A Cyclic AMP Receptor Protein-Regulated Cell-Cell Communication System Mediates Expression of a FecA Homologue in *Stenotrophomonas maltophilia*[▽]

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Stenotrophomonas maltophilia WR-C possesses an *rpf*/diffusible signal factor (DSF) cell-cell communication system. It produces *cis*-Δ2-11-methyl-dodecenoic acid, a DSF, and seven structural derivatives, which require *rpfF* and *rpfB* for synthesis. Acquisition of iron from the environment is important for bacterial growth as well as the expression of virulence genes. We identified a gene homologous to *fecA*, which encodes a ferric citrate receptor that transports exogenous siderophore ferric citrate from the environment into the bacterial periplasm. Western blot analysis with anti-FecA-His$_6$ antibody showed that the FecA homologue was induced in the iron-depleted medium supplemented with a low concentration of ferric citrate. Deletion of *rpfF* or *rpfB* resulted in reduced FecA expression compared to the wild type. Synthetic DSF restored FecA expression by the Δ*rpfF* mutant to the wild-type level. Reverse transcription-PCR showed that the *fecA* transcript was decreased in the Δ*rpfF* mutant compared to the wild type. These data suggest that DSF affected the level of *fecA* mRNA. Transposon inactivation of *crp*, which encodes cyclic AMP (cAMP) receptor protein (CRP) resulted in reduced FecA expression and *rpfF* transcript level. Putative CRP binding sites were located upstream of the *rpfF* promoter, indicating that the effect of CRP on FecA is through the *rpf*/DSF pathway and by directly controlling *rpfF*. We propose that CRP may serve as a checkpoint for iron uptake, protease activity, and hemolysis in response to environmental changes such as changes in concentrations of glucose, cAMP, iron, or DSF.

Stenotrophomonas maltophilia is found in a wide variety of environments such as soil, sewage, foods, plant rhizospheres, and hospital disinfectant solutions (5, 16, 17). It was previously identified as *Pseudomonas maltophilia* and then renamed *Xanthomonas maltophilia* before being reclassified in a new genus, *Stenotrophomonas* (35, 42). *S. maltophilia* is recognized as an emerging human pathogen and is associated with endocarditis, bacteremia, cystic fibrosis, and infections of the respiratory and urinary tracts, central nervous system, eyes, skin, and soft tissue (16).

Bacteria use small molecules in response to cell density to coordinate physiological processes, a process termed quorum sensing. A cell-cell communication system mediated by a diffusible signal factor (DSF), *cis*-Δ2-11-methyl-dodecenoic acid, was first identified in *Xanthomonas campestris* pv. *campestris* (13, 22, 48). This system is encoded by genes within an *rpf* (regulation of pathogenicity factors) cluster. RpfB, a putative long-chain fatty acyl coenzyme A (CoA) ligase, and RpfF, a putative enoyl CoA hydratase, are required for DSF biosynthesis (4). In *X. campestris* pv. *campestris*, RpfC and RpfG, a two-component system, regulate DSF production and interact with RpfF (2, 29). The *rpf*/DSF system in *X. campestris* pv. *campestris* is involved in the regulation of various biological functions and processes such as extracellular enzyme and exopolysaccharide production, biofilm formation, flagellum synthesis, resistance to toxins and oxidative stress, aerobic respi-

ration, cross-kingdom communication, and virulence (13, 21, 30, 51). We previously isolated *S. maltophilia* strain WR-C from the clogged infiltrative area of a septic system used for wastewater disposal (45) and showed that it possesses a DSF cell-cell communication system (33). In addition to DSF, *S. maltophilia* strain WR-C produces seven structural derivatives that are not shared by *X. campestris* pv. *campestris*. These eight compounds have signaling functions and also act as wetting agents for flagellum-independent translocation (33). Other physiological processes regulated by the DSF system in *S. maltophilia* have not been determined.

Iron is relatively abundant on earth, but it is biologically unavailable in its oxidized form (ferric oxyhydroxide) and when sequestered by iron storage proteins (43). Acquisition of iron from the environment is important for bacterial growth as well as expression of some virulence genes (39, 43). Ferric citrate is one of the heterologous siderophores that serves as an iron source for *Escherichia coli* and other gram-negative bacteria (28, 41, 49). Many bacteria possess *fecIRA* homologues, but the ferric citrate transport system and *fecIRA*-induced regulation have been studied only in *Escherichia coli* strain K-12 (8, 28). Ferric citrate is transported into *E. coli* through outer membrane receptor FecA, which also mediates the transcription of *fecABCDE* transport genes. Located upstream are the regulatory genes *fecIR* (25, 37). In the periplasm, the C-terminal region of FecR interacts with the N-terminal region of FecA and transfers the signal across the cytoplasmic membrane into the cytoplasm, where the N-terminal portion of FecR interacts with the sigma factor FecI. FecI binds to RNA polymerase and initiates the transcription of *fecABCDE* (37). Iron transport systems in *S. maltophilia* have not been identified.

The *cyclic AMP (cAMP) receptor protein (CRP)* is a tran-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
DH5α	λ⁻ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r$_K$⁻ m$_K$⁻) *phoA supE44 thi-1 gyrA96 relA1*	Invitrogen
BL21(DE3)	F⁻ *ompT hsdS*$_B$ (r$_B$⁻ m$_B$⁻) *gal dcm* (DE3)	Novagen
S. maltophilia		
WR-C	WT	45
fecA::EZTN	Km^r, *fecA*::EZTN transposon mutant, WR-C derivative	This work
crp::EZTN	Km^r, *crp*::EZTN transposon mutant, WR-C derivative	This work
Δ*rpfB*	Cm^r, *rpfB*::*cat rpfB* null mutant, WR-C derivative	33
Δ*rpfF*	Km^r, *rpfF*::*nptII rpfF* null mutant, WR-C derivative	33
Plasmids		
pBBR1MCS5	Gm^r, broad-host-range cloning vector	38
pGEM-T Easy	Ap^r, *lacZ'* cloning vector	Promega
pET22b(+)	Ap^r, protein expression vector carrying N-terminal *pelB* signal sequence and C-terminal His tag sequence	Novagen
p*fecA*::ET22b(+)	Ap^r, 3,138-bp *fecA* coding sequences and 1 bp upstream of *fecA* start codon sequence in frame translational fusion in pET22b(+)	This work
p*crp*	Gm^r, 828-bp *crp* native promoter and coding sequences in pBBR1MCS5	This work
p*rpfB*	Gm^r, 1,787-bp *rpfB* native promoter and coding sequences in pBBR1MCS5	33
p*rpfF*	Gm^r, 975-bp *rpfF* native promoter and coding sequences in pBBR1MCS5	33

scriptional regulator present in bacteria that responds to environmental conditions (6). CRP controls gene expression by binding cAMP and interacts at the promoters of targeted genes (14). It is well known as a regulator for genes involved in carbohydrate catabolism. The reduced levels of intracellular CRP and cAMP caused by glucose result in catabolite repression (36). CRP is reported to directly or indirectly regulate a variety of genes such as those for fatty acid utilization, iron uptake, and acyl homoserine lactone (AHL)- or autoinducer 2 (AI-2)-mediated quorum-sensing regulation or synthesis (1, 10, 20, 23, 44).

In this study, we investigated other targeted genes or biological processes controlled by the *rpf*/DSF system in *S. maltophilia*. The *rpf*/DSF system regulated the expression of a FecA homologue, which has not been shown to be regulated by any other known quorum sensing system. The *rpf*/DSF system was shown to be regulated by RpfC through protein-protein interaction with RpfF in *Xanthomonas* spp. (2, 29), but no transcriptional regulator has yet been identified. Here, an activator that is homologous to CRP was identified and shown to positively regulate *rpfF*, possibly by binding to an *rpfF* upstream region. In addition, CRP indirectly controlled FecA expression. It also controlled protease and hemolytic activity independently of the *rpfF*/DSF system.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are shown in Table 1. *S. maltophilia* WR-C and its mutant derivatives were routinely grown in Trypticase soy (TS) broth (Becton Dickinson, Sparks, MD) at 30°C unless otherwise stated. *S. maltophilia* wild type (WT) and mutants harbored the vector pBBR1MCS5 unless specified otherwise. All *E. coli* strains were grown in Luria-Bertani (LB) broth (Becton Dickinson) at 37°C. For FecA expression and reverse transcription-PCR (RT-PCR) analysis, strains were grown in *fecA* medium (1 g NH$_4$Cl, 3 g KH$_2$PO$_4$, 6 g Na$_2$HPO$_4$ · 7H$_2$O, 4 g glucose, and 0.02% L-methionine per liter, supplemented with 0.1 mM 2,2'-dipyridyl and 0.5 mM ferric citrate). When required, antibiotics were added to

the medium at the following concentrations: ampicillin (Ap), 100 μg ml⁻¹; carbenicillin, 50 μg ml⁻¹; chloramphenicol, 5 μg ml⁻¹; gentamicin (Gm), 50 μg ml⁻¹; kanamycin (Km), 50 μg ml⁻¹.

Generation of transposon mutants. *S. maltophilia* WR-C transposon mutants were generated using EZ::TN <R6Kγori/KAN-2> Tnp Transposome (Epicentre, Madison, WI) as described by Huang et al. (34). The transposon flanking regions were rescued by "rescue cloning," as described by the manufacturer, and sequenced using primers KAN-2 FP-1 and R6KAN-2 RP-1. Additional primers were used to sequence the full genes and flanking regions. Twenty microliters of overnight cultures in TS broth was spot inoculated on skim milk or blood agar plates to screen for defects in hemolysis and protease activity. For protease assays, TS agar plates containing 2% skim milk were incubated at 30°C for 1 day. The appearance of a clear zone around the inoculum indicates protease activity. Hemolytic activity was tested with blood agar plates that contained 5% defibrinated rabbit red blood cells in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and 1.5% Bacto agar and that were incubated at 30°C for 2 days. The appearance of a greenish zone around the inoculum indicates alpha-hemolysis.

Construction of plasmids and strains. To construct the plasmid for complementation of the *crp* mutant, the coding region, ribosomal binding site, and native promoter of *crp* in *S. maltophilia* WR-C were amplified by PCR with primers crp-F and crp-R (Table 2). The product was cloned into pGEM-T Easy

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5'→3')[a]	Restriction site
KAN-2 FP-1	ACCTACAACAAAGCTCTCATCAACC	—[b]
R6KAN-2 RP-1	CTACCCTGTGGAACACCTACATCT	—
crp-R	CGTCATCGGGTGCCGTACAGGACGAC	—
HindIII-fecA2	AAGCTTGAACCTGTAACGAACGC	HindIII
SacI-fecA2	GAGCTCGAGCCGGAAGCTGGCGA	SacI
T7 promoter	TAATACGACTCACTATAGGG	—
T7 terminator	GCTAGTTATTGCTCAGCGG	—
20F4-RT	CTGCAGAACAGCAACTACACCGAT	—
20R6-RT	CAACAGCACGCGGAAGTCATTGTTC	—
rmlA-F-RT	AGCGTAAAGGCATCATTCTTGCCG	—
rmlA-R-RT	TAGCGCTGGTTGAGATCGGTGATT	—
rpfB-RW3	TATTCCCACGGCTATGCGACCATC	—
rpfF-R	TACGCGGCATCGGACTGGGCACG	—

[a] Underlining indicates restriction sites.
[b] —, no restriction site was incorporated within the oligonucleotide.

(Promega, Madison, WI). The fragment containing *crp* (828 bp) was excised with EcoRI and ligated into pBBR1MCS5 to generate p*crp*. For complementation, p*crp* was electroporated (12.5 kV/cm, 25 μF, 400 Ω) into the *crp* mutant. Electroporation, restriction endonuclease digestion, PCR, cloning, DNA extraction, and DNA purification were performed using standard procedures (46).

To construct the plasmid for FecA overexpression, the *fecA* coding sequence (3,138 bp) and 1 bp upstream of the *fecA* start codon were PCR amplified with primers HindIII-fecA2 and SacI-fecA2 and cloned into pGEM-T Easy. The product was excised with HindIII and SacI and cloned into pET22b(+) (Novagen, San Diego, CA) to generate p*fecA*::ET22b(+). The construct was sequenced with T7 promoter and terminator primers (Novagen, Madison, WI) and electroporated into *E. coli* strain BL21(DE3).

Overexpression of FecA by the pET system for antibody production. *E. coli* BL21(DE3) harboring p*fecA*::ET22b(+) was grown in LB broth containing 50 μg ml^{-1} carbenicillin at 37°C. Transcription of the fusion gene was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C and 175 rpm for 2 h. *E. coli* BL21(DE3) harboring pET22b(+) was used as the control. After induction, cells were harvested and resuspended in lysis buffer (100 mM NaH$_2$PO$_4$, 10 mM Tris-HCl, 6 M urea, pH 8.0). Solubilized proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% polyacrylamide gels, 1× Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, pH 8.3, and 0.1% SDS, wt/vol), and Mini-PROTEIN II electrophoresis cells (Bio-Rad). The minigels were stained with Coomassie brilliant blue R-250 (0.25%, wt/vol) or were used for protein blotting. The band containing the FecA-His$_6$ fusion protein was excised from the gel and submitted to GeneTel Laboratories (Madison, WI) for immunization of chickens and generation of polyclonal immunoglobulin Y (IgY) antibodies in eggs.

Western blot analysis. Whole-cell lysates of *E. coli* BL21(DE3) harboring pET22b(+) and the FecA-His$_6$ fusion protein prepared as described above were separated by SDS-PAGE. The separated proteins were analyzed by immunoblotting with anti-His tag monoclonal antibodies and anti-mouse IgG-alkaline phosphatase (AP) conjugate (Novagen, Madison, WI). The protocol, as described in the manual of the Immuno-Star AP chemiluminescent protein detection systems (Bio-Rad, Hercules, CA), was followed, except that 0.5% casein (Sigma-Aldrich, St. Louis, MO) and CDP-star (Roche Diagnostic Corp., Indianapolis, IN) were used as the blocker and chemiluminescence substrate, respectively.

For FecA expression in *S. maltophilia*, strains were grown in a low-iron medium (fecA medium) at 30°C and 175 rpm for 3 days. Cells (optical density at 620 nm [OD$_{620}$] ≈ 0.6) were centrifuged, resuspended in 2× SDS gel loading buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 10% β-mercaptoethanol), boiled at 100°C for 10 min, and separated by SDS-PAGE. The separated proteins were analyzed by immunoblotting with anti-FecA-His$_6$ antibody (1:20,000) and rabbit anti-chicken IgY–AP conjugate (1:15,000) (GeneTel Laboratories) according to the manufacturer's instructions, except that 1.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and CDP-star were used as the blocker and chemiluminescence substrate, respectively. The experiment was repeated four times. Data presented are from one representative experiment.

RT-PCR. For determining *fecA* and *rmlA* transcripts, strains were grown at 30°C in fecA medium for 6 (OD$_{620}$ ≈ 0.01) and 18 h (OD$_{620}$ ≈ 0.1) unless stated otherwise; for the *rpfF* transcript, strains were grown in fecA medium without glucose for 2 days (OD$_{620}$ ≈ 0.1). Total RNA was extracted from 30 ml of culture with Tri Reagent LS (Molecular Research Center, Inc., Cincinnati, OH) and subjected to DNase I treatment with the TURBO DNA-*free* kit (Ambion, Inc., Austin, TX). RNA concentration was determined by measuring absorbance at 260 nm (A_{260}) with a Synergy HT multidetection microplate reader (Bio-Tek Instrument, Winooski, VT). RT-PCR was performed with the OneStep RT-PCR kit (QIAGEN, Valencia, CA) in a 20-μl reaction mixture containing 20 ng of total RNA, 0.6 μM of each gene-specific primer, 1× QIAGEN OneStep RT-PCR buffer, 400 μM of each deoxynucleoside triphosphate, and 2 units of QIAGEN OneStep RT-PCR enzyme mixture. Primers 20F4-RT and 20R6-RT were used for amplifying *fecA* (994 bp), primers rmlA-F-RT and rmlA-R-RT were used for amplifying *rmlA* (613 bp), and primers rpfB-RW3 and rpfF-R were used for amplifying *rpfF* (845 bp). Primer sequences are listed in Table 2. *rmlA* was constitutively expressed under the conditions tested in this study and was used as an internal control. RT was performed at 50°C for 30 min, followed by 15 min of reverse transcriptase inactivation at 95°C. The PCR cycling conditions were 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min for a total of 32 cycles and an additional 2-min extension at 72°C. To test for genomic DNA contamination, a negative-control PCR without the RT step was performed by using the PCR Master Mix (Promega, Madison, WI). Ten microliters of RT-PCR mixture was electrophoresed on 1% agarose gels, and the gels were stained with ethidium
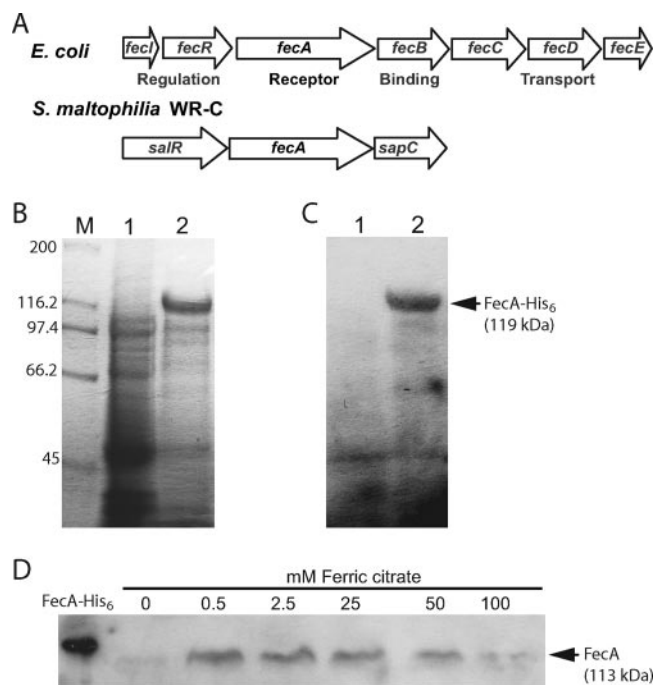

FIG. 1. Genetic organization and expression of FecA. (A) *fecA* and flanking genes in *Escherichia coli* K-12 (*E. coli*) (7) and *S. maltophilia* WR-C (GenBank accession no. EF011626). Genes depicted are not proportional to their respective sequence lengths. (B) SDS-PAGE of cell lysates from *E. coli* BL21(DE3) harboring pET22b(+) (lane 1) or p*fecA*::ET22b(+) (lane 2). M, SDS-PAGE standards, high range (Bio-Rad). (C) Anti-His tag Western blot of cell lysate from *E. coli* BL21(DE3) harboring pET22b(+) (lane 1) or p*fecA*::ET22b(+) (lane 2). (D) Anti-FecA-His$_6$ Western blot of cell lysates from *E. coli* BL21(DE3) harboring p*fecA*::ET22b(+) and *S. maltophilia* WR-C grown under different concentrations (0, 0.5, 2.5, 25, 50, and 100 mM) of ferric citrate in fecA medium at 30°C and 175 rpm for 3 days.

bromide for visualization under UV. The experiment was repeated four times. Data presented are from one representative experiment.

Nucleotide sequence accession numbers. Sequences obtained in this study were deposited in the GenBank database under accession numbers EF011625 and EF011626.

RESULTS

FecA expression is induced by ferric citrate. A *fecA* homologue encoding the ferric citrate receptor FecA was identified in the transposon library of *S. maltophilia* WR-C. The translated amino acid sequence exhibits 67% identity to the FecA in *X. campestris* pv. *campestris* (GenBank accession no. NP_638176). The *fecA* homologue is flanked upstream by *salR*, which encodes the *sal* operon transcriptional repressor and downstream by *sapC*, which encodes a SapC-related protein (Fig. 1A). SalR is an LacI family transcriptional regulator; SapC forms part of a paracrystalline surface layer and confers serum resistance in *Campylobacter fetus*. This gene organization is in part similar to one of the nine *X. campestris* pv. *campestris fecA* homologues (GenBank accession no. NP_638176), where *sapC* is also located downstream. This is in contrast to *E. coli*, where the binding and transport genes *fecBCDE* are adjacent to *fecA*. The full sequences and flanking
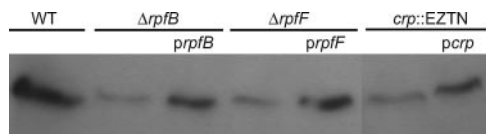
FIG. 2. Anti-FecA-His$_6$ Western blot of cell lysates from *S. malto-philia* WT and Δ*rpfB*, Δ*rpfF*, and *crp*::EZTN mutants and the respective complemented strains. Strains were cultured in fecA medium at 30°C and 175 rpm for 3 days.
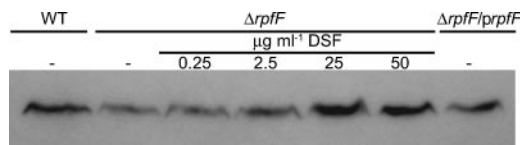

FIG. 3. Effect of synthetic *cis*-Δ2-11-methyl-dodecenoic acid (DSF) on FecA expression. Cell lysates from *S. maltophilia* WR-C WT, the Δ*rpfF* mutant, and the Δ*rpfF* mutant harboring p*rpfF* (Δ*rpfF*/p*rpfF*) were analyzed by Western blotting using anti-FecA-His$_6$ antibody. Strains were cultured at 30°C and 175 rpm for 3 days in fecA medium without DSF (−) and with different concentrations of DSF (0.25, 2.5, 25, and 50 μg ml^{-1}).

regions of the *S. maltophilia fecA* were deposited in the GenBank database (accession number EF011626).

To specifically determine FecA expression in *S. maltophilia* WR-C by Western blot analysis, full-length *fecA* (3,135 bp, encoding 1,045 amino acid residues with a molecular mass of 113 kDa) was cloned into the expression vector pET22b(+) containing a C-terminal His$_6$ tag and expressed in *E. coli* BL21(DE3). The fusion protein was used for antibody production. *E. coli* BL21(DE3) harboring p*fecA*::ET22b(+) expressed a 119-kDa FecA-His$_6$ fusion protein under IPTG-induced conditions, which was absent in the control strain harboring pET22b(+) (Fig. 1B). The FecA-His$_6$ fusion protein was confirmed by Western blot analysis with anti-His tag monoclonal antibody (Fig. 1C). FecA expression in *S. maltophilia* WR-C was induced by low concentrations (0.5 and 2.5 mM) of ferric citrate and inhibited when the concentration was increased to 100 mM (Fig. 1D). This apparent regulation of *S. maltophilia* FecA expression by ferric citrate implies that FecA may function as a ferric citrate receptor and possibly a transporter. Whether this FecA homologue can be induced by or bind to other types of substrate remains to be determined.

***rpfB* and *rpfF* are required for FecA expression.** We have identified an *rpf* system in *S. maltophilia* WR-C and generated mutants with deletions in *rpfB* and *rpfF* (33). *rpfF* was shown to affect siderophore production in *Xanthomonas oryzae* pv. *oryzae* (11). To determine if the *rpf*/DSF system plays a role in the putative ferric citrate transport system in *S. maltophilia* strain WR-C, the WT and its Δ*rpf* mutants were assessed for FecA expression. Both Δ*rpfB* and Δ*rpfF* mutants had a reduced level of FecA expression compared to the WT (Fig. 2). FecA expression was restored in the *rpfB*- and *rpfF*-complemented strains to levels similar to that in the WT. These results suggest both *rpfB* and *rpfF* participated in FecA expression.

DSF is a signal for FecA expression and affects the level of *fecA* mRNA. *rpfF* is involved in the synthesis of DSF as well as seven structurally related fatty acids (33). They are Δ2-tridecenoic acid, 10-methyl-dodecanoic acid, 11-methyl-dodecanoic acid, Δ2-12-methyl-tridecenoic acid, Δ2-tetradecenoic acid, Δ2-12-methyl-tetradecenoic acid, and Δ2-13-methyl-tetradecenoic acid. These eight compounds can activate the promoter of *engXCA* (encodes endoglucanase), as determined by a DSF bioassay using the DSF reporter strain *X. campestris* 8523/pL6engGUS (47), suggesting a signaling role for them. In addition, deletion of *rpfB* or *rpfF* resulted in reduced levels of FecA expression. We therefore hypothesized that DSF and its seven derivatives might be signals for FecA expression. To test this, a commercially available synthetic *cis*-Δ2-11-methyl-dodecenoic acid (DSF) was added to cultures of the Δ*rpfF* mutant, which were then incubated for 3 days. The synthetic DSF (25

or 50 μg ml^{-1}) restored FecA expression by the Δ*rpfF* mutant to the WT level (Fig. 3), suggesting that it is a signal for FecA expression, possibly through affecting levels of *fecA* mRNA.

RT-PCR analysis showed that the Δ*rpfF* mutant had a reduced level of the *fecA* transcript compared to the WT after 18 h of incubation (OD$_{620}$ ≈ 0.1) in fecA medium (Fig. 4), while no significant difference was observed between the Δ*rpfF* mutant and the WT after 6 h (OD$_{620}$ ≈ 0.01). There are no reports of what genes are constitutively expressed in *S. maltophilia*. We tested housekeeping gene *rmlA* and found that it was constitutively expressed in fecA medium, TS broth, and LB broth at 30°C for 6 and 18 h, and thus *rmlA* was used as the control. Deletion of *rpfF* did not affect the level of *rmlA* transcript at both times in fecA medium (Fig. 4). These results suggest that the *rpf*/DSF system plays a role in affecting the level of *fecA* mRNA.

CRP controls FecA expression. CRP encoded by *crp* was reported to regulate the iron uptake regulator Fur in *E. coli* (15). We hypothesized that a putative iron transport system mediated by FecA in *S. maltophilia* might be controlled by CRP. We obtained a *crp* transposon insertion mutant in a separate study to identify virulence-associated genes. The *S. maltophilia* CRP exhibits 87% sequence identity to *Xanthomonas campestris c*atabolite activation-*l*ike *p*rotein (Clp; GenBank accession no. NP_65866) and 44% identity to *E. coli* CRP (GenBank accession no. NPp_417816). FecA expression in the WT and *crp* mutant was examined. Transposon mutation in *crp* reduced FecA expression (Fig. 2), suggesting that CRP regulates FecA. The transposon mutants were screened for defects in proteolytic activity and hemolysis, because production of these two virulence factors was shown to correlate with cyto-
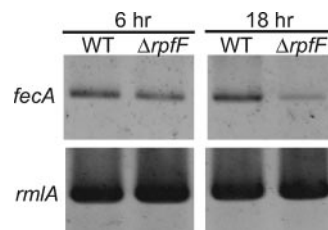

FIG. 4. Steady-state levels of *fecA* and *rmlA* mRNA in *S. maltophilia* WT and the Δ*rpfF* mutant. RNA was isolated from cultures of *S. maltophilia* WT and the Δ*rpfF* mutant in fecA medium after 6 h and 18 h at 30°C and 175 rpm. RT-PCR was performed with primers specific for *fecA* (994 bp) and *rmlA* (613 bp). The experiment was repeated four times with reproducible results. Data from one representative experiment are presented.
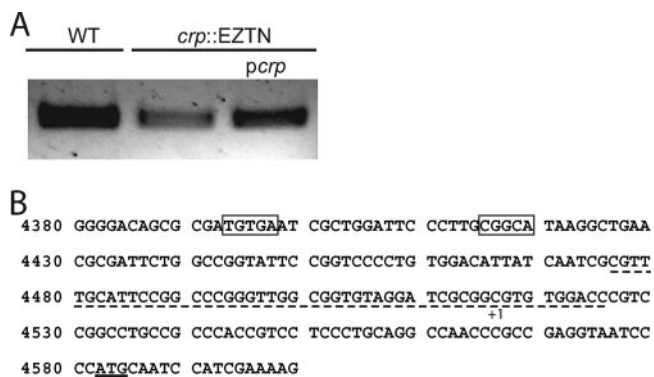
FIG. 5. Levels of *rpfF* transcript determined by RT-PCR in the WT, *crp* mutant, and its complemented strain (A) and predicted CRP binding sites upstream of the *rpfF* promoter (B). *rpfF* mRNA level was determined by RT-PCR. RNA was isolated from cultures of *S. maltophilia* WT, *crp* mutant (*crp*::EZTN), and *crp*-complemented (*crp*::EZTN/*pcrp*) strains grown in fecA medium without glucose at 30°C and 175 rpm for 2 days. RT-PCR was performed with primers specific for *rpfF* (845 bp). The experiment was repeated four times with reproducible results. Data from one representative experiment are presented. The partial *rpfF* sequence and upstream regions flanking *rpfF* were extracted from GenBank accession no. EF011627 nucleotides 4380 to 4600. Boxes, predicted CRP binding sites; solid underline, *rpfF* start codon; dashed underline, predicted *rpfF* promoter; +1, *rpfF* transcriptional start site.

toxicity of clinical *S. maltophilia* isolates to HeLa and Vero cells (26). A clear zone of proteolysis on skim milk agar and zone of alpha-hemolysis on blood agar were obtained with the WT, while no zones of proteolysis or hemolysis were observed with the *crp* mutant. Both phenotypes were restored in the complemented strain. The full sequences and flanking regions of *crp* were deposited in the GenBank database (accession number EF011625).

CRP positively regulates *rpfF* transcripts, possibly by binding upstream regions of the *rpfF* promoter. *rpfF* encodes enoyl CoA hydratase, which is an enzyme reported to be involved in fatty acid metabolism in *E. coli* (20). Genes involved in fatty acid metabolism and homoserine lactone or AI-2 synthesis or regulation have been shown to be directly or indirectly controlled by CRP (1, 10, 20, 23, 44). RT-PCR analysis was conducted to test whether *rpfF* in *S. maltophilia* WR-C is controlled by CRP. Reduced *rpfF* transcript levels in the *crp* mutant compared to the WT were observed; WT *rpfF* transcript levels were restored in the complemented strain (Fig. 5A). Two potential CRP binding sites are located upstream of the *rpfF* promoter and exhibit 100% identity with the consensus sequences identified in *X. campestris* (32) (Fig. 5B). The 5′TG TGA3′ sequence is conserved among other bacteria and critical for CRP binding (14). These results suggest that CRP is a transcriptional activator of *rpfF*, possibly by binding upstream regions of the *rpfF* promoter, which in turn affects the expression of FecA.

DISCUSSION

Iron plays important roles in bacterial growth and pathogenesis (43). In this study, a homologue of the ferric citrate receptor FecA was identified in *S. maltophilia* WR-C. In contrast

to *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Photorhabdus luminescens fecA* homologues, but similar to 8 of the 18 *P. aeruginosa fecA* homologues, the *fecIR* regulatory genes are not located upstream of *fecA* (8, 41, 43, 49). These genes may be located elsewhere, or the ferric citrate transport system in *S. maltophilia* may be controlled differently. Our results suggest that the FecA homologue is regulated by the *rpf*/DSF cell-cell communication system.

A link between quorum sensing and iron uptake has been postulated for *P. aeruginosa*, *Vibrio harveyi*, and *X. campestris*, although the molecular mechanisms of the interaction vary (12, 30, 40). Unlike *P. aeruginosa* and *V. harveyi*, which use AHL, *S. maltophilia* WR-C utilizes *cis*-Δ2-11-methyl-dodecenoic acid and seven other structural derivatives (collectively called DSFs) for cell-cell communication (33). Our results from Western blot and RT-PCR analysis of the WT and Δ*rpf* mutants suggest that DSFs act as signals that positively affect the *fecA* mRNA level. Interaction between the *rpf*/DSF system and ferric citrate uptake has not been reported previously, although positive regulation of iron uptake by cell-cell signaling has been observed in a variety of systems. Transcriptome analysis of *X. campestris* pv. *campestris* demonstrated that DSF upregulated genes encoding receptors for catecholate (e.g., FepA, CitA, and IroN) (30). The quinolone signal upregulates genes for pyoverdin biosynthesis and the pyochelin receptor FptA in *P. aeruginosa* (9), while transcriptome and proteome analysis showed that AHL activated iron uptake mediated by the ferripyoverdine receptor FpvA, FptA, and the hemin receptor PhuR (3, 53). The response regulator LuxO of the AI-1 and AI-2 signaling system positively regulates siderophore production in *Vibrio harveyi* (27, 40). LuxS, which is involved in AI-2 synthesis, positively affects expression of *fecBCDE* in *Actinobacillus actinomycetemcomitans* (27, 40). In contrast, iron acquisition is down-regulated by the signal molecule phenazine pyocyanine in *P. aeruginosa* (18).

Mutation in *crp* of *S. maltophilia* WR-C resulted in reduced levels of *rpfF* transcript and FecA expression. CRP binding sites are located upstream of the *rpfF* promoter. These results suggest that CRP directly regulates synthesis of DSFs and in turn indirectly controls FecA through the *rpf*/DSF cell-cell communication system. *Erwinia chrysanthemi* CRP was shown to bind *expI*, which is responsible for the synthesis of *N*-(3-oxohexanoyl)-homoserine lactone and *N*-(hexanoyl)-homoserine lactone (44). In contrast to the positive regulation by CRP observed in *S. maltophilia*, *E. chrysanthemi* CRP acts as a repressor of *expI* transcription. CRP was also shown to control AI-1, AHL, and AI-2 and mediated the quorum sensing system by activating expression of *luxR* in *Vibrio fischeri*, *lasR* in *P. aeruginosa*, *expR* in *Erwinia chrysanthemi*, and *lsrR* in *E. coli* (1, 10, 24, 44, 50). Unlike the AI-1 and AHL signaling systems, DSF synthesis in *X. campestris* is controlled by two-component regulatory proteins RpfC, RpfG, and RpfH (47). RpfC and RpfG were shown to interact with RpfF (2, 29). In our study, we showed that an activator of *rpfF*, CRP, regulates the synthesis of DSFs, possibly by protein-DNA interaction.

We were not able to individually isolate each of the DSFs produced by *S. maltophilia* WR-C, and six of them are not commercially available. These DSFs may display different levels of activity in controlling physiological processes. A relatively high concentration (25 μg ml^{-1}; ≈118 μM) of synthetic
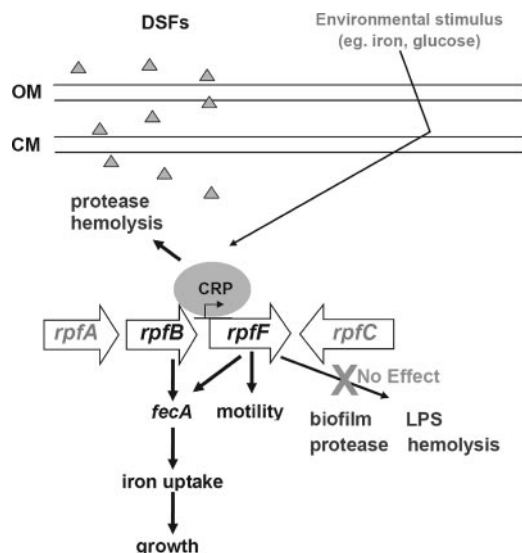
FIG. 6. A proposed model for the roles of CRP and DSFs in the regulation of various physiological processes in *S. maltophilia* WR-C. The organism produces DSFs (triangles), which require *rpfF* and *rpfB* for synthesis and are secreted outside the cell. The DSF system is involved in iron uptake mediated by FecA, ultimately affecting growth. It does not play a role in biofilm formation, lipopolysaccharide (LPS) biosynthesis, protease production, and hemolysis of rabbit blood cells. The divergent regulation of bacterial growth controlled by the DSF system and virulence factors in response to environmental stimuli may be mediated by CRP. CRP responds to environmental changes, such as iron and glucose levels, and binds to the predicted CRP binding site upstream of *rpfF*, activating the *rpf* system. OM, outer membrane; CM, cytoplasmic membrane.

DSF was required to restore FecA expression by the Δ*rpfF* mutant. Whether lower concentrations of some of the other DSFs may exert the same effect cannot be determined at this time. As demonstrated in *X. campestris* pv. *campestris* by Wang and coworkers, the minimum concentrations of *cis*-Δ2-11-methyl-dodecenoic acid and Δ2-tridecenoic acid required for activating the endoglucanase promoter were 0.5 μM and 30 μM, respectively (51). The DSFs produced by *S. maltophilia* WR-C varied in acyl chain length, ranging from 12 to 14 carbons. We speculate that RpfB and RpfF may exhibit acyl chain specificity or that other enzymes may modulate the chain length. RpfB is homologous to FadD, which is a long-chain fatty acyl CoA ligase (4). The 25-amino-acid-residue signature motif (residues 431 to 455) of *E. coli* FadD was shown to promote fatty acid chain specificity (19). *Pseudomonas* AHL synthases were reported to exhibit acyl chain specificity (52), and the β-ketoacyl acyl carrier protein reductase (FabG) might affect the acyl chain length (31).

We propose a model of CRP and DSFs in the regulation of some physiological processes in *S. maltophilia* WR-C (Fig. 6). CRP may serve as a checkpoint for controlling bacterial growth or expression of virulence factors in response to extracellular signals such as DSF, ferric citrate, and glucose concentrations and intracellular conditions such as levels of ferrous iron and cAMP. Under unfavorable nutrient conditions (e.g., low iron concentration), *rpfF* in *S. maltophilia* is activated to synthesize DSFs. The DSFs act as wetting agents for flagellum-independent translocation (33) and as signals for expression of a FecA

homologue, which may mediate ferric citrate uptake and in turn promotes bacterial growth. Notably, the Δ*rpfB* mutant exhibited a growth defect in fecA medium, but not in TS broth (data not shown). The *rpf*/DSF system does not play a role in biofilm formation, lipopolysaccharide biosynthesis (data not shown), protease production, and hemolysis of rabbit blood cells. Protease production and hemolytic activity in *S. maltophilia* are controlled by CRP. *S. maltophilia* and *X. campestris* pv. *campestris* share DSF as a signal molecule, suggesting that they could use it for intergenus communication when present in the same environment such as rhizospheres. The role of the *rpf*/DSF system in biofilm formation and production of extracellular enzymes is different in these two microorganisms, which may imply that they occupy distinct biological niches.

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