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Diffusible Signal Factor-Dependent Cell-Cell Signaling and Virulence in the Nosocomial Pathogen *Stenotrophomonas maltophilia*▽

Yvonne Fouhy,[1] Karl Scanlon,[1]† Katherine Schouest,[2] Charles Spillane,[2] Lisa Crossman,[3] Matthew B. Avison,[4] Robert P. Ryan,[1] and J. Maxwell Dow[1]*

BIOMERIT Research Centre, Department of Microbiology, BioSciences Institute, National University of Ireland, Cork, Ireland[1]*; Department of Biochemistry, BioSciences Institute, National University of Ireland, Cork, Ireland*[2]*; Pathogen Sequencing Unit, Wellcome Trust Sanger Institute, Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom*[3]*; and University of Bristol, Department of Cellular and Molecular Medicine, School of Medical Sciences, University Walk, Bristol BS8 1TD, United Kingdom*[4]

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The genome of *Stenotrophomonas maltophilia* encodes a cell-cell signaling system that is highly related to the diffusible signal factor (DSF)-dependent system of the phytopathogen *Xanthomonas campestris*. Here we show that in *S. maltophilia*, DSF signaling controls factors contributing to the virulence and antibiotic resistance of this important nosocomial pathogen.

Stenotrophomonas maltophilia is a gram-negative bacterium that is widespread in the environment and that has become important in the last 15 years as an emerging opportunistic pathogen associated with nosocomial colonization and infection (9, 23, 36). *S. maltophilia* is frequently isolated from clinical specimens and is implicated in catheter-related bacteremia and septicemia, urinary and respiratory tract infections, and endocarditis (9, 23, 36). Infections occur in cystic fibrosis and burn patients and are common in individuals with impaired defenses who are susceptible to opportunistic infections. The treatment of *S. maltophilia* infections is problematic, as isolates are resistant to many clinically useful antibiotics. A number of laboratories have begun to address the molecular bases for the broad antibiotic resistance and for virulence in *S. maltophilia* (14, 25, 29, 31, 34, 48). Cell-cell signaling is known to regulate diverse functions that contribute to the virulence and persistence of bacterial pathogens of both animals and plants (43, 45). However cell-cell signaling systems in *S. maltophilia* have not yet been described, and their role (if any) in regulation of these properties has therefore not been tested.

S. maltophilia is related to plant pathogens in the bacterial genera *Xanthomonas* and *Xylella* (26). In *Xanthomonas campestris*, cell-cell signaling mediated by the diffusible signal molecule diffusible signal factor (DSF) controls virulence factor synthesis and virulence to plants (3). DSF has been characterized as *cis*-11-methyl-2-dodecenoic acid (44). DSF synthesis is fully dependent on RpfF, which has some amino acid sequence similarity to enoyl coenzyme A hydratases and is partially dependent on RpfB, a long-chain fatty acyl coenzyme A ligase (3). DSF perception involves a two-component regulatory system, comprising the complex sensor RpfC and response regulator RpfG (37). The *rpfG* and *rpfC* genes are transcribed as the *rpfGHC* operon, although RpfH has no apparent role in signaling. A similar signaling system involving DSF or a DSF-like molecule occurs in *Xylella fastidiosa* (6, 27, 35). These Rpf/DSF signaling systems control interactions of *Xanthomonas* spp. with plants (4, 20, 28, 41), the interaction of *Xylella* with both its plant host and insect vector (27), the production of extracellular enzyme virulence factors and antibiotic resistance mechanisms in *Xanthomonas* (3, 15, 37, 40), and the formation of biofilms and adhesion in both genera (7, 11, 27). The relatedness of *S. maltophilia* to these plant pathogens prompted us to examine this organism for the presence and role of a DSF-dependent signaling system.

Evidence for the occurrence of the DSF signaling system in *S. maltophilia* was provided by both bioinformatic and experimental studies of the clinical isolate K279a (Table 1). The genome sequence of this organism (http://www.sanger.ac.uk/Projects/S_maltophilia/) was interrogated with the RpfF amino acid sequence of *X. campestris* by using tBLASTn (1), and a DNA sequence of approximately 8 kb (to include flanking genes) was analyzed using FramePlot (21). This indicated the presence of an *rpfBFCG* gene cluster, related to that found in *X. campestris* (Fig. 1). In BLASTP comparisons, the *S. maltophilia* proteins showed very high amino acid sequence similarity to their homologues in *X. campestris*; E values were all lower than 10^{-127}. The percentage of identical amino acids ranged from 65% (RpfC) to 85% (RpfG), and the percentage of similar amino acids ranged from 77% (RpfC) to 93% (RpfG). No homologue of *rpfH* was found in *S. maltophilia* (Fig. 1).

DSF can be assayed by measuring the restoration of endoglucanase activity to the *X. campestris rpfF* mutant strain 8523 by extracts from culture supernatants (Table 1) (3). Using this bioassay, DSF activity was detected in culture supernatants of *S. maltophilia* K279a (Fig. 2A). Furthermore, the *rpfF* gene from *S. maltophilia* K279a when introduced into the *rpfF* mutant of *X. campestris* directed DSF production and concomitantly restored the synthesis of the extracellular enzymes endoglucanase and protease (Fig. 2B). For these experiments,

* Corresponding author. Mailing address: BIOMERIT Research Centre, Department of Microbiology, BioSciences Institute, National University of Ireland, Cork, Ireland. Phone: 353-21-4901316. Fax: 353-21-4275934. E-mail: m.dow@ucc.ie.

† Present address: Ashtown Food Research Centre, Teagasc, Ashtown, Dublin 15, Ireland.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Source and relevant characteristics	Reference
S. maltophilia		
e-p3	Biocontrol agent, rhizosphere of oil seed rape	26
e-p10	Rhizosphere of oil seed rape	26
e-p14	Rhizosphere of potato	26
e-p13	Environmental isolate, rhizosphere of potato	26
e-p19	Environmental isolate, geocaulosphere of potato	26
e-p20	Environmental isolate, rhizosphere of potato	26
e-a1	Aquatic isolate, brackish lagoon	26
e-a21	Aquatic isolate, sewage treatment plant	26
e-a23	Eye care solution	26
c6	Clinical isolate	26
c20	Clinical isolate	26
W81	Biocontrol agent	12
K279a	Clinical isolate	2
K279a *rpfF*	*rpfF* mutant of K279a, created using pEX18Tc	This work
S. rhizophila e-p17	Environmental isolate, rhizosphere of potato	47
X. campestris		
8004	Wild type; Rif^r	42
8523	*rpfF* mutant; DSF^-	3
Δ*rpfF* mutant	*rpfF* deletion mutant; DSF^-	15
8557	*rpfC* mutant; DSF overproducer	37
Pseudomonas aeruginosa		
PA14	Wild type	32
PAO1	Wild type	18
Escherichia coli		
JM109	*endA recA1 gyrA96 thi-1 hsdR17 lacI^{q}ZΔM15 relA1*	33
OP50	Maintenance of *C. elegans*	8
Plasmids		
pCR-Blunt-TOPO	Cloning vector for blunt-ended PCR products	Invitrogen, Carlsbad, CA
pLAFR3	Broad-host-range cloning vector	39
pEX18Tc	Broad-host-range allelic exchange vector; Tc^r	17

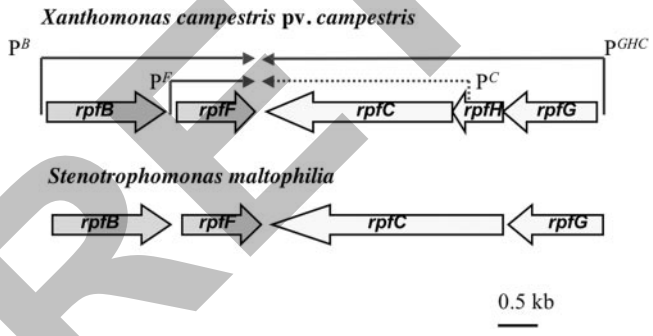

FIG. 1. Physical map of the part of the *rpf* gene cluster from *rpfB* to *rpfG* in *Xanthomonas campestris* and *Stenotrophomonas maltophilia* K279a. The organization of ORFs predicted by sequence analysis together with predicted directions of transcription are indicated by the broad arrows. The positions of the experimentally determined transcriptional start sites in *X. campestris* (37) together with the predicted transcripts are shown as single arrows. The sequence data for *S. maltophilia* were produced by the *S. maltophilia* K279a Sequencing Group at the Sanger Institute and can be obtained from http://www.sanger.ac.uk/Projects/S_maltophilia/).

the *rpfF* gene with its promoter was amplified by PCR using the primers RPFFCOMF (5′-GGATCCGGGTCTTTTTATTGC CGGAAC-3′) and RPFFCOMR (5′-AAGGCTTTCAATGG TGATGGTGGTGGTCCGGGTCGCCATTGC-3′) and the DNA fragment cloned into the TOPO vector (Table 1). The *rpfF* gene was excised as a BamHI-HindIII fragment and ligated into pLAFR3 (39) cut with the same enzymes. This resulting construct was introduced into *X. campestris* by triparental mating.

To assess the role of DSF signaling in *S. maltophilia* K279a, the *rpfF* gene was inactivated by directed insertion of a suicide vector. An internal fragment of the *rpfF* gene was amplified using the primers PEX18RPFF-F (5′-TGACATCGTCGACG ACTACCAGC-3′) and PEX18RPFF-R (5′-GGCTTTCCTTG ATCACCTGT-3′) and was cloned into the TOPO (Invitrogen) vector (Table 1). This fragment was excised with EcoRI and ligated into the suicide plasmid pEX18Tc. This construct was introduced into *S. maltophilia* K279a by triparental mating. The mating mixture was plated on NYGA medium containing tetracycline (125 μg ml^{-1}) to select for mutants. Candidate strains were analyzed by colony PCR using the primers Con-F (5′-TTGCGTATTGGGCGCTCTTCC-3′) and Con-R (5′-AC

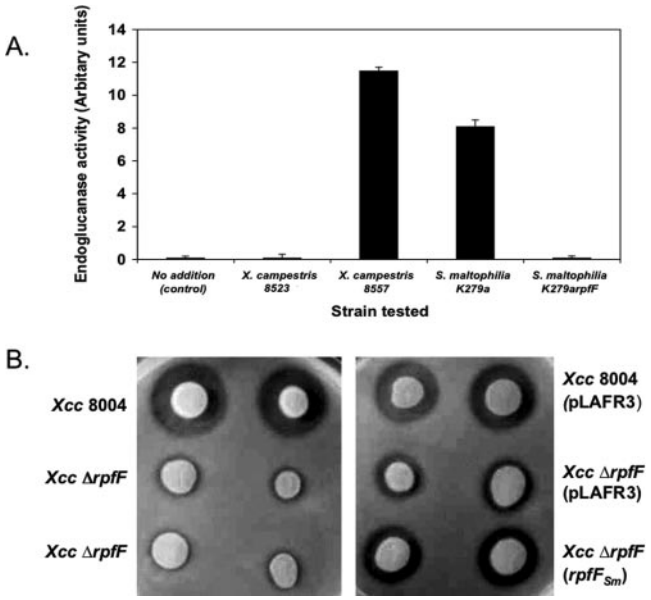

FIG. 2. (A) DSF activity in culture supernatants of strains of *S. maltophilia* K279a and *X. campestris*. Extracts were assayed using a *Xanthomonas* bioassay in which restoration of endoglucanase activity to an *rpfF* mutant is measured (3). Error bars indicate standard deviations. (B) Introduction of the *rpfF* gene from *S. maltophilia* K279a restores the synthesis of protease to the *rpfF* mutant of *X. campestris*. Enzyme activity was assessed by zones of clearing produced after growth of bacteria on skim milk agar plates. Left panel, protease production in wild-type *X. campestris* (*Xcc* 8004) and the *rpfF* mutant (*Xcc* Δ*rpfF*). Right panel, protease production by *X. campestris* Δ*rpfF* carrying the *S. maltophilia rpfF* gene cloned in pLAFR3. The vector alone had a small effect.

GATGATCGGCCTGTCGCT-3′) to confirm disruption of the *rpfF* gene by the suicide vector. As expected, disruption of *rpfF* in *S. maltophilia* K279a led to a loss of DSF synthesis as assayed using the *X. campestris rpfF* mutant reporter strain 8523(Fig. 2A).

The disruption of DSF signaling had pleiotropic effects in *S. maltophilia* K279a. The *rpfF* mutant had severely reduced motility (Fig. 3A), reduced levels of extracellular protease (Fig. 3B), and altered lipopolysaccharide (LPS) profiles (Fig. 3C) and formed aggregates when grown in L medium (Fig. 3d). Mutation of *rpfF* also led to reduced tolerance to a range of antibiotics and heavy metals (Table 2), as measured by growth of bacteria on agar plates supplemented with these agents at a range of concentrations. Effects on aggregative behavior were further tested by examination of microcolony formation in artificial sputum medium (ASM+ medium), which has been developed to mimic growth of bacteria (in particular *Pseudomonas aeruginosa*) in the cystic fibrosis lung (38). Under these growth conditions, the wild-type *S. maltophilia* formed microcolonies, although the *rpfF* mutant did not (Fig. 4).

The phenotypic effects of *rpfF* mutation in *S. maltophilia* could be reversed by addition of exogenous DSF. Addition of synthetic DSF from *X. campestris* (44) at 1 μM or extracts from wild-type *S. maltophilia* to cultures of the *S. maltophilia rpfF* mutant of an equivalent volume restored microcolony formation in ASM+ medium (Fig. 4). Addition of DSF to cultures of the *rpfF* mutant also allowed wild-type planktonic growth in L

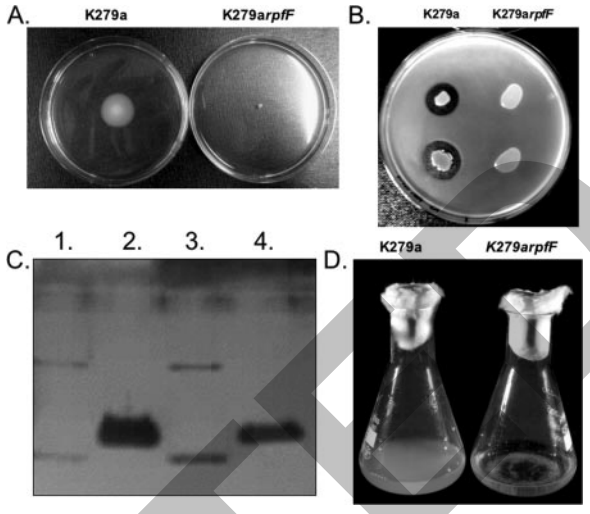

FIG. 3. Loss of DSF signaling through mutation of *rpfF* has a pleiotropic effect in *S. maltophilia*. The *rpfF* mutant shows reduced swimming motility in 0.1% Eiken agar (A), reduced production of extracellular protease (B), altered LPS as revealed by different mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of LPS extracted with hot phenol from the wild type (lanes1 and 3) and the *rpfF* mutant (lanes 2 and 4) and detected by silver staining (the bands of high mobility are probably lipid A attached to core oligosaccharides, whereas those with lower mobility have an additional O-antigen attachment) (C), and aggregation when grown in L medium, where the wild type grows in a dispersed fashion (D).

medium (data not shown), restored swimming motility (Fig. 5A), and restored the production of extracellular protease to wild-type levels (Fig. 5B).

The above findings demonstrated the influence of DSF signaling on LPS structure, protease synthesis, and aggregative behavior, which are functions that are known or suspected to be involved in *S. maltophilia* virulence (10, 13, 19, 25, 46). This prompted us to test the effect of *rpfF* mutation on *S. malto-*

TABLE 2. Influence of *rpfF* mutation on antibiotic and heavy metal tolerance of *S. maltophilia* K279a

Antimicrobial compound	MIC (μg/ml) for:	
	S. maltophilia K279a	*S. maltophilia* K279a *rpfF*
Kanamycin	500	8
Ampicillin	>1,500	8
Tetracycline	20	125[a]
Erythromycin	>700	120
Gentamicin	350	120
Spectinomycin	>2,000	8
Streptomycin	600	80
Nalidixic acid	40	40
Rifampin	10	5
Zinc chloride	>400	80
Copper chloride	>300	200
Nickel chloride	380	220
Cobalt chloride	250	150
Chromic chloride	>900	700
Cadmium chloride	80	55

[a] The suicide vector pEX18Tc used to create the mutant carries a tetracycline resistance determinant.

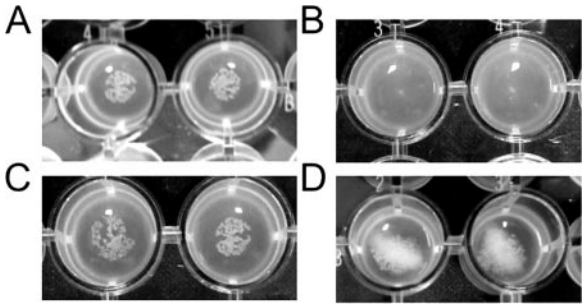

FIG. 4. DSF has a role in microcolony formation by *S. maltophilia* K279a. Bacteria were grown in ASM+ medium (30) in microtiter plates. (A) *S. maltophilia* K279a; (B) *S. maltophilia* K279a *rpfF*; (C) *S. maltophilia* K279a *rpfF* with added DSF; (D) *Pseudomonas aeruginosa* PAO1 (positive control).

philia virulence using a nematode model (8, 22, 24). Wild-type *S. maltophilia* K279a killed almost all of the N2 *Caenorhabditis elegans* in the assay within 24 h (Table 3). As judged by measurements after 12 h, the killing effect was similar to that caused by *P. aeruginosa* PA14. In contrast the *rpfF* mutant of *S. maltophilia* K279a did not kill any nematodes after 12 h and produced relatively limited killing after 24 h. These findings suggest that DSF signaling contributes to the virulence of *S. maltophilia*.

A number of other isolates of *S. maltophilia* and one of *Stenotrophomonas rhizophila* obtained from both clinical and environmental sources (Table 1) were surveyed for the presence of the *rpfF* gene by PCR and for the production of the DSF signal using the *Xanthomonas* bioassay. PCR analysis indicated the presence of the *rpfF* gene in all strains tested. DSF production was also detected in all strains of *S. maltophilia* with the exception of e-p20, although there was variation in the level, with some strains (c6 and e-p3) having little detectable activity (data not shown). Taken together, these findings indicate that DSF signaling is conserved in *Stenotrophomonas* isolates.

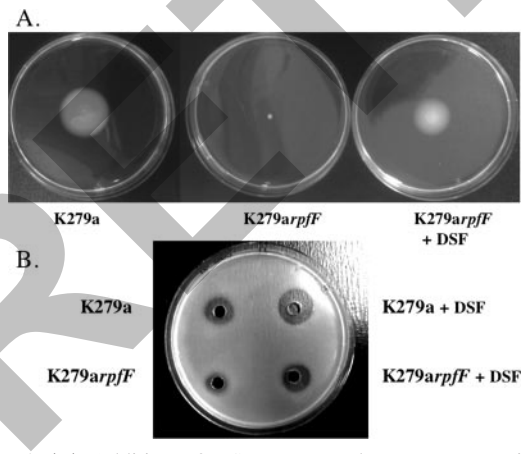

FIG. 5. (A) Addition of DSF to agar plates restores swimming motility to the *S. maltophilia rpfF* mutant. (B) Addition of DSF to cultures grown in NYG medium (42) restores protease production to the *S. maltophilia rpfF* mutant to wild-type levels. Addition of DSF to the wild type further increased protease production. Enzyme activity in culture supernatants grown to an optical density at 600 nm of 2.0 was assayed by development of a clear zone on skim milk agar plates.

TABLE 3. Virulence of bacterial strains on *Caenorhabditis elegans* N2[a]

Strain	Mean % killing ± SD after:	
	12 h	24 h
S. maltophilia K279a	45 ± 5	96 ± 6
S. maltophilia K279a *rpfF*	0 ± 0	10 ± 3
P. aeruginosa PA14	70 ± 7	99 ± 3
E. coli OP50	0 ± 0	2 ± 1

[a] Each strain was assayed at least three times for killing of adult nematodes. *E. coli* OP50 is routinely used for nematode maintenance.

The work in this study suggests that DSF signaling in *S. maltophilia* has a role in the regulation of a number of functions that contribute to antibiotic resistance and to the virulence of this organism in a nematode model. Our findings thus add to a body of work that indicates a role for cell-cell signaling in the virulence of diverse bacterial pathogens. Interference with such signaling processes affords a rational approach to aid the treatment of bacterial infections (5, 16). However, one limitation of such an approach is that strain-dependent differences in the role of cell-cell signaling can occur. In this context, a study of DSF signaling and its role in a wider number of *S. maltophilia* isolates is warranted.

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