 and 3.7.5, respectively). With none of these three techniques, DSF production was never detected in M30 supernatants, indicating that RpfC/F-2 does not produce DSF under the assayed conditions (Fig. 4, 5, 6, 7) (these experiments are described in the next sections where we will work with mutants derived from the model strain M30).

Initially, the significant differences between the N-terminal regions of the two RpfF variants made us hypothesize that this region could play a direct role in DSF synthesis. However, the residues conforming the substrate binding pocket (Leu136, Gly137, Gly138, Gly85, Leu276, Met170 and Trp258) as well as those involved in catalysis (Glu141, Glu161) in *Xcc* (107) are conserved in the two variants (Fig. 2B).

Then, this observation made us think that the synthase variant RpfF-2 could be however functional, but some other mechanism such as RpfC-2 repression, which has been described in many DSF-producer bacteria (107, 117), was responsible for the absence of DSF production in this variant group. For this reason we will also study the role of the RpfC in DSF synthesis repression in the next sections.

4.1.4 Both RpfF variants are able to synthesize DSF

We have generated the Δ*rpfF* mutant in the strains E77 and M30 to firstly demonstrate that the blue halo observed in the bioassays corresponded to the DSF produced by the synthase RpfF. It was indeed corroborated with the E77 Δ *rpfF* mutant (RpfC/F-1 variant), which does not produce the DSF molecule and obviously neither does the M30 Δ*rpfF* mutant (Fig. 5). In order to test the intrinsic capacity of the RpfF-2 variant to produce DSF, we introduced a plasmid construct containing the *rpfF* gene from M30 (RpfF-2 variant) in the E77 Δ *rpfF* mutant (RpfF-1 variant) to test heterologous complementation. The obtained results demonstrated that RpfF-2 was functional on DSF synthesis, since the E77 Δ*rpfF* mutant complemented with M30 *rpfF* displayed a big blue halo of DSF diffusion (Fig. 5A). Additionally, insertion of extra copies of its own *rpfF* in the M30 wt and M30 Δ *rpfF* also resulted in DSF production (Fig. 5B), indicating that RpfF-2 is able to produce DSF but it could be repressed in the wild type strain under the assayed conditions.

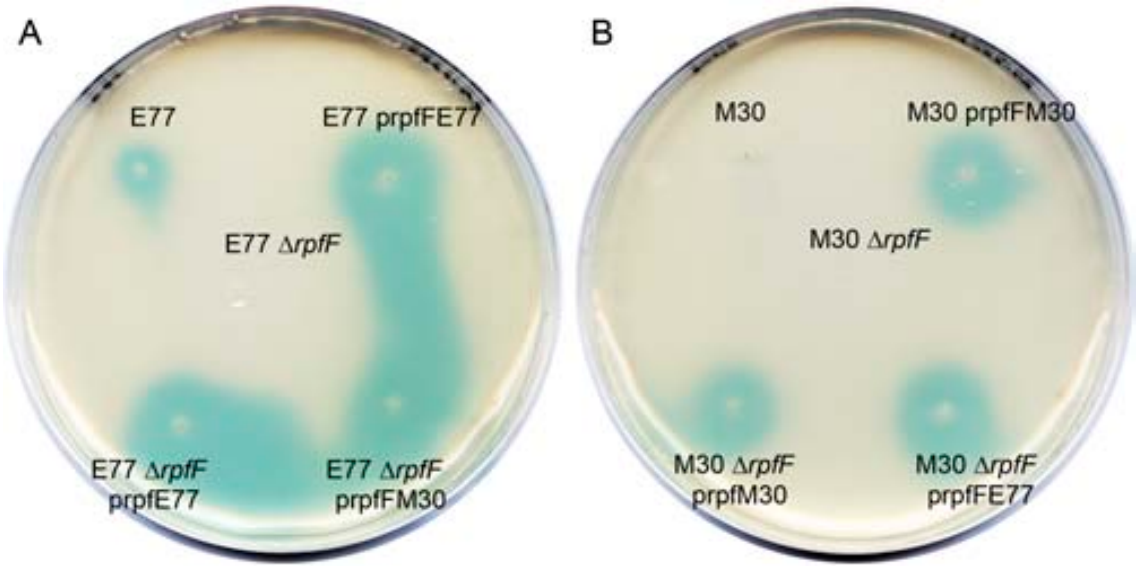

FIG 5 DSF bioassay of E77 (A) and M30 (B) with their respective Δ*rpfF* mutants and the homologous and heterologous complemented strains, using the *Xcc* 8523 pL6engGUS reporter strain.

Moreover, TLC-bioassay analysis of culture supernatants of E77 and M30, their Δ*rpfF* mutants and the complemented strains suggested that DSF is the only fatty acid with signalling activity that depends on the RpfF synthase function, since no other differential blue spot was observed when comparing M30 with E77 and with their respective Δ*rpfF* mutants and complemented strains (Fig. 6).

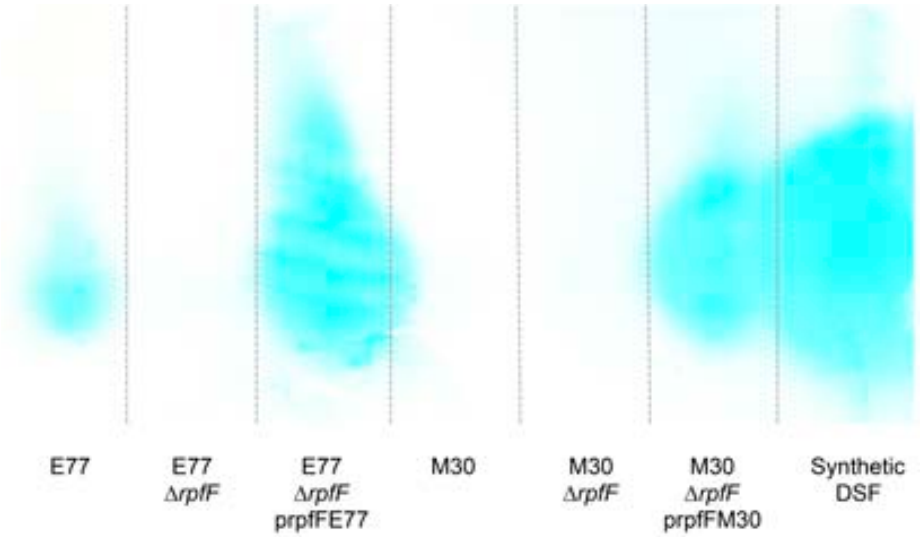

FIG 6 Scanned image of a TLC coupled to DSF bioassay of culture supernatants from E77 and M30, their respective Δ*rpfF* mutants and complemented strains. 5 µl of each concentrated supernatant and 2 µl of synthetic DSF were spotted onto TLC silica plate and separated with ethyl acetate:hexane (20:80, vol/vol) as running solvents. Once the TLC was run and dry, 100 ml of DSF reporter solution was added to the plate and incubated for 24 h at 28ºC. Blue spots indicate the presence of DSF molecules.

All concentrated supernatants plus the synthetic DSF were dissolved in toluene and total fatty acids were methylated prior to the Gas Chromatography/Mass Spectrometry (GC/MS) identification. Each sample was separated by GC and compared to the synthetic DSF, which showed a retention time of 17.8 minutes. Only the concentrated supernatants from the strains displaying DSF production in the supernatant bioassay showed a peak at the same retention time (Fig. 7). In addition, these peaks showed the same mass spectra as the synthetic DSF (Fig. 8), concluding that both RpfF variants produce DSF. MS analysis further supported that both *S. maltophilia* groups only produce the canonical DSF 11-*cis*-2methyl-dodecenoic acid since no other differential GC peak with a mass spectra fatty acid-compatible was observed comparing each other the DSF-producer strains (Fig. 7, 8).

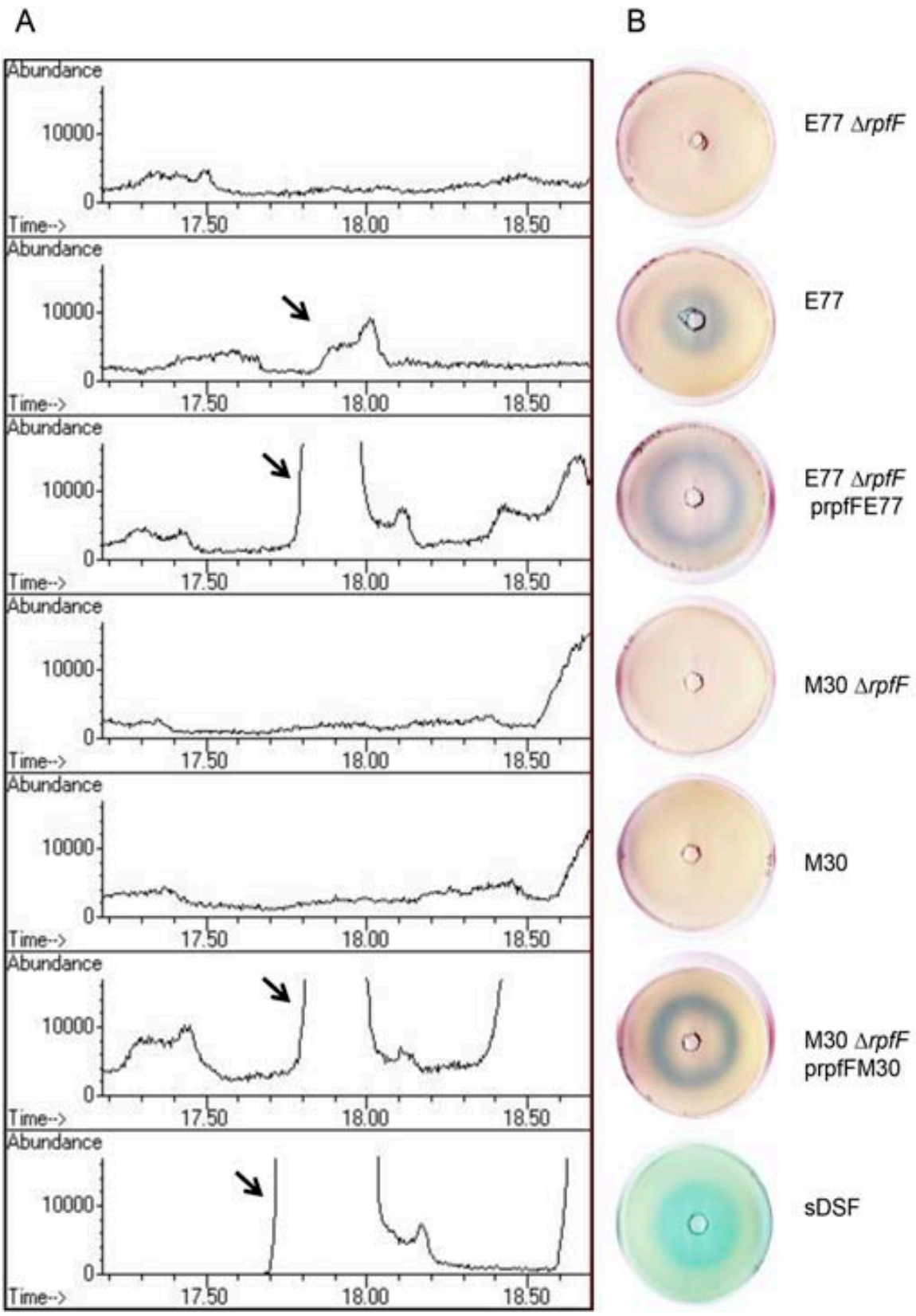

FIG 7 (A) Gas Chromatography analysis of culture supernatants of E77 and M30, their respective Δ*rpfF* mutants and complemented strains. (B) DSF bioassay of concentrated supernatants of the same strains from independent extraction.

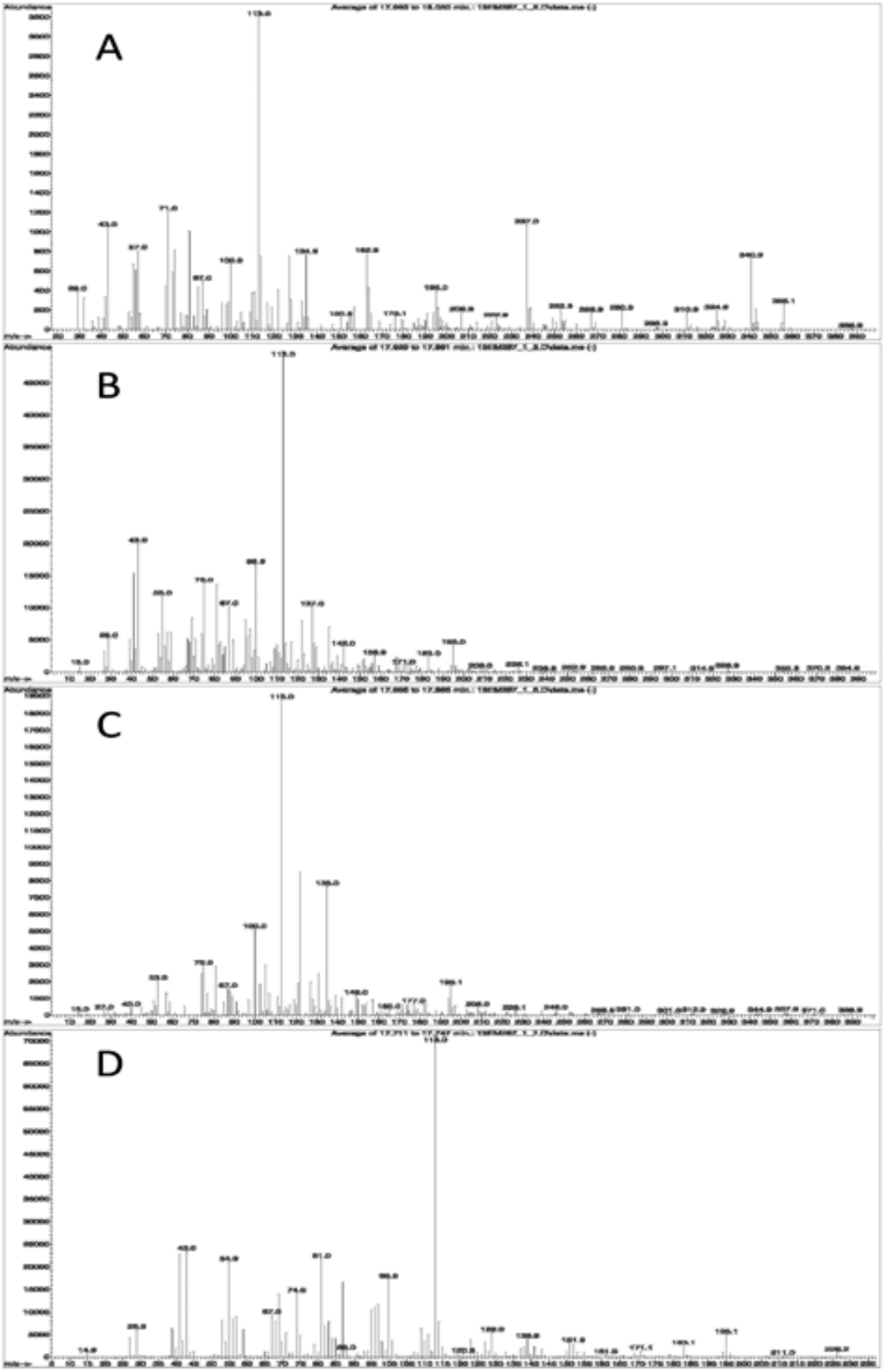

FIG 8 Mass spectra of the Gas Chromatography peaks with DSF activity (17.8 min.) (See also Fig. 7). (A) E77, (B) E77 Δ*rpfF* prpfFE77, (C) M30 Δ*rpfF* prpfFM30 and (D) Synthetic DSF.

4.1.5 RpfF-2 is permanently repressed by RpfC-2

Since both RpfF variants were shown to be functional on DSF production, we wanted to study the implication of each RpfC variant in DSF synthesis repression. To test the hypothesis that RpfF-2 was permanently blocked by RpfC-2, the DSF-producer strain E77 was separately transformed with plasmids expressing each of the RpfC variants. Since *rpfC* is expected to be co-transcribed jointly with *rpfG* in both *rpf*-1 and *rpf*-2 clusters, we generated the vectors prpfGCE77 and prpfGCM30 harbouring the full *rpfGC* operon. These vectors were effective repressors since in both cases resulted in a reduction of DSF synthesis in E77 strain (Fig 9). However, while E77 harbouring the prpfGCE77 vector showed only a small decrease in DSF synthesis, provision of prpfGCM30 resulted in a strong inhibition of DSF production (Fig. 9A), suggesting that RpfC-2 is a stronger repressor of the RpfF activity. In order to corroborate this hypothesis, we generated the Δ*rpfC* mutant in the M30 strain (RpfC-2). Consistently with the previous result, M30 Δ*rpfC* became a DSF-producer strain (Fig. 9B). In trans complementation of M30 Δ *rpfC* with vectors prpfGCE77 and prpfGCM30 led to a scenario similar to that observed for the E77 strain (Fig. 9B). While the plasmid construct derived from E77 (*rpfC-1*) slightly reduce DSF production in the M30 Δ*rpfC* mutant, homologous complementation of *rpfC-2* abolished the DSF synthesis in the M30 Δ*rpfC* mutant (Fig. 9B).

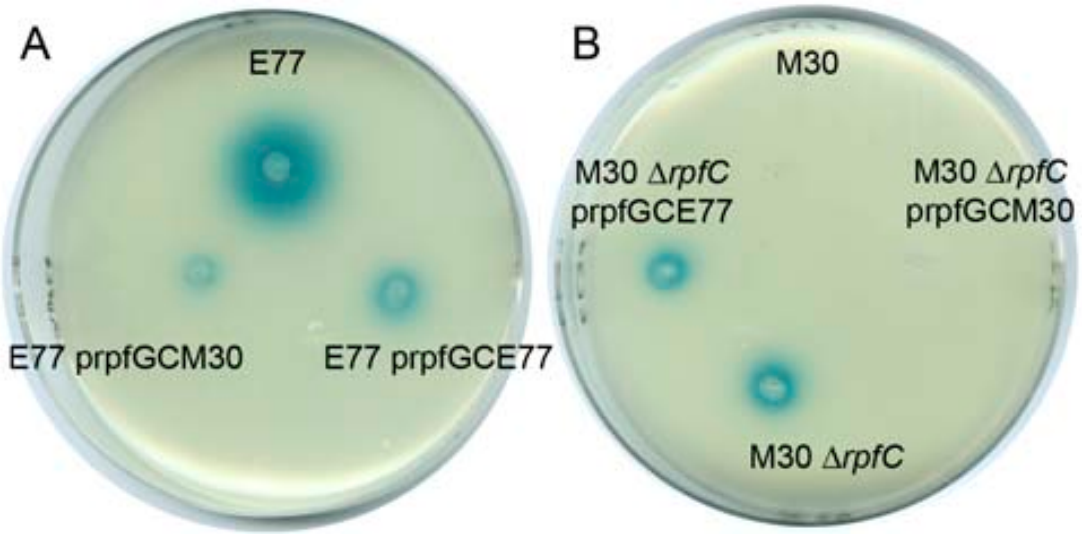

FIG 9 (A) DSF bioassay of E77 wt and E77 complemented with vectors prpfGCE77 and prpfGCM30. (B) DSF bioassay of M30 wt, M30 Δ *rpfC* and M30 Δ *rpfC* complemented with vectors prpfGCE77 and prpfGCM30.

To further characterize the relation between RpfF and RpfC in the two variants, gene expression of pairs *rpfF*-1/*rpfC*-1 and *rpfF*-2/*rpfC*-2 was quantified by qRT-PCR, taking the cultures a the same optical density as for DSF identification. Thus, expression in E77 wt was 5,16 ± 0,59-fold for *rpfF*-1 and 2,69 ± 0,29-fold for *rpfC*-1 (*rpfF*-1/*rpfC*-1 ratio of 1,92), while in M30 wt it was 1,57 ± 0,23-fold for *rpfF*-2 and 1,65 ± 0,25-fold for *rpfC*-2 (*rpfF*-2/*rpfC*-2 ratio of 0,95) (Fig. 10). This results indicate that the repression that RpfC exerts on RpfF is not only at protein level (RpfF-RpfC interaction), but also at genetic level, where RpfC could also act repressing the expression of the *rpfF* gene.

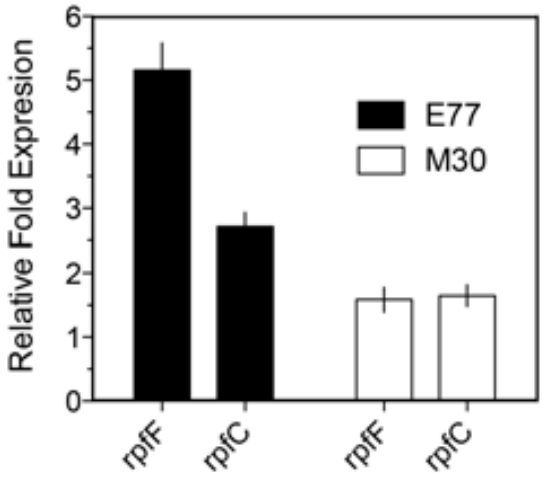

FIG 10 Quantification by qRT-PCR of gene expression of *rpfF* and *rpfC* from strains E77 and M30 normalized with the gene expression of the housekeeping gene *gyrA*, using the $2^{-\Delta\Delta Ct}$ method.

4.1.6 An improved liquid bioassay for accurately quantification of DSF molecules.

Since we observed that both RpfF variants were able to produce DSF, we wanted to study the conditions that would facilitate such production, especially in the RpfF-2 strain. Thus, we have developed a 96-well plate DSF bioassay method, with a sensitivity of 2 ng/ml, in order to accurately quantify DSF amount produced by *S. maltophilia* strains. Synthetic DSF was added to wells containing DSF-reporter solution (see Materials and Methods, section 3.8.3) with increasing concentrations (0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mM) and a calibration curve (rI=0.9787) was generated (Fig. 11). The resultant formula was used to quantify the levels of DSF in culture

supernatants of *S. maltophilia* strains. Concentrated *S. maltophilia* supernatants were dissolved in 20 µl of 30% methanol and 0.25, 0.5, 1, 2, 4 and 8 µl were tested in this new liquid bioassay. The 96 well-plate was incubated for an overnight at 28ºC and the absorbance of the wells was read at 620 nm.

This new methodology not only allows us to test a large number of samples in a single 96 well plate but also reduce time and costs. Altogether, this new DSF bioassay method becomes a useful tool in order to study the conditions that modulate DSF production in *S. maltophilia.*

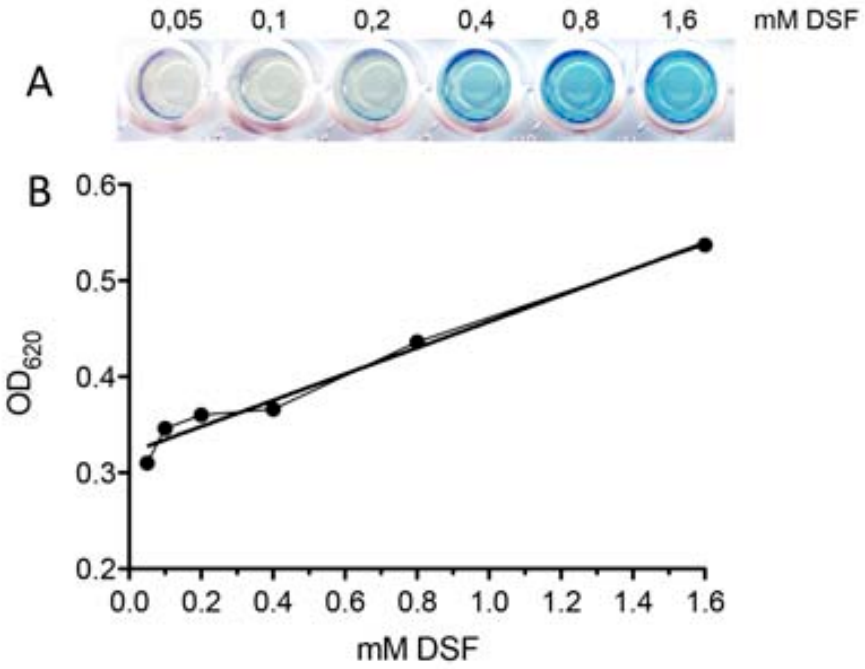

FIG 11 Liquid DSF bioassay. (A) Wells containing DSF-reporter solution (See Materials and Methods, section 3.8.3) inoculated with increasing concentrations of synthetic DSF. (B) Calibration curve generated by reading the absorbance of the wells at 620 nm.

4.1.7 DSF production is temperature and media-dependent

Little is known about which conditions facilitate DSF production in *S. maltophilia*. Hence, we have quantified the DSF levels from culture supernatants grown in LB medium in different temperatures, 20ºC, 25ºC, 30ºC, 37ºC and 42ºC for 48 h. In E77 strain (RpfC/F-1 variant), the growth temperature is critical in such production. Then, using the liquid DSF bioassay, we have observed that cultures grown in 30 ºC are the

most productive ones, since their supernatants showed a production of 1.4 +- 0.2 µg/ml of DSF, followed by those grown in 25ºC (0.6 ± 0.07 µg/ml) and 37 ºC (0.25 ± 0.03 µg/ml) (Table 13). On the other hand, DSF production is not detected when E77 is grown at 20ºC or 42ºC (Table 13). We again observed that DSF is never detected in M30 (RpfC/F-2 variant) supernatants (Table 13).

TABLE 13 Quantification of DSF production in culture supernatants of E77 and M30 grown in LB medium at different temperatures for 48 h.

	DSF production (µg/mL)				
	20ºC	25ºC	30ºC	37ºC	42ºC
E77	ND	0.6 ± 0.07	1.4 ± 0.2	0.25 ± 0.03	ND
M30	ND	ND	ND	ND	ND

ND: Not Detected

Since we observed that 30ºC was the most optimum temperature for DSF production in the E77 strain, we then wanted to study the implication of the media in such production at the most optimum temperature. We quantified the DSF production of E77 grown in another rich medium (NYG) and two minimal mediums (BM2 and modified M9-salts). The obtained results indicate that the DSF production is clearly higher in the rich media supernatants followed by those grown in BM2 and modified M9-salts (Table 14). Again, none of these media facilitated DSF production in the M30 strain (RpfC/F-2 variant) (Table 14).

TABLE 14 Quantification of DSF production in culture supernatants of E77 and M30 grown in different media conditions at 30ºC for 48 h.

	DSF production (µg/mL)			
	M9*	BM2	NYG	LB
E77	0.3 ± 0.03	0.4 ± 0.03	1.1 ± 0.15	1.4 ± 0.2
M30	ND	ND	ND	ND

ND: Not Detected
M9: Modified M9-salts medium*

4.1.8 13-methyl-tetradecanoic acid (C:15 iso), a possible DSF precursor, is the most abundant fatty acid in *S. maltophilia*

We have analysed the total cellular fatty acid for the representative strains *S. maltophilia* E77 by gas chromatography (See Materials and Methods, section 3.7.7).

The results revealed a high diversity and heterogeneity with near 35 different fatty acids. From three independent identifications, the most abundant fatty acid was found to be 13-methyl-tetradecanoic acid (15:0 iso) (Table 15), a highly DSF-related fatty acid (Fig. 12).

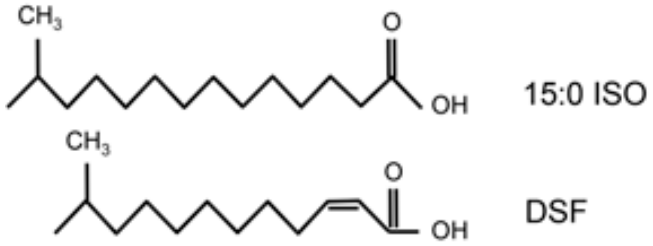

FIG 12 Illustration of the fatty acids 13-methyl-tetradecanoic (15:0 iso) and 11-*cis*-methyl-dodecenoic (DSF).

Since the representation of the 15:0 iso fatty acid is near to 33%, it is easy to think that this branched fatty acid is part of the cell membrane phospholipids, being one of the two nonpolar "legs". Total fatty acids identified in *S. maltophilia* with a relative abundance over 1% are listed in the Table 15.

TABLE 15 Distribution of the total cellular fatty acids in *S. maltophilia*.

RT (min)	Peak name	Percentage*
7,873	15:0 ISO	32,3 ± 0,8
9,774	15:0 ISO 2OH	12,6 ± 0,6
8,007	15:0 anteiso	11,9 ± 0,5
10,074	16.00	5,95 ± 0,5
10,786	ISO 17:1 w9c	4,22 ± 0,2
9,703	16:1 w9c	3,86 ± 0,3
6,935	14.00	3,77 ± 0,2
6,212	12:0 3OH	3,29 ± 0,3
7,099	13:0 ISO 3OH	3,27 ± 0,1
3,376	11:0 ISO	2,9 ± 0,1
11,151	17:0 ISO	2,49 ± 0,2
4,606	11:0 ISO 3OH	1,66 ± 0,2
13,126	18:1 w9c	1,47 ± 0,1
9,464	16:0 ISO	1,46 ± 0,06
4,331	Unknown	1,23 ± 0,1
6,429	14:0 ISO	1,06 ± 0,04

RT: Retention Time
** From three independent identifications (only FA with a relative abundance over 1% are listed).*

4.1.9 Unspecific medium-length fatty acids modulate DSF production in RpfC/F-1 variant strains

In order to determine the potentiality of some fatty acids in the DSF stimulation, we have evaluated the DSF production from supernatants of the strains E77 and M30 grown in LB medium supplemented with different commercial medium and large-length saturated fatty acids at 5 μM final concentration at 30ºC for 48 h. These fatty acids were 12:0 (lauric), 14:0 (myristic), 15:0 iso (13-methylmyristic or 13-methyl-tetradecanoic acid), 16:0 (palmitic) and 18:0 (stearic).

While in the M30 strain (RpfC-2 variant), none of these fatty acids neither stimulated DSF production (Fig. 13), in the E77 strain, supplementation of 12:0, 14:0 and 15:0 iso clearly potentiated the DSF synthesis. The maximum stimulation was found in the supernatants supplemented with 15:0 iso (DSF production of 2,4 μg/mL) followed by those supplemented with 14:0 (1,8 μg/mL DSF) and 12:0 (1,6 μg/mL DSF) (Fig. 13). Interestingly, while supplementation of 16:0 fatty acid did not show DSF stimulation, supplementation of 18:0 showed an inhibitory effect on DSF synthesis (Fig. 13). The same volume of the media supplemented with the mentioned fatty acids was also extracted and tested in the bioassay as negative control. No DSF activity was detected in those concentrated supernatant (data not shown).

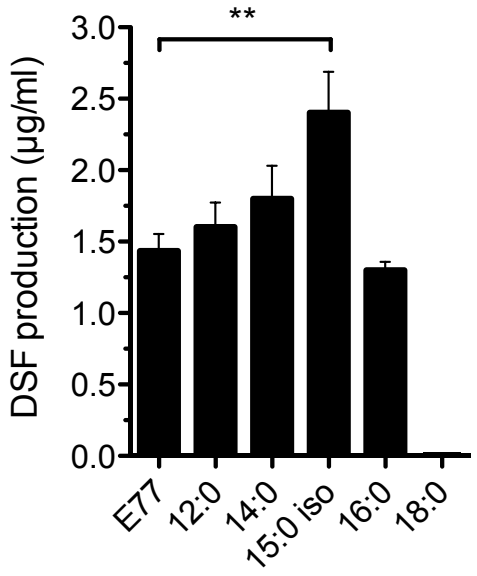

FIG 13 DSF quantification of E77 supernatant supplemented with different fatty acids at 5 μM final concentration. **, $P < 0.05$.

These results would indicate that the presence of different medium-length fatty acid (12-14 carbons) induce the dissociation of the RpfC/F-1 complex, potentiating the RpfF-1 activity and also suggest that the lack of the 5 TMR in the RpfC-2 variant would render this sensor incompetent for the promiscuous perception, resulting in a permanent repression of the RpfF-2 synthase even when these fatty acids are supplemented.

4.1.10 *S. maltophilia* RpfC/F-1 and RpfC/F-2 variant strains cross-talk each other, producing DSF in a positive feedback-manner.

Since we observed that RpfF-2 variant was functional when the stoichiometric imbalance was favorable to RpfF-2 (RpfF-2>RpfC-2), we were challenged to determine in which conditions RpfC/F-2 complex would dissociate and synthesize DSF in a wild type background. We have tested many growth conditions combining media and temperatures as well as supplementing different saturated fatty acids without success. However, we observed that, when *S. maltophilia* E77 and M30 are grown close enough that the halo of DSF production of E77 invades the growing zone of M30, it displays clearly DSF production, indicating that DSF stimulates itself-production in a positive feedback manner (Fig. 14).

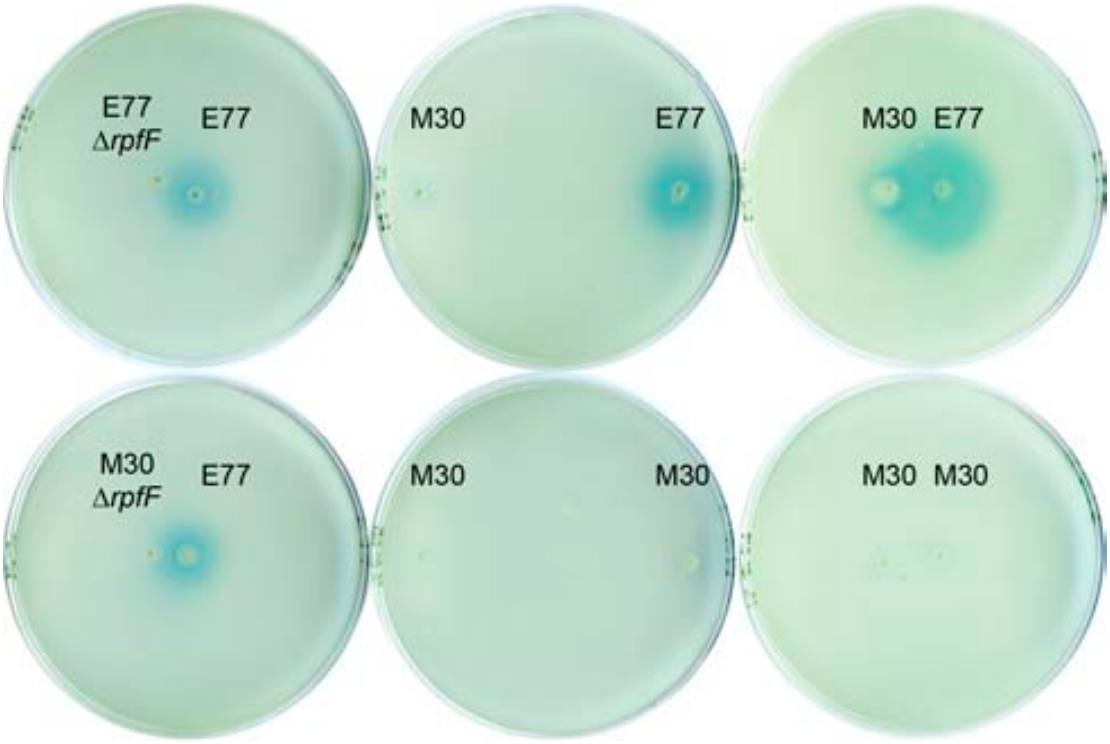

FIG 14 Colony-based DSF bioassay of E77, M30 and their respective Δ*rpfF* strains seeded at different distances one from the other.

In addition, mixed cultures of E77 and M30 grown in LB at 30ºC for 48 h displayed a higher DSF production than E77 axenic cultures (Fig. 15). Finally, supplementation of LB with 0,05 µM DSF triggers DSF production of M30 up to a maximum point of 0,6 µg/mL production after 48 h of growth (Fig. 15).

These results indicate, in one hand, that the sensor RpfC-2 is much more specific, liberating active-RpfF-2 only upon detection of DSF molecules, in the other, that DSF is produced in a positive feedback-manner in *S. maltophilia*.

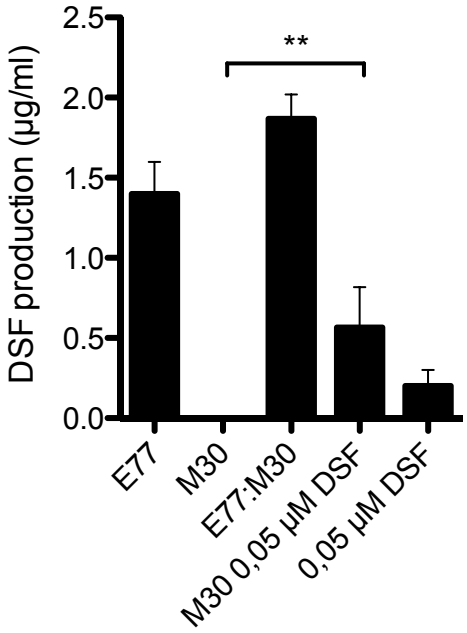

FIG 15 DSF production in cultures of axenic E77, axenic M30, a mixed culture of E77 and M30 and a culture of M30 supplemented with 0.05 µM DSF. **, $P < 0.05$.

4.1.11 Discussion

In this chapter, we have proposed to study the molecular mechanisms that govern DSF production in *S. maltophilia*.

We have characterized 78 *S. maltophilia* clinical strains, isolated from diverse sources in different European hospitals, for the *rpf* cluster. We have first demonstrated that the 78 strains contain the *rpfF* gene but the RpfF product is distributed into two different variants that we have named RpfF-1 and RpfF-2 (Fig. 1, 2). We have also shown that the isolates present two RpfC variants, each associated to the RpfF variants (Fig. 1A). The two RpfC variants are different in their N-terminal region, which corresponds to a

transmembrane region (TMR) (Fig. 3) thought to participate in DSF sensing in several *Xanthomonas* species (105). In *S. maltophilia* the RpfC-1 variant contains 10 TMR, displaying high similarity to the putative *Xcc* RpfH-RpfC TMR complex (Fig. 3). On the other hand, the RpfC-2 variant presents only 5 TMR, which appear to be related to *Xcc* RpfH rather than to the *Xcc* RpfC TMR domain (Fig. 1A, 3). This phenomenon is also observed in *Xylella fastidiosa, Xanthomonas oryzae* and *Pseudoxanthomonas* species, suggesting that the RpfC-2 variant is widely distributed among *Xanthomonadales* sharing the DSF-QS system. Nevertheless, protein-sequence comparison shows a high similarity between the RpfC and RpfH TMR domains (Fig. 1A), suggesting that a duplication event (for *Xcc rpfC*/*rpfH* and *S. maltop*hilia *rpfC-1*) or a deletion (for *S. maltophilia rpfC-2*) may have occurred.

A previous *S. maltophilia* population study suggested that an important group of *S. maltophilia* isolates lack *rpfF* (128). In this mentioned study, a PCR-based typing of 89 strains showed an *rpfF*+ prevalence of 61.8% while the remaining 38.2% was considered to be *rpfF*-. Based on our sequence analysis we can conclude that the work of Pompilio and collaborators (128) failed to detect *rpfF* because primers were designed to hybridize within the most variable region in this gene; more specifically, those primers do not amplify *rpfF* in strains carrying what we have defined as variant 2. Accordingly, we hypothesize that all *S. maltophilia* strains analysed in the study by Pompilio et al. showing a *rpfF*+ genotype belong to the RpfF-1 variant group, whereas *rpfF*- strains would belong to the RpfF-2 variant group. Interestingly, our analysis of *rpfF* from a collection of 82 *S. maltophilia* strains shows similar RpfF-variant frequencies in the population. RpfF-1 is present in 59.75% of the strains (including K279a and R551), whereas RpfF-2 is present in 40.25% (including D457 and JV3). Taking the two studies together (171 isolates), strains carrying the RpfF-1 variant appear to be more commonly isolated than those carrying the RpfF-2 variant, with a relative prevalence of ca. 60% and 40%, respectively.

Surprisingly, we have observed that only strains carrying the RpfC/F-1 pair produce DSF under wild type conditions (Fig. 4), while strains belonging to the RpfC/F-2 variant group require extra copies of its own *rpfF* (Fig. 5, 6, 7) or the absence of the repressor component RpfC-2 (Fig. 9) to achieve detectable levels of DSF production. These results indicate that RpfF-2 is able to synthesize DSF but production of this signalling molecule might be permanently repressed by RpfC-2 under the assayed conditions. It has been shown that the stoichiometric balance between RpfF and RpfC is

crucial for DSF production in many *Xanthomonadales*. In *Xcc*, RpfC physically interacts with the RpfF active site, inhibiting the DSF-synthesis activity (104, 105, 107). RpfC has been also shown to repress the RpfF activity in *X. fastidiosa* (117). Furthermore, analysis of mRNA levels in the strains E77 and M30 by qRT-PCR shows that the *rpfF* to *rpfC* expression ratio in the DSF-producer strain (variant 1) doubles that found in the non-producer one (variant 2) (Fig. 10). Together with the observation that complementation of extra copies of *rpfF-2* and/or deletion of Δ*rpfC-2* result in clearly DSF production in RpfC/F-2 strains, it is evident that the stoichiometry balance between these two components is also a crucial factor in DSF production in *S. maltophilia.*

Although DSF-QS system has been deeply studied among *Xanthomonadales* species, the biosynthetic pathway and catalytic mechanisms involved in DSF production as well as how the sensor RpfC perceive DSF signals remain to be a mystery. We here have generated an improved liquid DSF bioassay (Fig. 11), a useful tool to investigate the implication of the temperature and the media as well as the potentiality of some fatty acids in the modulation of DSF production in *S. maltophilia*. While these factors were not involved in DSF production in the M30 strain, they were clearly involved in the regulation of DSF production in the E77 strain (Table 13, 14). Moreover, while lauric (12:0) and myristic (14:0) acid moderately stimulate DSF production, the most abundant fatty acid in *S. maltophilia* 15:0 iso (Fig. 12) (Table 15) strongly stimulates DSF production in RpfC/F-1 strain E77 (Fig. 13). Since RpfF has an enoyl-CoA hydratase/isomerase function, and there is not a putative fatty acid methyl-transferase in the *rpf* cluster, we hypothesize that the only fatty acid that would really represent a DSF precursor is 15:0 iso, which already contains a branched methyl in the penultimate carbon (C13) (Fig. 12). 15:0 iso would become 11-*cis*-2methyl-dodecenoic acid by the hydratase activity of the RpfF -liberating one acetil-coA-, followed by the isomerase activity -also done by the RpfF-, generating the typical unsaturation between the alpha-beta carbons (Fig. 12). The analysis of the total cellular fatty acids further supported that 15:0 iso could represent the principal DSF precursor in *S. maltophilia* since its relative amount was 33% followed by 15:0 iso 2OH (13%) (Table 15), a hypothetical intermediate in the DSF biosynthetic pathway, between 15:0 iso and DSF. The idea that the principal DSF precursor comes from the membrane phospholipids would give sense to the fact that the sensor component RpfC, which is expected to sense diffusible

signals, is anchored to the cell membrane, because it is also controlling somehow the homeostasis of the cell membrane. Nevertheless, a lot of work remains to clarify this hypothesis.

However, since the RpfC has also a RpfF-repression function, we hypothesize that the DSF stimulation observed in the other supplemented supernatants (12:0 and 14:0) is due to the presence of a competent sensor input domain (composed of 10 TMR) (Fig. 3), which promiscuously sense DSF-related fatty acids, allowing free-active RpfF with the subsequent synthesis of DSF. In addition, this promiscuous sensing only occurs upon detection of fatty acids with 12-14 carbons length, since palmitic acid (16:0) didn't show DSF stimulation and stearic acid (18:0) showed a drastic inhibitory effect of DSF production (Fig. 13). Nevertheless, more efforts are needed to clarify such phenomenon.

It has been suggested that, in *Xcc*, RpfC could play a positive-feedback role in DSF synthesis, liberating active RpfF upon detection of DSF molecules (107). Accordingly with this hypothesis and taking all results together, we hypothesize that DSF production in RpfC/F-1 strains is due to the presence of a competent sensor input domain composed of 10 TMR (Fig 3) capable of detect not only DSF molecules but also some fatty acid derivatives, or DSF precursors, that would enable the liberation of active RpfF-1 and the subsequent synthesis of DSF. On the other hand, the missing TMR in RpfC-2 would render this factor incompetent for this promiscuous sensing leading to a permanent inhibition of RpfF-2 by RpfC-2 until the presence of few DSF molecules trigger the dissociation of this complex enabling free-active RpfF-2 to synthetize DSF. This indicates that the complex RpfC/F-2 is much more specific than RpfC/F-1, sensing only DSF molecules. Recent findings reported that, in *X. fastidiosa* (RpfC-2 situation, 5 TMR), RpfF is also required for DSF detection (117), something that might also be occurring in *S. maltophilia* strains harbouring the RpfC/F-2 combination.

With all these observations, it is evident that some initial DSF production must occur in order to triggers the DSF production in RpfC/F-2 strains. However, the specific conditions that would facilitate this initial DSF production in axenic cultures have not been yet elucidated and will require further studies. Nevertheless, it is well established that, in their natural niches, there exist competence and communication between populations of the same and different species (104), even between organisms from different kingdoms (100), which could generate this initial levels of DSF and induce the activation of the entire DSF system in the RpfC/F-2 variant population.

It is known that many *Xanthomonadales* produce more or even different signalling molecules than the canonical DSF. Although DSF was found to be the unique signalling molecule in *Xanthomonas campestris* pv. *campestris* (104) and *Xanthomonas axonopodis* pv. *citri* (*Xac*) (94, 95), in *Xanthomonas orizae* pv *orizae* (*Xoo*), three signalling fatty acids molecules have been described: DSF, BDSF (*cis*-2-dodecenoic acid) and CDSF ((2Z, 5Z)-11-methyldodecadienoic acid) (96). Recently, 2(Z)-tetradecenoic acid (xfDSF) was reported to be produced by *Xylella fastidiosa* strain temecula1 (99). More complex is the DSF-like pattern produced by *S. maltophilia* strain WR-C, which includes DSF and seven derivatives (127). Although their exact structure remains unclear and were not probed to be synthesized by RpfF, GC/MS analysis of pyrrolidide derivatives suggest that they could be: Δ2-tridecenoic acid, 10-methyl-dodecanoic acid, Δ2-methyl-tridecanoic acid, Δ2-tetradecenoic acid, Δ2-12-methyl-tetradecenoic acid and Δ2-13-methyl-tetradecenoic acid (127). But DSF-like production is not only restricted to *Xanthomonadales* since *cis*-2-decenoic acid is produced by *P. aeruginosa* (142, 143) and BDSF is produced by *B. cenocepacea* (100). Assuming a high degree of heterogeneity in DSF-like synthesis among bacteria, it wouldn't be strange to hypothesize that some DSF-derivative molecules are strain-dependent synthesized.

We have also been working for a long time under the premise that the RpfF-2 variant could produce a different DSF signalling molecule. Using several approaches, I have not been able to identify an alternative signal molecule. Although we cannot totally reject such hypothesis, the results from the experiments of TLC coupled to DSF bioassay (Fig. 6) and GC/MS (Fig. 7, 8) strongly suggest that the canonical DSF is the unique fatty acid with signalling activity produced by both RpfF-variants and consequently by *S. maltophilia*.

4.2 Phenotypic implications of DSF-QS in *S. maltophilia*

After demonstrate that two variants of the cluster *rpf* are distributed among the population of *S. maltophilia* and characterize the molecular mechanisms involved in DSF synthesis in each variant, we wanted to determine the role that each RpfF variant have in the regulation of virulent-associated processes. To address this question, we have studied some virulence-related phenotypes including swarming motility, biofilm formation and virulence capacity in the *C. elegans* and zebrafish models for the two mutant model strains E77 Δ*rpfF* (variant 1) and M30 Δ*rpfF* (variant 2).

4.2.1 Optimization of swarming motility assay for *S. maltophilia*

One important virulent factor that has been subject of extensive work in related bacteria is swarming motility. In addition, in *Burkholderia cenocepacea* it has been demonstrated that DSF-QS regulates this kind of motility (178). However, few articles are published relating swarming motility and *S. maltophilia*. In fact, there is only one paper reporting swarming motility in this bacterium, which was published by our group last year (72). However, in this mentioned study, a slow and particular kind of motion is reported. Therefore, we have tried to optimize the conditions that could facilitate this motility in *S. maltophilia*, by studying various factors such as the media, temperature, incubation time and inoculation process in the model strain E77. From four different media (LB, YEB, modified-M9 and BM2) we have observed that swarming motility takes place in minimal media (modified-M9 salts and BM2) (Fig. 16), whereas it is not observed in rich media conditions (LB, YEB) (data not shown). In addition, we have noted that it is facilitated in modified M9-salts medium more than in BM2, although no drastic differences are observed (Fig. 16). We have also noted that swarming activation is faster with streak inoculation than with pin inoculation, suggesting that a high-density population facilitates the initiation of this type of motion (Fig. 16). Regarding the incubation temperature, it seems that only 30ºC allows *S. maltophilia* to move in a swarming-manner, since it was not observed when plates were incubated neither 25ºC nor 37ºC (data not shown).

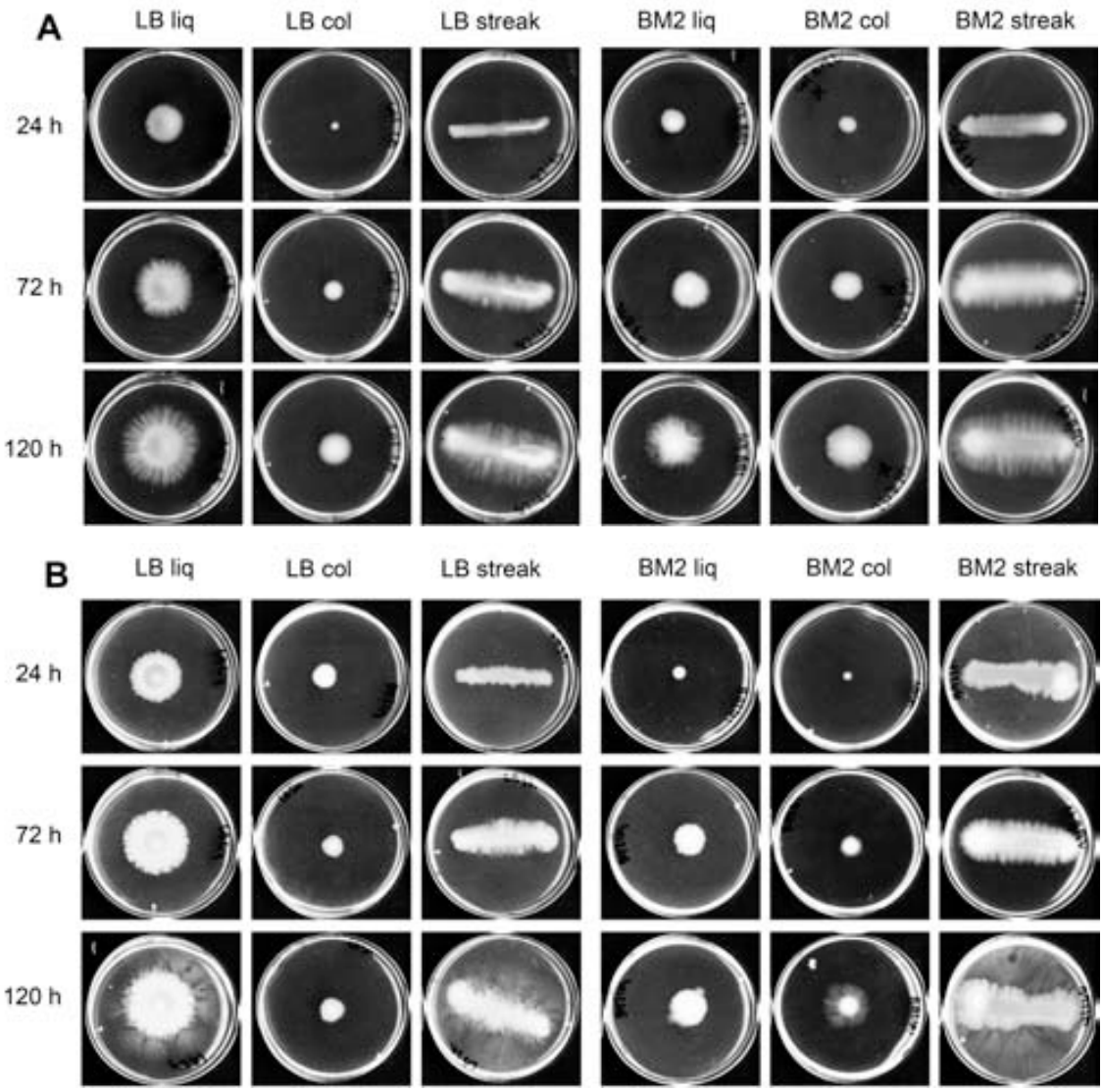

FIG 16 Optimization of swarming motility assay in *S. maltophilia* E77. (A) *S. maltophilia* seeded in BM2 0.5% agar after inoculation with different techniques from different media and growth conditions. (B) *S. maltophilia* seeded in modified M9-salts 0.5% agar after inoculation with different techniques from different media and growth conditions. *liq*: overnight liquid culture. *col*: pin inoculation from a bacterial colony. *streak*: "streak" inoculation from an overnight plate culture.

4.2.2 Δ*rpfF-1* but not Δ*rpfF-2* mutant shows alteration in swarming motility

We have investigated the implication of each RpfF variant in swarming motility. Aforementioned optimization experiments supported the idea that QS could be involved in swarming activation in *S. maltophilia*. To corroborate this hypothesis, we tested the ability of E77 and M30 Δ*rpfF* mutants to swarm under previously defined conditions (modified M9-salts medium with 0.5% agar concentration), relative to the wt strains.

As shown in the Figure 17, E77 wt displays tendril-like motility, whereas M30 wt hardly swarms, likely due to its DSF deficiency. The E77 Δ*rpfF* mutant showed a clear loss of motility and a phenotype restoration when *rpfF* was in-trans complemented. On the contrary, the swarming motility of the M30 Δ *rpfF* mutant was not significantly different from that of the M30 wt strain, suggesting that RpfF does not intervene in swarming control in M30. However, this behaviour does not seem to be strictly linked to the RpfF variant because, in the one hand, E77 Δ *rpfF* displayed an atypical non-tendril swarming morphology when it was heterologous complemented with the M30 *rpfF* gene. On the other, heterologous complementation of M30 Δ *rpfF* with the E77 *rpfF* gene resulted in motility similar to that of the M30 Δ*rpfF* mutant strain.

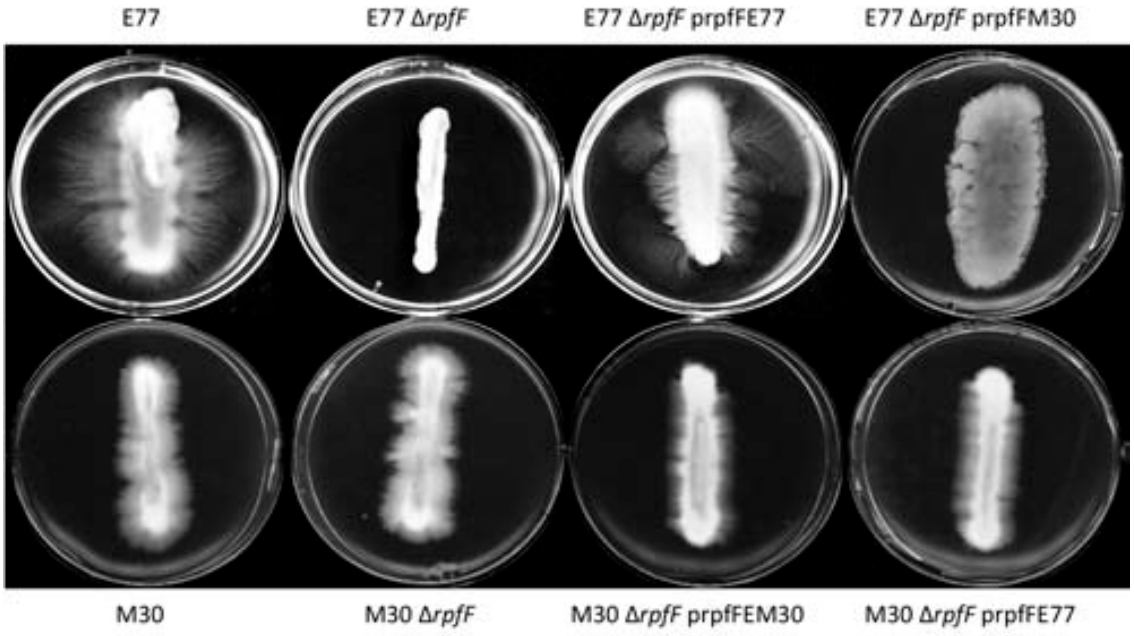

FIG 17 Swarming motility assay for E77 and M30, their Δ *rpfF* mutants and the homologous and heterologous complemented strains, on modified M9-salts medium solidified with 0.5% noble agar and incubated at 30 ºC for 4 days.

Since RpfF-1 was shown to be essential in swarming motility in E77, we wanted to get more insights into the physiological and molecular mechanisms involved in this kind of motility. Thus, we have initially evaluated the surfactant production and the cell morphology of active-bacteria taken from the edge of the swarming tendril after motility experiments, for the E77 wt, the Δ*rpfF* mutant and the complemented strain. The obtained results demonstrated that RpfF-1 was directly involved in the surfactant-like production, since the mutant strain showed a loss of the surfactant halo and it was

restored when the *rpfF* gene was in-trans complemented (Fig. 18A). In order to observe and describe the active-swarmer cells, microscopic TEM visualization was done. Curiously, while the E77 wt and the complemented Δ *rpfF* mutant showed a characteristic *Stenotrophomonas* bacillus shape, the Δ*rpfF* mutant displayed bigger and atypical cell morphology (Fig. 18B).

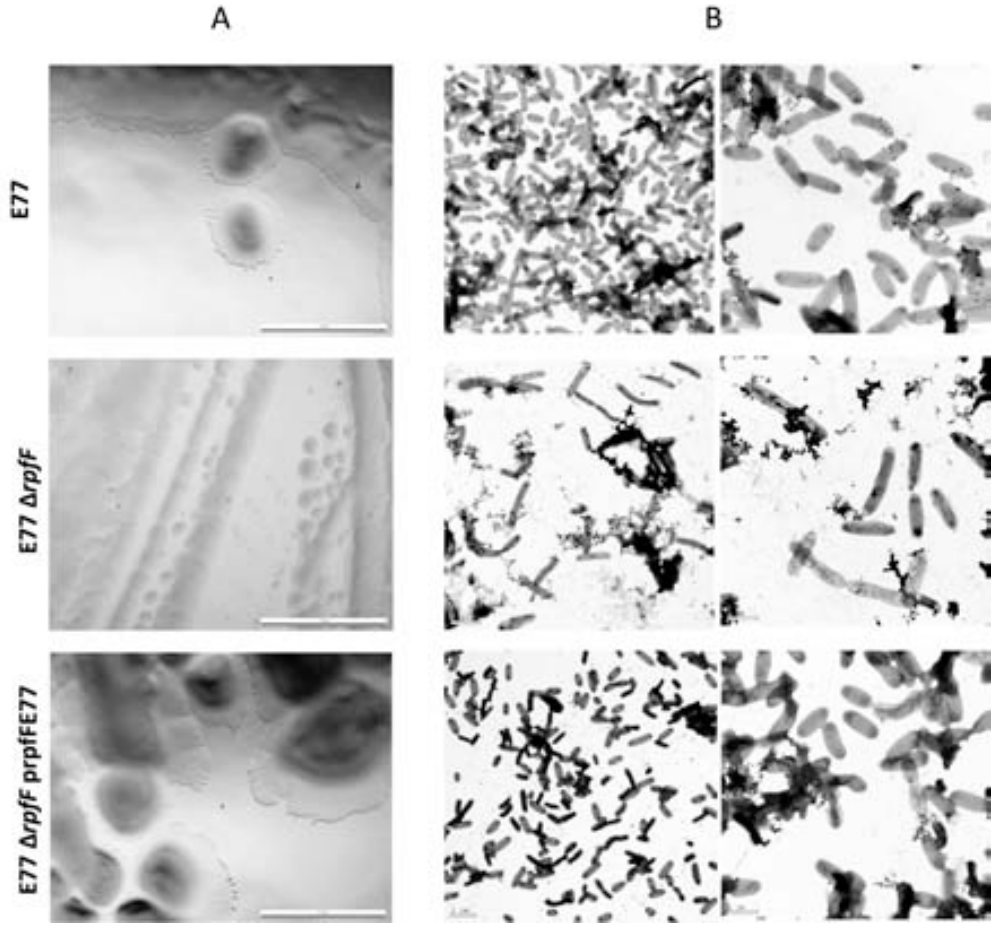

FIG 18 Surfactant imaging (A) and TEM micrographs (B) of E77 (top panels), E77 Δ*rpfF* mutant (middle panels) and the complemented Δ*rpfF* mutant (bottom panels).

4.2.3 Δ*rpfF-1* but not Δ*rpfF-2* mutant shows alteration in biofilm formation

Biofilm formation in abiotic polystyrene or glass surface was also evaluated for E77 and M30, their respective Δ*rpfF* mutants and complemented variants in modified M9-salts and BM2 media. The results showed that M30 has a higher capacity to form biofilm than E77 under both growth conditions (Fig. 19), contrary to the ability to swarm, suggesting that DSF production may inversely regulate these two behaviours in *S. maltophilia*. Additionally, the results also indicated that biofilm formation is only altered in E77 Δ*rpfF*, showing a significant increment relative to E77 wt. Homologous

and heterologous complementation with the respective RpfF variants almost restore E77 wt levels of biofilm formation in both plastic ($P < 0.001$) and glass surfaces (Fig. 19A, B). On the other hand, the M30 strain, the M30 Δ*rpfF* mutant and the homologous and heterologous complemented strains showed similar levels of biofilm formation (Fig. 19C, D). As for the regulation of swarming motility or the ability to produce DSF, it therefore appears that the regulation of biofilm formation is not strictly dependent on the RpfF variant but on one or more components associated to this variant, in particular RpfC.

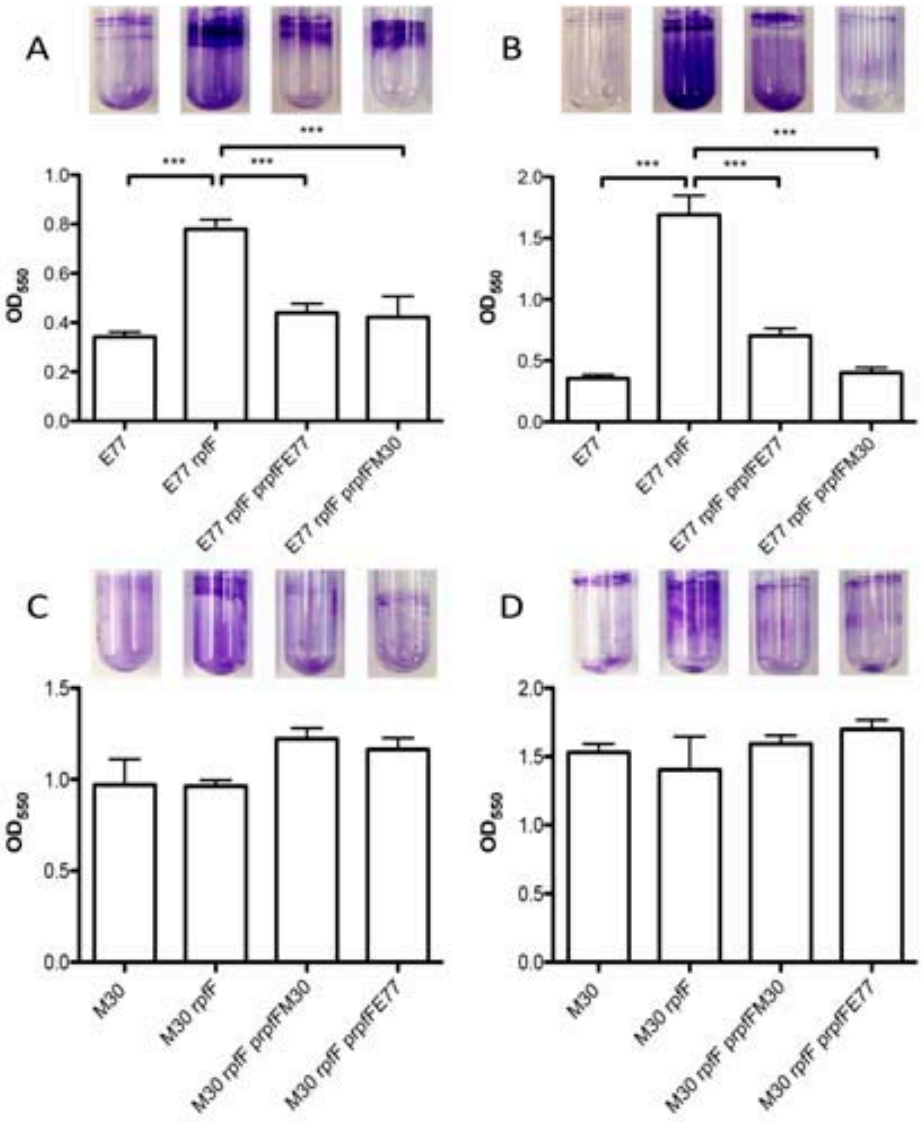

FIG 19 Biofilm formation of E77 and M30, their respective Δ*rpfF* mutants and the homologous and heterologous complemented strains on polystyrene (column bars graphics) or glass surface (test tubes photographs) in modified M9-salts (A and C) and BM2 (B and D) minimal media. ***, $P < 0.001$.

4.2.4 Only the Δ*rpfF*-1 mutant shows attenuation in *C. elegans*

To elucidate the direct implication of each RpfF variant in *S. maltophilia* virulence *in vivo*, the killing ability of E77 and M30, their respective Δ*rpfF* mutants and complemented strains were tested in *C. elegans*. Although both wild type E77 and M30 strains showed similar virulence capacity in the *C. elegans* model (LT50 or time of 50% death of 6.04 and 4.99 days respectively), significant attenuation was observed here for E77 Δ*rpfF* (Fig. 20). In line with the observations made for the phenotypes analysed previously, the virulence of E77 Δ*rpfF* is restored after complementation with either its own *rpfF* (RpfF-1 variant) or the M30 *rpfF* (RpfF-2 variant), indicating once more that E77 is able to respond to heterologous DSF production in a RpfC-1-variant background (Fig. 20). Infection with the M30 Δ *rpfF* mutant showed no significant differences relative to M30 wt.

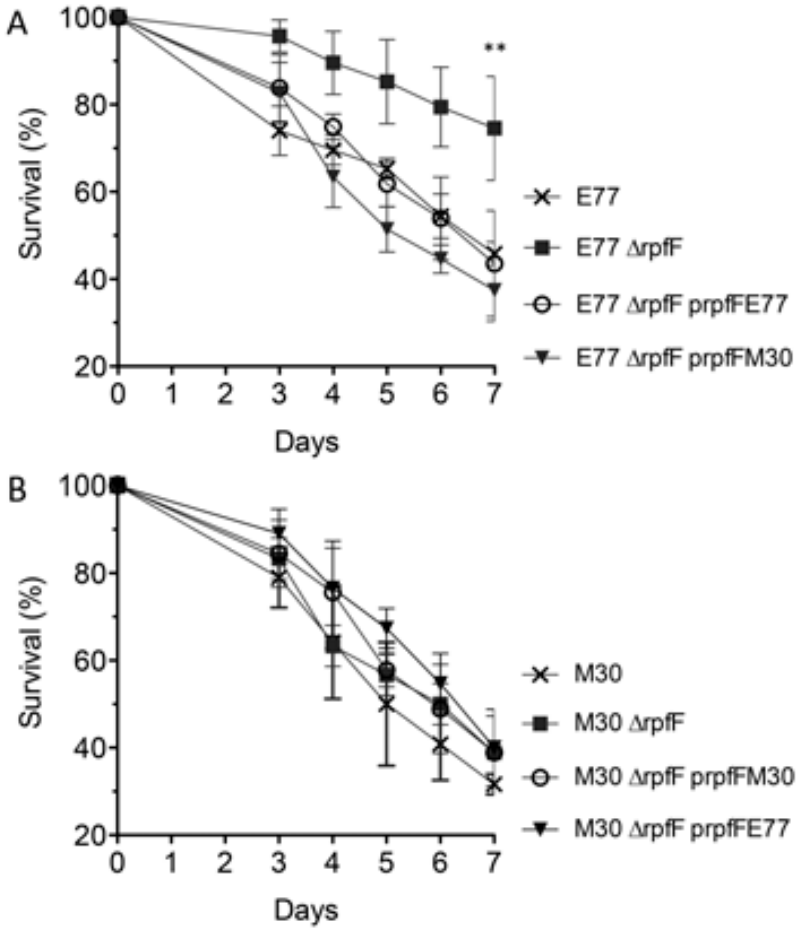

FIG 20 Virulence determination of E77 (A) and M30 (B), their respective Δ *rpfF* mutants and the homologous and heterologous complemented strains in the *C. elegans* CF512 model of infection. *E. coli* OP50 was used as negative control and showed no mortality within the firsts five days. **, $P < 0.05$.

4.2.5 Both Δ*rpfF* mutants show attenuation in the adult Zebrafish infection model

In order to corroborate that only Δ*rpfF*-1 mutant showed an attenuated phenotype in *in-vivo* models, both E77 and M30 strains with their Δ*rpfF*-1 and Δ*rpfF*-2 mutants and complemented strain were also evaluated in an adult zebrafish infection model. 12 fishes per sample were injected with a dose consisting of $5 \cdot 10^8$ cfu/mL and the mortality was periodically scored during five days. Surprisingly, and contrary to the previous results, in this animal model both Δ *rpfF*-1 and Δ *rpfF*-2 mutants showed attenuation compared to the wild type strains. In addition, when E77 Δ*rpfF* and M30 Δ*rpfF* were *in-trans* complemented with their respective *rpfF*, their virulence capacity was partially restored, indicating that in this animal model, the RpfF-2 is participating in the regulation of the virulence capacity (Fig. 21).

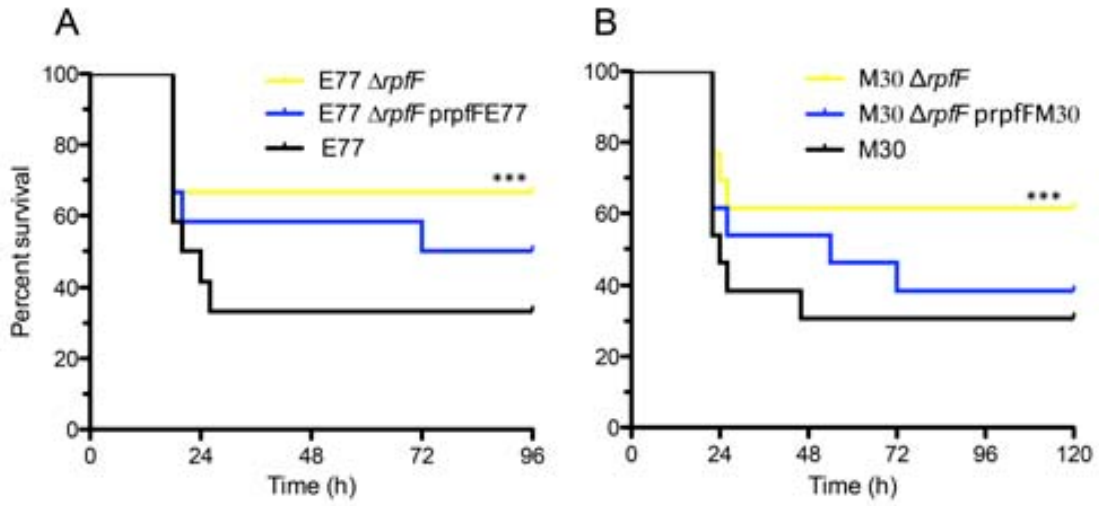

FIG 21 Virulence determination of *S. maltophilia* strains E77 (A) and M30 (B) with their respective Δ *rpfF* mutants and the complemented strains in the adult zebrafish model. ***, $P < 0.001$.

4.2.6 Attenuation of Δ*rpfF-1* mutant is due to its incapacity to disseminate throw the fish tissues

To get more insights into the dissemination capacity of the E77 strain during zebrafish infection, two fishes from each tank were sacrificed after 72 h post-injection and divided into three sections (anterior, abdominal and posterior) prior to CFU counting.

Interestingly, bacterial recovery showed the ability of E77 wt to disseminate through the fish body from the abdominal to the anterior and posterior regions. On the contrary, the

E77 Δ *rpfF* mutant does not seem to be able to colonize those regions effectively. Complementation of *rpfF* partially restores the body dissemination capacity (Fig. 22).

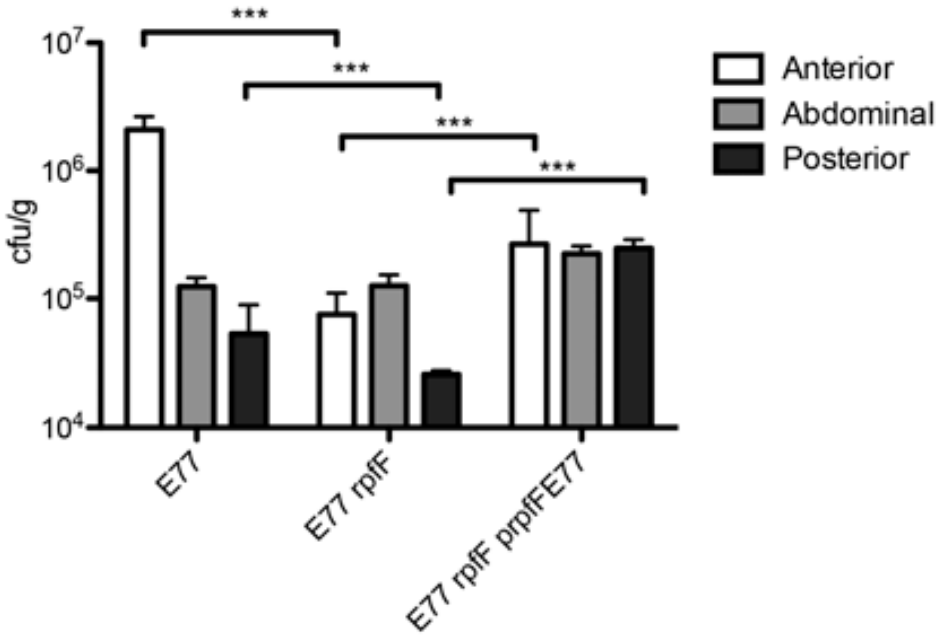

FIG 22 Bacteria recovery from different regions of the body of sacrificed fishes 72 h post infection with E77, its Δ*rpfF* mutant and complemented strains. ***, $P < 0.001$.

4.2.7 RpfF-1 and RpfF-2 strains act synergistically in virulence ability in the zebrafish infection model.

The evidence that both populations produce DSF in a positive feedback manner as well as not only RpfF-1 but also RpfF-2 regulate the virulence ability of *S. maltophilia,* pushed us to determine if in *in-vivo* model, both variant strains would act synergistically in the virulence capacity of the mixed population. Following this goal, we performed infection experiments in the adult Zebrafish model by injecting 16 fishes per sample, inoculationg an axenic inoculum ($5 \cdot 10^8$ cfu/mL) of E77, an axenic inoculum ($5 \cdot 10^8$ cfu/mL) of M30, and a mixed inoculum ($2.5 \cdot 10^8$ cfu/mL, each) of E77 and M30 (1:1) wild type and Δ *rpfF*-1:Δ*rpfF*-2. The obtained results showed that, when fishes were infected with the mixed 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% after 120 h post-injection (Fig. 23). Moreover, when fishes were infected with a mixed inoculum consisting of E77 Δ *rpfF*-1 and M30 Δ *rpfF*-2, they

showed a survival near to 40% after 120 hpi, demonstrating that RpfF is an essential trait for the full virulence capacity of *S. maltophilia* population (Fig. 23). Symptoms of disease as well as the fish behavior were also evaluated during the days of infection. Interestingly, fishes infected with the mixed wild type inoculum showed slightly more signs of disease compared to the other tanks, but showed a drastic change in their behaviour (spasms, compromised swimming and sudden death), especially between 24 and 48 h (data not shown).

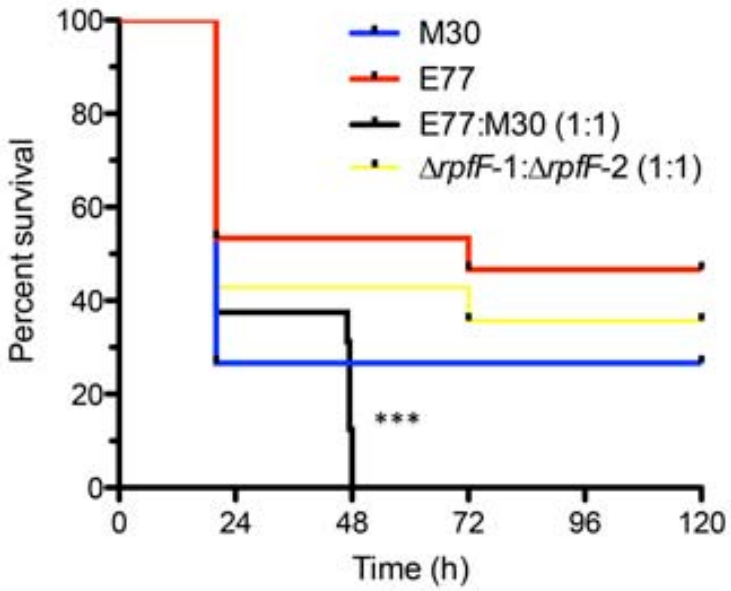

FIG 23 Virulence determination of E77, M30, a mixed inoculum of E77 and M30 (1:1) and a mixed inoculum of E77 Δ*rpfF*-1 and M30 Δ*rpfF*-2 (1:1) in the adult zebrafish model. ***, $P < 0.001$.

4.2.8 The full virulence capacity observed in the mixed inoculum correlates with in-vivo DSF production

In order to corroborate that both RpfF were active during the *in-vivo* infection process and also that they had an implication on the lethal virulence capacity observed in the fishes infected with the mixed inoculum, *in-situ* DSF quantification was done. Two fishes from each tank were sacrificed after 48 h post-injection and *in-situ* DSF production was determined (see Materials and Methods, section 3.8.4). Interestingly, the condition that showed the fulminant mortality (mixed inoculum of E77 and M30 wt) was the only condition that showed clearly DSF production in the *in-vivo* infection

environment (Fig. 24). Accordingly, the DSF production correlated with the virulence capacity observed in the fishes infected with the mixed inoculum.

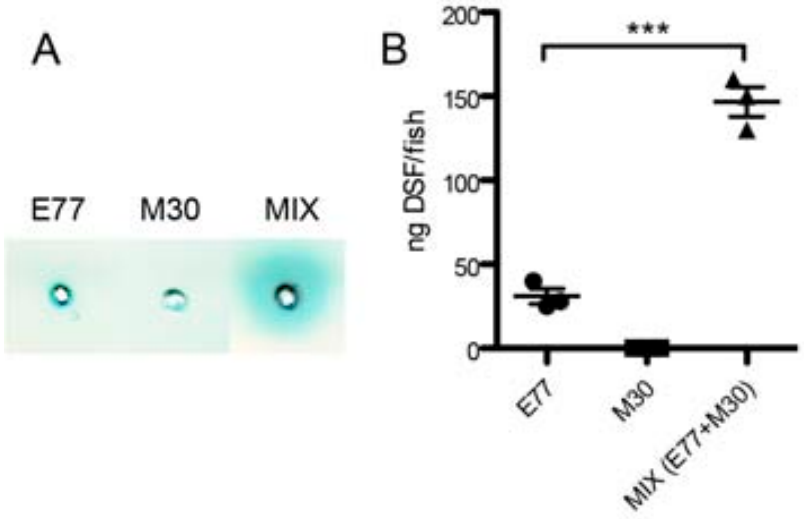

FIG 24 (A) Regular bioassay of DSF extracted from sacrificed fishes after 48 hpi with E77, M30 and the MIX inoculum (E77:M30, 1:1). (B) Quantification of DSF with the liquid bioassay, from the same samples. ***, $P < 0.001$.

4.2.9 Discussion

It is well known that the DSF-QS system regulates certain virulence traits in many bacteria (148, 179–185), including *Stenotrophomonas* (17, 65, 127). However, the regulation that each RpfF variant exerts on the physiology and pathogenicity of this bacterium remains uncertain. Specifically, in this chapter, we have focused into the implications of each RpfF variant into the regulation of virulence factors.

To test that, we have generated a Δ*rpfF* mutant for a strain representative of each variant group, i.e., E77 for the RpfF-1 variant group and M30 for the RpfF-2 one.

The E77 Δ*rpfF* mutant showed attenuation in both *C. elegans* (Fig. 20) and zebrafish (Fig. 21) infection models, proving that DSF-mediated regulation affects the virulence capacity of RpfC/F-1 strains. This is in line with the results reported for the K279a Δ*rpfF* (variant 1), which showed a loss of virulence in the *C. elegans* model (65). Moreover, the recovery of bacteria from sacrificed fishes 72 h post-injection

(intraperitoneal injection) showed that E77 is able to disseminate to anterior and posterior regions through the fish body, while E77 Δ *rpfF* was impaired in crossing intraperitoneal barriers (Fig. 22). This is in concordance with the results showing a loss of swarming motility (Fig. 17) and a drastic increment in the biofilm formation capacity (Fig. 19A, B) of the E77 Δ*rpfF* mutant, two important virulence-related traits that could be related to the virulence attenuation observed in the animal models.

We have studied for the first time the environmental factors that could modulate swarming motility in *S. maltophilia.* Although much work is needed to elucidate the exact conditions that influence this motility behaviour, we here propose the modified M9-salt medium 0.5% agar and 30ºC as a good approach to test swarming ability of *S. maltophilia* strains (Fig. 16). Many evidences of the implication of RpfF and DSF-like fatty acids in bacterial motility have been indeed reported (179, 181, 186, 187). Our results reinforce previous evidence that one of the main functions of DSF-QS is to regulate bacterial motility, especially in those carrying the RpfF-1 variant. Moreover, we have showed the implication of the RpfF-1 variant in surfactant production and cell morphology during swarming motility in *S. maltophilia* strain E77 (Fig. 18).

Many studies have also studied the implication of DSF-like molecules in biofilm regulation. There is however some controversy on whether DSF-like molecules may act stimulating or inhibiting the sessile or motile bacterial lifestyles. Thus, DSF molecules have been shown to positively regulate biofilm formation in *Xanthomonas oryzae* pv. *oryzae* (188), *Burkholderia cenocepacea* (181, 184) and *Xylella fastidiosa* (182, 189). On the contrary, in *Xanthomonas campestris* pv. *campestris* (*Xcc*) the DSF-mediated QS acts as a negative regulator of biofilm development (190–192). Additionally, fatty acid-mediated biofilm dispersion is not restricted to species sharing the DSF-QS system. For example, the fatty acid *cis*-2-decenoic produced by *P. aeruginosa* PAO1 stimulates biofilm dispersion in several gram-positive and gram-negative bacteria (193, 194). Our findings indicate that the DSF-QS system in *S. maltophilia* E77 has a similar regulatory function than that described for *Xcc,* where DSF also plays an important role in preventing biofilm formation and stimulating bacterial motility.

On the contrary, all virulence-related phenotypes evaluated on M30 were unaltered in the Δ *rpfF* mutant and in the corresponding complemented strain, suggesting that RpfC/F-2 variant strains may not use the DSF-QS system to regulate these virulence factors. However, while the M30 Δ *rpfF* mutant didn't show attenuation in the

C. elegans model, it certainly showed attenuation in the Zebrafish infection model (Fig. 21). It is evident that *C. elegans* and zebrafish models are totally different. While *C. elegans* is a very simple worm showing only innate immune response (195, 196), zebrafish is a vertebrate with a more developed immune system, which presents innate and adaptive immune responses (197). It is also known that the bacterial processes required for the infection as well as the inoculation in these two models are completely different. Together, it seems that under this environment, RpfC/F-2 variant strains could use the DSF-QS to regulate, somehow, its virulence ability.

In the chapter 4.2 it is reported that both RpfC/F-1 and RpfC/F-2 variant strains act synergistically in DSF production (Fig. 14). Here, we have evaluated the implication of this DSF synergism in the virulence ability of the entire population. Accordingly, the results strongly suggest that both populations act synergistically in the development of infection processes (Fig. 23) and also that the fulminant virulence ability is DSF-dependent (Fig. 24).

Together, our findings indicate, in one hand, that the RpfC/F-2 complex regulates virulence processes but only in very specific conditions (i. e. in Zebrafish infection). On the other hand, that both populations communicate each other, throw the DSF-QS, potentiating the DSF production and the virulence capacity of the whole population.

Since bacterial species sharing DSF-QS are almost ubiquitous and frequently share ecological niches, it is easy to think that RpfC/F-2 variant strains could often be in contact with DSF-producer bacteria (i. e., *Xcc* or *S. maltophilia* RpfC/F-1 variant). In this situation, a DSF-producer strain would act as a starter strain, triggering the reciprocal DSF-communication by synthesising the initial DSF molecules. It seems that RpfC/F-2 strains have evolved as a receptor group in this DSF communication, showing a lethargic DSF-deficient phenotype in axenic conditions, something that would render energetic saving as well as a more fine regulation of processes related to DSF-communication.

Furthermore, interspecies communication is not restricted to *Xanthomonadales*. Recently, It has been reported that the DSF molecule produced by *S. maltophilia* influenced biofilm formation of *P. aeruginosa* (198) Another study showed that the DSF-family signal C10:Δ2 (*cis*-DA) produced by *P. aeruginosa* not only dispersed its own biofilm but also induced the biofilm dispersion of numerous bacterial species including *E. coli, Klebsiella pneumoniae, Proteus mirabilis, Streptococcus pyogenes,*

Bacillus subtilis, and *Staphylococcus aureus* (142). In addition, BDSF from *B. cenocepacia* was found to be a functional substitute of the canonical DSF signal, and regulate biofilm formation and virulence factor production in *Xcc* (101). In this line, a novel signal 11-Me-C12:Δ2,5 identified from *B. multivorans* could also substitute DSF and C12:Δ2 and influence the biofilm development and virulence factor production in *Xcc* and *B. cenocepacia*, respectively (101). Interestingly, beside interspecies communication these mentioned DSF-derived signals (DSF, C12:Δ2 and 11-Me-C12:Δ,2,5) have been shown to inhibit the morphological transition of *Candida albicans* through interkingdom interference (100, 101, 104). Likewise, a fatty acid produced by the gram-positive *Streptococcus mutans* -*trans*-2-decenoic, designated SDSF- is also a potent inhibitor of morphological transition in *C. albicans* (103). Moreover, the signal C10:Δ2 produced for *P. aeruginosa* was found to be able to induce biofilm dispersion in *C. albicans*, as well as diverse bacteria (142).

Together with our results, it is evident that complex population processes, such as QS, are not well addressed when they are studied in axenic conditions.

4.3 *cis*-DA-mediated Quorum Sensing System in *S. maltophilia* and *P. aeruginosa*

New recent discoveries reported that the fatty acid *cis*-decenoic (*cis*-DA) is involved in biofilm dispersion in the important pathogen *P. aeruginosa* (142, 143). It has been shown that the putative enoyl-CoA hydratase (ECH) DspI (PA0745) is required for *cis*-DA synthesis (143), but also for the full virulence ability of *P. aeruginosa* strain PA14 (144). Interestingly, a preliminary sequence analysis revealed that *S. maltophilia* harbours a DspI orthologous, the putative ECH Smlt0266, displaying a significant identity with PA0745. In addition, we have recently reported that Smlt0266 is overexpressed in the *S. maltophilia* clinical-isolate M30 compared to the attenuated strain ATCC13637 (72). For this reason, in this chapter we will try to determine if this new *cis*-DA QS is also present and functional in *S. maltophilia*, taking M30 as a model strain.

4.3.1 *smlt0266* is the *dspI* orthologous in *S. maltophilia*

A preliminary genomic analysis using the Web-based comparative genomic tools (http://www.microbesonline.org) (199) revealed that in the genome of MPAO1, the gene encoding for the cis-DA synthase DspI (*PA0745*) (143) is organized into an operon, conformed by genes encoding for typical beta-oxidation components. Thus, *dspI* is flanked by upstream genes encoding for the putative aldehyde-dehydrogenase PA0747 and the hypothetical acyl-CoA dehydrogenase PA0746, and flanked by a downstream gene encoding for the putative enoyl-CoA hydratase/isomerase PA0744 (Fig. 25). Interestingly, a subsequent comparative genome analysis showed that the genome of *S. maltophilia* strain M30 harbours an orthologous operon composed by the genes *smlt0264*, *smlt0265*, *smlt0266* and *smlt0267* (Fig. 25). Protein blast analysis revealed a high homology between Smlt0266 and the *P. aeruginosa cis*-DA synthase DspI (68%) (Fig. 25). The elevated similarity observed between these components suggested that this operon likely regulate similar functions in both bacterial species. As mentioned, in *P. aeruginosa* the ECH DspI (PA0745) apart of being responsible for the cis-DA synthesis (143), it is also involved in many virulence-related processes (144). However, nothing is known about the alternative enoyl-CoA hydratase/isomerase PA0744, which displays significant similarity with the ECH Smlt0267 in *S. maltophilia*.

Since several synthases of fatty acids signalling molecules (i.e., RpfF/DSF) often present enoyl-CoA hydratase/isomerase activity, we wanted to study the role of these two ECH in the synthesis and regulation of the new lipid-mediated *cis*-DA QS in *S. maltophilia* and *P. aeruginosa*.

To do that, we have generated the *S. maltophilia* Δ*smlt0266,* Δ*smlt0267* and Δ*smlt0266-smlt0267* mutants and we have compared their phenotype with the respective *P. aeruginosa* orthologous mutants Δ*dspI* (Δ*PA0745*) and Δ*dspII* (Δ*PA0744*), obtained from the PAO1 transposon mutant library at the University of Washington (149).

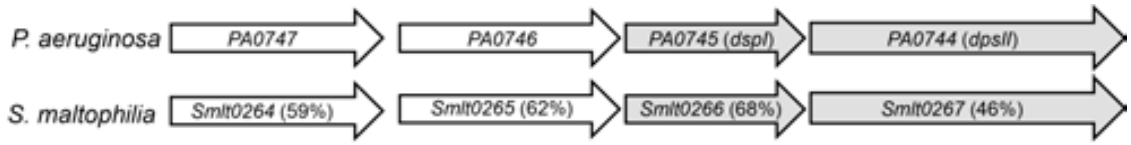

FIG 25 Comparison of the *dsp* operon between *P. aeruginosa* and *S. maltophilia.* Protein identity is indicated in percentage in brackets and grey arrows indicate the genes subject of this study.

4.3.2 Smlt0266 and Smlt0267 inversely regulate biofilm formation in *S. maltophilia*.

It is well known that DspI is involved in biofilm dispersion in *P. aeruginosa*, by synthesizing the dispersal inducer fatty acid *cis*-DA (143). To test if the orthologous gene *smlt0266* similarly regulates this behaviour in *S. maltophilia*, we have analysed the biofilm formation of the strains M30 wt, M30-derivative mutants Δ*smlt0266*, Δ*smlt0267* and the double mutant Δ*smlt0266-0267* and the M30 transformed with pBADSMdsp (Table 5). The later strain is the wt strain overexpressing the putative *cis*-DA synthase Smlt0266 under the control of the arabinose-inducible promoter (induced with arabinose at 0.2% concentration) (See Materials and Methods, section 3.6.3). As for *P. aeruginosa*, Smlt0266, but even more, Smlt0267, are directly involved in the biofilm formation capacity in *S. maltophila.* As shown in the Figure 26, deletion of the *smlt0266* gene led to a significant increase of biofilm formation. On the other hand, deletion of the *smlt0267* resulted in a drastic reduction of biofilm formation (Fig. 26), suggesting that these two components inversely regulate biofilm formation in *S. maltophilia.* However, while the absence of *smlt0266* resulted into a little increase of biofilm formation, deletion of *smlt0267* resulted into strongly reduced levels, almost abolition, of biofilm formation (Fig. 26).

To corroborate that the product of *smlt0266* was involved in biofilm dispersion, we overexpressed the orthologous *cis*-DA synthase Smlt0266 under the control of the arabinose-inducible promoter (M30 pBADSMdsp), resulting in a significant reduction of biofilm formation compared to the M30 wt strain (Fig. 26). Altogether, the results suggested that Smlt0267 could process the hypothetical dispersal fatty acid, preventing its accumulation and consequently the biofilm dispersion. Thus, when the alternative ECH Smlt0267 is absent (Δ*smlt0267*), the dispersal fatty acid accumulates and induces biofilm dispersion (Fig. 26).

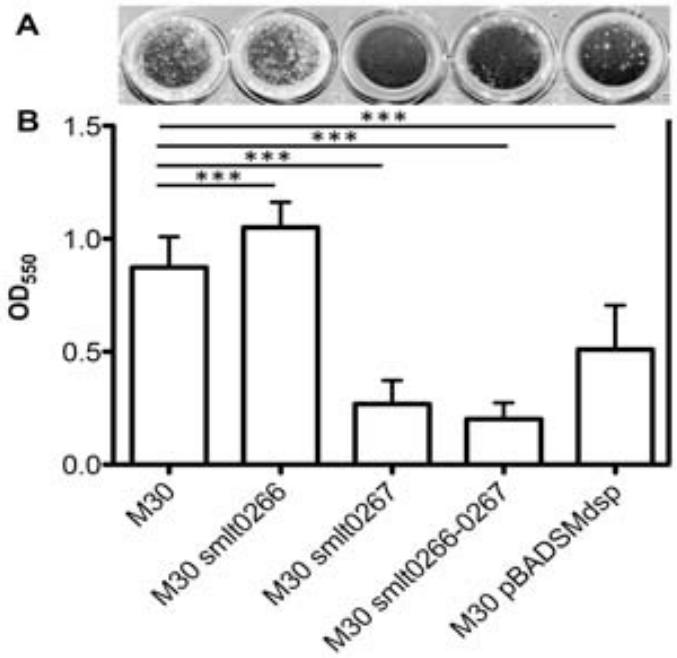

FIG 26 (A) Representative images of biofilm formation of *S. maltophilia* strain M30 wt, and the M30-derived mutants on a 24-wells plate after incubation in LB at 37ºC for 72 h. Wells were directly observed after washing three times with distilled water. (B) Quantification of the biofilm formation in 96-well plates under the same conditions, revealed using the crystal violet method and reading the absorbance at 550nm. ***, $P <$ 0,001.

4.3.3 Preliminary identification of *cis*-DA-like fatty acid produced by the DspI orthologous Smlt0266

Once we observed that overexpression of Smlt0266 resulted also in biofilm dispersion in *S. maltophilia*, we were challenged to identify the product of the mentioned ECH. Thus, we initially extracted the M30 culture supernatant grown at 37ºC in LB for 48 h,

using the ethyl acetate method (92). The concentrated dry samples were fractionated using HPLC and compared to fractions resulting from the synthetic *cis*-DA (Sigma) subjected to the same purification process. It is known that, in *P. aeruginosa*, *cis*-DA fatty acid is produced in very small amount. Accordingly, similar results were obtained from the supernatant purification of the wild type strain M30 (Fig. 27A). However, when the expression of Smlt0266 is induced (0,2% arabinose) an increased peak with the same retention time that synthetic *cis*-DA (min. 19) is observed in their supernatant chromatogram (Fig. 27B, C).

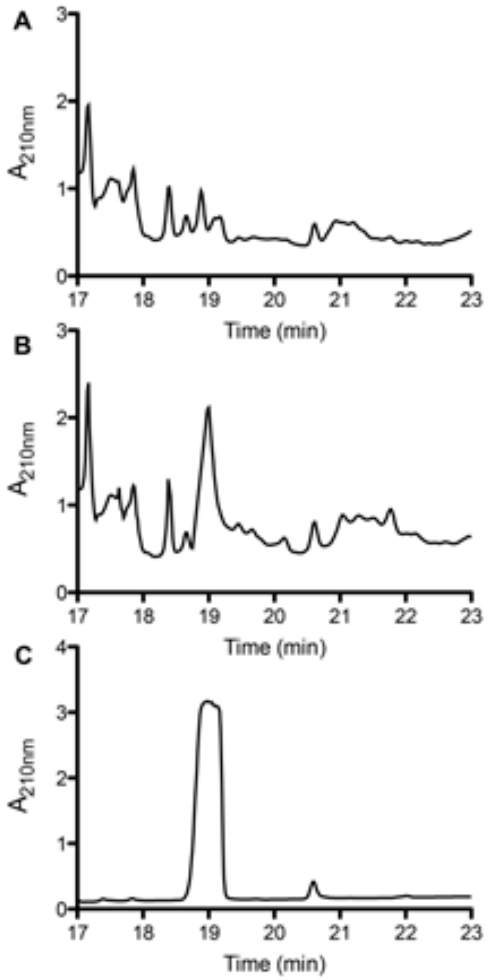

FIG 27 (A) HPLC chromatogram of purified M30 wt supernatant. (B) HPLC chromatogram of purified M30 pBADSMdsp supernatant after arabinose induction (0.2%) for 48 h at 37ºC. (C) HPLC chromatogram of purified synthetic *cis*-DA. Fractionation was performed using a C18 reverse-phase column (100 Å, pore size) (Phenomena) developed with a Methanol gradient 20-99 % in 30 minutes at a flow rate of 1 mL/min and monitored at 210 nm.

Together, the results indicate that, in one hand, the fatty acid *cis*-DA-like produced by *S. maltophilia* is structurally related, if not identical, to the synthetic *cis*-DA, since they showed the same retention time. In the other, that the production level of this signalling molecule is very low but seems to be functional, under the tested conditions.

4.3.4 Smlt0267 is involved in antibiotic resistance and virulence regulation.

Since fatty acid-mediated QS systems have emerged as a virulence regulation processes in many bacteria, we have also studied the implication of the both ECH Smlt0266 and Smlt0267 into the regulation of several virulence-related phenotypes. Thus, *S. maltophilia* Δ*smlt0266* and Δ*smlt0267* mutants have been evaluated for their ability in bacterial motility, antibiotic resistance and exopolysaccharides production. As mentioned before, the *cis*-DA synthase DspI has been shown to be involved in biofilm regulation and virulence (142–144), however, little is known about what phenotypic features makes Δ *dspI* (Δ*PA0745*, *smlt0266* orthologous) an attenuated strain in *P. aeruginosa* (144) and nothing is known about the implication of the alternative ECH *dspII* (*PA0744, smlt0267* orthologous) in such behaviour. Curiously, while deletion of *smlt0267* resulted into an alteration of all these phenotypes, none of them were significantly altered in the Δ*smlt0266* mutant. Hence, the Δ*smlt0267* mutant showed an alteration of exopolysaccharides production (observed as red spots on the top of the bacterial colony) and colony morphology (Fig. 28), an increased susceptibility to antibiotics, specially sulfamethoxazole and trimethoprim, (Table 16) as well as a reduction of swimming (Fig. 29) and twitching motility (Fig. 30). Swarming motility was not evaluated for the M30 strain, since it doesn't seem to be a swarmer strain (102).

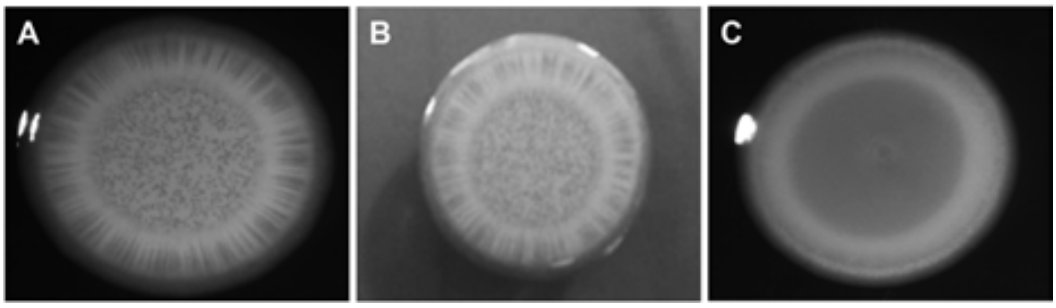

FIG 28 Representative images of the analysis of exopolysaccharide production and colony morphology of *S. maltophilia* strains M30 (A), Δ*smlt0266* (B) and Δ*smlt0267* (C) mutant, seeded in Congo Red plates and incubated at 25ºC for 72 h.

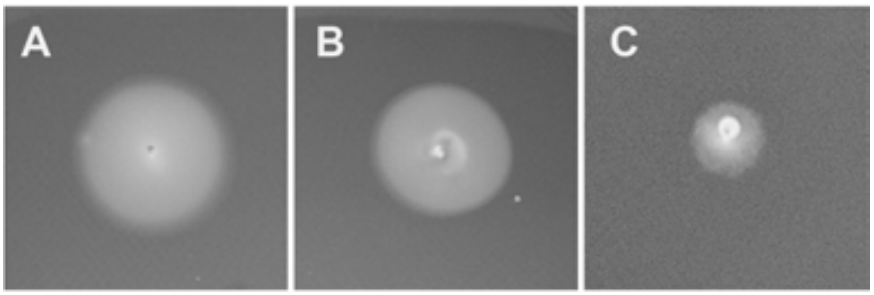

FIG 29 Representative images of swimming motility assay of *S. maltophilia* strains M30 wt (A) and the mutants Δ*smlt0266* (B) and Δ*smlt0267* (C), seeded in Tra medium solidified with 0.25% agar.

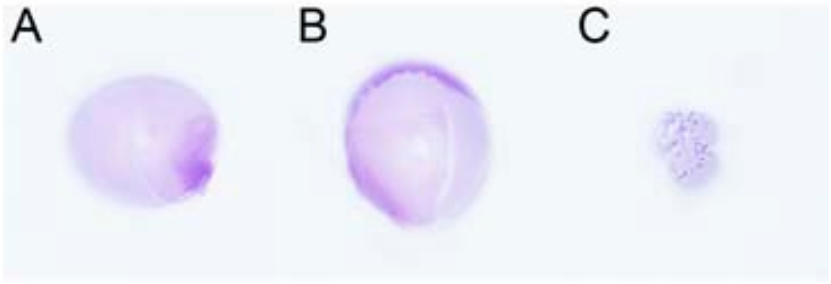

FIG 30 Representative images of twitching motility assay of *S. maltophilia* strains M30 wt (A) and the mutants Δ *smlt0266* (B) and Δ *smlt0267* (C) seeded in LB medium solidified with 1% agar, incubated at 30ºC for 24 h. After removing the agar medium, plates were revealed with crystal violet 0.1%.

TABLE 16 Antibiotic susceptibilities of *S. maltophilia* wild type strain M30 and the Δ*smlt0266* and Δ*smlt0267* mutants.

	MIC (μg/ml) for strains		
Antibiotics	M30	Δ*smlt0266*	Δ*smlt0267*
Tetracyclines			
Tetracycline	12.08	12.08	6.04
Minocycline	0.13	0.13	0.13
Aminoglicosides			
Gentamicine	12.08	12.08	12.08
Kanamicine	128	128	128
Sulfamides			
Sulfamethoxazole	256	256	8
Cotrimoxazole	16	16	4
Quinolones			
Ciprofloxacin	0.08	0.08	0.04
Norfloxacin	2	2	1
Levofloxacin	0.04	0.04	0.02
Chloramphenicol	6.04	6.04	6.04
Trimethoprim	4	4	2

R: Resistant, harbouring the *Erm-resistance cassette.*
MIC: Minimal inhibitory concentration determined by micro-titre assay.

4.3.5 Δ*smlt0267* but not Δ*smlt0266* mutant showed attenuation in the *C. elegans* model.

C. elegans strain CF512 was used as a model of infection to determine the implication of both ECH into *in-vivo* virulence regulation. In line with the observations made for the virulence-related phenotypes, only the Δ *smlt0267* showed diminution of virulence capacity in this model of infection (Fig. 31). This is in concordance with the results obtained in bacterial motility and biofilm formation, two clearly related virulence factors.

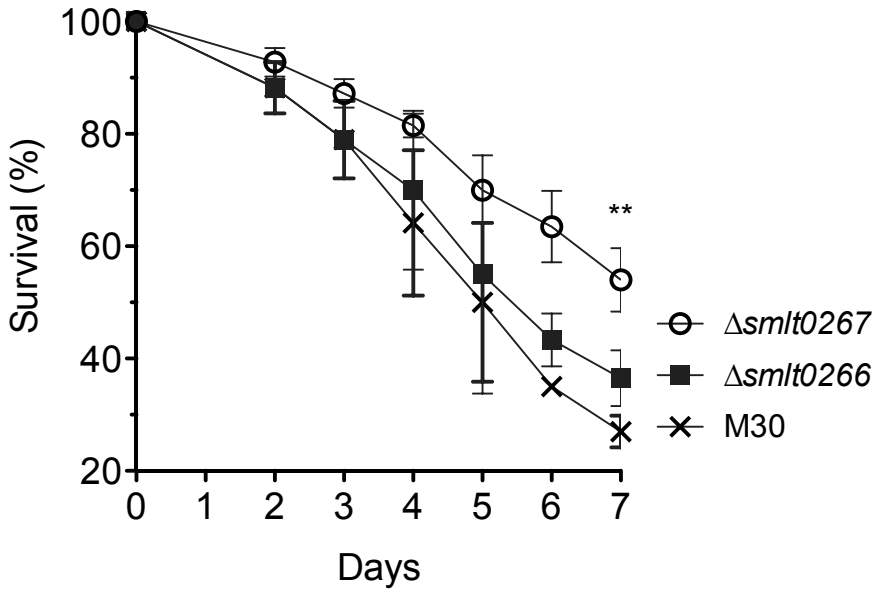

FIG 31 Virulence determination of *S. maltophilia* wild type strain M30 and the Δ*smlt0266* and Δ*smlt0267* mutants in the *C. elegans* CF512 model of infection. **, $P < 0.05$.

4.3.6 *P. aeruginosa* DspI and DspII inversely regulate biofilm formation as occurs in *S. maltophilia*

It has been reported that mutation of the synthase *dspI* (*PA0745*) resulted in reduced biofilm dispersion (143). We here have quantified the biofilm formation of the *P. aeruginosa* wt. strain MPAO1 and the mutant strains for the orthologous genes *dspI* (*PA0745*) and *dspII* (*PA0744*). We have compared these results with the obtained for *S. maltophilia* and with those reported in the literature. Thus, our results reinforce these previous evidences since the Δ*dspI* mutant showed a significant increment in biofilm formation compared to the wild type strain (Fig. 32), proving that the absence of the signalling molecule *cis*-DA lead to potentiate the cell adherence to polystyrene surface in *P. aeruginosa*. In addition, as happened in *S. maltophilia*, mutation of *dspII* (*PA0744*) resulted into a significant reduction of biofilm formation suggesting that both ECH DspI and DspII inversely regulate this behaviour (Fig. 32). Nevertheless, complementation of the Δ*dspI* mutant with the whole operon (dspI pPAdsp) did not restore to wild type levels its biofilm formation likely because the presence of the component *dspII* might process the dispersal fatty acid impeding the biofilm dispersion. Contrary, complementation of the Δ*dspII* with the whole operon (dspII pPAdsp) did restore its

biofilm formation to the wild type levels, perhaps due to the same phenomenon. This suggests that the alternative ECH DspII could play an important role in the processing of the *cis*-DA signal and consequently in the prevention of biofilm dispersion.

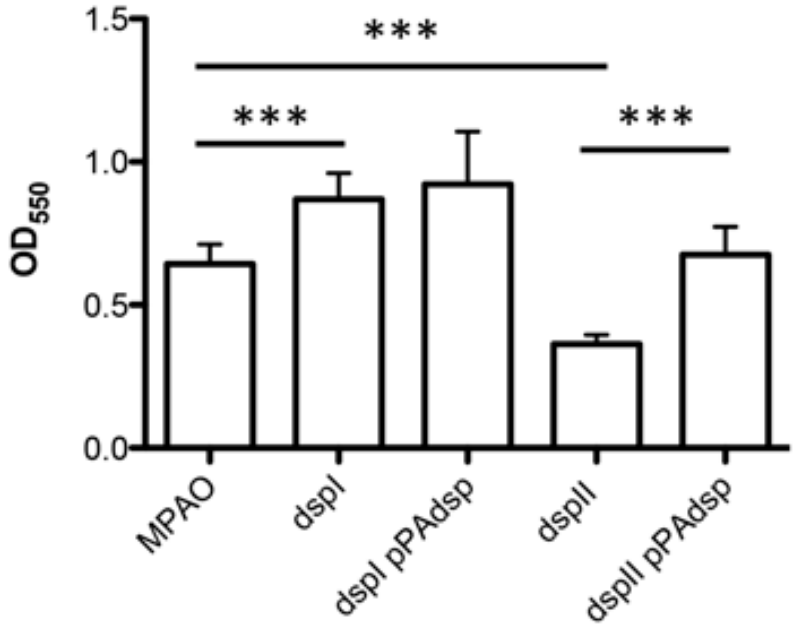

FIG 32 Biofilm formation of *P. aeruginosa* MPAO1 wt, the Δ*dspI* and Δ*dspII* mutants and the complemented strains on polystyrene surface, grown in LB medium at 37ºC for 24 h. ***, $P < 0{,}001$.

4.3.7 *P. aeruginosa* DspI and DspII regulate virulence-related phenotypes similarly to *S. maltophilia* Smlt0266 and Smlt0267

We have evaluated the same virulence-related phenotypes that were altered in the Δ*smlt0266* and Δ*smlt0267* mutants in *S. maltophilia*. These included, bacterial motility (swimming, swarming and twitching), exopolysaccharide production and colony morphology as well as the susceptibility to the same antibiotics that were tested for the *S. maltophilia* mutants. Interestingly, the Δ *dspI* and Δ*dspII* mutants showed an alteration of its motility as well (Fig. 33, 34), indicating that *cis*-DA system is also involved in the control of the population motility. Particularly, the Δ*dspII* was shown to have an alteration of swarming (Fig. 33) and twitching motility (Fig. 34), whereas the Δ*dspI* did not show alteration in these motilities compared to the parental strain MPAO1 (Fig. 33, 34). The alteration in the swarming motility observed for the Δ*dspII* mutant consisted in a drastic change of its morphology and the absence of pigmentation.

While the wild type strain MPAO1 and the Δ*dspI* mutant showed the characteristic solar swarming morphology typically observed in the strain PAO1 (200), the Δ*dspII* showed an atypical dendritic-like morphology (Fig. 33), which is commonly observed in other *P. aeruginosa* strains, i.e., strain PA14 (200). These results are in concordance with the obtained in the biofilm formation, since both phenotypes are commonly inversely regulated (135). It has been reported that the reduction of biofilm formation is usually linked to an increment of swarming motility (135, 201).

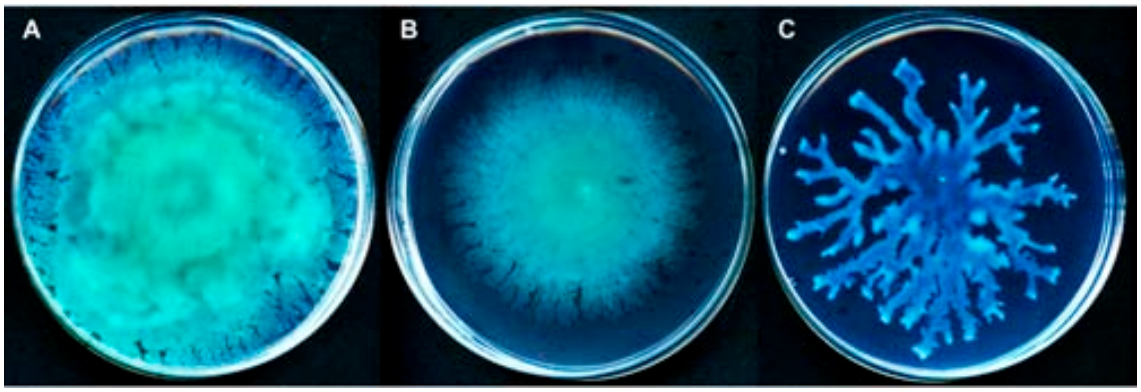

FIG 33 Representative images of swarming motility of *P. aeruginosa* wild type strain MPAO1 (A) and the Δ*dspI* (B) and Δ*dspII* (C) mutants, seeded in modified M9-salts medium solidified with 0.5% noble agar and incubated for 16 h at 37ºC.

Regarding to the twitching motility, similar results to *S. maltophilia* analysis were obtained for the *P. aeruginosa* mutants. Thus, the only strain that showed an alteration, particularly a reduction, was again the Δ*dspII* mutant (Fig. 34), corroborating that bacterial motility is under the control of the components that comprise this new *cis*-DA system. On the other hand, no differences were observed in the swimming motility comparing the wild type strain MPAO1 with the Δ*dspI* and Δ*dspII* mutants (data not shown).

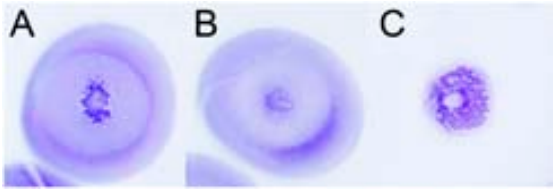

FIG 34 Representative images of twitching motility of *P. aeruginosa* wild type strain MPAO1 (A) and the Δ*dspI* (B) and Δ*dspII* (C) mutants, seeded in LB medium solidified with 1% agar and incubated for 24 h at 37ºC.

Similar results were obtained in the exopolysaccharide and colony morphology assay, in which only the Δ*dspII* mutant showed clearly alteration of this phenotype (Fig. 35). In addition, the Δ*dspII* mutant showed a kind of swarming motility on Congo Red plates (0.5% agar) (Fig. 35), corroborating that this motility behaviour was up regulated in this mutant strain. However contrary to the results obtained for the *S. maltophilia* mutants, the Δ*dspI* and Δ*dspII* showed the same antibiotic resistance profiles to that obtained for the MPAO1 wild type strain (data not shown). Taking all the results together, it is evident that these two ECH play an important role in the regulation of several commonly QS-related virulence phenotypes in both bacterial species. Moreover, it seems that the alternative ECHs DspII and Smlt0267 take more prominence in the virulence factor regulation than the synthases DspI and Smlt0266.

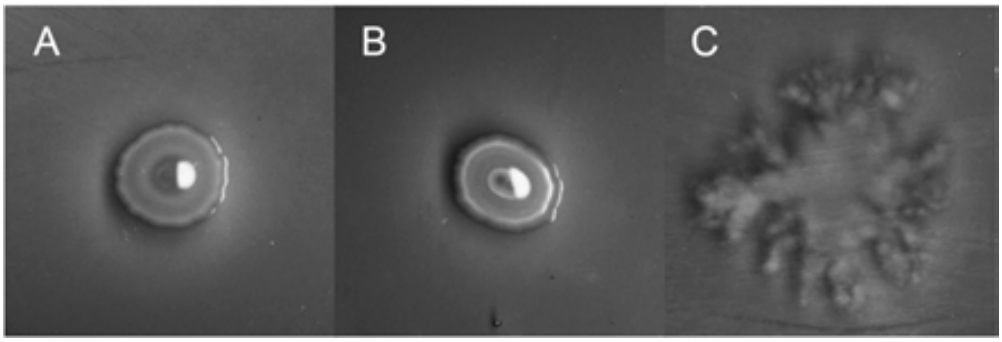

FIG 35 Representative images of the analysis of exopolysaccharide production and colony morphology of *P. aeruginosa* strains MPAO1 (A), Δ*dspI* (B) and Δ*dspII* (C) mutants, seeded in Congo Red plates and incubated at 25ºC for 72 h.

4.3.8 *P. aeruginosa* Δ*dspI* and Δ*dspII* showed attenuation in the *C. elegans* model

It has been reported that the mutation of *dspI* (*PA0745*) lead to an attenuation of virulence in *P. aeruginosa* (144). Therefore, we here have corroborated this in the MPAO1 strain, since the Δ*dspI* showed reduction of its virulence ability (Fig. 36). Interestingly, the Δ*dspII* mutant showed even more attenuation than Δ*dspI* (Fig. 36). This is in line with all the previously obtained results, since the Δ*dspII* (Δ*PA0744*) showed more drastic alteration of its virulence phenotype, which lead to a stronger attenuation compared to the Δ*dspI* mutant in the in-vivo model of infection. These results are also similar to that obtained for the mutants of the orthologous genes in *S. maltophilia* which further validate the implication of these two ECH in virulence regulation in both bacterial species.

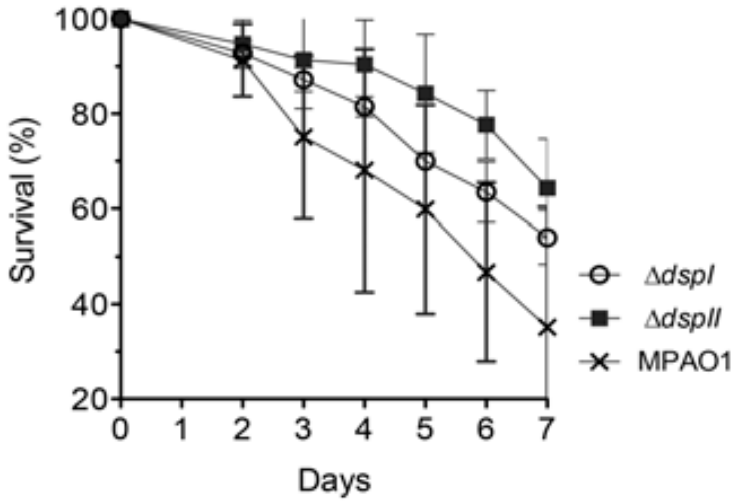

FIG 36 Virulence determination of *P. aeruginosa* wild type strain MPAO1 and the Δ*dspI* and Δ*dspII* mutants in the *C. elegans* CF512 model of infection. E. coli OP50 was used as negative control and showed no mortality within the first five days (data not shown).

4.3.9 Discussion

As mentioned in the introduction, the signalling network of *P. aeruginosa* is probably the most complex systems reported among bacteria. Although in this particular bacterium there coexist various QS-systems (e.g., AHL, PQS, PVD, among others) in this chapter, we have focused on the new *cis*-DA system, described for the first time few years ago in the laboratory-associated strain MPAO1 (142).

The *cis*-DA system has aroused attention recently, since this new small fatty acid produced by *P. aeruginosa* has been show to stimulate biofilm dispersion in several gram-negative and gram-positive bacteria (142). Inspired in the DSF-QS system described in the related bacteria *Xanthomonas*, *Stenotrophomonas*, *Burkholderia* and *Xylella*, the *cis*-DA has emerged as a new fatty acid-mediated communication system that control not only biofilm development, but also virulence in the *C. elegans* model (144). However, little is known about the molecular mechanisms involved in the synthesis and perception of the *cis*-DA molecule as well as which other physiological processes are under the regulation of this new QS system.

The identification of the homologue of the *cis*-DA synthase in *S. maltophilia* was not the only motivation that pushed us to investigate this new QS system in *S. maltophilia*, since in a recent work we reported that the ECH Smlt0266 is overexpressed (3.1-fold) in the *S. maltophilia* clinical-isolate M30 compared to the attenuated strain ATCC13637 (72). Moreover, we have also recently shown that a new *rpf-2* variant cluster govern the DSF system in the *S. maltophilia* strain M30 (102), which appears to regulate only some virulence related processes in a very specific environment (See section 4.2). For all that, we have studied the *cis*-DA QS system in *S. maltophilia* and we have compared the obtained results to those reported in the literature and also to the obtained in our laboratory for the *P. aeruginosa* MPAO1 wt and mutant-derived strains.

To do that, we have generated the mutants Δ*smlt0266,* Δ*smlt02667* and Δ*smlt0266-0267* in *S. maltophilia* and we have requested the *P. aeruginosa* Δ*dspI* (Δ*PA0745*) and Δ*dspII* (Δ*PA0744*) mutants to the PAO1 transposon mutant library at the University of Washington (202). We have evaluated the implication of these ECH into the virulence regulation in *S. maltophilia* and *P. aeruginosa*, respectively.

As happens in *P. aeruginosa*, overexpression of Smlt0266 also reduced the biofilm formation in *S. maltophilia* (Fig. 26), likely to the production of a related *cis*-DA fatty acid (Fig. 27). Nevertheless, more studies are needed to conclude the exact structure of the dispersal fatty acid produced by *S. maltophilia.* In the case of *P. aeruginosa*, our results corroborate those reported in the literature, since the Δ*dspI* (Δ*PA0745*) showed a significant increment of biofilm formation, due to the absence of the dispersal *cis*-DA molecule (Fig. 32).

Furthermore, a new interesting target has emerged from the study reported in this chapter, the enoyl-CoA hydratase Smlt0267 for *S. maltophilia* and its respective orthologous DspII (PA0744) for *P. aeruginosa*. Although its exact function remains unclear, we here have demonstrated the implication of this ECH into the regulation of more virulence factors than DspI (PA0745, for *P. aeruginosa*) and Smlt0266 (for *S. maltophilia*). Taking all together, the alternative ECH PA0744/Smlt0267 would represent a new and promising target for the design of new antibiotics in the pathogens *S. maltophilia* and *P. aeruginosa*. Moreover, PA0744 seems to be widely distributed among gram-negative bacteria, with homologues in several gram-negative species including *Chromobacterium violaceum, Pseudoxanthomonas spadix, Achromobacter xylosoxidans, Bordetalla bronchiseptica, Bordetella parapertussis, Xanthomonas*

campestris, Xanthomonas axonopodis and Ralstonia eutropha, among much other, as predicted by nucleotide BLAST tool (203), which makes this target even more interesting.

It is well known that *S. maltophilia* and *P. aeruginosa* are capable of forming biofilm easily, which complicates the treatment of colonized patients. Thus, the study of the molecular aspects that potentiates the biofilm formation, and more interestingly the biofilm dispersion, is mandatory to develop new treatments against infections caused by these two important pathogens.

P. aeruginosa is perhaps one of the most studied gram-negative bacteria and many virulence-traits that this bacterium exploits during infection process have been elucidated. Discovery of new genes and processes involved in the virulence regulation of this bacterium is therefore an arduous work. In a published high-throughput study for the identification of all the *P. aeruginosa* genes involved in virulence in the *C. elegans* model, the authors reported that the mutant Δ*PA0745* was one of the most attenuated strains (144). In a recent work, designed to identify the *cis*-DA synthase, all the annotated putative ECH were evaluated for the synthesis of the *cis*-DA signalling molecule with the subsequent biofilm dispersion, finally identifying PA0745 (DspI) as the synthase (143). In any of these reports, the alternative ECH PA0744 was included in the study. Both mentioned works were performed using the other well-studied *P. aeruginosa* strain PA14. Although in both cases authors also requested the required mutants in the transposon mutant library of University of Washington (202), in this strain, the mutant Δ*PA0744* is not available. It is evident that, since this mutant is available for the transposon library of MPAO1, this particular gene doesn't have vital function is *P. aeruginosa*.

Accordingly, we here report for the first time the role of the ECHs PA0744/Smlt0267 in the virulence regulation of *P. aeruginosa* and *S. maltophilia*, and propose a new and promising target to develop new strategies against bacterial infections caused by these two important human pathogens.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

5. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In this work, we have investigated two quorum-sensing systems mediated by fatty acid molecules in *S. maltophilia* and *P. aeruginosa* species.

A fatty acid (FA) is a carboxylic acid with an aliphatic tail (chain), which could be either saturated or unsaturated -those that present carbon–carbon double bonds-. In nature, FAs generally have an even number of carbon atoms, from 4 to 28. FAs are usually conforming triglycerides or phospholipids, however, when they are not attached to these molecules, they are termed "free" fatty acids. Usually, FAs are categorized depending on the chain length, as follows. Short-chain fatty acids (SCFA) are fatty acids with aliphatic tails of fewer than six carbons. Medium-chain fatty acids (MCFA) are fatty acids with aliphatic tails of 6–12 carbons. Long-chain fatty acids (LCFA) are fatty acids with aliphatic tails 13 to 21 carbons. Very long chain fatty acids (VLCFA) are fatty acids with aliphatic tails longer than 22 carbons.

FAs play three typical roles within biological systems. These include energy storage, phospholipid membrane formation, and signalling pathways.

Catabolism of FAs occurs by a pathway called β-oxidation and yields more adenosine triphosphate (ATP) per gram than carbohydrates or proteins. The oxidation process of fatty acids to obtain energy typically occurs in four steps:

1- Dehydrogenation by acyl-CoA dehydrogenase, yielding 1 FADH2.

2- Hydration by enoyl-CoA hydratase.

3- Dehydrogenation by 3-hydroxyacyl-CoA dehydrogenase, yielding 1 NADH.

4- Cleavage by thiolase, yielding 1 acetyl-CoA and a fatty acid that has now been shortened by 2 carbons (acyl-CoA).

This cycle repeats until the fatty acid has been completely reduced to acetyl-CoA or, in the case of fatty acids with odd numbers of carbon atoms, acetyl-CoA and 1 molecule of propionyl-CoA per molecule of fatty acid.

Fatty acids have also an important structural function in the cell, since they are essential in the formation of the membrane phospholipids. The organization of a gram-negative bacterial cell wall consist of an outer membrane and an inner membrane, separated by a region known as the periplasmic space, which has very important functions in the

survival and operation of the cell. Bacterial membranes are mainly composed of different phospholipids, which consist of a hydrophilic head and a hydrophobic tail. The major phospholipids in *E. coli* include the zwitterionic phosphatidylethanolamine (PE, 70% of the membrane), the anionic phosphatidylglycerol (PG, 20%), and cardiolipin (CL, 10%) (204).

Polyunsaturated fatty acids (e.g., 18:3 ω3c) are found in plants, algae and cyanobacteria, but are often not present in bacteria. Monounsaturated fatty acids (particularly at the omega-7 position), odd-chain saturated fatty acids (e.g., 15:0), branched-chain fatty acids (mainly iso/anetiso and 10-methyl) and cyclopropane fatty acids (e.g., 19:0 cyclo ω7c) are mostly synthesized by bacteria.

The third function described for fatty acid is cellular signalling. Prostaglandins were the first fatty acid-derived molecules to be discovered as signalling molecules in eukaryotic organisms (205). In bacteria, cellular fatty acid-mediated communication has been deeply studied in the last years. As mentioned along this work, quorum-sensing systems mediated by fatty acid molecules have been shown to regulate several virulence-related biological functions especially in *Xhantomonadales* species (206). This is the case of the DSF quorum sensing, which is indeed the main research line that I have been developing during my thesis.

During this PhD thesis period we have been able to elucidate that, in *S. maltophilia* there exist two populations that differentially control the DSF-QS (Sections 4.1 and 4.2). These two *S. maltophilia* groups -here designated *rpf*-1 and *rpf-2* variant groups- differentiate each other basically in the molecular mechanisms responsible of synthesize and sense DSF molecules. Taking one model strain from each group, we have also observed that the DSF-QS could also differentially regulate virulent processes between the two populations, as a consequence of the variance in the molecular regulation.

Since this is the first time that two different mechanisms govern the DSF-QS in same specie, many questions arise. It is important to know the frequencies found for each DSF-QS variant in the population of clinical *S. maltophilia* isolates. Taking two recent *S. malotphilia* population studies (102, 128) from a total of 171 isolates (mainly clinical strains), *rpf-1* variant have been more commonly isolated with ca. 60% of representation. In concordance with the results published for the model organism *Xcc*, we here have observed similar traits in terms of DSF production and detection, as well as a comparable biological regulation between *S. maltophilia rpf-1* variant strains and

Xcc. It has been reported that in *Xcc*, the DSF-QS regulates several virulence-related behaviours such as bacterial motility, biofilm development and virulence (91), something that we have also observed in *S. maltophilia rpf-1* variant strains (Section 4.1 and 4.2). Moreover, we have studied the conditions that modulate DSF production in *S. maltophilia*.

Regarding the *rpf-1* variant strains, we have observed that the temperature and the media composition are important factors involved in the DSF synthesis. In addition, we have observed that some medium and long-chain fatty acids are capable of stimulate DSF production, suggesting that the sensor RpfC-1 (which contains ten TMR in its input domain) is more promiscuous enabling free-active RpfF-1 to synthesize DSF upon detection not only DSF molecules but also 12 to 14-carbon length fatty acids (Section 4.1). Additionally, we have analysed the total cellular fatty acid in *S. maltophilia*. As shown in the Table 15 (Section 4.1), the most abundant fatty acid in *S. maltophilia* (≈30%) is 13-methyl-tetradecanoic (15:0 iso) a highly related DSF fatty acid. Although it needs to be verified, we postulate that 15:0 iso takes part in the conformation of the cell membrane phospholipids, being one of the two nonpolar "legs". Interestingly, it has been reported that in several *Xanthomonas* species (including *Xcc, Xoo* and *Xac*, among many others), the most abundant fatty acid is also 15:0 iso (207). Curiously, iso-branched fatty acids have been found to be common in gram-positive but unusual in gram-negative bacteria. Thereby, I think it is remarkable that 15:0 iso is present in these well-established DSF producer bacteria such as *Xanthomonas* sp. and *S. maltophilia*, whereas it is not present in non DSF-producer bacteria such as *P. aeruginosa* and *E. coli* (Data not shown, unpublished results).

There are three aspects that make me think that DSF communication appears as a consequence of the membrane homeostasis regulation. The first one is that 15:0 iso was the most stimulating fatty acid tested in DSF production in *S. maltophilia.*

The second one is that the upstream gene of *rpfF* the long-chain fatty acyl CoA ligase *rpfB*, is indeed annotated as *fadD.* Hence, RpfB displays 60% identity with the *E. coli* FadD. Interestingly, a recent report shows that, in *Sinorhizobium meliloti,* FadD plays a major role in the activation of endogenous fatty acids released from membrane lipids during stationary phase of growth (208). In this line, we hypothesize that RpfB could be responsible of conferring the precursor15:0 iso to RpfF to synthesize DSF.

The third one relies in that the sensor RpfC -which is expected to sense diffusible molecules- is anchored in the membrane. It doesn't make sense that a molecule able to diffuse the cell membrane needs a membrane receptor to be detected.
Thus, I think that the sensor RpfC senses possible alterations on the cell membrane stability and liberate RpfF that -jointly with RpfB- synthesize DSF from the membrane precursor 15:0 iso fatty acid. However, it is evident that extensive work is needed to validate such hypothesis.

On the other hand, the DSF-QS regulation is uncertain and seems to be particular in the *S. maltophilia rpf-2* population. DSF production was never detected in the *rpf-2* axenic cultures. However, we have showed that *rpf-2* variant strains are able to synthesize DSF only upon detection of DSF-itself, which indicates that DSF is produced in a positive feedback-manner in *S. maltophilia rpf-2* strains. This also suggests that the sensor RpfC-2 (containing only five TMR) is much more specific only allowing the dissociation of RpfF-2 when sensing DSF. It has been reported that in the related bacterium *Xylella fastidiosa* (which harbours a similar *rpf-2* cluster), the synthase RpfF is also participating in DSF perception, something that might also be occurring in these *S. maltophilia* variant strains.
Regarding the biological regulation, from all the phenotypes evaluated for the Δ*rpfF-2* mutant, only virulence attenuation in the zebrafish model was observed. Furthermore, we have also showed that there exist a synergism in DSF production and virulence ability between both *S. maltophilia* groups. Considering that *rpf-2* variant strains usually cohabit with DSF-producer bacteria (e.g., *Xanthomonadales*) it is easy to think that *rpf-2* triggers the DSF regulation upon detection of DSF molecules produced by neighbour bacteria.
Consequently, these results have revoked our initial hypothesis, which was that the DSF-QS was permanently switched off in this variant group. Although we have not been able to elucidate how, it is evident that DSF-QS regulate biological functions in this important *S. maltophilia* group. A hypothetic model of DSF regulation for each *S. maltophilia* variant group is proposed in the figure v.

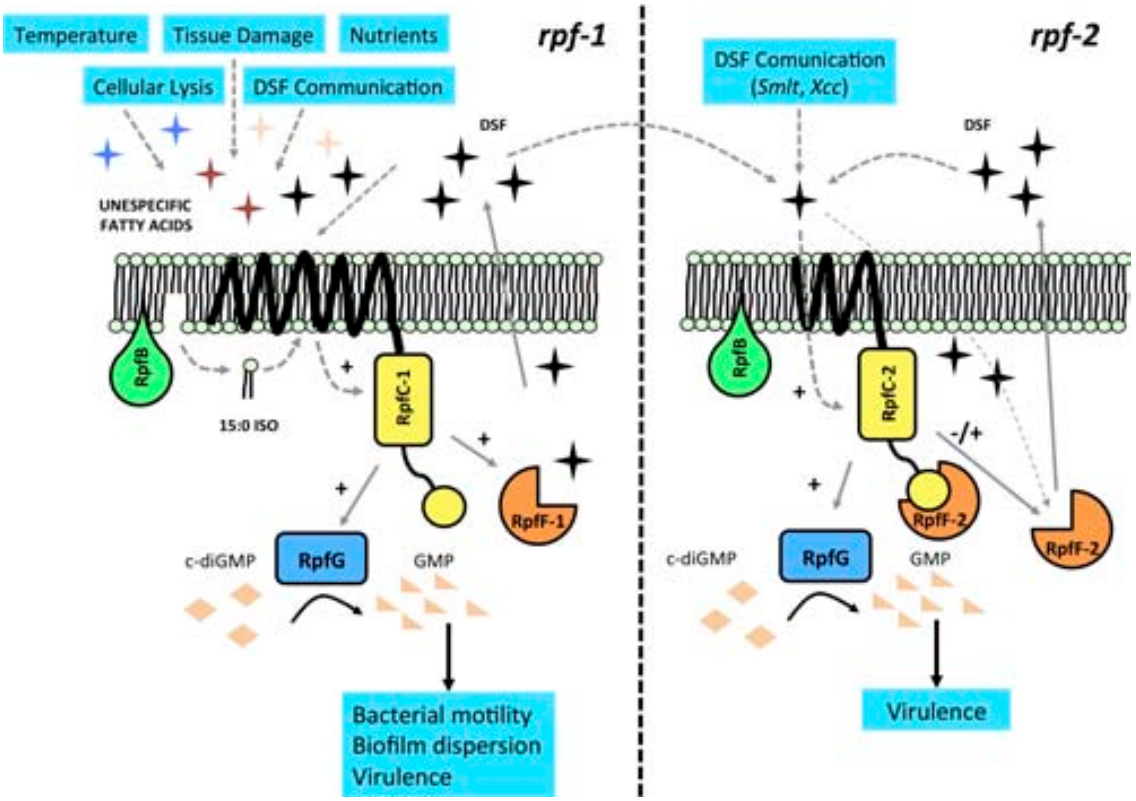

FIG v Schematic model of DSF-QS network proposed for both *S. maltophilia rpf-1* (left) and *rpf-2* (right) variant strains. (*rpf-1)* RpfC-1 (containing 10 TMR) allows basal activation of RpfF-1 with the subsequent DSF production. Several factors modulate the DSF production including temperature, media nutrients and the presence of medium and long-chain fatty acids. The presence of unspecific fatty acids likely coming from cellular lysis and tissue damage as well as the DSF produced by neighbour bacteria also stimulate the dissociation and activation of RpfF-1 with the corresponding DSF production. The main fatty acid in the phospholipids 15:0 ISO also stimulates DSF production likely as a consequence of the control of membrane homeostasis. The DSF-QS system controls bacterial motility, biofilm dispersion and virulence in *rpf-1* strains. (*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. Liberation of active RpfF-2 with the subsequent DSF production happen only upon detection of DSF-itself, likely coming from neighbour DSF-producer strains (e.g., *Xcc* or *S. maltophlia rpf-1*). In this situation, *rpf-2* strains have been shown to regulate virulence capacity in the zebrafish model. Solid arrows indicate confirmed pathway and dashed arrows indicate proposed pathways.

What does each *rpf* variant confers to *S. maltophilia* pathogenic strains during human infections is a crucial and complicated question that must be addressed in the future.
As mentioned, to the date, *S. maltophilia rpf-1* strains are more commonly isolated, but it will be interesting to follow the evolution of *S. maltophilia* infections in a long term in order to establish a presumed correlation between *rpf* variants and infection success.

Regarding to the *rpf*/DSF-QS evolution, "the chicken or the egg" dilemma is also here present. It would be interesting to define which *rpf* variant appeared first in *S. maltophilia*. This would additionally help us to get insights into the evolution processes that have occurred in *Xanthomnadales*. In my opinion, I opted to think that *rpf-1* variant strains are more evolved, since it seems that their promiscuous DSF production and perception allow them to activate the entire virulence-related machinery under a high variety of environments (Fig. v). Nevertheless it is evident that these hypotheses will require further study.

In the third block of this thesis, I have been working with a new fatty acid-mediated QS system in *S. maltophilia* that has been recently identified in the important pathogen *P. aeruginosa* (Section 4.3). In this case, the cellular communication is mediated by a smaller 10-carbon FA *cis*-decenoic (*cis*-DA). It has been reported that this new FA stimulates biofilm dispersion not only in *P. aeruginosa,* but also in many gram-negative and gram-positive bacteria (142). Although much work is needed to complete the study, I have been able to preliminary characterize two important components in the cluster that control *cis*-DA synthesis in *S. maltophilia*. In *P. aeruginosa*, it has been reported that the enoyl-CoA hydratase (ECH) DspI (PA0745) is responsible for the synthesis of this new signalling FA (143). Sequence genome analysis has revealed that *S. maltophilia* harbours the homologous synthase Smlt0266. Mutation of *smlt0266* lead to a similar alteration that is observed in *P. aeruginosa* Δ*dspI*. Additionally, a new interesting target for both *S. maltophilia* and *P. aeruginosa* has emerged from this work, the alternative ECH DspII (PA0744) in *P. aeruginosa* and the corresponding Smlt0267 in *S. maltophilia.* While mutation of the *dspI* (in *P. aeruginosa*) and *smlt0266* (in *S. maltophilia*) lead to alteration mainly in the biofilm development, mutation of the alternative ECH *dspII* (in *P. aeruginosa*) and *smlt0267* (in *S. maltophilia*) lead to a drastic effect in the virulent phenotype of both pathogens. These common alterations include exopolysaccharide production, biofilm development, bacterial motility and

virulence in the *C. elegans* model (Section 4.3). These results are as interesting as complex to understand, by the moment. Initially, I was reluctant to think that the synthase of a new QS system was organized in a clear β-oxidation operon. The gene that encodes for the synthase *dspI/smlt0266* is upstream flanked by components involved in the first steps of classic fatty acid oxidation pathway such as a putative aldehyde-dehydrogenase and a hypothetical acyl-CoA dehydrogenase, and downstream flanked by a putative enoyl-CoA hydratase/isomerase (Fig. 25). However, the evidence that the mutation of *dspI/smlt0266* but specially *dspII/smlt0267* lead to an alteration of several typically QS-regulated processes, led me reconsider my initial doubts. Although I still have some concerns, it is evident that the two ECH are involved in the regulation of virulence processes in both *P. aeruginosa* and *S. maltophilia*, which make them a promising targets to design new antibacterial strategies.

The fatty acid signalling function in bacteria communication has been studied for no more than two decades. Nevertheless, it has aroused rapidly the attention of the scientific community since it has been demonstrated that this kind of signalling systems regulate several virulent processes in many important pathogens. Proof of this is DSF-QS described in several human and plant pathogens such as *Xanthomonas* sp, X. *fasidiosa*, *B. cepacea* and *S. maltophilia*. In addition, fatty acid-mediated signalling molecules have been also described in the gram-positive *Streptococcus mutans*, in the yeast *Candida albicans* and more recently in *P. aeruginosa*. Taking all together, it is evident that FA-mediated communication systems are a phenomenon widely distributed among microorganisms and will yield new important discoveries in a recent future.

Along this work we have tried to participate in increase the knowledge of the FA-mediated QS system in the important nosocomial pathogen *S. maltophilia* by studying in detail the DSF-QS and providing the first strokes of the new *cis*-DA QS system. However, more efforts will be required to completely elucidate the complexity of these cellular communication systems, especially in the well-established human pathogens.

CONCLUSIONS

6. CONCLUSIONS

1. Two variants of the cluster *rpf* (here named *rpf-1* and *rpf*-2) with significant differences in the genes encoding the DSF synthase RpfF and the sensor component RpfC are distributed among the population of *Stenotrophomonas maltophilia*.
2. Only strains harbouring the cluster variant *rpf-1* display detectable levels of DSF under wild type conditions, and they represent 60% of total isolates.
3. In *rpf-1* variant strains, RpfC-1 contains ten transmembrane regions (TMR) highly related to the sensor input domain present in *Xanthomonas campestris* pv. *campestris* composed by RpfH and RpfC. In *rpf-2* variant strains, the sensor input domain of RpfC-2 only contains five TMR.
4. The absence on DSF production observed in *rpf-2* variant strains is likely due to the permanent repression that RpfC-2 exerts on RpfF-2, although both RpfF variants are functional and are able to synthesize the canonical DSF molecule.
5. RpfC-1 variant -containing ten TMR- liberates free active-RpfF-1 with the subsequent DSF synthesis upon detection not only DSF, but also saturated medium length fatty acids. These include lauric (12:0), myristic (14:0) and 13-methylmyristic (15:0 iso), which is indeed the most abundant fatty acid in *S. maltophilia*.
6. RpfC-2 only allows activation of RpfF-2 upon detection of DSF-itself, indicating that this sensor component is much more specific and, at the same time, that DSF is produced in a positive feedback manner in *S. maltophilia*.
7. DSF quorum sensing system seems to regulate more biological functions including biofilm formation, swarming motility, and virulence in *C. elegans* and zebrafish models in *S. maltophilia* strains harbouring the cluster variant *rpf-1*, than in those harbouring the *rpf-2* variant, which seems to regulates only virulence in an infection model.
8. DSF inversely regulate biofilm formation and swarming motility in the *rpf-1* variant strain E77.
9. Both *S. maltophilia rpf*-variant groups act synergistically in DSF production both *in*-vitro and *in-vivo* and in their virulence capacity in the zebrafish infection model.

10. *S. maltophilia* also uses the *cis*-DA quorum sensing system to regulate numerous virulence-related processes. While mutation of the gene encoding the putative *cis*-DA synthase Smlt0266 results in an increment of biofilm formation, mutation of the gene encoding the alternative enoyl-coA hydratase Smlt0267 lead to reduction of several virulence factors including biofilm formation, twitching and swimming motility, antibiotic resistance and virulence.
11. The *smlt0267* orthologous gene *PA0744* also regulates similar functions in *P. aeruginosa,* demonstrating that Smlt0267/PA0744 represent a novel potential target for the development of new antimicrobial therapies.

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7. REFERENCES

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ANNEX

8. ANNEX

8.1 PUBLICATIONS

Journal of Bacteriology

Two Different *rpf* Clusters Distributed among a Population of Stenotrophomonas maltophilia Clinical Strains Display Differential Diffusible Signal Factor Production and Virulence Regulation

Pol Huedo, Daniel Yero, Sònia Martínez-Servat, Iratxe Estibariz, Raquel Planell, Paula Martínez, Àngels Ruyra, Nerea Roher, Ignasi Roca, Jordi Vila, Xavier Daura and Isidre Gibert

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Two Different *rpf* Clusters Distributed among a Population of *Stenotrophomonas maltophilia* Clinical Strains Display Differential Diffusible Signal Factor Production and Virulence Regulation

Pol Huedo,[a,b] Daniel Yero,[a,b] Sònia Martínez-Servat,[a,b] Iratxe Estibariz,[a,b] Raquel Planell,[a] Paula Martínez,[a,b] Àngels Ruyra,[a] Nerea Roher,[a] Ignasi Roca,[c] Jordi Vila,[c] Xavier Daura,[a,d] Isidre Gibert[a,b]

Institut de Biotecnologia i de Biomedicina (IBB), Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès (Barcelona), Spain[a]; Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès (Barcelona), Spain[b]; Department of Clinical Microbiology, School of Medicine, IDIBAPS and Barcelona Centre for International Health Research (CRESIB, Hospital Clínic-Universitat de Barcelona), Barcelona, Spain[c]; Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain[d]

The quorum-sensing (QS) system present in the emerging nosocomial pathogen *Stenotrophomonas maltophilia* is based on the signaling molecule diffusible signal factor (DSF). Production and detection of DSF are governed by the *rpf* cluster, which encodes the synthase RpfF and the sensor RpfC, among other components. Despite a well-studied system, little is known about its implication in virulence regulation in *S. maltophilia*. Here, we have analyzed the *rpfF* gene from 82 *S. maltophilia* clinical isolates. Although *rpfF* was found to be present in all of the strains, it showed substantial variation, with two populations (*rpfF*-1 and *rpfF*-2) clearly distinguishable by the N-terminal region of the protein. Analysis of *rpfC* in seven complete genome sequences revealed a corresponding variability in the N-terminal transmembrane domain of its product, suggesting that each RpfF variant has an associated RpfC variant. We show that only RpfC–RpfF-1 variant strains display detectable DSF production. Heterologous *rpfF* complementation of Δ*rpfF* mutants of a representative strain of each variant suggests that RpfF-2 is, however, functional and that the observed DSF-deficient phenotype of RpfC–RpfF-2 variant strains is due to permanent repression of RpfF-2 by RpfC-2. This is corroborated by the Δ*rpfC* mutant of the RpfC–RpfF-2 representative strain. In line with this observations, deletion of *rpfF* from the RpfC–RpfF-1 strain leads to an increase in biofilm formation, a decrease in swarming motility, and relative attenuation in the *Caenorhabditis elegans* and zebrafish infection models, whereas deletion of the same gene from the representative RpfC–RpfF-2 strain has no significant effect on these virulence-related phenotypes.

Quorum sensing (QS) is a bacterial cell-cell communication process that allows bacteria to synchronize particular behaviors on a population-wide scale. Within current knowledge, QS in *Stenotrophomonas maltophilia* depends on the diffusible signal factor QS (DSF-QS) system, which is based mainly on the fatty acid DSF (*cis*-11-methyl-2-dodecenoic acid) (1, 2). DSF synthesis is fully dependent on RpfF, an enoyl coenzyme A hydratase encoded by the *rpf* (regulation of pathogenicity factors) cluster, a set of genes that includes all of the components necessary for the synthesis and detection of DSF molecules. In addition to RpfF, *rpf* encodes the aconitase RpfA, the fatty acid ligase RpfB, the two-component sensor-effector hybrid system RpfC, and the cytoplasmic regulator element RpfG (1, 2). The DSF-QS system was first described in the phytopathogen *Xanthomonas campestris* pv. *campestris*, where it plays an important role in virulence regulation (3). Since then, this system has been described in several members of the order *Xanthomonadales*, including the genera *Xanthomonas*, *Xylella*, and *Stenotrophomonas*, as well as in members of the order *Burkholderiales* (1, 3–5). The specific functions regulated by the DSF-QS system are dependent on the species, but it has been suggested that it controls several virulence-related phenotypes (6). In the case of *S. maltophilia*, little is known about the mechanisms implicated in DSF-QS regulation. It has been demonstrated that disruption of DSF signaling has a drastic effect on *S. maltophilia* K279a, since the *rpfF* mutant shows reduced swimming motility, reduced exoprotease production, altered lipopolysaccharide, reduced tolerance to a range of antibiotics and to heavy metals, and reduced virulence in a *Caenorhabditis elegans* infection model (1). In addition, FecA, a ferric citrate receptor, has been shown to be positively regulated by the DSF-QS system. This receptor contributes to the internalization of iron, an essential element for the expression of virulence-related genes (7). In the *S. maltophilia* WR-C wild-type (WT) strain and a flagellum-defective *xanB* mutant, flagellum-independent translocation was stimulated not only by the main DSF but also by its derivative 11-methyl-dodecanoic acid (2). Regarding the interaction of *S. maltophilia* with plants, DSF seems to be involved in oilseed germination, plant colonization, and biofilm architecture in the environmental strain R551-3 (8). Recently, the BDSF system (a DSF variant in *Burkholderia* species) has also been shown to contribute to the swarming motility phenotype of *Burkholderia cenocepacia* (9).

In a recent *S. maltophilia* population study, the authors detected *rpfF*$^+$ genotypes in 61% of the 89 strains tested, suggesting that an important population of *S. maltophilia* lacks the *rpfF* gene

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Address correspondence to Xavier Daura, Xavier.Daura@uab.cat, or Isidre Gibert, Isidre.Gibert@uab.cat.
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(10). With the rapid increase in the number of *S. maltophilia* sequenced genomes, it is now possible to compare the *rpf* clusters of different strains. A preliminary analysis showed that all of the genomes sequenced contain the *rpfF* gene. In addition, at least two *rpf* cluster variants can be detected on the basis of sequence and genomic organization, with main differences found in the *rpfF* and *rpfC* genes. The genetic variation observed in the *rpfF* gene translates into two distinct protein variants, here named RpfF-1 and RpfF-2. Furthermore, we can associate each of these RpfF variants with a corresponding RpfC variant, i.e., RpfC-1 and RpfC-2, respectively. We have also investigated the DSF production of representative strains from each variant group, revealing that only the strains carrying the RpfF–RpfC-1 variants show detectable DSF production under the conditions assayed. Moreover, characterization of the Δ*rpfF* mutant of a strain from each RpfF variant group indicates that the virulence-related phenotypes are differently regulated in the two populations.

MATERIALS AND METHODS

Strains and growth conditions. A panel of 78 *S. maltophilia* clinical isolates were collected from point prevalence studies in the intensive care units of different European hospitals. For the name, geographic origin, hospital, and isolation source of each strain, see Table S1 in the supplemental material. From this collection, E77 (RpfF-1 variant group) and M30 (RpfF-2 variant group) (11) were used as model strains to characterize Δ*rpfF* mutants (see Table S2). *Escherichia coli* OP50 was provided by the Caenorhabditis Genetics Center (CGC). *X. campestris* pv. *campestris* 8523/pL6engGUS was obtained from the authors of reference 12.

Bacteria were routinely grown at 37°C in Luria-Bertani (LB) medium on a rotary shaker. When needed, LB was supplemented with tetracycline (Tc) at 17 μg/ml, chloramphenicol (Cm) at 3.2 μg/ml, erythromycin (Erm) at 500 μg/ml, and ampicillin (Ap) at 20 μg/ml. For phenotypic analysis in minimal medium, strains were grown in BM2 medium (62 mM potassium phosphate buffer, pH 7, 2 mM $MgSO_4$, 10 μM $FeSO_4$, supplemented with glucose 0.4%) or a modified M9-salts medium without NH_4Cl (0.5% Casamino Acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) and supplemented with 0.2% glucose.

Sequence determination and analysis. PCR products of 682 to 721 bp containing the *rpfF* promoter and the region encoding the N-terminal fragment were amplified from all 78 *S. maltophilia* strains with primers PrpfFtypeUp and PrpfFTypeDw (see Table S3 in the supplemental material) and directly sequenced (Macrogen Inc.). Translation of partial open reading frames (ORFs) to amino acids and sequence alignments were done with MEGA V5.2 (13) and BioEdit, respectively. A phylogenetic tree was constructed with MEGA V5.2 on the basis of a trimmed alignment with the 108 N-terminal residues of RpfF from strain K279a. In parallel, the genomes of strains E77, M30, and UV74 were sequenced and a first draft was constructed (to be reported upon completion). RpfC variant determination was then based on the RpfC sequences from the publicly available sequenced genomes (strains K279a, R551-3, D457, and JV3, with GenBank accession numbers AM743169.1, CP001111.1, HE798556.1, and CP002986.1, respectively) and our draft genome sequences (strains E77, M30, and UV74), by using SMART (14) for the identification and annotation of protein domains.

Generation and complementation of Δ*rpfF* and Δ*rpfC* mutants. For the primers and plasmids used for cloning, see Tables S3 and S4 in the supplemental material, respectively. *S. maltophilia* E77 Δ*rpfF* and M30 Δ*rpfF* and Δ*rpfC* mutants were obtained by allelic-exchange recombination with an Erm resistance cassette. Briefly, *rpfF* upstream and downstream flanking regions were amplified by PCR (see Table S3 in the supplemental material) and inserted, flanking the Erm resistance cassette, into the pEX18Tc vector (15), thus generating plasmids pEXE77*rpfF* and pEXM30*rpfF* for E77 and M30, respectively. Both strains were electroporated (16) with the respective suicide vectors, and transformants were selected on LB plates containing 500 μg/ml Erm and subsequently streaked onto LB plates containing 17 μg/ml Tc to discard single-crossover events. *rpfF* deletion was also verified by PCR and DNA sequencing. To generate a Δ*rpfC* mutant of the M30 strain, the same strategy was used. For the primers used to amplify upstream and downstream regions of *rpfC* from M30, see Table S3 in the supplemental material. Both fragments were inserted, flanking the Erm resistance cassette, into pEX18Tc, generating pEXM30*rpfC*. Strain M30 was electroporated, and the mutant candidates were screened and verified with the corresponding primers (see Table S3) as described above.

A fragment of ca. 1,100 bp containing either the E77 or the M30 *rpfF* ORF and the predicted promoter was amplified by PCR, ligated to pBBR1MCS-Cm (17), and introduced into E77 and/or M30 for either homologous or heterologous *trans* complementation of Δ*rpfF*. On the other hand, a fragment of ca. 3,000 bp was amplified from M30 and E77 to generate complementation vectors prpfGCM30 and prpfGCE77 (see Table S4), respectively. These fragments contained the *rpfG* and *rpfC* operon with its own promoters. Both fragments were digested with the respective restriction enzymes and ligated into pBBR1MCS1-Cm. Finally, prpfGCM30 and prpfGCE77 were introduced into E77, M30, and the M30 Δ*rpfC* mutant for either homologous or heterologous *trans* complementation.

Supernatant DSF extraction. DSF extraction from culture supernatants was carried out by the ethyl acetate method (3). Briefly, overnight bacterial cultures grown on LB medium were harvested by centrifugation and the supernatant was extracted with the same volume of ethyl acetate. The organic phase was evaporated to dryness with a rotary evaporator, and the residues were dissolved in an appropriate volume of methanol (for supernatant DSF bioassay and analysis by thin-layer chromatography [TLC]) or dichloromethane (for analysis by gas chromatography-mass spectrometry [GC-MS]).

DSF bioassay and TLC analysis. DSF determination was performed with *X. campestris* pv. *campestris* 8523/pL6engGUS (DSF reporter strain) as previously described (12), with a few modifications. Briefly, the DSF reporter strain was grown in 10 ml of NYG medium (0.3% yeast extract, 0.5% peptone, 2% glycerol) supplemented with Tc (10 μg/ml) to an optical density at 600 nm (OD_{600}) of 0.7. Cells were harvested, reconstituted with 1 ml of fresh NYG, added to 100 ml of cold NYG medium containing 1% BD Difco Noble agar (NYGA) supplemented with 80 μg/ml X-Glu (5-bromo-4-chloro-3-indolyl β-D-glucuronide sodium salt; Sigma), and plated into petri plates upon solidification.

For colony-based DSF bioassays, candidate strains were pin inoculated onto plates of NYGA containing X-Glu (80 μg/ml) seeded with the DSF reporter strain and incubated for 24 h at 28°C. The presence of a blue halo around the colony indicates DSF activity.

For supernatant-based DSF bioassays, bacterial cultures were grown in 250 ml of LB for 48 h at 30°C (OD_{600} of about 4). Supernatants were extracted by the ethyl acetate method, and residues were dissolved in 200 μl of methanol. A 3-μl volume of each sample was deposited into a hand-generated well in a 5.5-cm plate containing NYGA supplemented with 80 μg/ml X-Glu and seeded with the DSF reporter strain to a final OD_{600} of 0.07. Plates were incubated for 24 h at 30°C. DSF activity was determined by the presence of a blue halo around the well.

For supernatant TLC analysis, 3-μl aliquots of dissolved methanol residues were spotted onto a silica gel 60 TLC plate (20 by 20 cm; Merck) and separated with ethyl acetate-hexane (20:80, vol/vol) as running solvents. TLC plates were subsequently air dried for at least 1 h and overlaid with 100 ml of unsolidified NYGA containing 80 μg/ml X-Glu and the DSF reporter strain at an OD_{600} of 0.07. TLC plates were incubated overnight at 28°C, and DSF activity was identified by the presence of blue spots.

Identification of DSF molecules from culture supernatants by GC-MS. Bacterial cultures were grown in 2 liters 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. DSF molecules were identified by GC (Agilent Technologies 6890) with an Agilent 19091S-433 column coupled to an MS detector (Hewlett-Packard 5973).

Determination of virulence in a *C. elegans* model. *C. elegans* CF512 [fer-15(b26)II; fem-1(hc17)IV], a strain showing temperature-dependent sterility, was provided by CGC. Nematodes were routinely maintained on NGM plates (1.7% agar, 50 mM NaCl, 0.25% peptone, 1 mM $CaCl_2$, 5 μg/ml cholesterol, 25 mM KH_2PO_4, 1 mM $MgSO_4$) seeded with *E. coli* OP50 at 16°C.

Determination of the virulence of *S. maltophilia* strains in the *C. elegans* CF512 infection model was based on the "slow killing" method (18). Strains were grown in brain heart infusion broth overnight at 30°C, and 100 μl of each strain culture was spread onto a 5.5-cm-diameter NGM agar plate and incubated at 30°C for 24 h. Each plate was then seeded with 15 to 20 adult hermaphrodite CF512 worms, incubated at 25°C (sterility conditions), and scored for live worms every 24 h. *E. coli* OP50 was used as a negative control. A worm was considered dead when it no longer responded to touch. Three replicates per strain were prepared.

Determination of virulence in a zebrafish model. Adult (9- to 12-month-old) WT zebrafish (*Danio rerio*) were subjected to a 12-h light-dark cycle at 28°C and fed twice daily with dry food. All of the fish used in infection experiments were transferred to an isolated system and acclimated for 3 days before infection. Adult zebrafish ($n = 12$ per condition) were infected by intraperitoneal injection (19) with 20 μl of a 5×10^8-CFU/ml suspension of each *S. maltophilia* strain. The strains were previously grown at 28°C on 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 there were no deaths. Fish were observed daily for signs of disease and death.

One fish from each tank was sacrificed at 72 h postinfection and divided into three sections (anterior, abdominal, and posterior regions) with a sterile surgical blade. All weights were annotated, and every section was homogenized in 3 ml of PBS. After serial dilution, bacteria were plated onto LB medium containing 20 μg/ml Ap (for WT E77), LB containing 500 μg/ml Erm (for the E77 Δ*rpfF* mutant), or LB supplemented with Cm (for the complemented E77 Δ*rpfF* mutant). Finally, CFU were counted and divided per gram of tissue. All of the isolates obtained postmortem from infected zebrafish were identified as *S. maltophilia* on the basis of cell and colony morphology, the analytical profile index, and the 16S rRNA gene sequence (data not shown).

Biofilm formation. To analyze biofilm formation on a polystyrene surface, 200-μl volumes of bacterial cultures grown to an OD_{600} of 0.1 in modified M9 or BM2 medium were inoculated into the wells of untreated 96-well microtiter plates (BrandTech 781662) and incubated for 24 h at 30°C. The plates were then washed three times with water, fixed at 60°C for 1 h, and stained for 15 min with 200 μl of 0.1% crystal violet. The dye was discarded, and the plates were rinsed in standing water and allowed to dry for 30 min at 37°C. Crystal violet was dissolved in 250 μl of 95% ethanol for 15 min, and the OD_{550} of the extracted dye was measured.

Biofilm formation on a glass surface was assayed by inoculating 2 ml of the same medium and adjusted OD as described above into glass tubes and incubating them for 24 h at 30°C with agitation (250 rpm). Biofilm formation was measured by crystal violet staining as described above.

Swarming assay. Swarm agar was made on the basis of modified M9 salts medium without NH_4Cl (0.5% Casamino Acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 0.4% glucose and solidified with 0.5% BD Difco Noble agar. Plates containing 20 ml of fresh swarm medium were dried under a laminar-flow hood for 20 min before inoculation. Inoculation was performed with a sterile Drigalski spatula containing biomass from a fresh LB plate by softly depositing it on top of a semisolid modified M9 plate. Inoculated swarm plates were sealed to maintain the humidity and incubated at 28°C for 3 to 5 days.

Quantitative reverse transcription (qRT)-PCR. Gene expression analysis was performed to determine the ratios of *rpfF* to *rpfC* mRNAs in *S. maltophilia* E77 and M30. Total RNA was isolated from cultures grown under the same conditions as for DSF extraction with a GeneJet RNA purification kit (Thermo Scientific), and DNA was eliminated with TURBO DNase (Ambion, Life Technologies). One microgram of RNA was used to synthesize cDNA with an iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed with the CFX96 real-time PCR system (Bio-Rad), and PCR amplification was detected with SsoAdvanced SYBR green Supermix (Bio-Rad). PCR products of 80 to 110 bp were amplified for *rpfC*, *rpfF*, and *gyrA*; the latter was used as an endogenous gene to normalize gene expression (20). For the primers used, see Table S3 in the supplemental material. Differences in the relative amounts of mRNA for the *rpfF*-1, *rpfC*-1, *rpfF*-2, and *rpfC*-2 genes were determined by the $2^{-\Delta\Delta CT}$ method (21). RNA samples were extracted in three different experiments, and results are given as mean values.

Ethics statement. 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 of the Universitat Autònoma de Barcelona, Spain (reference number CEEAH-1968).

Nucleotide sequence accession numbers. All of the amplified *rpfF* sequences from this *S. maltophilia* strain collection have been deposited in the GenBank database and assigned accession numbers KJ149475 to KJ149552.

RESULTS

***S. maltophilia* harbors two RpfF variants that apparently differ in DSF production.** Amplification and sequencing of the corresponding DNA region demonstrated that all of the *S. maltophilia* strains in this study (see Table S1 in the supplemental material) contain the *rpfF* gene. However, slightly different *rpfF* fragment lengths were obtained because of the region's variability. In addition, alignment of the translated N-terminal regions and subsequent phylogenetic analysis revealed that RpfF of *S. maltophilia* may be distributed into two distinct variants, which we have named RpfF-1 and RpfF-2 (Fig. 1 and 2A and B). The RpfF-1 variant is present in 47 (60.26%) of the 78 strains, whereas RpfF-2 is present in the remaining 31 strains (39.74%) (Fig. 1). Of the additional four complete genome sequences available, K279a and R551-3 contain the RpfF-1 variant and D457 and JV3 contain the RpfF-2 variant.

Interestingly, no strain carrying the RpfF-2 variant showed DSF activity when tested with the *X. campestris* pv. *campestris* 8523/pL6engGUS bioassay (Fig. 1B; see Fig. S1 in the supplemental material). To corroborate the absence of DSF production in these strains, culture supernatants were analyzed with the DSF reporter bioassay, as well as by TLC and GC-MS (see Materials and Methods) with M30 as a representative strain. DSF production was never detected in M30 supernatants by any of these three techniques, indicating that RpfF-2 does not produce DSF under the conditions assayed (Fig. 3B and 4; see Fig. S2 in the supplemental material).

Initially, the significant differences between the N-terminal regions of the two RpfF variants made us hypothesize that this region could play a direct role in DSF synthesis. However, the residues that form the substrate binding pocket (Leu136, Gly137, Gly138, Gly85, Leu276, Met170, and Trp258), as well as those involved in catalysis (Glu141, Glu161), in *X. campestris* pv. *campestris* (22) are conserved in the two variants (Fig. 2B). In order to

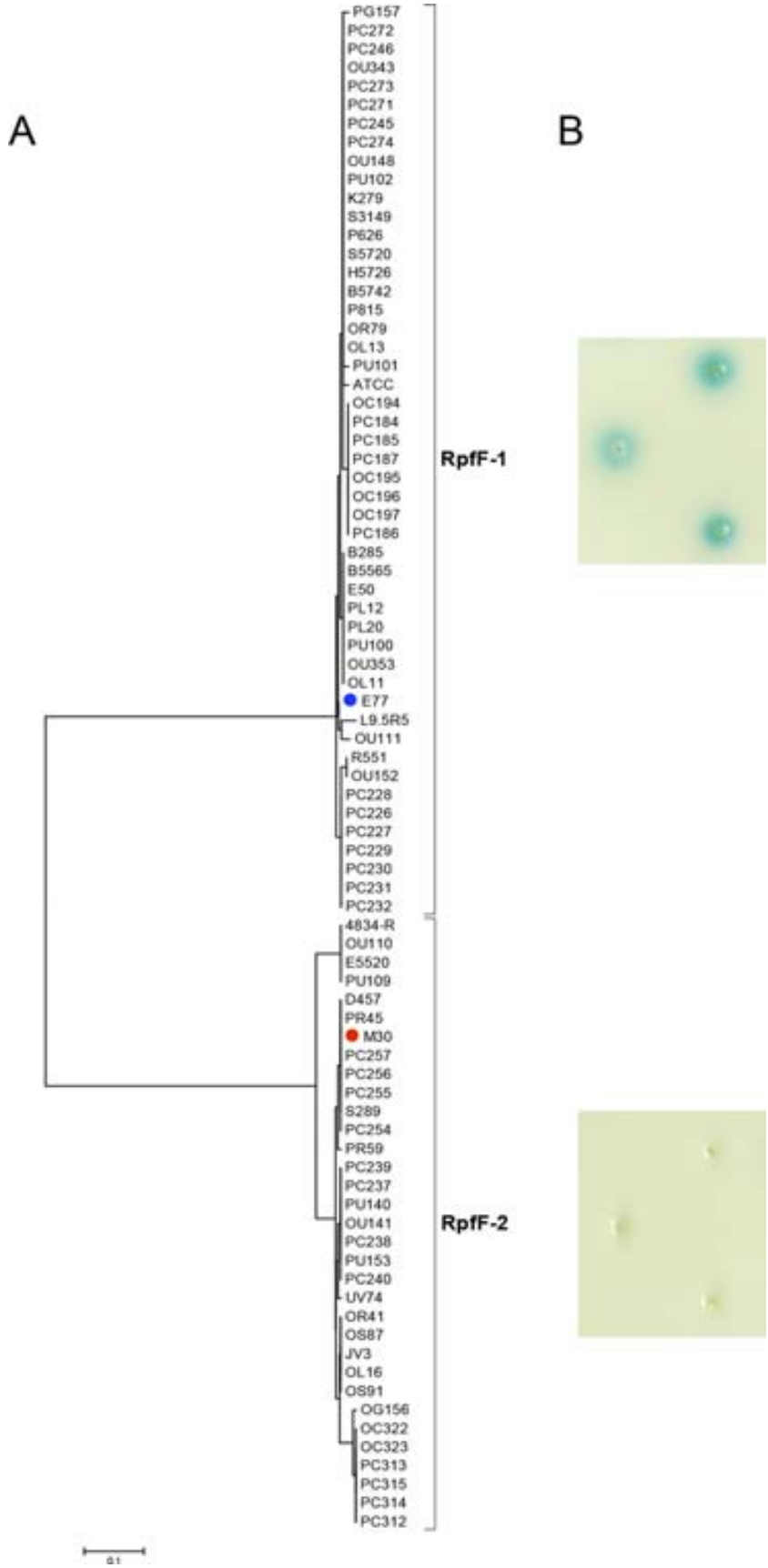

FIG 1 (A) Phylogenetic analysis of 82 *S. maltophilia* strains based on the first 108 amino acids of RpfF. (B) Colony DSF bioassay of three representative strains of each RpfF variant group. Top: E77, ATCC 13637, and K279a (RpfF-1). Bottom: M30, D457, and UV74 (RpfF-2).

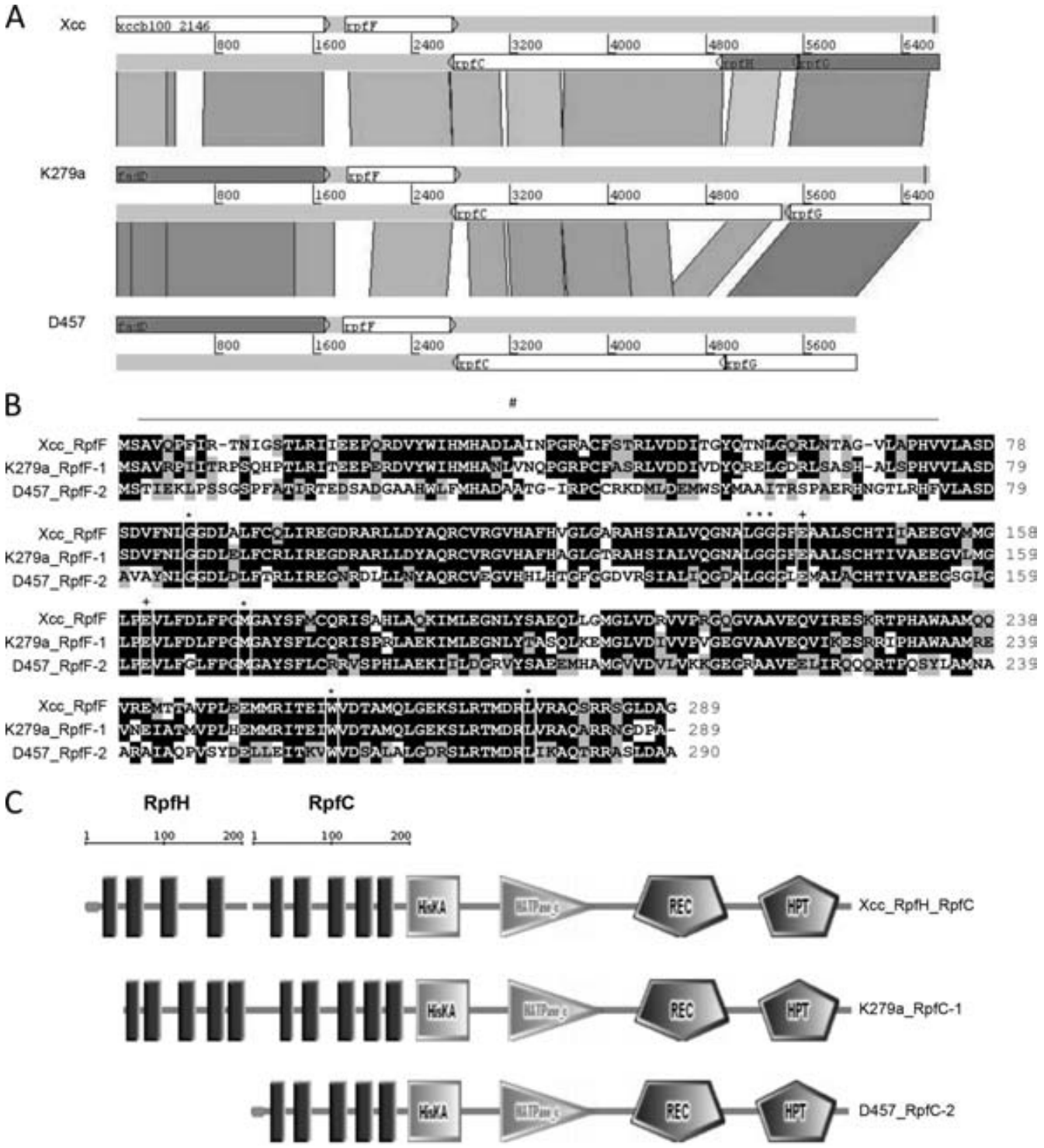

FIG 2 (A) Comparison of the *rpf* cluster in *X. campestris* pv. *campestris* and *S. maltophilia* K279a and D457. The alignment was performed with tblastx (percent identity cutoff, 45%) from the BLAST suite and visualized with the Artemis Comparison Tool. Conserved protein regions are paired by shaded blocks where color intensity is proportional to sequence identity. The scales are relative positions in base pairs. (B) Alignment of RpfF proteins from *X. campestris* pv. *campestris* and *S. maltophilia* K279a (RpfF-1) and D457 (RpfF-2). Symbols: #, hypervariable region; *, binding pocket residues; +, glutamate catalytic residues. (C) SMART software analysis of RpfC and RpfH from *X. campestris* pv. *campestris* and RpfC from *S. maltophilia* K279a and D457, where HisKA is a histidine kinase domain, HATPase_c is a histidine ATPase domain, REC is a CheY-like receiver domain, and HPT is a histidine phosphotransferase domain.

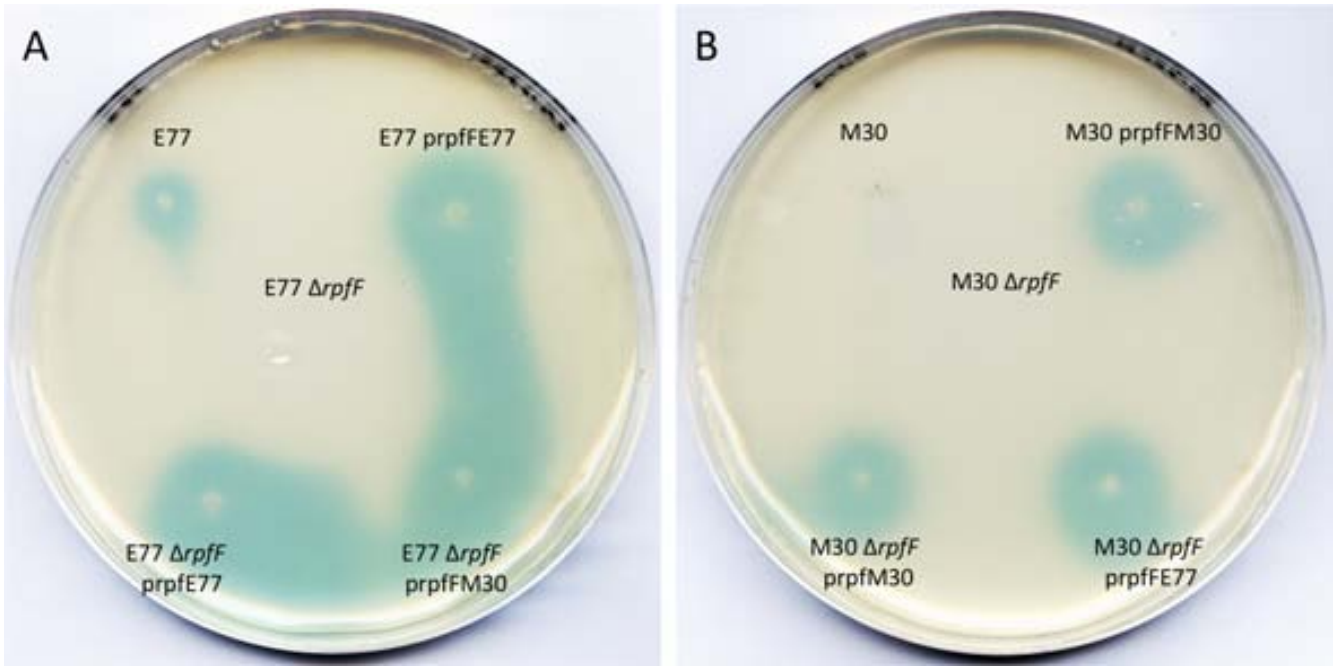

FIG 3 DSF bioassay of E77 (A) and M30 (B) with their respective Δ*rpfF* mutants and homologously and heterologously complemented strains and the *X. campestris* pv. *campestris* 8523/pL6engGUS reporter strain.

test the intrinsic capacity of the RpfF-2 variant to produce DSF, we inserted the *rpfF* gene from M30 (RpfF-2 variant) into an E77 Δ*rpfF* mutant (RpfF-1 variant) by heterologous complementation. The results obtained demonstrate that RpfF-2 is functional in DSF synthesis, since the E77 Δ*rpfF* mutant complemented with M30 *rpfF* showed a big blue halo of DSF diffusion (Fig. 3B). Additionally, insertion of extra copies of its own *rpfF* gene into WT M30 and the M30 Δ*rpfF* mutant resulted in DSF production (Fig. 3B), suggesting that RpfF-2 is able to produce DSF but it is repressed in the WT strain under the conditions assayed.

The experiments with deletion mutants and the corresponding complemented strains proved that the blue halo observed in the bioassays is due to the fatty acid produced by the *rpfF* product (Fig. 3). In addition, MS analysis demonstrated that the signaling factor is DSF (see Fig. S3 in the supplemental material). Moreover, DSF bioassays and TLC analyses of culture supernatants of E77 and M30, their Δ*rpfF* mutants, and the complemented strains suggested that DSF is the only fatty acid with signaling activity that depends on the RpfF synthase function, since no other differential blue spot was observed when M30 and E77 were compared with each other and with the respective Δ*rpfF* mutant and complemented strains (see Fig. S2).

Each RpfF variant has an associated RpfC variant, and RpfC-1 contains a TM sensor input domain highly related to the *X. campestris* pv. *campestris* RpfH-RpfC complex. Analyzing the complete *rpf* cluster in the four *S. maltophilia* complete genome sequences and in our three draft genome sequences (E77, UV74, and M30), we observed that *rpfC* also differed significantly between the two *S. maltophilia* variant groups defined by the *rpfF* gene (Fig. 2A). Thus, each RpfF variant group appears to have an associated RpfC variant. RpfC-1 (belonging to the RpfF-1 variant strains) and RpfC-2 (belonging to the RpfF-2 variant strains) differ in their N-terminal regions, corresponding to the transmembrane (TM) domain or sensor input domain (Fig. 2C) (23).

It has been postulated that in *X. campestris* pv. *campestris*, an additional integral membrane protein, RpfH, participates in DSF sensing (12). In *S. maltophilia*, the RpfH protein appears to be fused to RpfC-1, generating a sensor input domain with 10 TM regions, as would happen in a putative *X. campestris* pv. *campestris* RpfH-RpfC complex. However, the TM domain of the RpfC-2 variant contains only five TM regions. Interestingly, tblastx analysis revealed that the five TM regions present in the RpfC-2 variant are highly related to *X. campestris* pv. *campestris* RpfH, while the absent five regions would correspond to the *X. campestris* pv. *campestris* RpfC TM domain (Fig. 2A). This indicates that both RpfC variant groups produce a putative RpfH protein but only the RpfC-1 variant contains its own five TM regions in the sensor input domain. The loss of these regions in RpfC-2 could have an implication for DSF detection.

RpfF-2 is permanently repressed by RpfC-2. In order to study the implication of each RpfC variant in DSF synthesis repression, DSF producer strain E77 was provided with both RpfC variants in *trans*. Since *rpfC* is expected to be cotranscribed jointly with *rpfG* in the *rpfGC* operon in both the *rpf*-1 and *rpf*-2 clusters, we generated vectors prpfGC-E77 and prpfGC-M30. The in *trans* repression vectors resulted in a reduction of E77 DSF synthesis in both cases. However, while E77 harboring the prpfGC-1 vector showed only a small decrease in DSF synthesis, provision of prpfGC-2 resulted in strong inhibition of DSF production (Fig. 5A), suggesting that RpfC-2 is a stronger repressor of RpfF activity. In order to corroborate this hypothesis, we generated a Δ*rpfC*-2 mutant of strain M30. Consistent with the previous result, the M30 Δ*rpfC* mutant became a DSF producer strain (Fig. 5B). Complementation of the M30 Δ*rpfC* mutant with vectors prpfGC-E77 and prp-

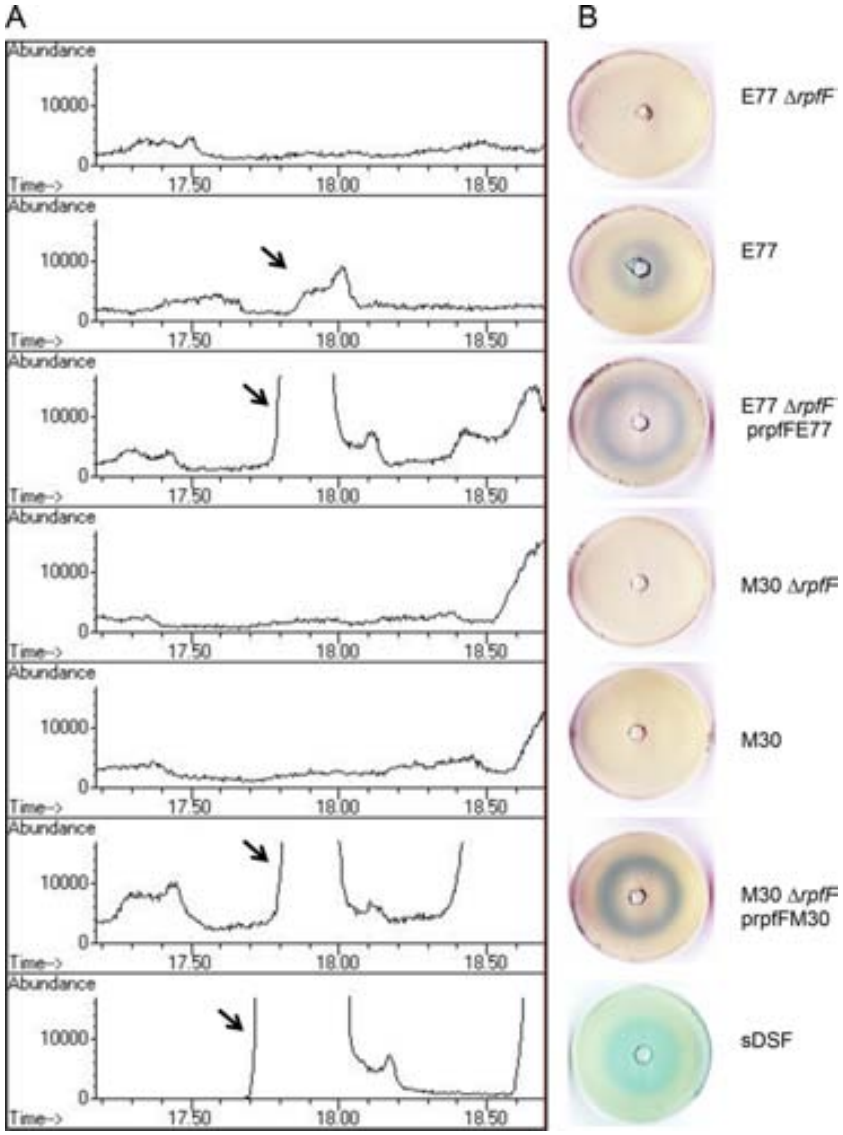

FIG 4 (A) GC analysis of culture supernatants of E77, M30, and their respective Δ*rpfF* mutants and complemented strains. (B) DSF bioassay of concentrated supernatants of the same strains from independent extractions.

fGC-M30 in *trans* led to a scenario similar to that obtained with the E77 strain (Fig. 5B). To further characterize the relationship between RpfF and RpfC in the two variants, the expression of the gene pairs *rpfF*-1–*rpfC*-1 and *rpfF*-2–*rpfC*-2 was quantified by qRT-PCR using the $2^{-\Delta\Delta CT}$ method with *gyrA* as an endogenous control. Thus, expression in WT E77 was 5.16-fold ± 0.59-fold for *rpfF*-1 and 2.69-fold ± 0.29-fold for *rpfC*-1 (*rpfF*-1/*rpfC*-1 ratio of 1.92), while in WT M30 it was 1.57-fold ± 0.23-fold for *rpfF*-2 and 1.65-fold ± 0.25-fold for *rpfC*-2 (*rpfF*-2/*rpfC*-2 ratio of 0.95) (see Fig. S4 in the supplemental material).

Δ*rpfF* mutants display different virulence-associated phenotypes as a function of the native RpfF variant. We have investigated the implication of the two RpfF variants for virulence-associated phenotypes such as biofilm formation and swarming motility. We had previously observed that swarming activation of *S. maltophilia* is faster with streak inoculation than with pin inoculation, suggesting that a high-density population facilitates the initiation of this type of motion (unpublished results). This supports the idea that QS could be involved in swarming activation in *S. maltophilia*. To corroborate this hypothesis, we tested the ability of E77 and M30 Δ*rpfF* mutants to swarm on modified M9 medium with a 0.5% agar concentration, relative to that of the WT strains. WT E77 displays tendril-like motility, whereas WT M30 hardly swarms, likely because of its DSF deficiency (Fig. 6). The E77 Δ*rpfF* mutant shows a clear motility loss and phenotype restoration when *rpfF* is complemented in *trans*. On the contrary, the swarming motility of the M30 Δ*rpfF* mutant is not significantly different from that of the WT M30 strain, suggesting that RpfF does not intervene in swarming control in M30. However, this behavior does not seem to be strictly linked to the RpfF variant. Thus, on the one hand the E77 Δ*rpfF* mutant displayed an atypical nontendril swarming morphology when heterologously complemented with the M30 *rpfF* gene. On the other, heterologous complementation of the M30 Δ*rpfF* mutant with the E77 *rpfF*

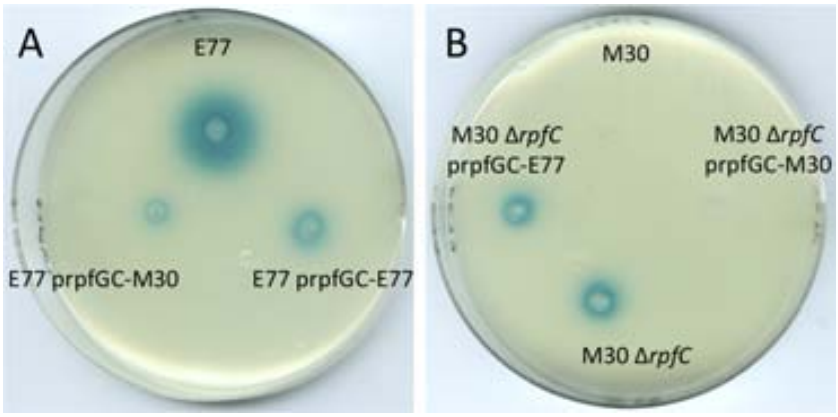

FIG 5 (A) DSF bioassay of WT E77 and E77 complemented with vectors prpfGCE77 and prpfGCM30. (B) DSF bioassay of WT M30, the M30 Δ*rpfC* mutant, and the M30 Δ*rpfC* mutant complemented with vectors prpfGCE77 and prpfGCM30.

gene resulted in motility similar to that of the M30 Δ*rpfF* mutant strain.

Biofilm formation by E77, M30, and the respective Δ*rpfF* mutants and complemented variants on a polystyrene or glass surface was also evaluated under two different medium conditions (M9 and BM2). The results show that M30 has a higher capacity than E77 to form biofilm under both growth conditions (Fig. 7), contrary to the ability to swarm, suggesting that DSF production may inversely regulate these two behaviors in *S. maltophilia*. Additionally, the results also indicate that biofilm formation is altered only in the E77 Δ*rpfF* mutant, showing a significant increase relative to that of WT E77. Homologous and heterologous complementation with the respective RpfF variants restores almost WT E77 levels of biofilm formation ($P < 0.0005$) on both glass and plastic surfaces (Fig. 7A and B). On the other hand, the M30 strain, the M30 Δ*rpfF* mutant, and the homologously and heterologously complemented strains show similar levels of biofilm formation (Fig. 7C and D). As for the regulation of swarming motility or the ability to produce DSF, it therefore appears that the regulation of biofilm formation is not strictly dependent on the RpfF variant but on one or more components associated with this variant, in particular, RpfC. Specifically, the results suggest that RpfF is involved in the regulation of biofilm formation and swarming motility only in strains that natively carry RpfF-1 (even when this is replaced with RpfF-2). This is likely connected to the ability of these strains to produce DSF.

The Δ*rpfF*-1 mutant, but not the Δ*rpfF*-2 mutant, shows attenuation in *C. elegans*. To elucidate the direct implication of each RpfF variant in *S. maltophilia* virulence *in vivo*, the killing ability of E77, M30, and the respective Δ*rpfF* mutants and complemented strains was tested in *C. elegans*. Although the WT E77 and M30 strains showed similar virulence capacities in the *C. elegans* model (with times required to kill 50% of the nematodes, 6.04 and 4.99 days, respectively), significant attenuation was observed here for the E77 Δ*rpfF* mutant (Fig. 8). In line with the observations made for the phenotypes analyzed previously, the virulence of the E77 Δ*rpfF* mutant is restored after complementation with either its own *rpfF* gene (RpfF-1 variant) or the M30 *rpfF*

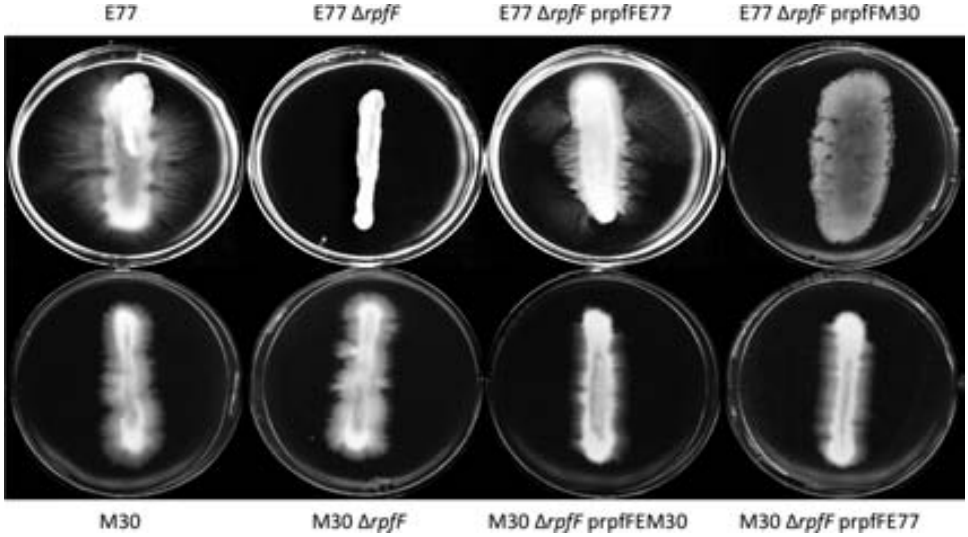

FIG 6 Swarming motility assay of E77, M30, and their Δ*rpfF* mutants and homologously and heterologously complemented strains on modified M9 medium solidified with 0.5% Noble agar and incubated at 30°C for 4 days.

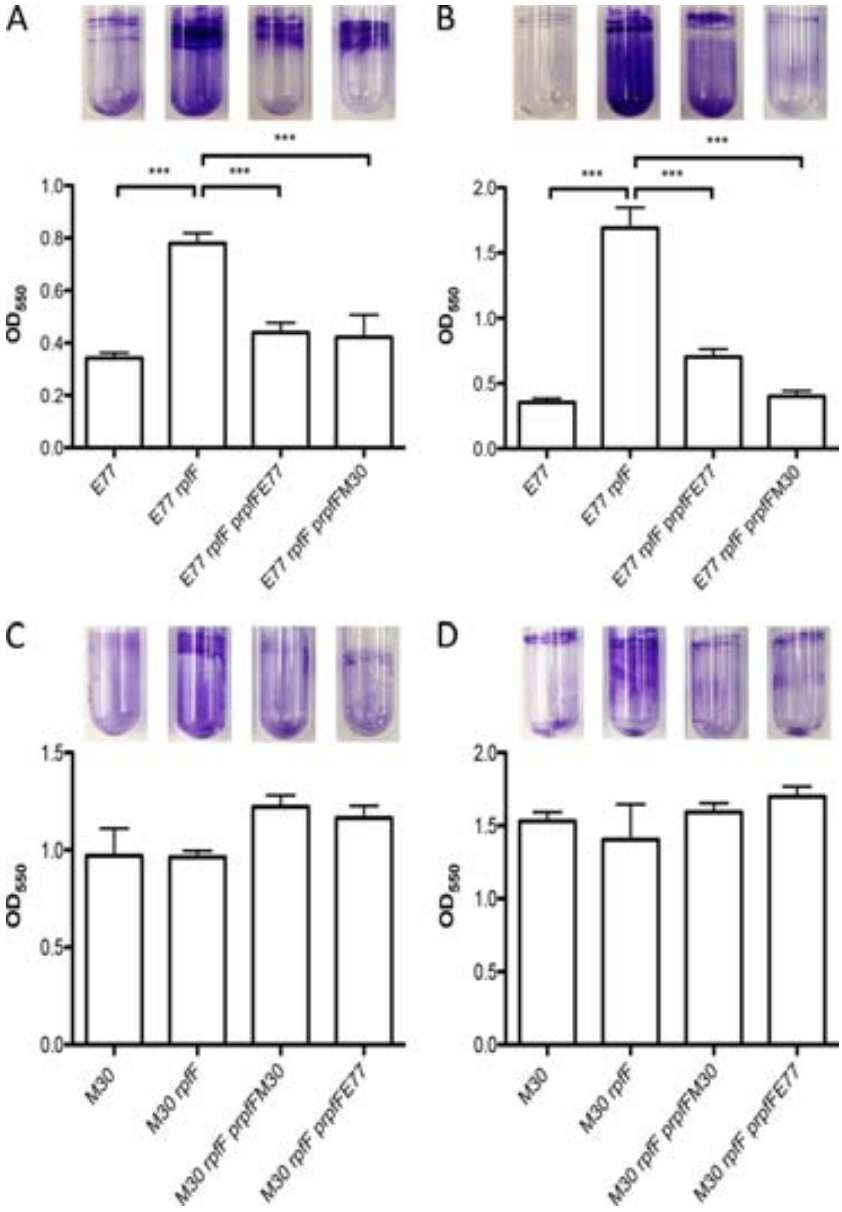

FIG 7 Biofilm formation by E77, M30, and their respective Δ*rpfF* mutants and homologously and heterologously complemented strains on polystyrene (plots) and glass (tubes) surfaces in M9 (A and C) and BM2 (B and D) minimal media. ***, $P < 0.0005$.

gene (RpfF-2 variant), indicating once more that E77 is able to respond to heterologous DSF production in an RpfC-1 variant background (Fig. 8). Infection with the M30 Δ*rpfF* mutant shows no significant differences from WT M30. These results suggest again that the RpfF-RpfC pair may regulate virulence only in those strains carrying the variant 1 combination.

The Δ*rpfF*-1 mutant shows attenuation in zebrafish due to its inability to disseminate through fish tissues. E77, its Δ*rpfF* mutant, and the complemented strain were evaluated in zebrafish as a vertebrate model. Similar results were obtained, corroborating that RpfF-1 is involved in virulence regulation (Fig. 9A). Interestingly, recovery of bacteria from sacrificed fish from each tank at 72 h postinjection showed the ability of WT E77 to disseminate through the fish body from the abdominal region to the anterior and posterior regions. On the contrary, the E77 Δ*rpfF* mutant does not seem to be able to colonize those regions effectively. Complementation of *rpfF* partially restores its body dissemination capacity (Fig. 9B).

DISCUSSION

We have characterized 78 *S. maltophilia* clinical strains isolated from diverse sources in different European hospitals for the *rpfF* gene. We have first demonstrated that the 78 strains contain the *rpfF* gene but the RpfF product is distributed into two different variants that we have named RpfF-1 and RpfF-2 (Fig. 1 and 2A). We also show that the isolates produce two RpfC variants, each associated with one of the RpfF variants (Fig. 2A). The two RpfC variants are different in the N-terminal region, which corresponds

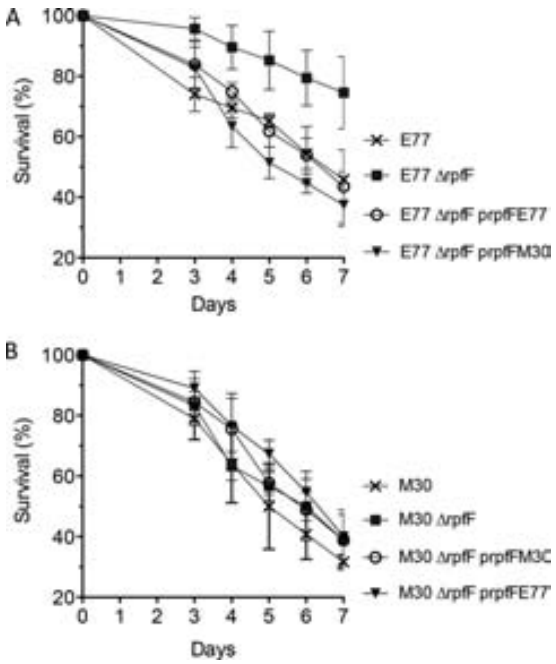

FIG 8 Determination of virulence of E77 (A), M30 (B), and their respective Δ*rpfF* mutants and homologously and heterologously complemented strains in a *C. elegans* CF512 model of infection.

to a TM domain (Fig. 2C) thought to participate in DSF sensing in several *Xanthomonas* species (12). In *S. maltophilia*, the RpfC-1 variant contains 10 TM regions that display high similarity to the putative *X. campestris* pv. *campestris* RpfH-RpfC TM complex (Fig. 2A and C). On the other hand, the RpfC-2 variant has only five TM regions, which appear to be related to the *X. campestris* pv. *campestris* RpfH TM domain rather than that of *X. campestris* pv. *campestris* RpfC (Fig. 2A). This phenomenon is also observed in *Xylella fastidiosa*, *Xanthomonas oryzae*, and *Pseudoxanthomonas* species, suggesting that the RpfC-2 variant is widely distributed among the members of the order *Xanthomonadales* that share the DSF-QS system. Nevertheless, protein sequence comparison shows a high similarity between the RpfC and RpfH TM domains, suggesting that a duplication event (for *X. campestris* pv. *campestris rpfC* to *rpfH* and *S. maltophilia rpfC*-1) or a deletion (for *S. maltophilia rpfC*-2) may have occurred.

A previous *S. maltophilia* population study suggested that an important group of *S. maltophilia* isolates lack *rpfF* (10). PCR-based typing of 89 strains showed an $rpfF^{+}$ prevalence of 61.8%, while the remaining 38.2% were considered to be *rpfF* mutants. On the basis of our sequence analysis, we can conclude that the work of Pompilio and collaborators (10) failed to detect *rpfF* because the primers they used were designed to hybridize within the most variable region of this gene; more specifically, those primers do not amplify *rpfF* in strains carrying what we have defined as

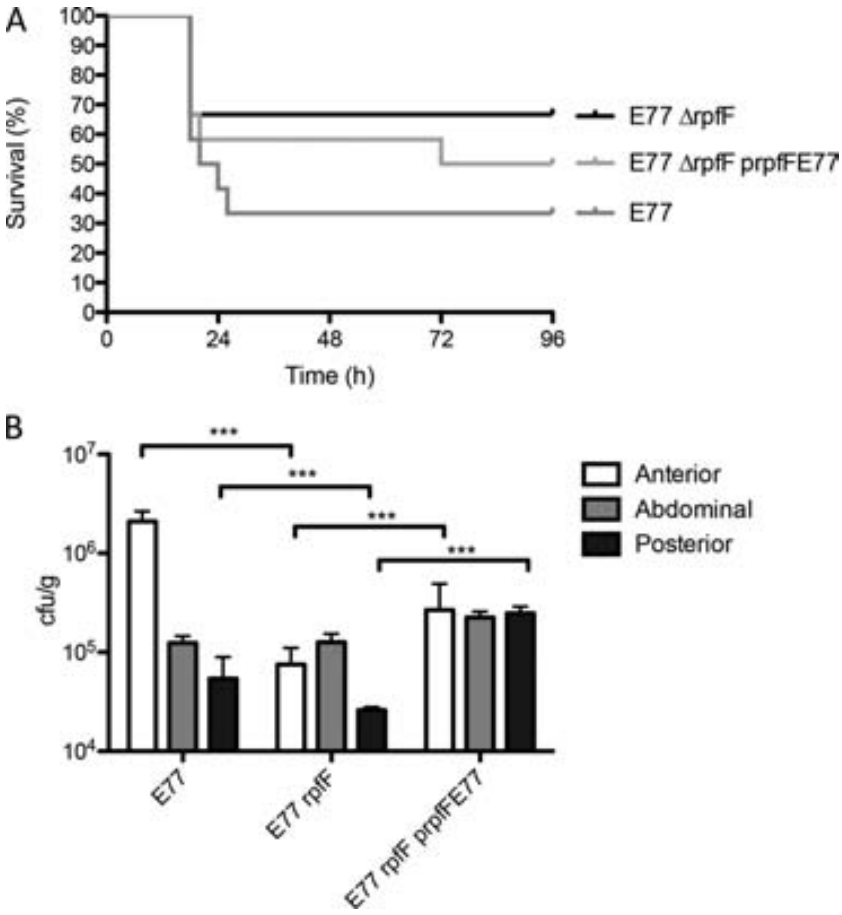

FIG 9 (A) Virulence of E77, the Δ*rpfF* mutant, and the complemented strain in 9-month-old zebrafish. (B) Bacterial recovery from different regions of the bodies of sacrificed fish at 72 h postinfection with E77, the Δ*rpfF* mutant, or the complemented strain. ***, $P < 0.0005$.

variant 2. Accordingly, we hypothesize that all of the *S. maltophilia* strains analyzed in the study by Pompilio et al. and showing an *rpfF*$^+$ genotype belong to the RpfF-1 variant group, whereas the *rpfF* mutant strains would belong to the RpfF-2 variant group. Interestingly, our analysis of *rpfF* from a collection of 82 *S. maltophilia* strains shows similar RpfF variant frequencies in the population. RpfF-1 is present in 59.75% of the strains (including K279a and R551), whereas RpfF-2 is present in 40.25% (including D457 and JV3). Taking the two studies together (171 strains), strains carrying the RpfF-1 variant appear to be more commonly isolated than those carrying the RpfF-2 variant, with relative prevalences of ca. 60 and 40%, respectively.

Surprisingly, we have observed that only strains carrying the RpfC–RpfF-1 pair produce DSF under WT conditions, while strains belonging to the RpfC–RpfF-2 variant group require extra copies of their own *rpfF* gene (Fig. 3 and 4) or the absence of the repressor component RpfC-2 (Fig. 5) to achieve detectable DSF production levels. These results indicate that RpfF-2 is able to synthesize DSF but the production of this signaling molecule is permanently repressed by RpfC-2 under the conditions assayed. It has been shown that the stoichiometric balance between RpfF and RpfC is crucial for DSF production in many members of the order *Xanthomonadales*. In *X. campestris* pv. *campestris*, RpfC physically interacts with the RpfF active site, inhibiting DSF synthesis activity (12, 22, 24). RpfC has also been shown to repress the RpfF activity of *X. fastidiosa* (25). Analysis of mRNA levels in E77 and M30 by qRT-PCR shows that the *rpfF*/*rpfC* expression ratio in the DSF producer strain (variant 1) is double that found in the nonproducer one (variant 2), suggesting, together with the observation that variant 2 strains complemented with extra *rpfF* copies produce DSF, that the different phenotypes of the two variants may be partly due to the different regulation of the stoichiometry of these two components. It has also been suggested that in *X. campestris* pv. *campestris*, RpfC could play a positive-feedback role in DSF synthesis, liberating active RpfF upon the detection of DSF molecules (22). Assuming similar mechanisms in *S. maltophilia*, we hypothesize that DSF production in RpfC–RpfF-1 strains is due to the presence of a competent sensor input domain, i.e., composed of 10 TM regions, in RpfC-1, which would enable the liberation of active RpfF-1 upon DSF detection and the subsequent synthesis of DSF. On the other hand, the missing TM regions in RpfC-2 would render this factor incompetent for DSF sensing, leading to permanent inhibition of RpfF-2 by RpfC-2 in a situation of equal numbers of copies. Demonstrating that RpfC-1 liberates free active RpfF after DSF detection and understanding its mechanism or unveiling why the *S. maltophilia* population produces two RpfC variants and what implications it may have for DSF-mediated regulation are questions that require further studies. The possibility that RpfC–RpfF-2 variant strains may produce DSF under specific environmental conditions or that RpfF-2 may produce a different yet undetected DSF derivative cannot be ruled out. Comparison of GC-MS spectra from M30, its Δ*rpfF* mutant, and the complemented strain did not, however, reveal any peak compatible with the mass of a DSF derivative.

It is well known that the DSF-QS system regulates certain virulence traits in many bacteria (1, 3, 9, 12, 26–29). To determine the possible implication of each RpfF variant for virulence regulation, we generated an *rpfF* deletion mutant for a strain representative of each variant group, i.e., E77 for the RpfF-1 variant group and M30 for the RpfF-2 group. All of the phenotypes evaluated in M30 were unaltered in the Δ*rpfF* mutant and in the corresponding complemented strain, suggesting that RpfC–RpfF-2 variant strains may not use the DSF-QS system to regulate these virulence factors, likely because of their inability to produce and sense DSF molecules under the conditions assayed. On the contrary, the E77 Δ*rpfF* mutant showed attenuation in both the *C. elegans* (Fig. 8A) and zebrafish (Fig. 9A) infection models, proving that DSF-mediated regulation affects the virulence of RpfC–RpfF-1 strains. Moreover, the recovery of bacteria from sacrificed fishes at 72 h postinjection showed that E77 is able to disseminate to the anterior and posterior regions through the fish body, while the E77 Δ*rpfF* mutant had serious problems in crossing intraperitoneal barriers (Fig. 9B). This is in concordance with the results showing a loss of swarming motility (Fig. 6) and a drastic increase in biofilm formation capacity (Fig. 7A and B) by the E77 Δ*rpfF* mutant, two important virulence-related traits that would explain attenuation in the animal models and especially in the zebrafish experiments. Much evidence of the implication of RpfF and DSF-like fatty acids in bacterial motility has indeed been reported (1, 2, 9, 30). Our results thus reinforce previous evidence that one of the main functions of DSF-QS is to regulate bacterial motility. Many studies have also demonstrated the implication of DSF-like molecules in biofilm regulation. There is, however, some controversy about whether DSF-like molecules may act by stimulating or inhibiting the sessile or motile bacterial lifestyle. Thus, DSF molecules have been shown to positively regulate biofilm formation in *X. oryzae* pv. *oryzae* (31), *B. cenocepacia* (9, 28), and *X. fastidiosa* (4, 26). On the contrary, in *X. campestris* pv. *campestris*, the DSF-mediated QS acts as a negative regulator of biofilm development (32–34). Additionally, fatty acid-mediated biofilm dispersion is not restricted to species with the DSF-QS system. For example, the fatty acid *cis*-2-decenoic acid produced by *Pseudomonas aeruginosa* PAO1 stimulates biofilm dispersion in several Gram-positive and Gram-negative bacteria (35, 36). Our findings indicate that the DSF-QS system in *S. maltophilia* E77 has a regulatory function similar to that described for *X. campestris* pv. *campestris*, where DSF also plays an important role in preventing biofilm formation and stimulating bacterial motility.

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Draft Genome Sequence of *Stenotrophomonas maltophilia* Strain M30, Isolated from a Chronic Pressure Ulcer in an Elderly Patient

Pol Huedo,[a,b] Óscar Conchillo-Solé,[a] Daniel Yero,[a,b] Sònia Martínez-Servat,[a,b] Xavier Daura,[a,c] Isidre Gibert[a,b]

Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Barcelona, Spain[a]; Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Barcelona, Spain[b]; Catalan Institution for Research and Advanced Studies, Barcelona, Spain[c]

P.H. and O.C.-S. contributed equally to this article.

***Stenotrophomonas maltophilia* is an emerging opportunistic pathogen with an increasing prevalence of multidrug-resistant strains. Here, we report the draft genome sequence of *S. maltophilia* strain M30, isolated from a pressure ulcer in an elderly patient.**

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Address correspondence to Xavier Daura, xavier.daura@uab.cat, or Isidre Gibert, isidre.gibert@uab.cat.

Stenotrophomonas maltophilia is an aerobic ubiquitous Gram-negative bacillus commonly isolated from hospital environments (1). Although *S. maltophilia* displays limited invasiveness and pathogenic capacity, it is capable of infecting a wide range of tissues and organs, especially in immunocompromised patients (2). Its disease patterns include bacteremia, catheter-related infections, pneumonia, biliary and urinary tract infections, and skin infections. The therapeutic agent of choice is typically trimethoprim-sulfamethoxazole (3, 4), but resistance to this drug is increasingly being reported (5). New insights into the mechanisms of drug resistance are needed in order to identify new effective drug targets.

S. maltophilia strain M30 was isolated from a pressure ulcer of an elderly patient in the Hospital Municipal de Badalona (Barcelona, Spain) in 2009. The M30 strain is a multidrug-resistant (MDR) organism, showing resistance not only to tetracycline, kanamycin, sulfamethoxazole, and erythromycin (6), but also to the complement-mediated bactericidal action of serum (6). Multilocus sequence type analysis (7) revealed that M30 belongs to a new sequence type (sequence type 76 [ST-76]) (6) clustering within a new genetic group (genogroup C) previously described by Kaiser et al. (7). This genetic group comprises clinical isolates from different geographic regions and includes the model MDR strain D457 (8). Recently, it was demonstrated that in strain M30, the diffusible signal factor (DSF)-mediated quorum-sensing system is regulated by a new *rpf* cluster variant (9).

Genomic DNA was extracted with GenElute bacterial genomics DNA kit (Sigma-Aldrich), and whole-genome sequencing was performed using Illumina MiSeq technology at the Universitat Autònoma de Barcelona Genomics core facility. The low-quality reads were filtered, and the remaining reads were *de novo* assembled using VelvetOptimiser version 2.2.5 (10) relaying on Velvet version 1.2.10 (11) and improved with the IMAGE program from the PAGIT package version 1 (12). The assembly resulted in 193 contigs (G+C content, 66.3%), with an N_{50} contig size of 46,399 nucleotides, covering a total of 4,902,008 bp. The average length of the contigs is 25.4 kb,nd a the biggest contig contains 142,025 bp.

Genome annotation was performed by the NCBI Prokaryotic Genome Annotation Pipeline version 2.5 (rev. 434060), and 4,515 genes were predicted, of which 4,392 are coding sequences (CDSs), 43 are pseudogenes, 11 are rRNAs (5S, 16S, and 23S), 68 are tRNAs, and 1 is a noncoding RNA (ncRNA). Among the predicted CDSs of M30, we found that 357 genes are not shared with the six strains of *S. maltophilia* (K279a, D457, JV3, R551-3, EPM1, and Ab55555) for which complete genome sequences were available at NCBI (http://www.ncbi.nlm.nih.gov/genome) at the time of analysis. Most of these unique genes encode hypothetical proteins, enzymes related to DNA metabolism and repair, and transposases and integrases, indicating that horizontal gene transfer may be an important source of genomic diversity in *S. maltophilia*. Notably, the set of genes exclusive to M30 also encoded proteins involved in host-microbe interactions, including ankyrin repeat (ANK)-containing proteins (13), two predicted hemolysins, one protease, and one amidohydrolase.

This draft genome will help improve our understanding of genome-associated resistance mechanisms in *S. maltophilia* and the virulence factors the bacterium exploits in order to become a pathogen.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. JELS00000000. The version described in this paper is JELS02000000.

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Abundance of the Quorum-Sensing Factor Ax21 in Four Strains of *Stenotrophomonas maltophilia* Correlates with Mortality Rate in a New Zebrafish Model of Infection

Mario Ferrer-Navarro[1☯], Raquel Planell[1☯], Daniel Yero[1], Elías Mongiardini[1], Gerard Torrent[1], Pol Huedo[1,2], Paula Martínez[1,2], Nerea Roher[1], Simon Mackenzie[1,3], Isidre Gibert[1,2]*, Xavier Daura[1,4]*

1 Institut de Biotecnologia i de Biomedicina (IBB), Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès, Barcelona, Spain, **2** Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès, Barcelona, Spain, **3** Institute of Aquaculture, University of Stirling, Stirling, Scotland, United Kingdom, **4** Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain

Abstract

Stenotrophomonas maltophilia is a Gram-negative pathogen with emerging nosocomial incidence. Little is known about its pathogenesis and the genomic diversity exhibited by clinical isolates complicates the study of pathogenicity and virulence factors. Here, we present a strategy to identify such factors in new clinical isolates of *S. maltophilia*, incorporating an adult-zebrafish model of *S. maltophilia* infection to evaluate relative virulence coupled to 2D difference gel electrophoresis to explore underlying differences in protein expression. In this study we report upon three recent clinical isolates and use the collection strain ATCC13637 as a reference. The adult-zebrafish model shows discrimination capacity, i.e. from very low to very high mortality rates, with clinical symptoms very similar to those observed in natural *S. maltophilia* infections in fish. Strain virulence correlates with resistance to human serum, in agreement with previous studies in mouse and rat and therefore supporting zebrafish as a replacement model. Despite its clinical origin, the collection strain ATCC13637 showed obvious signs of attenuation in zebrafish, with null mortality. Multilocus-sequence-typing analysis revealed that the most virulent strains, UV74 and M30, exhibit the strongest genetic similitude. Differential proteomic analysis led to the identification of 38 proteins with significantly different abundance in the three clinical strains relative to the reference strain. Orthologs of several of these proteins have been already reported to have a role in pathogenesis, virulence or resistance mechanisms thus supporting our strategy. Proof of concept is further provided by protein Ax21, whose abundance is shown here to be directly proportional to mortality in the zebrafish infection model. Indeed, recent studies have demonstrated that this protein is a quorum-sensing-related virulence factor.

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* E-mail: Xavier.Daura@uab.cat (XD); Isidre.Gibert@uab.cat (IG)

☯ These authors contributed equally to this work.

Introduction

Stenotrophomonas maltophilia is a non-fermentative Gram-negative bacterium with increasing incidence in hospital environments [1,2]. This obligate aerobic bacterium can be found in almost any aquatic or humid environment, including drinking-water supplies [3] and is now recognized as an emerging nosocomial pathogen. *S. maltophilia* has been associated with respiratory infections, septicemia, biliary sepsis, endocarditis, conjunctivitis, meningitis, urinary tract infections and various wound infections in immunocompromised patients as well as in cystic fibrosis (CF) patients [2,4,5]. Currently, *S. maltophilia* has been isolated from the lungs of approximately 10% of the CF patients in USA and up to 25% of those in Europe [1] and displays significant morbidity and mortality rates among debilitated patients [2,5,6,7,8].

S. maltophilia exhibits high-level intrinsic resistance to a variety of structurally unrelated antibiotics, including β-lactams, quinolones, aminoglycosides, tetracycline, disinfectants and heavy metals [9,10]. Intrinsic resistance may be due to reduced outer-membrane permeability, changes in LPS structure, the production of multidrug efflux pumps and the presence of integrons for site-specific insertion of resistance gene cassettes [11,12]. The production of melanin-like pigments and biofilms have also been linked to antimicrobial resistance [12]. Thus, the adhesion of *S. maltophilia* to medical implants, catheters and epithelial cells, leading to the formation of biofilms, confers natural protection against different antimicrobial agents and host immune defenses. In this regard, the development of therapies against *S. maltophilia* infection represents a significant challenge for both clinicians and microbiologists. In addition, knowledge of virulence factors is scarce and limited to homology relationships.

Recently, four *S. maltophilia* genomes have been fully sequenced and assembled (strains K279a, R551–3, JV3 and D457), and

putative virulence factors have been identified by homology relationships [11,13,14]. These factors include type I, II, IV, and V protein-secretion systems, various pili, fimbriae, putative adhesins, tissue-degradative exoenzymes, siderophores, quorum-sensing factors and proteins involved in polysaccharide synthesis and intracellular signaling. Some fimbrial structures have been identified and characterized and their role in adhesion to epithelial cells and abiotic surfaces has been demonstrated [15]. However, the level of understanding of this bacterium's pathogenicity and virulence is still limited and the number of *S. maltophilia* strains phenotypically and genotypically analyzed is minor. Furthermore, there is considerable uncertainty about the route(s) of infection of *S. maltophilia*. Additionally, the remarkable diversity of sources from which *S. maltophilia* strains have been isolated indicates that these bacteria exhibit a high level of genomic plasticity and metabolic heterogeneity, possibly allowing them to expand their pathogenic potential. Heterogeneity is also illustrated among *S. maltophilia* isolates recovered from a single patient, showing phenotypic variation over time as a consequence of horizontal gene transfer or high mutation rates [16].

In order to provide answers to some of the above the development of an appropriate infection models is essential. Previous studies suggest a limited invasiveness of *S. maltophilia* in mice, as indicated by a transient and minimal presence of the microorganism in animal organs. For example, *S. maltophilia* CF strains were shown to cause no mortality in a neonatal mouse model of respiratory tract infection [17]. Despite this lack of robust invasiveness, mouse models of *S. maltophilia* infection have provided information on the type of host immune response induced by this opportunistic pathogen [2]. More recently, a model of acute respiratory infection in DBA/2 mice following a single exposure to aerosolized bacteria enabled the investigation of bacterial clearance, histological damage, and inflammatory response in the lungs of infected mice [18]. However, while bacterial colonization and mortality were achieved in that model, infection disseminated at a very low rate even using high doses of a virulent strain and most of the animals were able to resolve *S. maltophilia* lung colonization in a relatively short time period. For that reason, animal-weight loss is often taken as the best criterion for the comparison of pathogenesis and virulence of tested strains [18,19]. In addition, lung infection models tend to be time-consuming, labor intensive and have associated welfare issues. Therefore, alternative, simple models of *S. maltophilia* infection are still needed to test the virulence of phenotypically and genotypically diverse strains.

In recent years the zebrafish (*Danio rerio*) has emerged as an important model of vertebrate development, human disease and microbial infection [20]. Nevertheless, to our knowledge, zebrafish has not yet been reported as a model of *S. maltophilia* infection. Our choice of adult zebrafish as a plausible model was motivated by a series of observations. The existing literature in relation to other bacterial pathogen models successfully developed in the zebrafish [20,21,22]. *S. maltophilia* as a natural pathogen of fish causing infectious intussusception syndrome in adult channel catfish [23]. The inclusion of a wild-type phenotypic population diversity as opposed to inbred mouse lines. The adaptive and innate immune system of zebrafish has significant similarities to mammalian systems [24,25]. The zebrafish is recognized as an important vertebrate model with genomic enablement and last, but not least relevant, ethical, economic and process-simplicity considerations.

In the study presented here, we have tested a combined approach that uses an adult-zebrafish model of *S. maltophilia* infection for the evaluation of relative virulence and the successive analysis by fluorescence-based two-dimensional Difference in-Gel Electrophoresis (DIGE) [26] of the underlying differences in protein expression, in a quest for virulence factors. This analysis was applied to three recent clinical isolates and the collection strain ATCC13637, as a reference. Although a colloidal Coomassie-stained 2DE proteomic analysis to find heat-induced changes in *S. maltophilia* protein abundance has been reported [27], to our knowledge no quantitative proteomic comparison between *S. maltophilia* strains with distinct virulence phenotypes has been previously performed.

Results

Zebrafish Infection Model Confirms Attenuation of Collection Strain and Points at Varying Virulence of the Clinical Isolates

Adult zebrafish were used as an infection model to determine the virulence of the individual strains. An intraperitoneal injection of 10^8 cfu resulted in strain-dependent mortality rates (Figure 1), where UV74 was the most aggressive, with 84% mortality 48 h post-injection (p.i.), followed by strains M30 (55%), E77 (5%) and ATCC13637 (0%). Although the experimental period was during 7 days, in all cases mortality occurred during the first 48 h. Clinical symptoms of dead fish included cutaneous hemorrhage under the lower jaw, on the belly, and around the anus; all dead fish had a distended abdomen containing bloody or clear fluid and severe enteritis with intussusception in the lower intestine. All isolates obtained from post-mortem UV74 infected zebrafish were identified as *S. maltophilia* based on morphology, API (analytical profile index) and 16S rDNA sequence (data not shown). No zebrafish died when injected with 20 µl of sterile PBS (data not shown). Interestingly, injection of 10^8 heat-inactivated UV74 bacteria did not produce mortality in zebrafish. This suggests that viable bacteria producing thermo-labile proteins are required for infection and that mortality is not caused by non-specific activation due to bacterial components such as lipopolysaccharides.

Strain-genotyping Analysis Reveals a Distant Genetic Relationship between the Strains

The genetic relationship of the four *S. maltophilia* strains was assessed using multilocus sequence typing (MLST). The isolates were compared and organized based on similarities among seven housekeeping genes (*atpD*, *gapA*, *guaA*, *mutM*, *nuoD*, *ppsA* and *recA*).

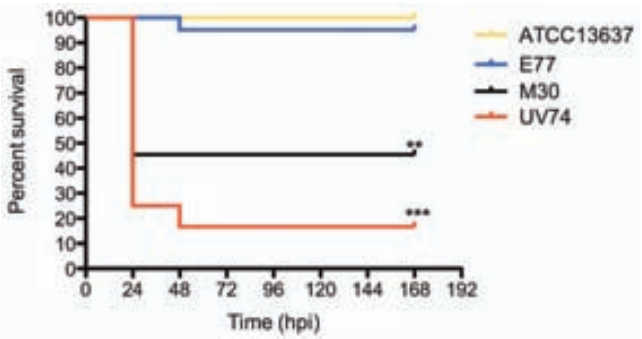

Figure 1. Survival curves of adult zebrafish injected with *S. maltophilia* ATCC13637, E77, M30, UV74 (10^8 cfu in 20 µl). Control groups injected with heat-inactivated UV74 (10^8 cfu in 20 µl) and sterile PBS (20 µl) presented no mortality (not shown). Zebrafish mortalities were recorded for 168 h post-infection (hpi) and survival curves analyzed using the Kaplan-Meier method (log-rank test: ***, $p \leq 0.001$; **, $p \leq 0.01$).
doi:10.1371/journal.pone.0067207.g001

This analysis resulted in the three clinical strains being classified into new, different sequence types (M30 as ST-76, UV74 as ST-77 and E77 as ST-81), indicating they are not clonally related despite being isolated from a common hospital setting. Nevertheless, strains M30 and UV74 share the same allele for genes *atpD* and *mutM*. The collection strain ATCC13637 had been already characterized by MLST and assigned the ST-14 [28]. In addition, thirty-five concatenate sequences from different *S. maltophilia* strains, isolated from human clinical cases, were obtained from public databases and used to provide the context for the genotypic classification of the three clinical isolates and the collection strain. Strains were chosen such that they cover as far as possible the full genetic breadth of the species [28]. The phylogenetic analysis revealed distant genetic relationships between the three clinical strains and the reference that according to a previous classification based on AFLP fingerprinting [29] would belong to a different genomic group (Figure 2). Strains M30 and UV74 show the closest genetic relationship, clustering within genomic group C as previously described by Kaiser *et al.* [28]. The MLST results imply that the three pair wise (collection:clinical) proteomic analyses described below are non-redundant.

The Most Virulent Strain, UV74, also shows the Most Resistant Phenotype

MIC determination included the following antibiotic families: tetracyclines, aminoglycosides, macrolides, sulfonamides, chloramphenicol and fluoroquinolones (Table 1). UV74 presented the most resistant phenotype to the antibiotics tested. The most distinguishing feature of this strain is its significantly higher MIC for ciprofloxacin (16 to 64-fold). In addition, the new clinical isolates were significantly more resistant to kanamycin than the ATCC13637 strain (8 to 32-fold).

The Collection Strain shows Reduced Biofilm-formation and Swimming Capacities

The strains presented similar growth curves except for E77, whose doubling time during the exponential phase was 1.3 h as opposed to 1 h for the other three strains (Figure S1). The capacity to form biofilms on polystyrene plates was tested (Table 2) and statistical analysis revealed that the collection strain had a reduced ability to form biofilms in comparison to the clinical strains. M30 presented the greatest biofilm-formation capacity.

The analysis of different types of bacterial motility (swimming, twitching, swarming) showed that ATCC13637 displays significantly reduced swimming compared to the clinical strains

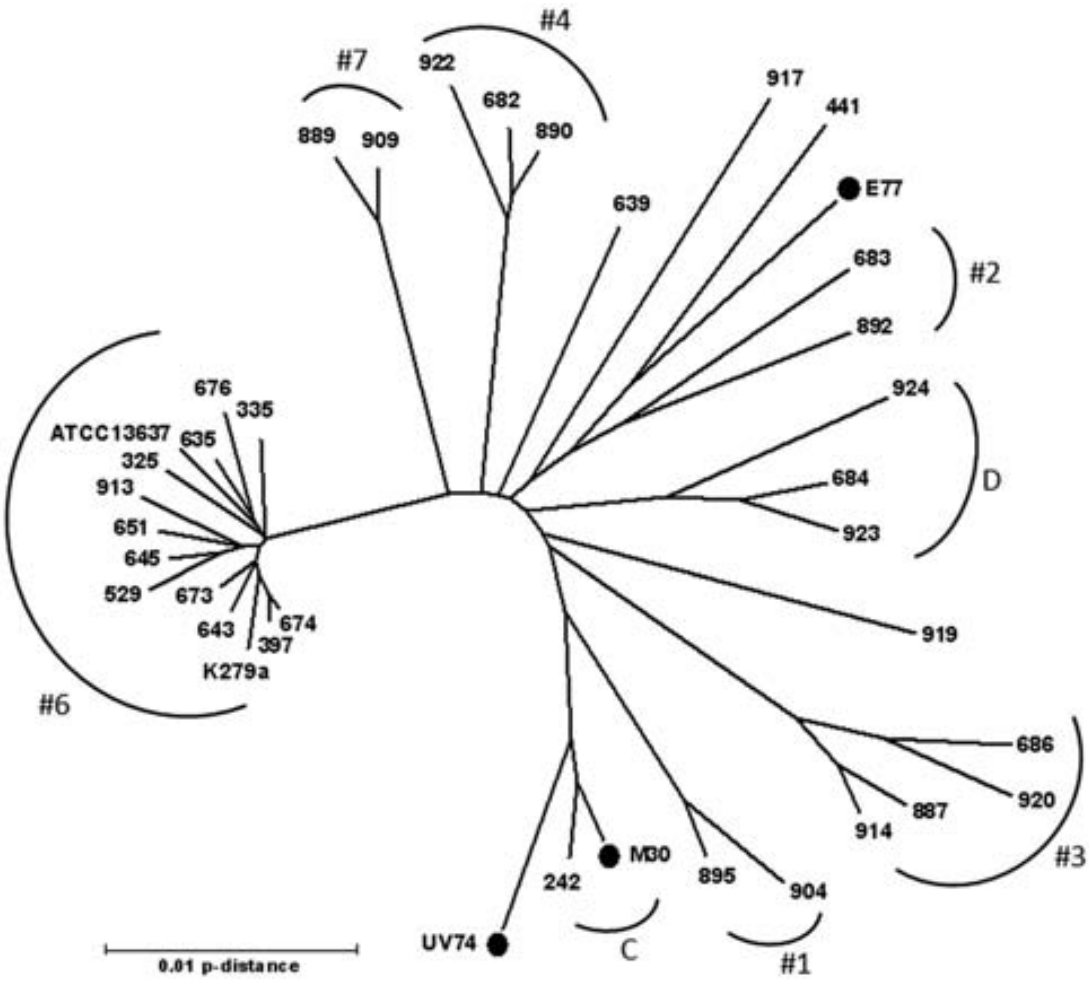

Figure 2. Neighbor-joining radiation tree based on the concatenated sequences of the MLST loci, showing relationships among the three recent clinical isolates (solid circles), the ATCC13637 strain and 35 previously characterized *S. maltophilia* pathogenic strains [**28**]. Previously defined genomic groups [28,29] are indicated (#1 to #7, C and D).
doi:10.1371/journal.pone.0067207.g002

Table 1. MICs for several antibiotics against the *S. maltophilia* collection and clinical strains.

	MIC (μg/ml)										
Strain	**Tc**	**Mino**	**Gent**	**Kan**	**Trim**	**Sul**	**Cipro**	**Nor**	**Levo**	**Ery**	**Cm**
ATCC13637	12.8	0.2	3.2	16.0	16.0	512.0	0.2	12.8	0.8	6.4	6.4
M30	12.8	0.1	3.2	128.0	4.0	256.0	0.8	2.0	0.4	12.8	6.4
UV74	25.6	0.8	3.2	512.0	32.0	512.0	12.8	12.8	0.8	12.8	12.8
E77	6.4	0.2	3,2	256.0	8.0	64.0	0.4	12.8	0.2	12.8	6.4

Tc, tetracycline; Mino, minocycline; Gent, gentamicin; Kan, kanamycin; Trim, trimethoprim; Sul, sulphamethoxazole; Cipro, ciprofloxacin; Nor, norfloxacine; Levo, levofloxacin; Ery, erythromycin; Cm, chloramphenicol.
doi:10.1371/journal.pone.0067207.t001

(Table 2). Twitching migration capabilities however were similar among the four strains. Finally, all strains presented swarming motility consisting of branches or tentacles radiating from the inoculation point. Although E77 presented swarming under the conditions described in materials and methods, observation of this type of motility in the other strains required 12 days of incubation at 30°C in the medium described by Kholer *et al.* [30]. To our knowledge, this is the first time swarming has been described for *S. maltophilia*.

Serum Sensitivity Correlates with Virulence in Zebrafish

Resistance of the four strains to antibody/complement-mediated bactericidal action of serum was also tested (Figure 3). The ATCC13637 and E77 strains clearly showed a higher sensitivity to the bactericidal action of serum (0.0044% and 0.082% survival, respectively) compared to the M30 and UV74 (5% and 16% survival, respectively). As a control, when the incubation was performed with Hank's balanced salt solution (HBSS) or inactivated serum no mortality was observed. Notably, these results correlate with zebrafish mortality ($p = 0.059$).

Adhesion to HeLa Cells Correlates with Serum Sensitivity

To test the relative adhesion capacities of the *S. maltophilia* strains to eukaryotic cells, adhesion experiments with HeLa cells were performed (Figure 4). For ATCC13637, the number of adhered bacteria to HeLa cells after 2 h of incubation was 1.7% of the initial bacterial load. This was significantly lower than the percentages found for M30 (12%), E77 (5%) and UV74 (45%). This results correlate with serum sensitivity ($p = 0.008$) and also follow the strain-virulence order.

Differential Proteomics Points at Specific Processes and Highlights a Protein that Correlates with Virulence

To analyze potential differences in the protein profiles of the collection and clinical strains, independent samples were taken from each strain in the exponential-growth phase (OD = 1) and were Cy-dye labeled and pairwise separated by 2DE. An example of the resulting fluorescence images is shown in Figure S2, with the ATCC13637 sample in red and the clinical sample in green. A total of 1807, 1776 and 1677 protein spots were detected in the ATCC13637-M30, ATCC13637-E77 and ATCC13637-UV74 gels, respectively. Using a 1.5-fold threshold for clinical:collection abundance ratio, ca. 100 spots displayed significant differential content of protein from collection and clinical sources ($p<0.05$, ANOVA test). These spots were excised and in-gel digested with trypsin. Following MALDI-MS time-of-flight analysis, 38 proteins were identified for which the absolute value of the ratio was ≥ 1.5 for at least two clinical isolates. The low identification rate (38 out of 100 differential spots) stems partly from the fact that the identification is performed on the analytical gels, to avoid uncertainties with mismatching spots on the preparative silver-stained gels. The protein amount loaded on the analytical gels is low (25 μg per sample), making the identification of weak-intensity spots impossible. Some of the proteins were identified in several spots, indicating the presence of different post-translationally modified forms. A detailed list of the identified proteins and quantified abundance differences is given in Table S1, with the corresponding statistical analysis provided in Table S2. Example fluorescence images and expression profiles of some of the identified proteins are shown in Figures S2 and S3, respectively. In addition, a survey on orthologs of the 38 proteins that have been linked to pathogenesis, virulence or resistance has been performed and summarized in Table S3.

Table 2. Swimming, twitching and biofilm formation of the *S. maltophilia* collection and clinical strains.

	Swimming[a] (cm)	Twitching[a] (cm)	Biofilm formation[a] (OD_{620nm})
Strain	**24 h**	**24 h**	**24 h**
ATCC13637	2.1±0.5	1.5±0.2	0.16±0.02
M30	3.1±0.5[b]	1.4±0.2	0.33±0.02[b]
UV74	3.8±0.5[b]	1.4±0.2	0.31±0.02[b]
E77	3.4±0.5[b]	1.6±0.2	0.19±0.02[b]

[a]Values represent the mean and standard deviation.
[b]$p \leq 0.001$ significance of difference with ATCC13637 by one-way analysis of variance (ANOVA) with a Bonferroni's multiple comparison post-test.
doi:10.1371/journal.pone.0067207.t002

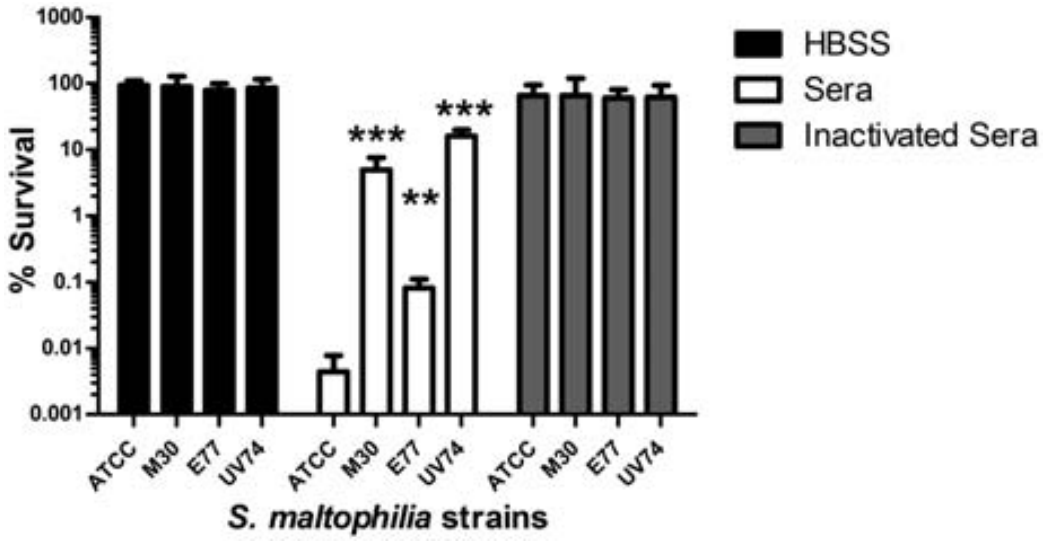

Figure 3. *S. maltophilia* serum-sensitivity assay. Percentage of surviving cells after 90 minutes of incubation in the presence of Hank's balanced salt solution, human serum or inactivated serum. The values represent the mean of three replicas of the three independent experiments. **, $p \leq 0.01$; ***, $p \leq 0.0001$ significance of difference with ATCC13637 by unpaired *t*-test with Welch correction for unequal variances.
doi:10.1371/journal.pone.0067207.g003

Among the proteins with altered abundance in clinical strains there are enzymes involved in the biosynthesis of fatty acids and other cell-wall components, enzymes involved in energy-related and other metabolic pathways, proteases, an RNA polymerase subunit, outer membrane proteins with receptor and transport activities and two completely uncharacterized proteins. The first 12 proteins (enzymes) in Table S1 are assigned to a common network by predicted pairwise interactions (see Table S3). Likewise, proteins 13 to 17 in Table S1 appear also cross connected. Indeed, some of them are known to share a role in biofilm formation and quorum sensing. Note that the ortholog information provided in Table S3 is not necessarily exportable to *S. maltophilia*. The molecular mechanisms underlying pathogenesis, virulence and resistance may vary not only between species but also between strains within a species. Furthermore, ortholog proteins involved in parallel mechanisms in two closely related species might still do so in different ways. For example, various proteins in Table S1, with known or suggested role in pathogenesis in other species appear significantly upregulated in one clinical isolate and downregulated in another, the most striking case being the LptD transporter. This protein is essential for the translocation of Lipid A across the outer membrane and, by extension, of LPS presence in the surface, and is strongly upregulated in E77 and downregulated in UV74 (remarkably the most virulent strain). While bearing this limitation in mind, comparison of Tables S1 and S3 highlights the following proteins as potentially relevant to pathogenesis, virulence or resistance: FabD, AccC, AcsA, PdhB, AcnB, enoyl-CoA hydratase, MurA, RpoA, LptD, putative TonB-dependent receptors Smlt3444 and Smlt4151, putative FadL, putative OmpA Smlt0955, PepO and putative Ax21. The latter protein, quorum-sensing factor Ax21, shows a strong correlation with the results of virulence in zebrafish challenges ($p = 0.022$) as well as with the strain order for adhesion to HeLa cells and serum sensitivity. In addition to these candidates, on the sole basis of the proteomics data (Table S1) the following proteins also deserve special attention: FadI, SucB, Bcd, OprP, putative exported peptidase S9, NuoG, UPF0234 family protein and hypothetical protein Smlt3796. Note that FadI and SucB, which are predicted to interact (Table S3), have a similar expression behaviour (Table S1), strongly downregulated in UV74 and weakly upregulated in M30 and E77.

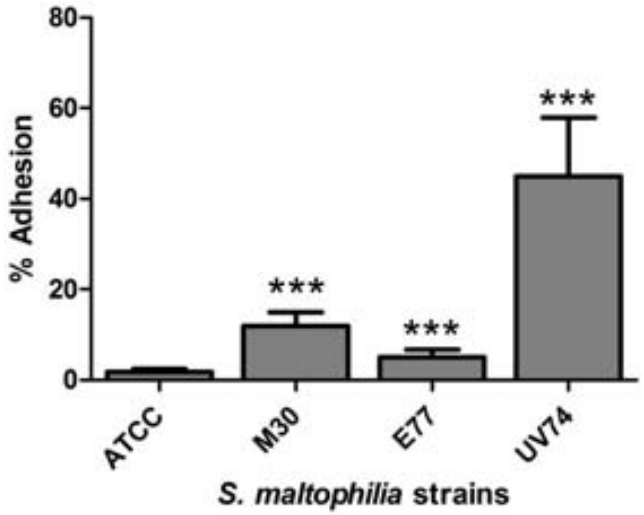

Figure 4. *S. maltophilia* adhesion to HeLa cells. Percentage of bacteria adhered to HeLa cells after 120 min of incubation in 24-well plates. The data correspond to the mean and standard deviation of three different assays carried out in triplicate. ***, $p \leq 0.001$ significance of difference with ATCC13637 by unpaired *t*-test with Welch correction for unequal variances.
doi:10.1371/journal.pone.0067207.g004

Discussion

The elucidation of molecular mechanisms leading to pathogenicity and virulence is essential to a better understanding of *S. maltophilia* infection and to the design of strategies to effectively combat it. The aim of the present study was to identify virulence factors in new clinical isolates of *S. maltophilia* by firstly establishing an alternative, simple animal model for the evaluation of relative virulence. This would then provide the basis for the analysis of

underlying differences in protein expression, in a quest to identify virulence factors. To our knowledge this is the first time the zebrafish has been used as a model of *S. maltophilia* infection.

The present study shows that the adult-zebrafish model has a high discrimination capacity, i.e. from very low to very high mortality rates for different strains of *S. maltophilia*, and correlates with serum sensitivity. This is also known to correlate with virulence in mouse and rat models [17,31]. Taken together, these properties point toward the zebrafish as a promising animal model for the study of *S. maltophilia* pathogenicity and virulence in humans.

Furthermore, the symptoms observed in the adult-zebrafish model after infection are very similar to those reported in a recent study on aquacultured adult channel catfish [23]. This includes ascites and enteritis, prolapse in the rectum and intussusception in the lower intestine, cutaneous hemorrhage in some cases. Affected fish were also listless, sought the water surface, became more lethargic as the disease progressed, and most died within a few days. These common symptoms suggest that the process following intraperitoneal infection is parallel to natural infection.

The collection strain ATCC13637 showed obvious signs of attenuation, with null zebrafish mortality in our experiments. This strain was isolated in 1960 from the oropharyngeal region of a patient with mouth cancer [32]. Since then, it has likely undergone a large number of *in vitro* serial passages. It is well known that the repeated *in vitro* sub-culturing of microorganisms during extended periods of time results in adaptation to the laboratory environment leading to changes in physiology, including pathogenic capacity and production of virulence factors. As a matter of fact, this property has been used in vaccine development to obtain live-attenuated strains [33]. This result points once more at the shortcomings of using standard laboratory strains in studies related to pathogenesis and virulence, even when their origin is clinical.

The three new *S. maltophilia* clinical isolates, M30, UV74 and E77, were chosen on the basis of their different clinical origin (decubitus ulcer, vascular ulcer and sputum, respectively). Despite being collected from patients of the same hospital and during the same year, the MLST analysis reported here demonstrates that they are not clonally related. Interestingly, the most virulent *S. maltophilia* strains (UV74 and M30) are the genetically most similar ones by means of MLST (Figure 2). Thus, M30 and UV74 cluster within the previously described genomic group C [28], which includes worldwide clinical strains isolated from tracheal secretion and blood cultures. Genetic and mutation-frequency analysis becomes particularly relevant when dealing with *S. maltophilia* clinical isolates, as the proportion of isolates showing high mutation frequencies (hypermutators) appears to be significantly higher for *S. maltophilia* than for other organisms [34]. However, whereas for different bacterial species the strains isolated from CF patients with chronic lung infections often show high mutation frequencies [35,36], hypermutators have rarely been found among isolates from other types of infections [35,37]. None of the strains tested in the present study were obtained from CF patients, for which reason mutation rates were not determined.

Phenotypic analysis of the four strains revealed that UV74 presents also the most resistant pattern to the set of antibacterials tested. In addition, the correlation found between zebrafish mortality, serum sensitivity and HeLa-cell adhesion points at the potential use of animal-free models for virulence-screening purposes. The correlation is particularly significant between serum sensitivity and HeLa-cell adhesion ($p = 0.008$), but also notable between zebrafish mortality and serum sensitivity ($p = 0.059$). As mentioned, a direct relationship between *S. maltophilia* virulence and serum sensitivity has been already described in a mouse model of respiratory tract infection [17] and a rat model of lung infection [31].

The demonstration of the attenuated character of ATCC13637 in the zebrafish infection model motivated the comparison of the proteomes of the three clinical isolates to the collection reference with the purpose of identifying potential virulence factors within the set of proteins showing a higher abundance in the clinical strains. Clearly, *S. maltophilia* will not necessarily show the same protein expression profiles under *in vitro* exponential-growth and *in vivo* infection conditions. It is very likely that a number of proteins relevant to pathogenicity and virulence will only be upregulated (or suppressed) in the latter case and will be therefore missed in this study. Nevertheless, there remains a certain possibility that proteins differentially expressed in the clinical and collection strains under *in vitro* exponential-growth conditions may also be related to pathogenicity and virulence, e.g. virulence factors that acquire a more constitutive character as result of adaptation to the clinical/infective environment.

Of the 38 proteins identified by differential proteomics, 24 are already documented for their potential implication in pathogenesis, virulence or resistance mechanisms in other bacteria (Table S3), validating the strategy presented here. Further proof of concept is provided by the identification of the putative quorum-sensing factor Ax21 (Smlt0387), whose abundance is shown to correlate with mortality in the zebrafish infection model ($p = 0.02$). It should be noted that this protein has been found in more than one spot in the gels, suggesting a different composition of isoforms between the collection and clinical isolates, being the more basic isoform the predominant in the latter. McCarthy *et al.* [38] have recently published a study in which deletion of *smlt0387* affects the transcription of genes encoding proteins involved in transcriptional regulation, antibiotic resistance and pilus assembly, and influences various phenotypes leading to reduced motility, reduced tolerance to some antibiotics, reduced biofilm formation and reduced virulence in the larval *Galleria mellonella* infection model. In addition, it has been shown that the homologous protein in *Xanthomonas oryzae* (60% identity), a microorganism closely related to *S. maltophilia*, triggers innate immunity in both plants and animals [39].

Ax21 has two conserved domains. One of them (pfam13505) is found in a wide range of outer-membrane-bound beta-barrel proteins. The other one (TIGR04273) is present in the sulfation-dependent quorum-sensing factor Ax21 protein family. This family consists of proteins closely related to Ax21 (Activator of XA21-mediated immunity), a 194-residue protein present in plant and animal pathogens [40] that is secreted by a type I secretion system (RaxABC) and appears to be sulfated at a tyrosine found in a motif LSYN in the N-terminal region. In *X. oryzae* the small protein Ax21 serves as a quorum-sensing factor, inducing density-dependent gene expression and thus regulating biofilm formation, bacterial motility and virulence. In addition, Ax21 is critical for *X. oryzae* virulence at low densities in rice leaves [40]. Contrary to the *Xanthomonas* case, Smlt0387 would apparently perform its intra-species signalling activity in a non-sulfation-dependent manner [38]. Our results further suggest that in *S. maltophilia* the relative abundance of this protein is directly proportional to virulence. Further studies (in progress) will be needed to address the potential role of the other proteins identified in the pathogenesis, virulence or resistance of *S. maltophilia*.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

The three clinical *S. maltophilia* strains characterized in this work were isolated from different patients at the Hospital Municipal de Badalona (Barcelona, Spain) during the year 2009. The clinical origin of M30, E77 and UV74 strains were decubitus ulcer, sputum and vascular ulcer, respectively. Species identification was confirmed biochemically using the API NE system (bioMérieux). The collection strain ATCC13637, isolated in 1960 from the oropharyngeal region of a patient with mouth cancer [32], was also included in the experiments. *S. maltophilia* strains were routinely cultured o/n in Luria-Bertani (LB) media at 37°C and 250 rpm unless otherwise stated, and growth curves were monitored following the optical density at 550 nm (Figure S1).

Zebrafish Infection Assay

Adult (9–12 months) wild-type zebrafish (*D. rerio*) were kept in a 12 h light:12 h dark cycle at 28°C and fed twice daily with dry feed. All fish used in infection experiments were transferred to an isolated system and acclimated for three days before infection. Adult zebrafish (n = 12/each condition) were infected by intraperitoneal injection (i. p.) [41] with 20 µl of a 5×10^9 cfu/ml suspension of *S. maltophilia* strain ATCC13637 and the clinical isolates M30, E77 and UV74. These strains were previously grown at 28°C in blood agar plates (BioMérieux) for 20 h and collected directly from the plates with phosphate buffered saline (PBS). Two control groups were injected with PBS and with a heat-inactivated UV74 strain (incubation at 100°C for 30 min, at which time no viable bacteria were detectable), respectively. Fish were observed daily for signs of disease and mortality. All living injected fish were sacrificed after 7 days by MS-222 overdose. All experiments were repeated independently twice.

Multilocus Sequence Typing and Phylogenetic Analysis

PCR amplification and sequencing of the seven housekeeping genes included in the MLST scheme was performed as previously described [28,42]. The detailed MLST procedure and set of primers used were obtained from the *S. maltophilia* MLST database (http://pubmlst.org/smaltophilia/). Briefly, genomic DNA was extracted with GenElute Bacterial Genomics DNA Kit (Sigma-Aldrich). Fragments of the seven genes *atpD*, *gapA*, *guaA*, *mutM*, *nuoD*, *ppsA*, and *recA* were amplified using FastStart Taq DNA Polymerase (Roche, Diagnostics) as follows: initial denaturation at 95°C for 9 min followed by 30 cycles of denaturation at 94°C for 20 s; annealing at the appropriate temperature for 1 min; extension at 72°C for 50 s; final elongation at 72°C for 5 min. PCR products were treated with ExoSAP-IT (USB Products) and sequenced at Macrogen Inc. (Seoul, Korea) in both directions using standard conditions. The allele numbers for each locus and sequence type (ST) were determined by comparison with the available sequences at the *S. maltophilia* MLST database, where the data has been deposited. Phylogenetic relationships were established on the basis of the concatenated seven gene sequences without further corrections. Cluster analysis was performed with the Neighbour-Joining method, using uncorrected p-distance. The phylogenetic tree of *S. maltophilia* strains was constructed by use of MEGA software version 4 [43], together with 35 already analyzed pathogenic *S. maltophilia* strains [28] with sequence information available at the *S. maltophilia* MLST database.

Determination of MICs

The susceptibility of *S. maltophilia* to the following antimicrobial agents was tested: tetracycline, minocycline, gentamicin, kanamycin, trimethoprim (from Apollo Scientific Ltd), sulfamethoxazole, norfloxacin, ciprofloxacin, erythromycin, levofloxacin (from Sigma-Aldrich), and chloramphenicol (from Roche Diagnostics). The MICs for these antibiotics were determined by microdilution test using 96-well plates by serial two-fold dilutions of each drug in 100 µl of LB. 100 µl of bacterial suspension (final $OD_{550nm} = 0{,}005$) were added and the antibiotic dilutions and the organism suspension were mixed and incubated at 37°C for 16 h before developing with resazurin (30 µl 0.01%) [44]. The MIC was defined as the lowest drug concentration that prevented bacterial growth. The microdilution assay followed the Clinical and Laboratory Standards Institute (CLSI, www.clsi.org) guidelines for antimicrobial susceptibility testing.

HeLa Cell Adherence Assay

HeLa cells were grown for 24 h on 24-well tissue culture plates (TPP Techno Plastic Products AG) containing 2 ml of Minimum Essential Medium α (Invitrogen) with 10% (v/v) inactivated fetal bovine serum (Invitrogen) and Glutamax™ (Invitrogen) to 90–95% of confluence. Bacterial cultures of ATCC13637, M30, E77 and UV74 strains were grown o/n in LB medium at 37°C without agitation and resuspended in HeLa medium. Bacteria were added at a multiplicity of infection (MOI) of 50:1 in triplicate to the confluent 24-well plates and were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide for 120 minutes. Wells were gently washed five times with 2 ml of Dulbecco's Phosphate-Buffered Saline (Invitrogen) to remove non-adherent bacteria. The number of cell-attached bacteria was quantified by lysis with 0.1% Triton X-100 and serial dilution were plated onto LB plates. Adhesion was measured as a percentage between adhered and the initial cells.

Serum Sensitivity Assay

Human serum sensitivity assay was performed following the protocol described by Waters *et al.* [17]. Briefly, bacteria were grown on LB medium to an OD_{550nm} of 0.5, washed in Hanks' balanced salt solution (HBSS) (Invitrogen), and incubated in HBSS, 60% serum or 60% heat-inactivated serum with agitation at 37°C with agitation for 90 min. The serum inactivation was performed at 56°C during 30 min. After incubation, bacteria were plated on LB plates after serial dilution. The survival was measured as a percentage between the surviving and initial cells.

Biofilm Formation on Polystyrene Plates

Quantification of *S. maltophilia* biofilm formation was assessed by crystal violet (CV) staining in 96-well polystyrene plates. Bacterial cultures of the four different *S. maltophilia* strains were grown o/n at 37°C. Absorbance at 550 nm was adjusted to 0.1 and 200 µl were grown in a 96-well plate during 24 h at 30°C. Cells were washed three times with water, fixed at 60°C for 1 h and stained during 15 min with 200 µl of 0.1% CV. The dye was discarded, and the plate was rinsed in standing water and allowed to dry for 30 min at 37°C. Stained biofilms were exposed to 250 µl of 95% ethanol for 15 min, and the OD of the extracted dye was measured at 620 nm.

Motility Assays

Overnight cultures of the different *S. maltophilia* strains were grown (plate or liquid medium) in LB under standard conditions. The swimming motility was determined in TrA plates (1% tryptone, 0.5% NaCl, 0.25% agar) [45]. Thus, 5 µl of adjusted o/n cultures of the different *S. maltophilia* strains were spotted on TrA plates. The twitching and swarming motility plates were LB plates

at 1% agar and BM2 at 0.5% agar [46], respectively. Twitching was assessed via subagar stab inoculations (stab assay) from o/n fresh plates as previously described [47]. The twitching zones were then visualized by staining with 1% (wt/vol) crystal violet and their diameters measured. Noble agar (Difco^{TM}) was used in the preparation of the three motility assay plates. The growth halos were measured in cm after 24 h of incubation at 30°C for the swimming and twitching motilities and after 7 days at 30°C for swarming.

Statistics

Statistical analyses were performed using the GraphPad Prism program version 5.00. Comparison of strain phenotypic data was performed by one-way analysis of variance (ANOVA) with a Bonferroni's multiple comparison post-test or unpaired *t*-test with Welch correction for unequal variances, as indicated in figure and table captions. Survival curves of zebrafish infection experiments were analyzed using the Kaplan-Meier method. Differences were evaluated using the log-rank test. The relationship between relative protein abundance in the different strains and pathogenicity variables, such as zebrafish mortality rates and adhesion to human cells, was evaluated with Pearson's chi-squared test. $p \leq 0.05$ was considered significant.

Sample Preparation for Two-dimensional Gel Electrophoresis

20 ml of exponential culture (OD = 1) were washed with PBS 1X three times and resuspended in lysis solution (8 M urea, 2 M thiourea, 2.5% 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS), 2% ASB-14, 40 mM Tris-HCl, pH 8.8). ASB-14 was used to increase the presence of membrane proteins in the 2DE [48]. Then, samples were disrupted by sonication and centrifuged in order to discard any insoluble cellular debris. In order to remove salts and other contaminants, samples were cleaned with 2D Clean-Up Kit (GE Healthcare). Resulting pellets were resuspended in the above-mentioned lysis solution. Protein concentration was determined with 2D-Quant Kit (GE Healthcare) and adjusted to 2 mg/ml by the addition of a DIGE labeling buffer (7 M urea, 2 M thiourea, 2.5% w/v CHAPS, 40 mM Tris, pH 8.8). A pool consisting of equal amounts of each of the two samples analyzed in the experiment was prepared as an internal standard for quantitative comparisons [49]. The clinical isolates were labeled with Cy3 and the collection strain ATCC13637 was labeled with Cy5. A third fluorescent dye, Cy2, was used to label the internal standard sample. Labeling was carried out by the addition of 400 pmol of the required Cydye in 1 µl of anhydrous N,N-dimethylformamide per 50 µg of protein. After 30 min of incubation on ice in the dark, the reaction was quenched with 10 mM lysine and the samples incubated for a further 10 min. Samples were combined according to the experimental design, using 50 µg of protein per Cy dye per gel, and diluted two-fold with isoelectric focusing (IEF) sample buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% dithiothreitol [DTT], 2% pharmalytes, pH 3–10). One clinical strain and the ATCC13637 strain sample, together with an aliquot of the internal standard pool, were then separated by two-dimensional electrophoresis (2-DE) in each of the gels. This experimental design allows the accurate quantification and statistical assessment of the differences in protein abundances observed between the two sample groups.

2D Difference Gel Electrophoresis

The 2-DE was performed using GE Healthcare reagents and equipment. First-dimension isoelectric focusing was performed on immobilized pH gradient strips (24 cm, pH 3–10) using an Ettan IPGphor System (GE Healthcare). Samples were applied near the basic end of the strips by cup-loading, after being incubated o/n in 450 µl of rehydration buffer (7 M urea, 2 M thiourea, 2.5% w/v CHAPS, 2% ASB-14 w/v, 0.5% pharmalytes, pH 3–10, 100 mM DeStreak reagent). After focusing at 70 kVh, strips were equilibrated, first for 15 min in 10 ml of reducing solution (6 M urea, 100 mM Tris-HCl, pH 8, 30% v/v glycerol, 2% w/v SDS, 5 mg/ml dithiothreitol [DTT]) and then in 10 ml of alkylating solution (6 M urea, 100 mM Tris-HCl, pH 8, 30% v/v glycerol, 2% w/v SDS, 22.5 mg/ml iodoacetamide) for 15 min on a rocking platform. Second dimension SDS-PAGE was performed by laying the strips on 12.5% isocratic Laemmli gels (24×20 cm), cast in low fluorescence glass plates, on an Ettan DALT Six system. Gels were run at 20°C at a constant power of 2.5 W per gel for 60 min followed by 17 W per gel until the bromophenol blue tracking front had run off the end of the gel. Triplicate gels were run for each sample using independent biological replicates. Fluorescence images of the gels were obtained on a Typhoon 9400 scanner (GE Healthcare). Cy2, Cy3 and Cy5 images were scanned at excitation/emission wavelengths of 488/520 nm, 532/580 nm and 633/670 nm, respectively, at a resolution of 100 µm. Both image analysis and statistical quantification of relative protein levels were performed using Progenesis SameSpots V.4 (Nonlinear Dynamics) (See Table S1 for detailed statistics of each spot). The data were analyzed as pairwise comparisons.

Protein Identification by Mass Spectrometry

In order to excise the spots of interest, gels were silver stained as described elsewhere [50]. Protein spots of interest were excised from the gel using a cut tip. The selected spots are those that are differentially expressed in at least two clinical isolates. In-gel trypsin digestion was performed as described previously [51]. MALDI-MS analysis of tryptic peptides was performed on an Ultraflex time-of-flight instrument (Bruker Daltonics). Samples were prepared using α-cyano-4-hydroxy-cinnamic acid. Calibration was performed in the external mode using a peptide calibration standard kit (Bruker Daltonics). The spectra were processed using Flex Analysis 2.2 software (Bruker Daltonics). Peak lists were generated using the signals in the 800–4000 mass:charge ratio (m/z) range, with a signal:noise threshold >3. The SNAP algorithm included in the software was used to select the monoisotopic peaks from the isotopic distributions observed. After removing m/z values corresponding to commonly observed matrix cluster ions, an internal statistical calibration was applied. Peaks corresponding to frequently seen keratin and trypsin autolysis peptides were then removed. The resulting final peak list was used for the identification of the proteins by peptide mass fingerprint. The Mascot 2.0 program (Matrix Science) was used to search the NCBI non-redundant database (http://ncbi.nlm.nih.gov, March 2010), with no limitation on taxonomy. Search parameters were as follows: trypsin cleavages excluding N-terminal to P, one missed cleavage permission, carbamidomethylation and methionine oxidation as variable modification, mass tolerance <50 ppm, monoisotopic mass values. Criteria for positive identification were a significant Mascot probability score ($p<0.05$), and at least five matching peptide masses. A minimum score of 83, and a >50-point difference between this score and the score of the second ranked non-homologous match was obtained for all the identified differentially expressed proteins.

Ethics Statement

Stenotrophomonas maltophilia strains were obtained from the internal collection of Hospital Municipal de Badalona (Barcelona, Spain) and have no link with patient data.

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 the Universitat Autònoma de Barcelona (UAB), Spain (ref # CEEAH-1968).

Supporting Information

Figure S1 Growth curves of the four *S. maltophilia* strains in Luria-Bertani (LB) media at 37°C and 250 rpm.
(TIF)

Figure S2 Representative image of the gels obtained for M30 vs. ATCC13637. Superimposed images in pseudo-color from Cy3 (green, clinical isolate) and Cy5 (red, ATCC13637 strain) labeled samples run on a two-dimensional DIGE gel. The horizontal dimension corresponds to isoelectric point (*pI*) and ranges from 3 (left) to 10 (right). The vertical dimension corresponds to mass and ranges from ≈15 kDa (bottom) to ≈200 kDa (top).
(TIF)

Figure S3 DIGE image analysis from the M30/ATCC13637 comparison. (a) Superimposed images in pseudo-color from Cy3 (green, clinical isolate) and Cy5 (red, ATCC13637 strain) labeled samples run on the two-dimensional gel. The horizontal dimension corresponds to isoelectric point (*pI*) and ranges from 3 (left) to 10 (right). The vertical dimension corresponds to mass and ranges from ≈15 kDa (bottom) to ≈200 kDa (top). The six spots with largest difference in protein abundance between the two samples are marked. Three-dimensional images representing the intensity of these spots, corresponding to the Cy3 image (M30 strain, left-hand panel for each protein) and the Cy5 image (collection strain, right-hand panel for each protein), are shown. (b) Standardized abundance plots for the six proteins. Each graph displays the abundance observed for the spot in each of the three gel images corresponding to the clinical isolate sample (pink) and the ATCC13637 strain sample (blue), after standardizing the values using the internal standard pool images (Cy2) of each of the three gels. The line links the average abundance values for each group of samples. ANOVA's test for the difference in abundance between each two groups results in p values <0.05 in all cases shown.
(TIF)

Table S1 *S. maltophilia* proteins presenting significant abundance difference in the clinical and ATCC13637 strains.
(DOCX)

Table S2 Statistical report for each identified DIGE spot.
(DOCX)

Table S3 Orthologs of the differentially abundant proteins reported to be involved in pathogenesis, virulence or resistance mechanisms.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MFN RP DY NR SM IG XD. Performed the experiments: MFN RP DY EM GT PH PM NR. Analyzed the data: MFN RP DY NR SM IG XD. Wrote the paper: MFN RP DY NR SM IG XD.

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