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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

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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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(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

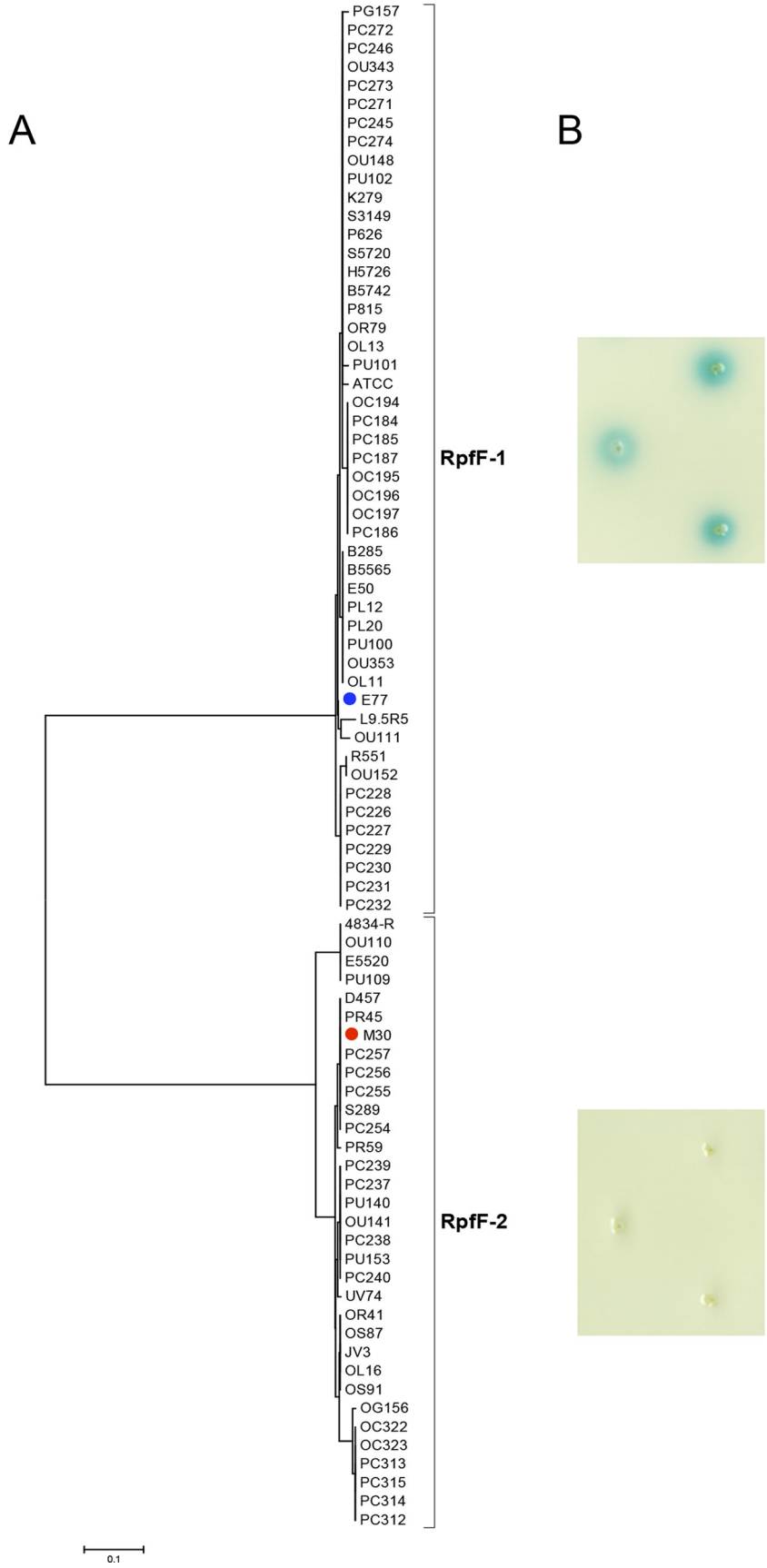

FIG 1 (A) Phylogenetic analysis of 82 *S. maltophilia* strains based on the first 108 amino acids of RpfF. (B) Colony DSF bioassay of three representative strains of each RpfF variant group. Top: E77, ATCC 13637, and K279a (RpfF-1). Bottom: M30, D457, and UV74 (RpfF-2).

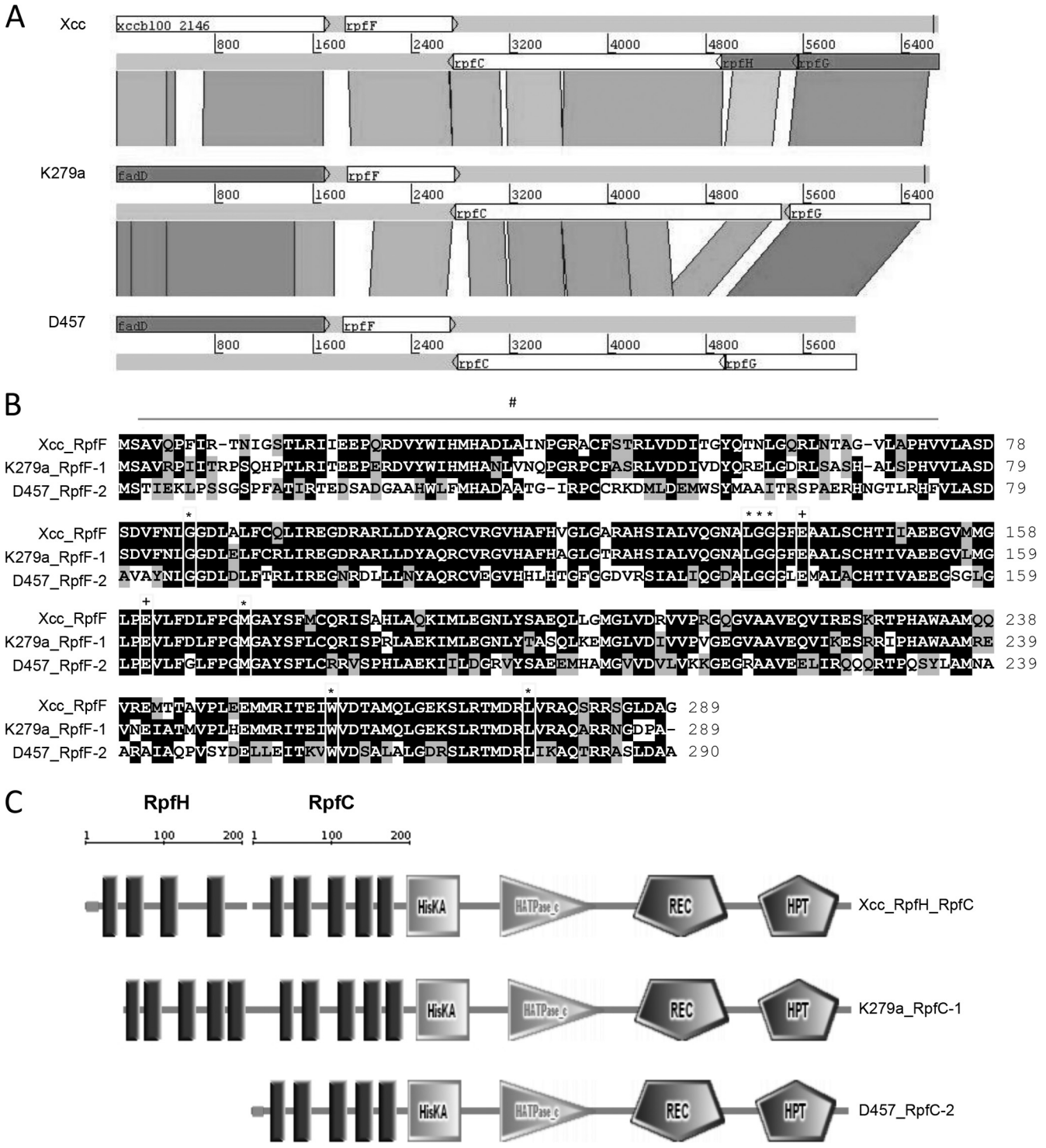

FIG 2 (A) Comparison of the *rpf* cluster in *X. campestris* pv. *campestris* and *S. maltophilia* K279a and D457. The alignment was performed with tblastx (percent identity cutoff, 45%) from the BLAST suite and visualized with the Artemis Comparison Tool. Conserved protein regions are paired by shaded 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.

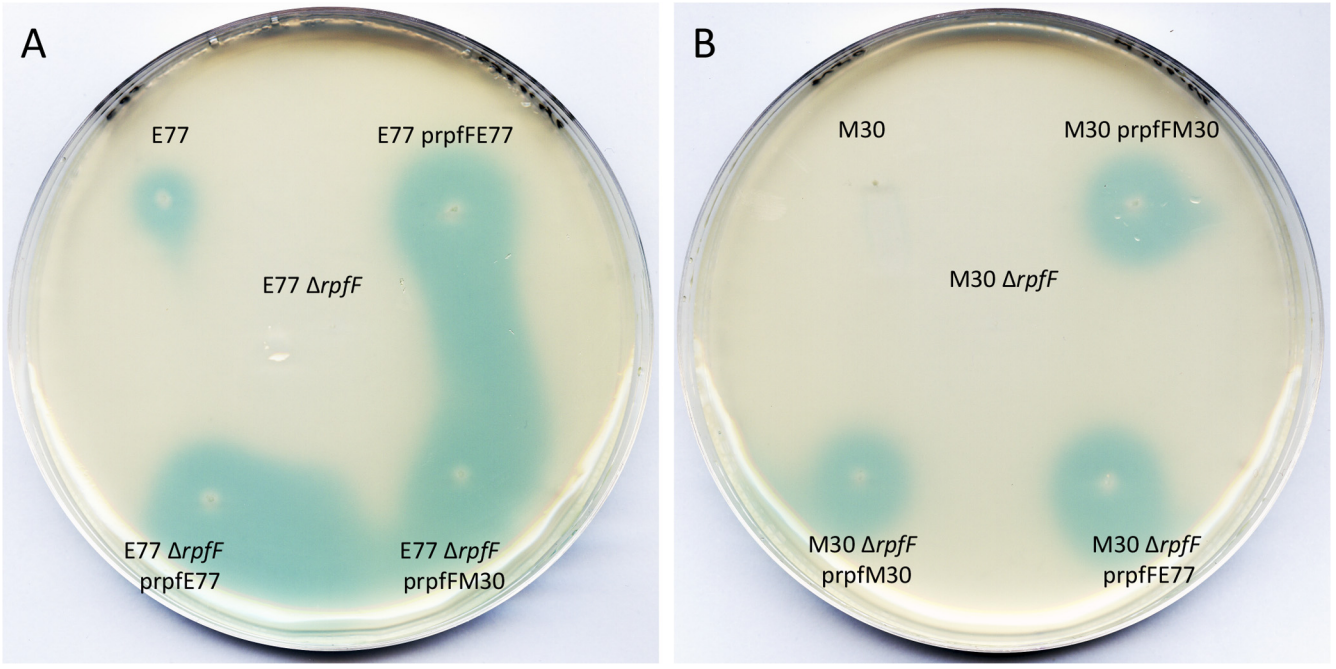

FIG 3 DSF bioassay of E77 (A) and M30 (B) with their respective Δ*rpfF* mutants and homologously and heterologously complemented strains and the *X. campestris* pv. *campestris* 8523/pL6engGUS reporter strain.

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-

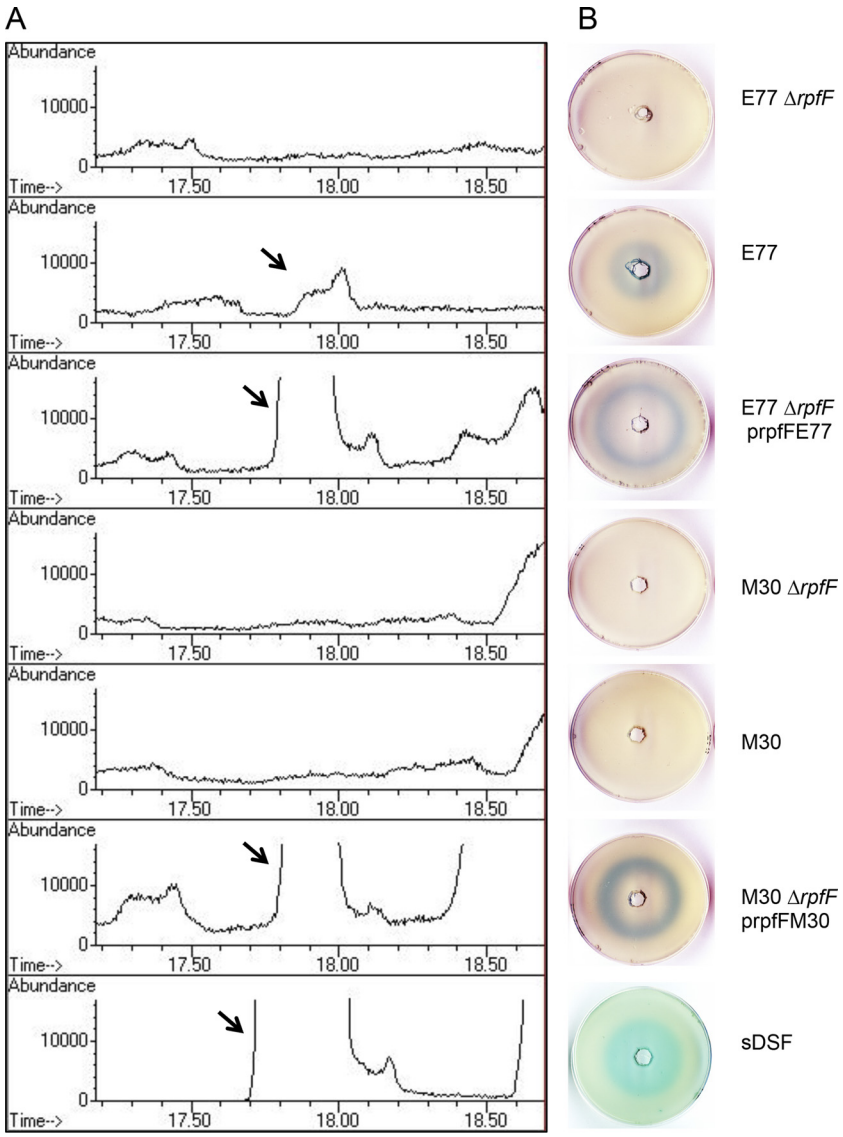

FIG 4 (A) GC analysis of culture supernatants of E77, M30, and their respective Δ*rpfF* mutants and complemented strains. (B) DSF bioassay of concentrated supernatants of the same strains from independent extractions.

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*

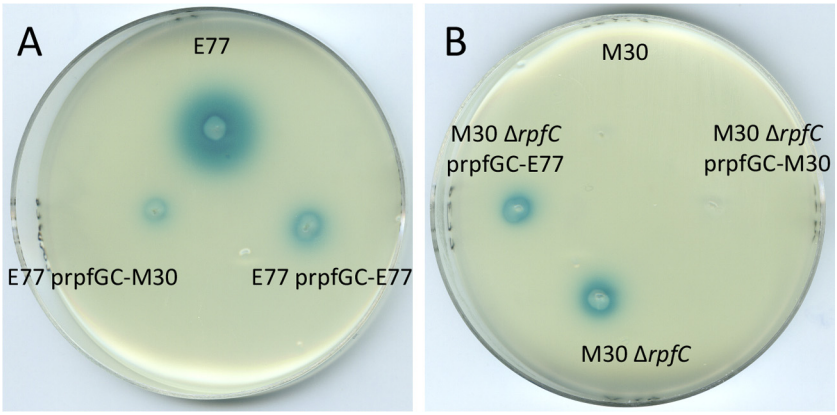

FIG 5 (A) DSF bioassay of WT E77 and E77 complemented with vectors prpfGCE77 and prpfGCM30. (B) DSF bioassay of WT M30, the M30 Δ*rpfC* mutant, and the M30 Δ*rpfC* mutant complemented with vectors prpfGCE77 and prpfGCM30.

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*

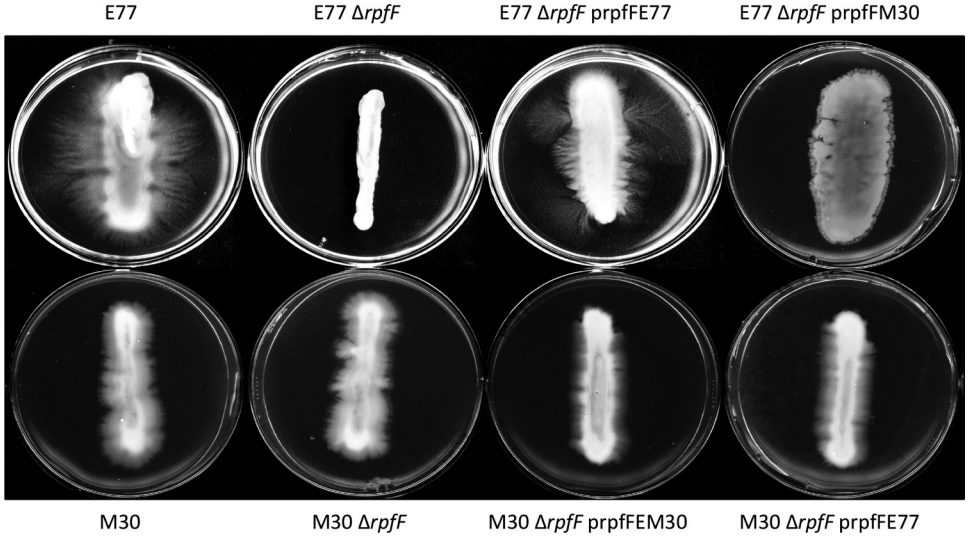

FIG 6 Swarming motility assay of E77, M30, and their Δ*rpfF* mutants and homologously and heterologously complemented strains on modified M9 medium solidified with 0.5% Noble agar and incubated at 30°C for 4 days.

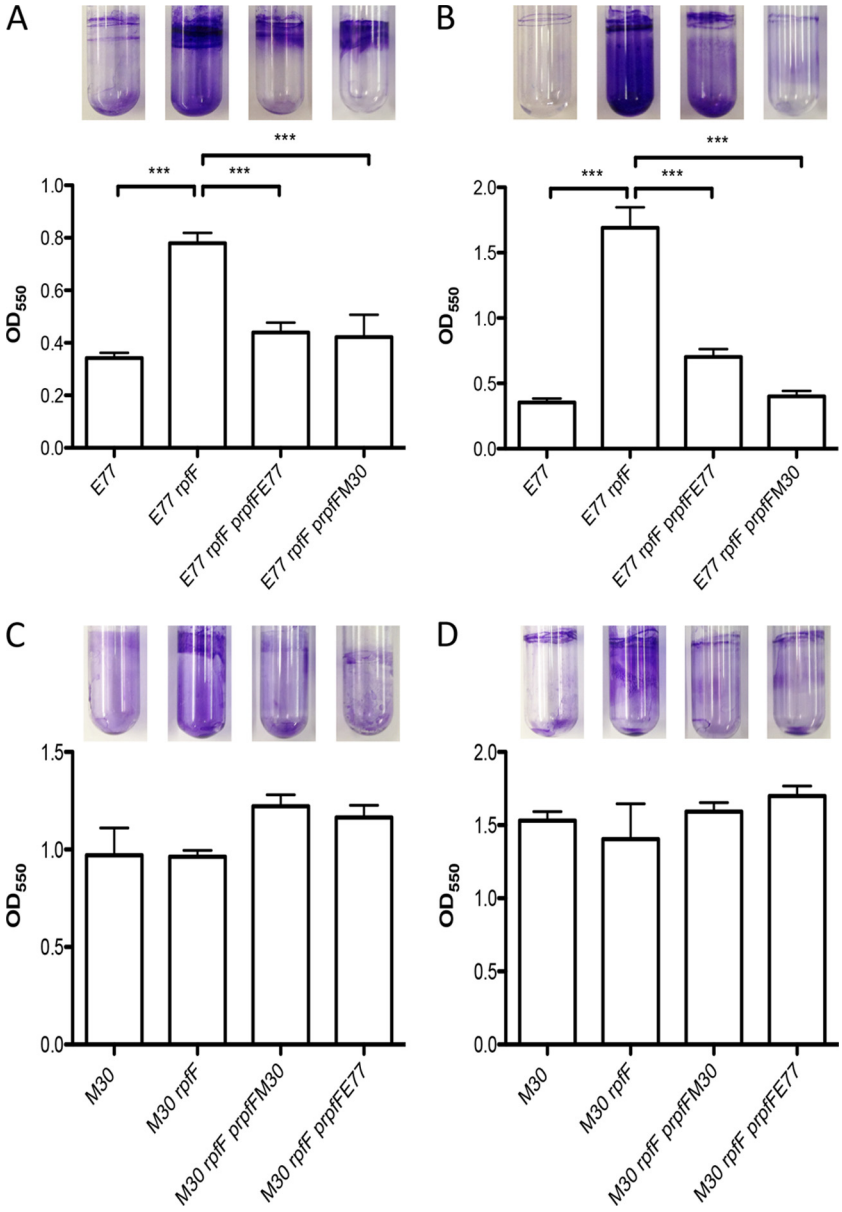

FIG 7 Biofilm formation by E77, M30, and their respective Δ*rpfF* mutants and homologously and heterologously complemented strains on polystyrene (plots) and glass (tubes) surfaces in M9 (A and C) and BM2 (B and D) minimal media. ***, $P < 0.0005$.

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

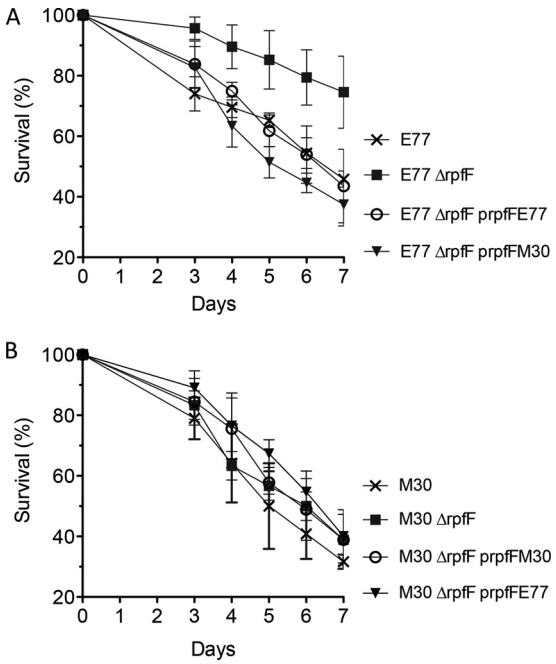

FIG 8 Determination of virulence of E77 (A), M30 (B), and their respective Δ*rpfF* mutants and homologously and heterologously complemented strains in a *C. elegans* CF512 model of infection.

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

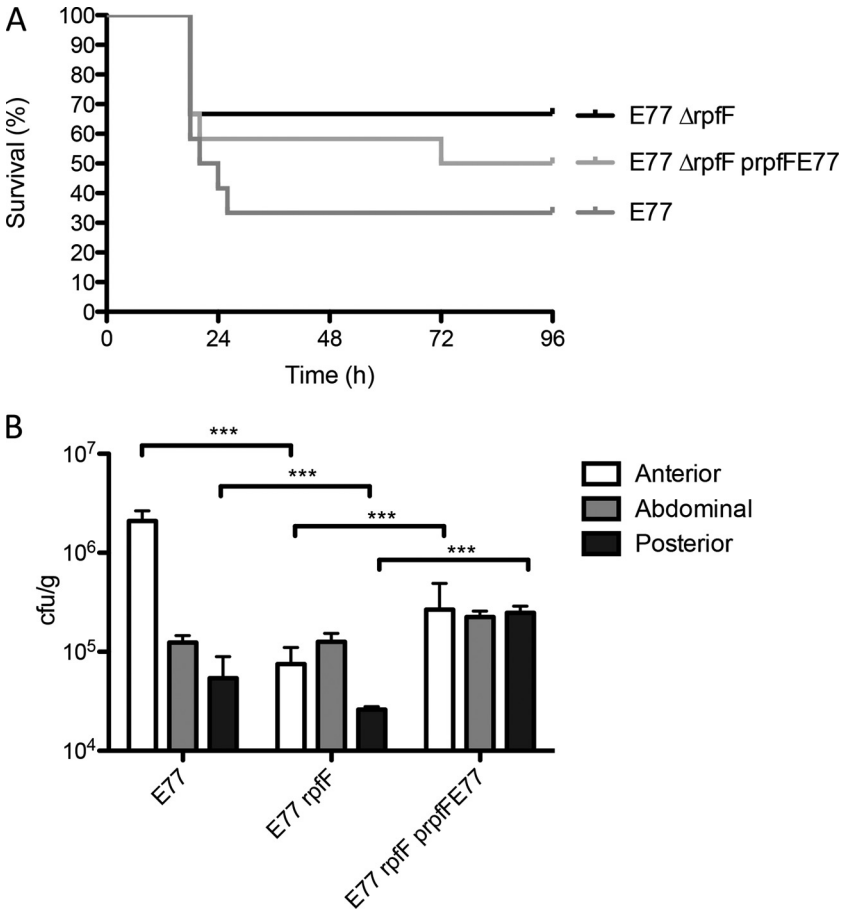

FIG 9 (A) Virulence of E77, the Δ*rpfF* mutant, and the complemented strain in 9-month-old zebrafish. (B) Bacterial recovery from different regions of the bodies of sacrificed fish at 72 h postinfection with E77, the Δ*rpfF* mutant, or the complemented strain. ***, $P < 0.0005$.

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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