Diffusible signal factor signaling regulates multiple functions in the opportunistic pathogen *Stenotrophomonas maltophilia*

Shi-qi An[1*] and Ji-liang Tang[2*]

Abstract

Objective: *Stenotrophomonas maltophilia* is a Gram-negative bacterium commonly isolated from nosocomial infections. Analysis of the genome of the clinical *S. maltophilia* isolate K279a indicates that it encodes a diffusible signal factor (DSF)-dependent cell–cell signaling mechanism that is highly similar to the system previously described in phytopathogens from the genera *Xanthomonas* and *Xylella*. Our objective was to study the function of DSF signaling in the clinical strain *S. maltophilia* K279a using genetic and functional genomic analyses.

Results: We compared the wild-type strain with a mutant deficient in the *rpfF* (regulation of pathogenicity factors) gene that is essential for the synthesis of DSF. The effects of disruption of DSF signaling were pleiotropic with an impact on virulence, biofilm formation and pathogenesis. The phenotypic effects of *rpfF* mutation in *S. maltophilia* could be reversed by addition of exogenous DSF. Taken together, we demonstrate that DSF signaling regulates factors contributing to virulence, biofilm formation and motility of this important opportunistic pathogen.

Keywords: *Stenotrophomonas maltophilia*, Cell–cell signaling, Diffusible signal factor, Antibiotic resistance, Biofilm formation, Virulence

Introduction

Stenotrophomonas maltophilia is a Gram-negative bacterium that is found ubiquitously in the environment and which has become an important opportunistic pathogen [1–3]. *S. maltophilia* infections occur in cystic fibrosis and burns patients and are common in individuals with compromised immune systems who are inclined to opportunistic infections [1–3]. The organism is commonly isolated from clinical specimens and is involved in urinary and respiratory tract infections, endocarditis and in catheter-related bacteraemia and septicaemia [1–3]. Isolates are resistant to the majority of clinically useful antibiotics making the treatment of *S. maltophilia* infections problematic [4]. A number of laboratories have

been addressing the molecular bases for virulence and antibiotic resistance in *S. maltophilia* [1–3, 5, 6].

Stenotrophomonas maltophilia is related to bacteria from the genera *Xanthomonas* and *Xylella* [7]. In Xanthomonads, cell–cell signaling mediated by molecules of the diffusible signal factor (DSF) family control virulence factor synthesis, development of biofilms as well as disease in plants (reviewed in Ryan et al. [8]). Furthermore, these signal molecules have been shown to play a role in *Xylella* interaction with its insect vector (reviewed in Ryan et al. [8]). DSF family signal molecules are *cis*-unsaturated fatty acids, the first of which to be identified was *cis*-11-methyl-2-dodecenoic acid from *Xanthomonas campestris* [9]. The synthesis and perception of the signals require products of the *rpf* gene cluster; DSF synthesis is dependent on RpfF, which has some amino acid sequence similarity to enoyl-CoA hydratases, whereas DSF perception involves a two-component regulatory system, including the complex sensor RpfC and response regulator RpfG [8–11]. In *X. campestris*, the *rpfG* and *rpfC* genes are co-transcribed as the *rpfGHC* operon,

*Correspondence: s.an@qub.ac.uk; jltang@gxu.edu.cn
[1] Wellcome Wolfson Institute for Experimental Medicine, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK
[2] College of Life Science and Technology, State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Guangxi University, 100 Daxue Road, Nanning 530004, Guangxi, China

although RpfH has no clear role in signaling and is not conserved in other *Xanthomonas* species or in *Xylella* [12]. The *rpfB* gene, which encodes a long-chain fatty acyl coenzyme A ligase, is linked to *rpfF* in *X. campestris* and has been implicated in DSF turnover [13]. The relatedness of *S. maltophilia* to these plant pathogens prompted us to examine this organism for the existence and role of a DSF-dependent signaling system. In 2007, it was reported that mutation of *rpfF* had effects on different phenotypes in *S. maltophilia* [14]. However, this paper was recently retracted due to errors in data presentation [15]. Here we report on the outcomes of repeated key experiments that indicate the pleiotropic nature of *rpfF* mutation and show that DSF signaling controls factors contributing to virulence, motility and biofilm formation of this nosocomial pathogen.

Main text

Methodology

Bacterial strains and growth conditions

For the majority of experiments, NYGB medium was used as growth media for *S. maltophilia* strains, which contains 20 g/L glycerol (Sigma-Aldrich, UK), 3 g/L yeast extract (Difco, UK) and 5 g/L bacteriological peptone (Oxoid, UK). The measurement bacterial biofilm formation was carried out in L medium, which comprises of sodium chloride, 5 g/L; yeast extract, 5 g/L; Bactotryptone (Difco, UK), 10 g/L and D-glucose (Sigma-Aldrich, UK), 1 g/L.

The strain of *S. maltophilia* used was K279a [4]. In this study in order to create a disruption of the *rpfF* gene in *S. maltophilia*, an internal fragment of the gene was amplified using the primers PEX18RPFF-F: 5′-TGACATCGT CGACGACTACCAGC-3′) and PEX18RPFF-R: 5′-GGC TTTCCTTGATCACCTGT-3′ and was cloned into the TOPO (Invitrogen) vector. This fragment was excised with *Eco*RI and ligated into the suicide plasmid pEX18Tc [16]. This construct was introduced into *S. maltophilia* K279a by triparental mating. The mating mixture was plated on NYGA medium containing tetracycline (125 μg/mL) to select for mutants. *S. maltophilia* K279a has low intrinsic level of tetracycline tolerance. Candidate strains were analyzed by colony PCR using the primers Con-F: 5′-TTG CGTATTGGGCGCTCTTCC-3′ and Con-R: 5′-ACG ATGATCGGCCTGTCGCT-3′ to confirm disruption of the *rpfF* gene by the suicide vector. For complementation studies, the *rpfF* gene was cloned into pBBR1MCS [17].

For the complementation of *X. campestris* strains, the *rpfF* gene from *S. maltophilia* K279a with its promoter was amplified by PCR using the primers RPFFCOMF: 5′-GAATTCAGACGGCGGGGTCTTT-3′ and RPFF-COMR: 5′-AAGCTTTCAGGCCGGGTCGCCATT-3′ and the DNA fragment cloned into the TOPO vector.

The *rpfF* gene was excised as a *Eco*RI–*Hin*dIII fragment and ligated into pLAFR3 [10] cut with the same enzymes. This resulting construct was introduced into selected *X. campestris* strains by triparental mating.

DSF extraction and synthetic DSF

DSF was extracted into ethyl acetate from culture supernatants of strains grown in NYGB as described by Barber and colleagues [10]. DSF was assayed by measuring the restoration of endoglucanase activity to an *X. campestris rpfF* mutant strain 8523 by extracts from culture supernatants [10]. Synthetic DSF from *X. campestris* (*cis*-11-Methyl-2-dodecenoic acid) was purchased from Merck (Sigma-Aldrich, UK).

Extracellular enzymes assays

For measurement of protease activity, strains were grown in NYG medium, 3 μL of the overnight cultures (OD$_{600}$ nm ≈ 1.0) were spotted onto an NYG plate containing 1% (w/v) skimmed milk and allowed to dry before growth at 30 °C for 24(h). For measurement of endoglucanase activity, strains were grown in NYG medium overnight. Enzyme activity in cell-free culture supernatants were measured by radial diffusion assays into substrate-containing agar plates using carboxymethyl cellulose (CMC) as substrate [10].

Motility assays

Bacterial motility assays were carried out on NYGB media that was solidified using 0.6% Eiken agar (Eiken Chemical, Tokyo). A sterile 200-μL tip was used to inoculate *S. maltophilia* strains to the centre of the plate. Plates were visualized after incubated at 30 °C for 48 h.

Biofilm formation assay

Bacterial strains were assessed for biofilm formation by aggregation in L medium as described previously [18]. Here log-phase-grown bacteria were diluted to OD$_{600}$ nm = 0.02 in L media broth, and 5 mL was incubated at 30 °C for 24 h in 14 mL glass tubes.

Virulence assay

Virulence was tested in *Galleria mellonella* larvae [19], which were stored at 4 °C in wood shavings. *G. mellonella* were injected with 10 μL of successively diluted bacteria (1×10^6 CFU). Infected *G. mellonella* were placed on Whatman paper lined Petri dishes and incubated at 37 °C. The *G. mellonella* were monitored for their survival after a 24 h period. Four separate tests were conducted consisting of 10 larvae for each strain. The control groups for each experiment consisted of *G. mellonella* injected with PBS alone.
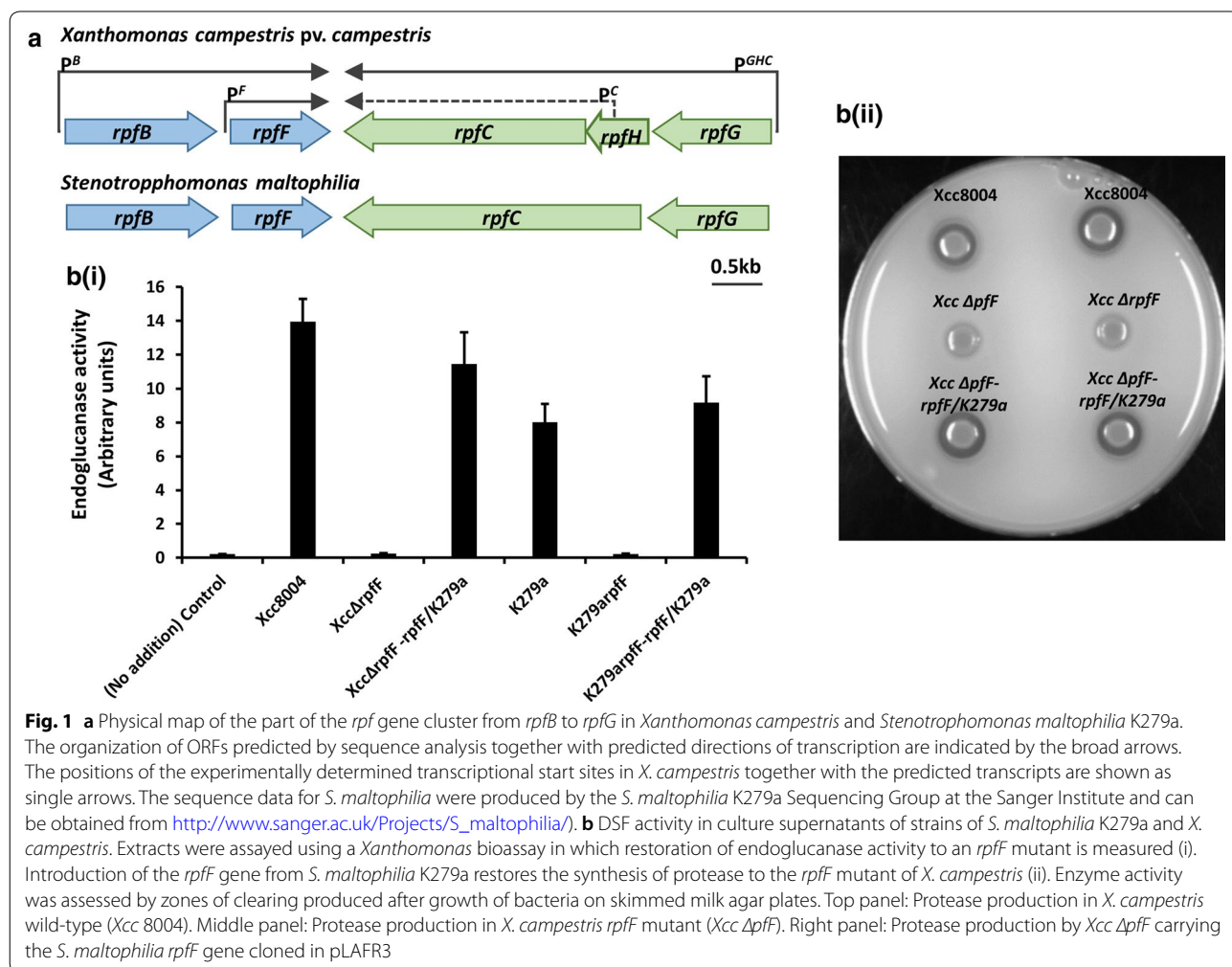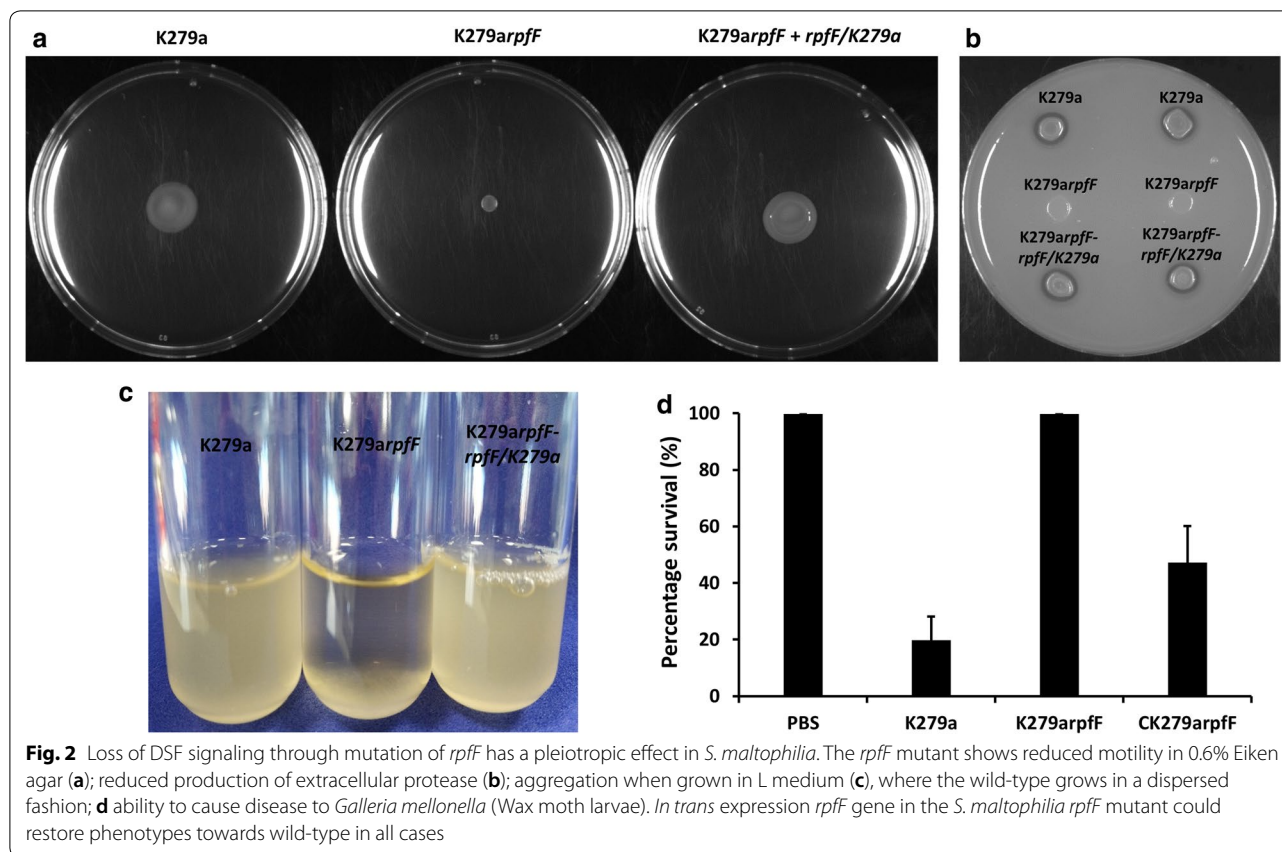
Results and discussion

Initial evidence for the occurrence of the DSF signaling system in *S. maltophilia* clinical isolate K279a was provided by bioinformatic analysis. Interrogation of the genome sequence of this organism (http://www.sanger.ac.uk/Projects/S_maltophilia/) using with the RpfF amino acid sequence of *X. campestris* in tBLASTn revealed a homolog of RpfF. Further analysis of a DNA sequence of approximately 8 Kb (to include flanking genes) indicated the presence of an *rpfBFCG* gene cluster, related to that found in *X. campestris* (Fig. 1). No homologue of *rpfH* was identified in *S. maltophilia* (Fig. 1). The *S. maltophilia* proteins showed very high amino acid sequence similarity to their homologues in *X. campestris*; in BLASTP comparisons, E values were lower than 10^{-127}.

These bioinformatic studies were supported by experimental studies to examine the production of DSF by the K279a strain. DSF in extracts of culture supernatants was assayed by measuring the restoration of endoglucanase activity to an *X. campestris rpfF* mutant (see "Methodology"). Using this bioassay, DSF activity was detected in culture supernatants of *S. maltophilia* K279a (Fig. 1). Further evidence that the product of the *S. maltophilia* K279a *rpfF* gene directs DSF production was obtained from experiments in which the cloned gene was introduced into the *rpfF* mutant of *X. campestris*. The transconjugant produced detectable DSF production and the production of the extracellular enzymes endoglucanase and protease was concomitantly restored (Fig. 1). Furthermore, inactivation of *rpfF* in *S. maltophilia* K279a by use of the pEX18Tc suicide vector (see "Methodology") led to a loss of DSF synthesis as (Fig. 1).

The effects of disruption of DSF signaling through inactivation of *rpfF* in *S. maltophilia* K279a were pleiotropic. The *rpfF* mutant had severely reduced motility (Fig. 2), decreased levels of extracellular protease (Fig. 2) and formed aggregates or biofilms when grown in L medium (Fig. 2). Importantly, *in trans* expression

Fig. 1 **a** 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* 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/). **b** 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 (i). Introduction of the *rpfF* gene from *S. maltophilia* K279a restores the synthesis of protease to the *rpfF* mutant of *X. campestris* (ii). Enzyme activity was assessed by zones of clearing produced after growth of bacteria on skimmed milk agar plates. Top panel: Protease production in *X. campestris* wild-type (*Xcc* 8004). Middle panel: Protease production in *X. campestris rpfF* mutant (*Xcc ΔpfF*). Right panel: Protease production by *Xcc ΔpfF* carrying the *S. maltophilia rpfF* gene cloned in pLAFR3

Fig. 2 Loss of DSF signaling through mutation of *rpfF* has a pleiotropic effect in *S. maltophilia*. The *rpfF* mutant shows reduced motility in 0.6% Eiken agar (**a**); reduced production of extracellular protease (**b**); aggregation when grown in L medium (**c**), where the wild-type grows in a dispersed fashion; **d** ability to cause disease to *Galleria mellonella* (Wax moth larvae). *In trans* expression *rpfF* gene in the *S. maltophilia rpfF* mutant could restore phenotypes towards wild-type in all cases

of the *rpfF* gene in the mutant could restore phenotypes towards wild-type in all cases.

The above findings demonstrated the impact of DSF signaling on aggregative behavior and protease synthesis in *S. maltophilia*, which are functions that are believed to be involved in virulence. This motivated us to test the effect of *rpfF* mutation on *S. maltophilia* virulence using the *Galleria mellonella* larvae model (see "Methodology"). Under the assay conditions used, the wild-type *S. maltophilia* K279a killed the majority of larvae after 24 h, whereas the *rpfF* mutant of *S. maltophilia* K279a produced no killing (Fig. 2). These findings suggest that DSF signaling contributes to the virulence of *S. maltophilia*. The pleiotropic effects of loss by mutation *rpfF* are consistent with previous observations in different strains of *S. maltophilia* and species of *Stenotrophomonas* such as *S. rhizophila*, where *rpfF* mutants have altered biofilm formation, extracellular polysaccharide synthesis and virulence [5, 6].

The phenotypic effects of *rpfF* mutation in *S. maltophilia* could be reversed by addition of exogenous DSF. Addition of synthetic DSF from *X. campestris* at 1 mM or extracts from wild-type *S. maltophilia* to cultures of the *S. maltophilia rpfF* mutant of an equivalent volume

restored wild-type planktonic growth in L medium and restored motility towards wild-type levels (Fig. 3). The work is consistent with recent studies that identify the importance of RpfF proteins and DSF signaling in regulation in other environmental strains of *S. maltophilia* [5, 6].

Conclusions

The work in this paper suggests that DSF signaling in *S. maltophilia* has a role in the regulation of a number of functions that contribute to aggregation or biofilm formation and to the virulence of this organism. 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. However, one limitation of such an approach is that differences in the operation of Rpf–DSF mediated cell–cell signaling have been reported in different strains of *S. maltophilia* to include both clinical and environmental isolates. In this context, a detailed study of DSF signaling and its role in a wider number of *S. maltophilia* isolates is very much warranted.

Fig. 3 Addition of DSF to agar plates restores motility to the *S. maltophilia rpfF* mutant. The addition of DSF to agar plates restores **a** motility; **b** production of extracellular protease; **c** wild-type planktonic growth in L medium to the *S. maltophilia rpfF* mutant
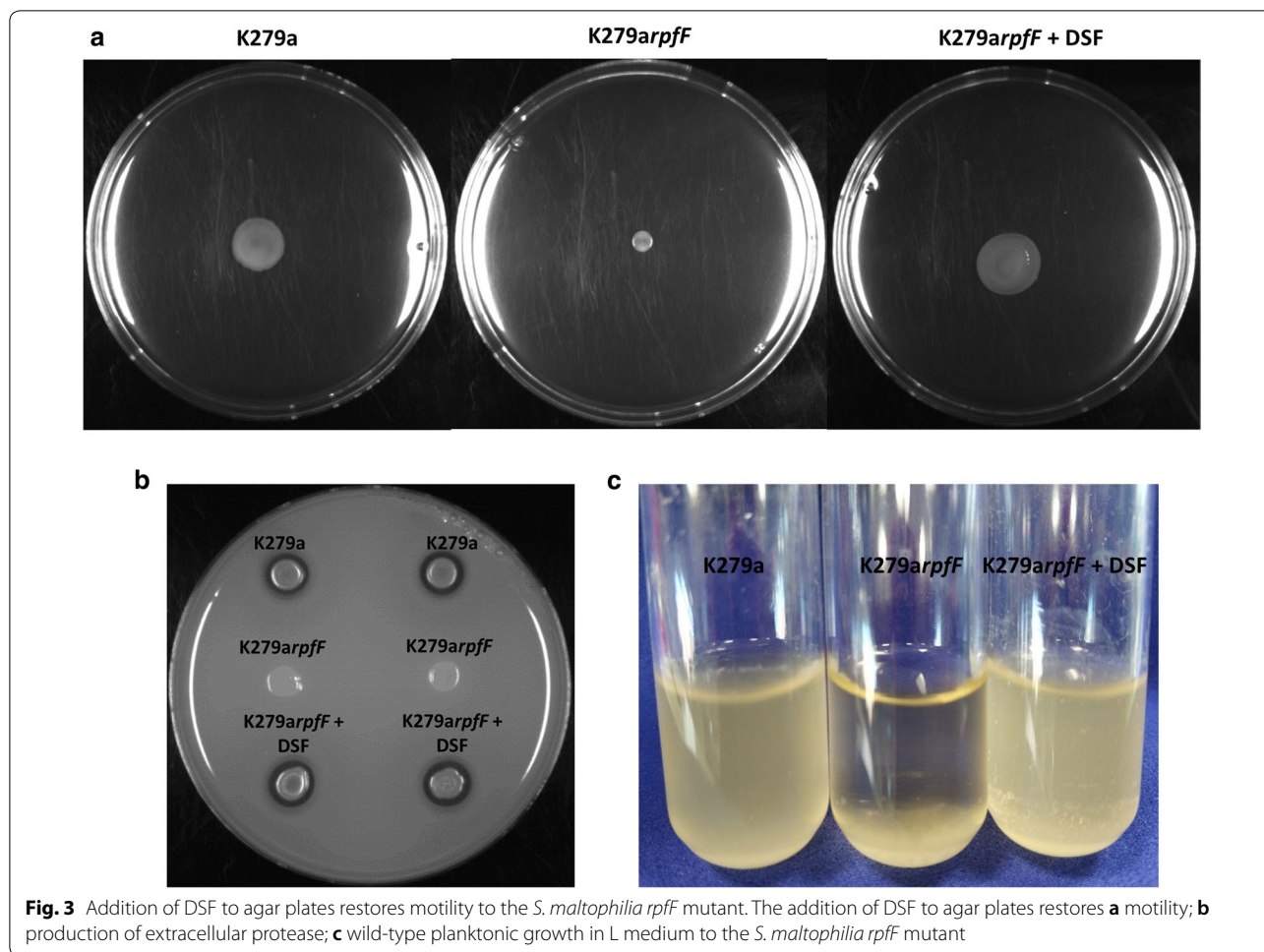
Limitations

- The study focuses on a single sequenced strain of *S. maltophilia*.
- The molecular mechanism for how the DSF signal is perceived by *S. maltophilia* and regulates the specific phenotypes observed is not investigated.
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Abbreviations
DSF: diffusible signal factor; Rpf: regulation of pathogenicity factors; OD: optical density; *Xcc*: *Xanthomonas campestris* pv. *campestris*.

Authors' contributions
Experimental design: SQA; JLT. Experimental work: SQA; JLT. Data analysis: SQA, JLT. Writing manuscript: SQA, JLT. Reviewing manuscript: all authors. Both authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data generated or analysed during this study are included in this published article.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

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