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Diffusible Signal Factor (DSF)-dependent quorum sensing in pathogenic bacteria and its exploitation for disease control

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RUNNING HEADLINE: DSF signalling in bacteria

Summary

Cell-to-cell signals of the Diffusible Signal Factor (DSF) family are *cis*-2-unsaturated fatty acids of differing chain length and branching pattern. DSF signalling has been described in diverse bacteria to include plant and human pathogens where it acts to regulate functions such as biofilm formation, antibiotic tolerance and the production of virulence factors. DSF family signals can also participate in interspecies signalling with other bacteria and interkingdom signaling such as with the yeast *Candida albicans.* Interference with DSF signalling may afford new opportunities for the control of bacterial disease. Such strategies will depend in part on detailed knowledge of the molecular mechanisms underlying the processes of signal

synthesis, perception and turnover. Here, I review both recent progress in understanding DSF signalling at the molecular level and prospects for translating this knowledge into approaches for disease control.

Keywords: Virulence; signalling; plant pathology; pseudomonads; microbial physiology.

Introduction

Many bacteria use cell-to-cell signalling, often called quorum sensing (QS), to modulate their activities in response to aspects of their environment such as population density or confinement to niches. Such communication depends upon the synthesis and release of diffusible signal molecules to the milieu, their perception by different sensors and signal transduction that leads to alteration in bacterial gene expression and behaviour. Cell-to-cell signalling regulates diverse processes that include the formation of biofilms and the production of virulence factors in pathogenic bacteria. Bacteria within biofilms are often much more tolerant to antibiotics than their planktonic counterparts, hence interference with signalling may afford routes to disease control, by reducing virulence and through improvement of efficacy of existing antibiotic therapies. The development of such strategies will depend in part upon a detailed knowledge of the molecular mechanisms of signal synthesis, perception and transduction.

Bacterial signal molecules belong to a wide range of chemical classes. The DSF family of signals comprises *cis*-2-unsaturated fatty acids of differing chain length and branching pattern (reviewed by Deng *et al*. 2011; Ryan *et al.* 2015)(Fig. 1). The first family member described was *cis*-11-methyl-2-dodecenoic acid (designated DSF) from the phytopathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*)(Barber et al., 1997; Wang et al., 2004).

Further family members have been described in *Burkholderia cenocepacia* (*cis*-2-dodecenoic acid; BDSF), *Xylella fastidiosa* (*cis*-2-tetradecenoic acid; XfDSF; *cis*-2-hexadecenoic acid; XfDSF2), *Xanthomonas oryzae* (*cis,cis*-11 methyldodeca-2,5-dienoic acid; CDSF) and *Pseudomonas aeruginosa* (*cis*-2-decenoic acid)(Boon *et al*. 2008; Beaulieu *et al*., 2013; Ionescu *et al*. 2016; He *et al*. 2010; Davies and Marques, 2009). The *cis* unsaturated double bond at the 2-position is regarded as the signature for DSF family signals and is a key structural feature for activity (Wang et al., 2004); where tested, *trans* derivatives have little or no signalling activity. With the exception of *P. aeruginosa*, many of these bacteria produce multiple DSF family signals, although each genus seems to be most responsive to the major signal that it produces (Ionescu *et al.* 2013; 2016). However, although *Pseudomonas aeruginosa* does not synthesize *cis*-11-methyl-2-dodecenoic acid (DSF) or *cis*-2-dodecenoic acid (BDSF), it is capable of sensing these molecules with consequences for bacterial behavior, including altered biofilm architecture and increased antibiotic tolerance (Ryan *et al.* 2008). It should be noted that *Burkholderia cenocepacia* and *Pseudomonas aeruginosa* have additional QS systems mediated by *N*-acyl homoserine lactones and alkyl quinolones, and there is evidence of regulatory interplay between these different systems (Schmid *et al.* 2012; Udine *et al.* 2013).

Signalling by the DSF family of signals positively influences the virulence of a number of plant and human pathogenic bacteria to include *Xanthomonas* spp., *Stenotrophomonas maltophilia*, *Burkholderia cenocepacia* and *Pseudomonas aeruginosa* (reviewed in Ryan *et al.* 2015). By way of contrast, signal-deficient mutants of *Xylella fastidiosa* show enhanced virulence to plants but a reduced capacity to colonize their insect vector and hence poor transmission to uninfected plants (Newman *et al*. 2004; Chatterjee *et al*. 2008 a,b). A role for

DSF family signalling in biofilm formation in a number of bacteria has also been described (Dow *et al.* 2003; Newman *et al.* 2004; Torres *et al.* 2007; Tao *et al.* 2010; Deng *et al.* 2012).

DSF family signals are also implicated in inter-species and inter-kingdom signalling. *Cis*-2-decenoic acid induces biofilm dispersal in a range of Gram-negative and Gram-positive bacteria that do not produce DSF family signals (reviewed by Marques *et al*. 2015), and also improves the efficacy of antibiotic action in a number of organisms (see below). BDSF can modulate the yeast-hyphal transition in the dimorphic fungus *Candida albicans* responds to (Boon *et al*. 2008) and DSF has been shown to induce defence-related responses in plants (Kakkar *et al*. 2015).

Here I briefly review the current understanding of DSF signalling in bacteria before going on to focus on prospects for translating the knowledge gained into methods for disease control through inhibition of signal synthesis, enhancement of signal turnover or interference with signal perception.

The two 'core' pathways of DSF signal transduction

The *rpf* gene cluster (for regulation of pathogenicity factors) encodes the components of the DSF signalling system in *Xanthomonas* spp. (reviewed in Ryan *et al.* 2015). The synthesis of DSF is totally dependent on RpfF, an enzyme of the crotonase superfamily that has amino acid sequence relatedness to enoyl CoA hydratase. Although *rpfF* has its own promoter, it is also transcribed as part of an operon with the upstream *rpfB* gene, which encodes a long chain fatty acyl CoA ligase. DSF sensing and signal transduction involves a two-component system comprising the sensor kinase RpfC and regulator RpfG, encoded by an adjacent operon. RpfC is a complex sensory kinase with a transmembrane sensory input domain,

histidine kinase, CheY-like receiver (REC) and HPt domains, whereas RpfG has receiver domain attached an HD-GYP domain, which is a cyclic di-GMP phosphodiesterase. Perception of DSF by RpfC is linked to an alteration in the cellular level of the second messenger cyclic di-GMP probably through auto-phosphorylation and subsequent phospho-transfer to RpfG, which activates this protein for cyclic di-GMP degradation (Fig.2). RpfC acts not only as a sensor for DSF but also in repression of DSF biosynthesis (Slater *et al*. 2000). Several mechanisms for this regulation of signal synthesis have been proposed. These include an influence of RpfC on *rpfF* expression (An *et al*. 2013) and repression of signal synthesis via a physical interaction of RpfF with RpfC (He *et al.* 2006). Bioinformatic analysis reveals that the key elements *rpfF-rpfC-rpfG* are widely conserved in bacteria from the genera *Xanthomonas* and *Xylella* that are plant pathogens, in the human pathogen *Stenotrophomonas maltophilia*, as well as in unrelated bacteria such as *Thiobacillus* and *Leptospirillum* species.

The second core pathway for DSF family signalling was first identified in *Burkholderia*. As for DSF in *Xanthomonas*, BDSF synthesis in *Burkholderia* depends on a homolog of RpfF (Boon *et al.* 2008). However BDSF perception depends upon RpfR, a protein with PAS, GGDEF and EAL domains (Deng *et al.* 2012)(Fig. 2). GGDEF and EAL domains are implicated in the synthesis and degradation respectively of the second messenger cyclic di-GMP (reviewed in Römling *et al*. 2013). *In vitro*, RpfR exhibits cyclic di-GMP phosphodiesterase activity that is modulated by binding of BDSF to the N-terminal PAS domain (Deng *et al*. 2012). The *rpfF* and *rpfR* genes are adjacent and convergently transcribed and are widely conserved not only in *Burkholderia* species but also in bacteria from related genera such as *Achromobacter*, and unrelated Enterobacteriacaeae including *Yersinia*, *Serratia*, *Cronobacter* and *Enterobacter.* Accordingly, the production of DSF

family signals has been shown to be widespread in *Burkholderia* spp. (Suppinger *et al*. 2016a). Moreover, a role for RpfR/RpfF in regulation of biofilm formation, colony morphology and virulence to zebrafish embryos of *Cronobacter turicensis* has been recently described (Suppinger *et al*. 2016b).

Additional DSF family signal transduction pathways have been described in *Xanthomonas* and *Burkholderia* (Ryan *et al.* 2015). The soluble histidine kinase RpfS of *Xcc,* binds DSF through the N-terminal PAS_4 domain to influence the expression of a sub-set of DSF-regulated genes. Likewise the complex sensor kinase BCAM0227 in *B. cenocepacia* involves, which is not a homolog of RpfC of *Xcc*, is involved in regulation of a subset of BDSF-dependent factors. Bioinformatic analysis suggests that these sensors should be considered as accessory as RpfS is not fully conserved in *Xanthomonas* species and, unlike RpfR, BCAM0227 is restricted to *B. cenocepacia* (Ryan *et al.* 2015).

The two "core" pathways both link sensing of a DSF family signal to cyclic di-GMP turnover, but by different mechanisms (Fig. 2). Cyclic di-GMP can exert a regulatory action at transcriptional, post-transcriptional and post-translational levels within the cell and is known to have key role in regulation of biofilm formation and virulence factor synthesis in a wide range of organisms (Römling *et al*. 2013). As a consequence, modulation of cyclic di-GMP signalling *per se* may allow control of these bacterial processes and a number of approaches to achieve these aims have been proposed. These will not be considered here but the reader is directed to several recent reviews (Sintim *et al.* 2010; Römling and Balsalobre 2012; Caly *et al.* 2015).

Cis-2-decenoic acid signalling in *Pseudomonas aeruginosa*

P. aeruginosa does not have an *rpfF-rpfC-rpfG* or *rpfF-rpfR* gene cluster but produces *cis*-2-decenoic acid through the RpfF homolog DspI (Davies and Marques, 2009; Amari *et al.* 2013). The *dspI* gene is located in a cluster of genes encoding enzymes implicated in fatty acid metabolism. The identity of the sensor for this signal and the transduction mechanism are not known however.

RpfF and signal biosynthesis

The RpfF proteins that are synthases for DSF signals are members of the crotonase superfamily of enzymes and have amino acid sequence similarity to enoyl CoA hydratases (Barber *et al*. 1997; Boon *et al*. 2008; Amari *et al*. 2013). *In vitro* studies have identified that the immediate substrate for BDSF synthesis by the RpfF homolog of *B. cenocepacia* is the 3-hydroxylated fatty acyl-ACP (acyl carrier protein), an intermediate in fatty acid biosynthesis (Bi *et al*. 2012). Generation of BDSF requires that the enzyme work initially as a dehydratase to convert 3-hydroxydodecanoyl-ACP to *cis*-2-dodecenoyl-ACP and then as a thioesterase to release free BDSF (*cis*-2-dodecenoic acid). RpfF from *B. cenocepacia* (or *Xcc*) can also generate free saturated fatty acids from any fatty acyl ACP substrate through its thioesterase activity (Bi *et al.* 2012; Zhou *et al.* 2015a). Indeed, the *in vitro* synthesis of BDSF requires the addition of an exogenous acyl-ACP synthetase to reverse this thioesterase reaction (Bi *et al*. 2012). RpfF is the only member of the crotonase superfamily with both dehydratase/desaturase and thioesterase activity. Mutation of *rpfF* in different bacteria affects the appearance in culture supernatants not only of unsaturated fatty acids of the DSF family but also of saturated fatty acids (see for example Beaulieu *et al*. 2013; Huang and Lee Wong 2007). This suggests that even *in vivo* the desaturase and thioesterase actions are not tightly co-ordinated to allow only DSF family signal production. Individual *Xanthomonas*, *Xylella*,

Stenotrophomonas and *Burkholderia* species can produce multiple DSF family signals that are all dependent on RpfF for their synthesis (Huang and Lee Wong 2007; He *et al.* 2010; Deng *et al.* 2010; Beaulieu *et al.* 2013). These observations suggest that the enzyme does not have a strict specificity for a particular substrate. The pattern of DSF family signals produced can depend upon the medium and growth conditions (He *et al.* 2010; Ionescu *et al.* 2016). Nevertheless, although *P. aeruginosa* produces *cis*-2-decenoic acid (via the action of the RpfF homolog DspI), detection of the longer chain derivatives such as BDSF or DSF has not been reported.

Crystal Structures of DSF synthases

Crystal structures of RpfF from *Xcc* and *B. cenocepacia* have been determined and have allowed insights into the molecular mechanisms of substrate binding, the different actions of the enzyme, and interactions with other Rpf proteins as well as providing a guide for the rational design of effective inhibitory molecules (Cheng *et al.* 2010; Spadaro *et al.* 2016). The structure of RpfF of *Xcc* (Cheng *et al.* 2010) showed the occurrence of two glutamate residues that are predicted to be involved in catalysis; alanine substitution of these residues completely abolished DSF production. In addition the structure showed that a number of residues that are highly conserved across different RpfF homologs form a hydrophobic pocket similar to the substrate binding pockets in *E. coli* methyl malonyl decarboxylase and rat enoyl CoA hydratase. However because of steric hindrance this cavity is too small to accommodate the fatty acyl chain of DSF (11-methyl *cis*-2-dodecenoic acid), leading to the suggestion that RpfF may need to undergo conformational change to bind its substrate. The crystal structure of RpfF from *B. cenocepacia* (BCAM0581 which was designated DfsA) showed the unexpected presence of dodecanoic acid in the catalytic site of the enzyme (Spadaro *et al.* 2016)(Fig.3). This is likely to have been generated as a product of the

thioesterase activity of DfsA on a dodecanoyl-ACP substrate. The very slow release of this reaction product from the DfsA active site means that it acts as an inhibitor. Furthermore, exogenous dodecanoic acid can effectively inhibit the thioesterase activity of DfsA on dodecanoyl-ACP, although dodecenoic acid was only inhibitory at much higher concentrations. In the crystal, the fatty acid molecule adopts an unusual elongated structure (Fig. 3), indicating how DSF family signal synthases can recognize their relatively long chain hydrophobic substrates without large conformational changes. Of the eleven substrate-contacting residues in DfsA, nine are identical in RpfF from *Xcc* (with two strongly conservative changes)(Fig. 4). In contrast in DspI, only three of these residues are identical, three showed strongly conservative changes and five were either not conserved or absent from the alignment (Fig. 4). Whether these differences are related to substrate specificity is as yet unknown. The elucidation of the substrate-binding mode in DfsA provides a starting point for structure-based drug discovery studies targeting BDSF signal generation and hence the control of synthesis of *B. cenocepacia* virulence factors.

Interaction between RpfF and RpfC

As outlined above, RpfC in *Xcc* acts not only as a sensor for DSF but also in repression of DSF biosynthesis. This action of RpfC may be mediated in part by protein-protein interaction between the REC domain of RpfC and RpfF, which sequesters RpfF to restrict synthesis of DSF (He *et al.* 2006). Conformational changes in RpfC upon DSF binding may allow rapid auto-induction of DSF synthesis as a result of release of RpfF. Structures of the co-crystal complex of the RpfC REC domain with RpfF have revealed the nature of the interaction and the participating residues (Cheng *et al*. 2010). These observations may also provide some molecular insight into the role of RpfF-RpfC interactions in *Xylella fastidiosa,* which appear to be key for XfDSF signal transduction (Ionescu *et al*. 2013). In *Xylella*, addition of the

DSF family signal does not restore the phenotype of the *rpfF* mutant to wild type (as it does in *Xanthomonas*). Instead signal transduction in *Xylella* requires both RpfC and RpfF. Moreover, enzymatically inactive variants of RpfF can also support XfDSF signal transduction, indicating that RpfF in *Xylella* is multifunctional (Ionescu *et al*. 2013). Whether RpfF interacts with other proteins as a part of the signal transduction mechanism is currently unknown. It remains to be seen whether the available structural information will allow the design of molecules that could interfere with these key RpfF-RpfC interactions.

Signal degradation and the role of RpfB

RpfB, which is a predicted fatty acid CoA ligase, was originally thought to be involved in signal synthesis in *Xanthomonas* but is now known to act in the mobilization of saturated fatty acids generated by the thioesterase action of RpfF (Bi *et al.* 2014). This action allows their recovery and use in phospholipid biosynthesis. In addition there are several reports that RpfB has a role in DSF degradation, although *in vitro* RpfB has little activity against BDSF or DSF (Almeida *et al.* 2012; Zhou *et al.* 2015b). These findings have led to the suggestion that the *in vivo* activity is modulated by additional factors or by an alteration in conformation perhaps driven by interactions with other proteins (Zhou *et al.* 2015b). Orthologs of RpfB occur widely in many bacteria suggesting that the ability to degrade DSF family signals may be widespread. In *Burkholderia* spp., the encoding genes are not linked to *rpfF* however, in contrast to what is seen in most xanthomonads.

A functional screen showed that many bacteria have the ability to degrade DSF, with some strains belonging to genera *Bacillus, Paenibacillus, Microbacterium, Staphylococcus,* and *Pseudomonas* capable of particularly rapid degradation (Newman *et al.* 2008; Caicedo *et al.* 2016). A mutational analysis in *Pseudomonas* species strain G indicated a role for *carAB* in

rapid DSF inactivation/degradation (Newman *et al.* 2008). The *carAB* genes encode enzymes responsible for the synthesis of carbamoylphosphate, which is a precursor in biosynthesis of pyrimidines and arginine. This may suggest an involvement of UDP-sugars in signal turnover. Whether orthologs of RpfB also contribute to DSF degradation in these bacteria is not yet known however.

The identification of environmental or plant-associated organisms capable of DSF degradation has allowed their assessment as potential biocontrol agents for particular plant diseases. Inoculation of bacteria able to degrade DSF can reduce virulence and symptom production by *Xylella fastidiosa* in grape, *Xcc* in brassica and *Xanthomonas citri* in citrus (Newman *et al.* 2008; Caicedo *et al.* 2016). Taken together, these findings suggest that it should be possible to select further strains for improved biocontrol of plant diseases, particularly those caused by xanthomonads.

Control of bacterial phytopathogenesis by over-expression of RpfF in plants

Work in both *Xylella* and *Xanthomonas* has indicated that DSF family signalling is normally finely balanced during the plant disease (Torres *et al.* 2007; Chatteerjee *et al*. 2008a,b) so that disruption of the balance by over-production of the signal may be a strategy for disease control. This process, which has been termed 'pathogen confusion' (Lindow *et al.* 2014), may be achieved by plant transgenic approaches. The expression of RpfF from *Xylella fastidiosa* in grape and citrus reduces the virulence of *Xylella fastidiosa* and *Xanthomonas citri* respectively (Lindow *et al.* 2014; Caserta *et al.* 2014) although the underlying mechanisms are not fully understood. RpfF expression in grape directs the production of *cis*-2-tetradecenoic acid (XfDSF) as well as *cis*-2-hexadecenoic acid (XfDSF2) (Lindow *et al.*

2014). The latter of these unsaturated fatty acids is only produced by *Xylella fastidiosa* grown on agar and was not previously detected in culture medium (Ionescu *et al.* 2016). It is plausible that expression of RpfF may generate further structural analogs that directly influence cell-cell signalling in a negative fashion. Equally the presence of DSF signals may activate the premature production of virulence factors, triggering plant defences which can overwhelm the smaller number of producing bacteria.

Recent work suggests that DSF *per se* may activate plant defence responses that could lead to impaired bacterial growth and virulence gene expression. DSF (but not the *trans* derivative of DSF) has been shown to trigger callose deposition, the induction of the *PR1* gene and plant cell death in leaves of *Arabidopsis* and *Nicotiana benthamiana* and roots of rice (Kakkar *et al.* 2015). Treatment with DSF leads to a decrease in *Xcc* virulence and disease severity. The concentrations of exogenous DSF required to induce these effects are quite high ($\geq$ 20 μM). However at lower concentrations, DSF can act to prime plant cells so that they respond more rapidly and/or to a greater extent to the flagellin peptide flg22, a model Microbial Associated Molecular Pattern (MAMP). During pathogenesis by *Xcc*, the triggering of plant defences by DSF may be countered by different mechanisms, to include the action of the extracellular polysaccharide xanthan (Yun *et al.* 2006; Aslam *et al.* 2008). This polyanionic polymer chelates divalent calcium ions, preventing their influx from the apoplast to the cytosol, an event that normally triggers defence (Yun *et al.* 2006; Aslam *et al.* 2008). In RpfF-transgenic plants however, priming or direct elicitation may lead to more rapid induction and/or enhanced levels of defence-related responses that may overwhelm any pathogen countermeasures.

Structural analogues of DSF that block signal sensing

In addition to blocking signal synthesis, small molecule modulation of signal sensing or downstream transduction steps could afford a route to influence disease severity and improve therapy (Njoroge and Sperandio 2009). Relatively small libraries of structural signal analogues or very large random libraries of chemical compounds can be screened for interesting lead compounds using high throughput methods. This strategy has been successfully used to target the sensor kinase QseC, a receptor for host signals as well as the bacterial AI-3 signal that is located in the cytoplasmic membrane of a range of Gram-negative pathogens (Rasko *et al.* 2008; Curtis *et al.* 2014). Sensory proteins located at the cell surface or in the cytoplasmic membrane are attractive as targets for interference since the inhibitory molecules do not need to enter the bacterial cytoplasm to exert an effect.

Recent unpublished work has examined the effect of a panel of structural analogues of DSF on the action of PA1396 of *P. aeruginosa*, a sensor kinase whose input domain has five predicted transmembrane helices and is related to that of RpfC of *Xanthomonas* PA1396 is involved in interspecies signalling with bacteria that produce BDSF or DSF. As outlined above, although *P. aeruginosa* does not synthesise DSF or BDSF, it is capable of sensing these molecules (through PA1396) to activate changes in gene expression, alter biofilm formation and increase antibiotic tolerance (Ryan *et al*. 2008). Interspecies signalling of this nature may occur in polymicrobial infections such as those associated with cystic fibrosis (CF) where *P. aeruginosa* is present together with *S. maltophilia* and *Burkholderia* species, which produce DSF and BDSF. PA1396 does not however respond to *cis*-2-decenoic acid, the intra-species signal of the DSF family found in *P. aeruginosa*. Particular structural analogs of DSF have been shown to reduce biofilm formation and antibiotic tolerance of *P. aeruginosa* both *in vitro* and in murine infection models (S.Q. An and R.P. Ryan personal

communication). These effects depend largely on the interaction of the molecules with PA1396. The analogs may thus represent lead compounds for novel antibiotic adjuvants. It remains to be seen whether the same compounds affect virulence gene expression in bacteria with the RpfC-RpfG two-component system or influence the action of RpfR. In the context of RpfR, determination of the crystal structures of the PAS domain in the presence or absence of the signal may aid the rational design of further inhibitory molecules.

DSF family molecules as potential therapeutics

A body of work has shown that the signal molecules themselves may also have applications in promoting antibiotic efficacy and biofilm dispersal in non-producing organisms. DSF and BDSF have been shown to enhance the antimicrobial efficacy of antibiotics against a range of bacteria to include *Bacillus cereus*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Neisseria subflava* and *Pseudomonas aeruginosa* (Tian *et al.* 2013; Deng *et al.* 2014). BDSF inhibits the formation and causes the dispersion of biofilms of *Francisella novicida*, a model organism for *Francisella tularensis*, the causal agent of tularemia in humans and animals (Dean *et al*. 2015). *Cis*-2-decenoic acid has also been shown to inhibit growth and biofilm formation by *S. aureus* (Davies and Marques 2009; Jennings *et al.* 2012). In addition, it can revert antimicrobial-insensitive persister cells of *Escherichia coli* and *P. aeruginosa* to a susceptible state (Marques et al. 2010) and in combination with antibiotics or antiseptics can eradicate pre-established bacterial or dual species fungal-bacterial biofilms (Rahmani-Badi *et al.* 2014; Sepehr *et al*. 2014; Rahmani-Badi *et al.* 2015).

DSF, BDSF and CDSF can all act to inhibit the yeast-to-hyphal morphological transition in *Candida albicans* (Boon *et al*. 2008; He *et al.* 2010; de Rossi *et al.* 2014). The ability to switch between yeast and hyphal forms is an important facet of *C. albicans* virulence, with the hyphal form having key roles in the infection process. Hence inhibition of this process by

DSF and derivatives could potentially have a role in therapy. It has also been reported that BDSF can inhibit *C. albicans* adherence to catheters, which is related to modulation of the yeast-hyphal morphological transition (Tian *et al*. 2013). Notably both BDSF and its *trans* derivative are able to block adherence and germ tube formation; the underlying signal transduction mechanisms are not yet known.

In addition to the practical applications, these observations also indicate the possibility that interspecies or interkingdom signalling between DSF-producing bacteria and non-producing microbes occurs in environments where they are present together. For example, interactions between DSF-producing bacteria and *C. albicans* may be important in polymicrobial communities, such as occur in the airway of cystic fibrosis (CF) sufferers. One caveat is that the concentrations of the signal molecules required for particular effects seen *in vitro* may not be within a physiologically range attained *in vivo*.

Conclusions

It is now evident that signal molecules of the DSF family play a significant role in regulation of diverse functions in a wide range of bacteria to include the virulence of pathogens of plants and animals. The targeting of these DSF-mediated pathways by the use of small molecule inhibitors of signal generation or sensing, use of biocontrol bacteria that degrade the signal or generation of transgenic plants that express signal synthases all have potential as new strategies for disease control. Furthermore, the action of these *cis*-2-unsaturated fatty acids in promotion of biofilm dispersal and improvement of the efficacy of existing antibiotics may be of considerable importance given the widespread problem of burgeoning antimicrobial resistance coupled with the limited development of new antibiotics.

In addition to these translational considerations, our expanding knowledge of DSF-mediated signalling poses new sets of basic research questions. What are the molecular details of DSF

signal recognition by different sensor kinases and the PAS domains? What are the molecular components of the *P. aeruginosa* signal-response network involving *cis*-2-decenoic? By what mechanism does *cis*-decenoic acid cause biofilm dispersal in a range of bacteria? Further work is clearly needed to address both fundamental and translational aspects of bacterial signalling mediated by members of the DSF family.

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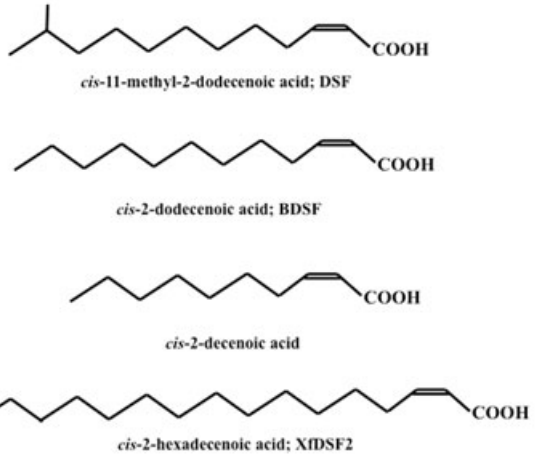
Figure legends

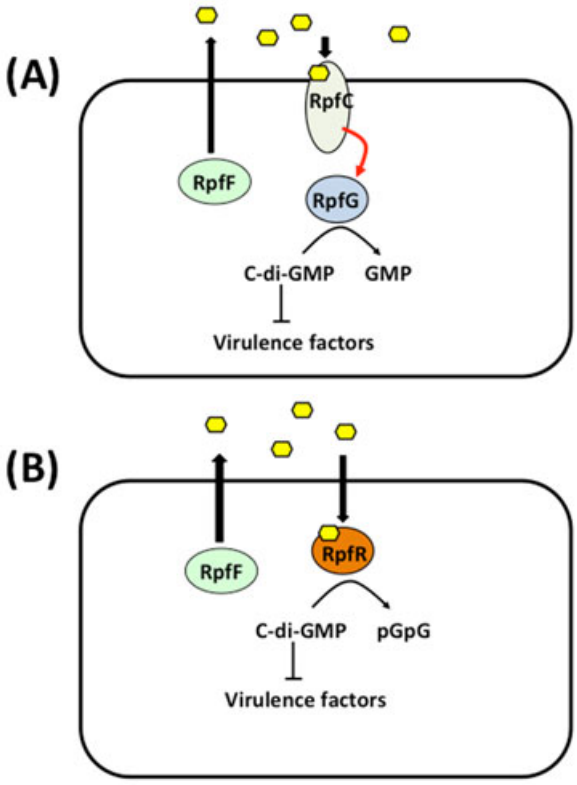
Figure 1. The DSF family of signals comprises *cis*-2-unsaturated fatty acids of different chain lengths and branching pattern. The first family member described was *cis*-11-methyl–dodecenoic acid in *Xanthomonas campestris*. Other family members include BDSF (*cis*-2-dodecenoic acid) from *Burkholderia cenocepacia*, *cis*-2-decenoic acid from *Pseudomonas aeruginosa* and the recently described XfDSF2 (*cis*-2-hexadecenoic acid) from *Xylella fastidiosa* (see text for details).

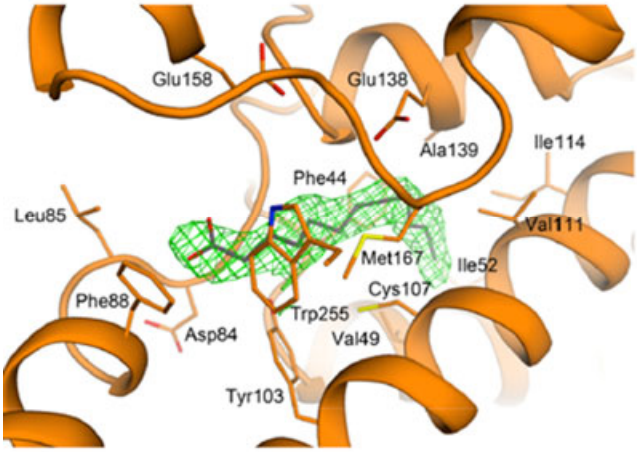
Figure 2. Two 'core' pathways of signalling involving DSF family signals are exemplified by *Xanthomonas* and *Burkholderia* species (A and B respectively). In both cases, the DSF signal molecules (hexagons) are synthesised by an RpfF homolog and signal tranduction is linked to the turnover of the second messenger cyclic di-GMP. In *Xanthomonas* (A), signal perception and transduction involves the sensor kinase RpfC and two component regulator RpfG, which is an HD-GYP domain cyclic di-GMP phosphodiesterase. In *Burkholderia* (B), signal sensing involves RpfR, a cytoplasmic GGDEF-EAL domain protein implicated in cyclic di-GMP degradation (see text for details).

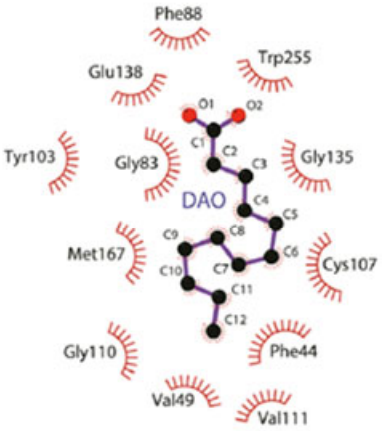
Figure 3. The structure of DfsA (BCAM0581) with bound lauric acid reveals the potential substrate-binding pocket for the hydroxylated fatty acyl-ACP substrates of RpfF DSF synthases. The left-hand panel represents an overview showing the presence of the elongated fatty acid molecule in the substrate-binding cavity, surrounded by hydrophobic amino acid residues. (The catalytic glutamate residues are Glu138 and Glu158). The right-hand panel is a schematic of the amino acid environment surrounding the lipid molecule, here indicated as DAO. Adapted with permission from: The Crystal Structure of *Burkholderia cenocepacia* DfsA Provides Insights into Substrate Recognition and Quorum Sensing Fatty Acid Biosynthesis by Francesca Spadaro, Viola C. Scoffone, Laurent R. Chiarelli, Marco Fumagalli, Silvia Buroni, Giovanna Riccardi, and Federico Forneris Biochemistry, 2016, 55 (23), pp 3241–3250. Copyright 2016 American Chemical Society.

Figure 4. Primary sequence alignment of RpfF proteins for which a role in synthesis of DSF family signals has been demonstrated, indicating key residues involved in catalysis and in creating the substrate binding pocket. The sequences (aligned by ClustalW) are of DspI from *Pseudomonas aeruginosa* (PA0745; Uniprot Q9I5I4_PSEAE); RpfF from *Xylella fastidiosa* (Uniprot Q87EB0_XYLFT), RpfF from *Xanthomonas campestris* (XC_2332; Uniprot A0A0H2X7S0_XANC8); RpfF from *Cronobacter turicensis* (CTU_23310; Uniprot C9XTL6_CROTZ); RpfF from *Stenotrophomonas maltophilia* (Uniprot B2FQ87_STRMK) and DfsA from *Burkholderia cenocepacia* (BCAM0581; Uniprot B4EKM5_BURCJ). The key residues involved in catalysis are two glutamate residues, indicated by red triangles and the oxyanion hole residues indicated by blue triangles. Residues that form the substrate-binding pocket and contact the substrate in DsfA (see Fig.3) are indicated in red in the DsfA sequence and in the other sequences where these are conserved. Substitutions at these positions by amino acids with similar properties are indicated in yellow.







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Xylella       -----msavhpip-hpicessiriieethrnvywiymhahlarttgaayfslkliddimn
Xanthomonas   -----msavqpfir-tnigstlriieepqrdvywihmhadlainpgracfstrlfdditg
Steno         -----msavrpiitrpsqhptlriteeperdvywihmhanlvnqpgrpcfasrlfddivd
Cronobacter   msvfnqstcklftd-tarftqlsgfyeeerriiwmmlr-----aqprpcfnhvlieeimn
Bcam0581      mqlqshpacrpfye-agelsqltafyeegrnvmwmmlr-----seprpcfnqqlftdiih

DspI          lrqlie-hlnrdddiya-lvvtgqgpkffsagafdlnmfadgdkarare----marrfg--
Xylella       yqsvlrqrlkeqtvqlpf-vvlasdsnvfnlggflqlfcdlirrkerealldyacrqvrg
Xanthomonas   yqtnlgqrlntagvlaph-vvlasdsdvfnlggflalfcqliregdrarlldyaqrqvrg
Steno         yqrelgdrlsashalsph-vvlasdsdvfnlggflelfcrliregdrarlldyaqrqvrg
Cronobacter   lsylvqearl----evdfwvtgslvpgmyntggflqffvdcirngkrealrayaracvds
Bcam0581      larvardsgl----tfdfwvtgslvpelfnvggflsffvdairsgrrdqlmayarsqids

DspI          -eafealrdfrgvsiaaingyamggglecalacdiriaerqaqmalpeaavgllpcaggt
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Xanthomonas   vhafhvglgarahsialvqgnalgggfeaalschtiiaeegvmmglpevlfdlfpgmgay
Steno         vhafhaglgtrahsialvqgnalgggfeaalschtivaeegvlmglpevlfdlfpgmgay
Bcam0581      vyelytgfgtgaisiamvegsalgggfeaalahhyvlaqkgvklgfpeiafnlfpgmggy

DspI          qalpwlvgegwakrmilcnervdaetalriglveqvvdsgeargaalllaakvarqspva
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Xanthomonas   sfmcqrisahlaqkimlegnlysaeqllgmglvdrvvprgqgvaaveqvire-skrtpha
Steno         sflcqrisprlaekimlegnlytasqlkemglvdivvpvgegvaaveqvike-srripha
Cronobacter   slvtrragmrlaeeliwqgeshtaewyqpqglvdqlfepgqgfvatrtfidt-lkprlng
Bcam0581      slvarkanrglaesliatgeahaaewyedcglidetfdagdaylatrtfidv-tkpklng

DspI          irtikpliqgarer-----------apntflpee-------rerfvdlfdaqdtregvna
Xylella       waam----qevkkiahevsleemiritelvdsalklsnkslrtmerliraqqthkntal
Xanthomonas   waam----qqvremttavpleemmriteivdtamqlgekslrtmdrlvraqsrrsglda
Steno         waam----revneiatmvplhemmriteivdtamqlgekslrtmdrlvraqarrngdpa
Cronobacter   vram----lrarqrvlrlsrnelmeitedvdaafslepkdvgymerlillqnrhtaaal
Bcam0581      iram----lrarervfqlsrselmditeavhaaftiepkdlaymerlvmlqnrrvsklr

DspI          flekrdpkwrnc
Xylella       kn----------
Xanthomonas   g-----------
Steno         ------------
Cronobacter   rkag--------
Bcam0581      tv----------
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