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Listening to a New Language: DSF-Based Quorum Sensing in Gram-Negative Bacteria

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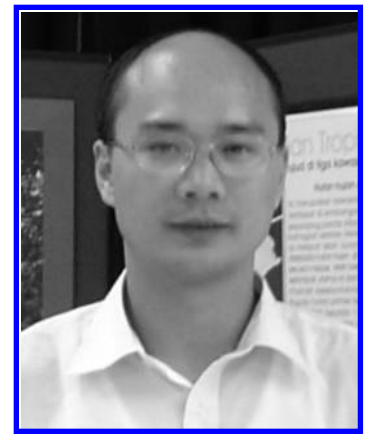
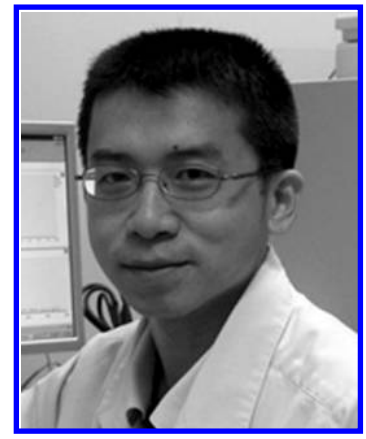


Yinyue Deng was born in 1980 in Jiangxi, China, and graduated from the Nankai University in 2001, where he obtained his M.S. degree three years later. He received his Ph.D degree in 2010 from the National University of Singapore under the mentorship of Professor Lian-Hui Zhang at the Institute of Molecular and Cell Biology. He works now as a Research Fellow in Zhang's laboratory. His research interests mainly focus on the BDSF-dependent quorum sensing in *Burkholderia cenocepacia*.



Ji'En Wu was born in 1976 in Beijing, China, and graduated from the Peking University in 1998. He received his Ph.D degree from the Department of Chemistry at National University of Singapore in 2002 under the supervision of Professor Leslie J. Harrison. In the same year, he joined the Institute of Molecular and Cell Biology as a research officer. In 2005, he joined Professor Thomas J. Simpson's laboratory at the University of Bristol as a Postdoctoral Assistant to study biosynthesis of mupirocin. He moved back to Singapore in 2007 and joined the research group of Professor Lian-Hui Zhang at the Institute of Molecular and Cell Biology as a Research Fellow. His study focuses on the biosynthesis of small molecules with potent bioactivities.

1. Introduction

It has become increasingly clear that bacterial cells can communicate with each other to facilitate their adaptation to changing environmental conditions. This cell−cell communication mechanism, known as quorum sensing (QS), relies on the production, detection, and response to diffusible signal molecules in a cell-density-dependent manner.[1−4] QS is of vital importance to many bacterial species in the regulation of a variety of functions such as biofilm formation, toxin production, exopolysaccharide synthesis, extracellular enzyme production, motility, and plasmid transfer.[5−9] At low cell density, each bacterial cell produces a basal level of QS signals, which are diffused or transported into extracellular environments. At high cell density, after a critical concentra-

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Lian-Hui Zhang is a Research Director/Professor at the Institute of Molecular and Cell Biology and an Adjunct Professor at the National University of Singapore (NUS). He received his Ph.D degree in 1993 under the mentorship of Professor Allen Kerr at the University of Adelaide, where he discovered an AHL-type quorum sensing signal essential for regulation of Ti plasmid conjugal transfer in *Agrobacterium tumefaciens*. He has been an Australian Research Council Postdoctoral Research Fellow and has received NUS Outstanding Researcher Award and Singapore National Science Award for his work in quorum sensing and quorum quenching. His research interests focus on cell−cell communication systems and signaling mechanisms implicated in regulation of bacterial virulence and biofilm formation and on developing quorum quenching strategies to control bacterial infections.

tion has been reached, the accumulated signals initiate a set of biological activities in a coordinated fashion.

Since the 1980s, several types of QS signals have been identified in Gram-negative bacteria. Of these, the best characterized quorum sensing signals belong to the *N*-acyl homoserine lactone (AHL) family. The first AHL-type QS signal was described in *Vibrio fischeri*,[10] in which the AHL signal synthesized by the enzyme LuxI interacts with its receptor LuxR and consequently induces the transcriptional expression of the *lux* genes encoding bioluminescence.[11,12] The AHL-family signals, which share a conserved homoserine lactone ring linked to a fatty acid side chain with variations in chain length and substitutions, have been found in more than 70 bacterial species.[13] Numerous biological functions are known to be AHL-dependent, including luminescence, plasmid transfer, virulence, biofilm formation, motility, and antibiotic production.[1,4] Interestingly, in addition to AHL-family signals, LuxR-type receptors can also be activated by diketopiperazines (DKPs), which are structurally unrelated to AHLs. DKPs have been identified in *Proteus mirabilis*, *Citrobacter freundii*, *Enterobacter agglomerans*, *Pseudomonas fluorescens*, and *Pseudomonas alkaligenes*.[14,15] Autoinducer 2 (AI-2) is another type of widely detected QS signal, which was identified originally in *Vibrio harveyi*[16] and subsequently in other Gram-negative bacteria species.[17] AI-2 type signals are involved in regulation of bioluminescence, type III secretion systems, and extracellular virulence factor production. In addition, there are other seemingly less widely distributed QS signals such as 3-hydroxypalmitic acid methyl ester (3-OH PAME),[18] 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS),[19] bradyoxetin,[20] autoinducer *in Escherichia coli* (AI-3),[21] and 2-heptyl-4-quinolone (HHQ),[22] which have been identified and characterized within the last two decades.

Apart from the above-mentioned QS signals, the diffusible signal factor (DSF), which was originally identified in *Xanthomonas campestris* pv *campestris* (*Xcc*),[23,24] represents another interesting type of QS signal found in Gram-negative bacterial pathogens. Evidence is accumulating that DSF-family signals are widespread.[23−25] In the past few years, research has not only identified a range of DSF-family signals but also unveiled unique QS signaling pathways and mechanisms distinct from other known QS systems. This review will discuss these interesting features with focus on the conservation and diversity of various DSF-dependent QS systems in Gram-negative bacteria. For the convenience of discussion, DSF will be used as a collective term for the DSF-family signals unless otherwise specified.

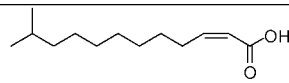
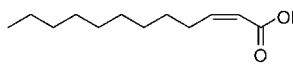
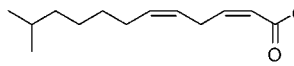
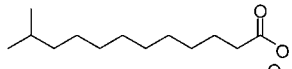
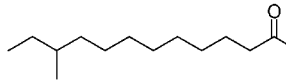
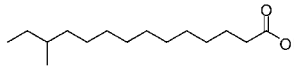
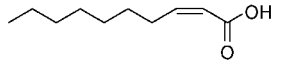
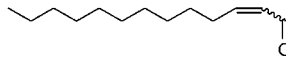
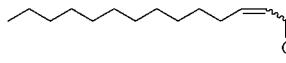
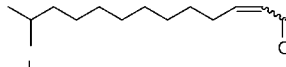
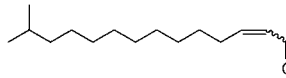
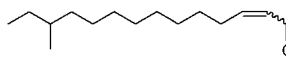
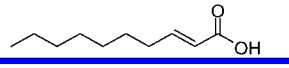
2. Chemistry of DSF-Family Signals

2.1. Detection of DSF-Family Signals

Detection of DSF activity was first reported a decade ago by M. J. Daniels' group when analyzing an *Xcc* gene cluster designated *rpf* (regulation of pathogenicity factors). It was found that the protease and endoglucanase activity of an *rpfF* mutant could be restored when cultivated in proximity to its wild-type parental strain.[23] This result led to speculation that wild-type *Xcc* might produce a diffusible signal factor (DSF) which induced protease and endoglucanase production and that the enzyme encoded by *rpfF* was associated with DSF biosynthesis. The protease-based bioassay of DSF activity uses an agar plate containing skimmed milk, in which DSF-induced production of extracellular proteases by *Xcc* strains is indicated by a clear zone around the bacterial colony. For detection of endoglucanase activity, an agar plate supplemented with carboxymethylcellulose (CMC) is used. The zone of CMC hydrolysis by endoglucanase becomes visible after developing with 0.1% Congo red followed by distaining with 1 M NaCl.[23]

Second-generation DSF-activity detection methods are based on engineered DSF biosensors. Typically, the promoter of a DSF-inducible operon *engXCA* is fused with a promoterless reporter *gusA* gene that encodes β-glucuronidase (GUS); then the P*engXCA-gusA* cassette is cloned in a broad-host-range vector, and the resultant plasmid is mobilized to a DSF-minus mutant of *Xcc* to generate the DSF biosensor strains.[24,26] The DSF activity is indicated by a blue halo around the test colony or DSF extract in an agar plate containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Table 1. List of DSF-Family Signals

Name	Structure	Original strain	References	Note
11-Me-C_{12}:Δ^2 (DSF)		*X. campestris, S. maltophilia, B. multivorans, X. oryzae pv. oryzae*	24,28,29,36	
C_{12}:Δ^2 (BDSF)		*B. cenocepacia, B. lata, B. multivorans, B. stabilis, B. vietnamiensis, B. dolosa, B. ambifaria, B. stabilis, B. anthina, B. pyrrocinia, X. oryzae pv. oryzae*	27-29	
11-Me-C_{12}:$\Delta^{2,5}$ (CDSF)		*B. multivorans, B. stabilis, B. anthina, B. pyrrocinia, X. oryzae pv. oryzae*	28,29	
11-Me-C_{12}		*S. maltophilia*	36	
10-Me-C_{12}		*S. maltophilia*	36	*a*
12-Me-C_{14}		*X. fastidosa*	32	*a*
C_{10}:Δ^2		*P. aeruginosa*	37	
C_{13}:Δ^2		*S. maltophilia*	36	*a,b*
C_{14}:Δ^2		*S. maltophilia*	36	*a,b*
12-Me- C_{13}:Δ^2		*S. maltophilia*	36	*a,b*
13-Me-C_{14}:Δ^2		*S. maltophilia*	36	*a,b*
12-Me-C_{13}:Δ^2		*S. maltophilia*	36	*a,b*
C_{19}:Δ^{2t} (SDSF)		*Streptococcus mutans*	40	

[a] These DSF signals have not been verified for their functions with the purified or synthetic molecules. [b] The configuration was not determined.

(X-Gluc) and DSF biosensor bacterial cells. As DSF is diffusible in agar plate, a dose−response plot of biosensor strain to DSF can be established, permitting semiquantitative determination of DSF activity.[24]

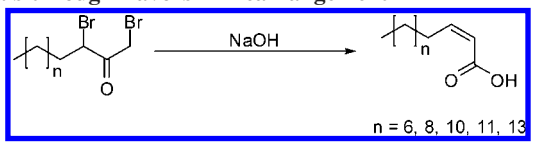
These engineered biosensor strains have been widely used in detection of DSF-family signals in other bacterial species, including the DSF-family signals produced by members of the *Burkholderia cepacia* complex and *Xanthomonas oryzae* pv *oryzae*.[27−29] The DSF-family signals from *Xylella fastidiosa* and *Xanthomonas axonopodis* pv *citri* were unveiled by their ability to restore the endoglucanase activity of *Xcc rpfF* deletion mutant.[30−32] In addition, another DSF biosensor, which was constructed using the promoter of DSF-inducible *engXCA* and a promoterless *gfp* gene,[33,34] has been used to detect the DSF-family signals from *Xy. fastidiosa* and *Xanthomonas axonopodis* pv *glycines*.[33−35]

2.2. Purification and Structural Characterization of DSF-Family Signals

Crude DSF extracts were initially prepared by extraction of culture supernatants of *Xcc* using 0.3 volumes of sodium bicarbonate-equilibrated ethyl acetate.[23] The extraction protocol was later optimized to improve DSF yield by using an equal volume of ethyl acetate.[24] Pure DSF was obtained by further purification with flash column chromatography and high-performance liquid chromatography (HPLC).[24] The chemical structure of DSF was determined using mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses.[24] The data from high-resolution electrospray ionization mass spectrometry (ESI-MS) suggest a molecular formula of $C_{13}H_{23}O_2$. NMR spectrometry analysis indicates that there are two terminal CH_3 groups at C-12 and C-13 sites and seven CH_2 groups from C-4 to C-10. Additionally, there is a double bond with a *cis*-configuration at C-2 to C-3. In combination, these data indicate that DSF is *cis*-11-methyl-2-dodecenoic acid, a novel α,β-unsaturated fatty acid (Table 1), which has been further verified using a synthetic DSF molecule.[24]

The findings in *Xcc* facilitate the purification and characterization of DSF-family signals in other bacterial species. By using ethyl acetate extraction, solid-phase extraction with an Oasis HLB cartridge, and HPLC, DSF and seven structural derivatives were identified by ESI-MS and gas chromatography−mass spectrometry (GC-MS) analysis from the culture supernatants of *Stenotrophomonas maltophilia*[36] (Table 1). Among them, the biological activities of *cis*-Δ2-11-methyldodecenoic and 11-methyldodecanoic acids have been verified using synthetic compounds.[36] In another study, crude ethyl acetate extract from the culture supernatants of *Xy. fastidiosa* was derivatized for GC-MS analysis. By using standard fatty acid methyl esters mixture as controls, GC-MS analysis led to identification of a DSF analogue 12-methyltetradecanoic acid[32] (Table 1), but its biological activity and role in *Xy. fastidiosa* cell−cell communication have not yet been established. In addition to ethyl acetate, chloroform has also been used for extraction of DSF-family

Scheme 1. Synthesis of the *cis*-Configuration DSF-Family Signals through Favorskii Rearrangement



signals from bacterial culture supernatants. A DSF analogue, i.e., *cis*-2-decenoic acid, was purified from *Pseudomonas aeruginosa* culture supernatants using chloroform extraction and subsequent HPLC fractionation[37] (Table 1). Considering the fact that fatty acids exist as anions in weak basic bacterial culture supernatants and that these anions are less soluble in organic solvent than corresponding free acids, a recent study has revised the extraction protocol by using acidified supernatants for solvent extraction.[28] The results showed that adjusting the pH value of supernatants to ~4.0 substantially improved the extraction efficiency of DSF-family signals.[27,28]

2.3. Synthesis of DSF-Family Signals

Biosynthesis of DSF signal in *Xcc* is dependent on *rpfF* and *rpfB*, which are located next to each other in the *rpf* gene cluster. These two genes encode a putative enoyl-CoA hydratase and a putative long-chain fatty acyl CoA ligase, respectively.[23,26] The *rpf* gene cluster has subsequently been identified in other bacteria such as *S. maltophilia*, *X. axonopodis* pv *citri*, *X. oryzae* pv *oryzae*, and *Xy. fastidiosa*.[25,29,30,33,34,38,39] The mechanism of DSF-family signal production appears to be widely conserved. In addition to RpfF in *Xcc*, the role of RpfF homologues in synthesis of DSF-family signals has also been established in other bacterial species, such as *B. cenocepacia*,[27] *X. oryzae* pv *oryzae*,[29] *Xy. fastidiosa*,[33,34] *S. maltophilia*,[36] and *X. axonopodis* pv *glycines*.[35] The role of RpfB is relatively less well characterized except for in *Xcc* and *S. maltophilia*, in which mutation of *rpfB* seemed to abrogate the production of DSF-family signals.[23,36] The putative catalytic mechanisms of these two enzymes are discussed in section 3.1.

Chemically, DSF and its *cis*-analogues have been synthesized by Favorskii rearrangement.[24] Briefly, ketones prepared with relevant fatty acid substrates are used to generate the intermediates 1,3-dibromo-2-ones, which are then used to produce *cis*-2-enoic carbolic acids (as shown in Scheme 1).

The *trans*-derivatives of DSF have been synthesized through preparation of 2-bromo fatty acids followed by elimination of hydrobromic acids using a strong base such as potassium *tert*-butoxide.[24] Synthetic DSF-family signals have also been used in several recent studies as standard controls,[32,36,37] but the information regarding their synthesis has not been described.

2.4. Nomenclature

Like the AHL-type QS signals, the list of DSF-family signals is expanding rapidly. *cis*-11-Methyl-2-dodecenoic acid was identified from *Xcc* as the first member of DSF-family signals in 2004 and is known as DSF.[24] For convenience, *cis*-2-dodecenoic acid and *trans*-2-decenoic acid, the DSF structural analogues identified from *B. cenocepacia* and *Streptococcus mutans*, were named BDSF and SDSF, respectively.[27,40] However, the shortcoming of this nomenclature system soon becomes obvious as a range of DSF-like signals have been identified from different bacterial species[28,29,37] (Table 1), and especially since one bacterial species may produce more than one DSF-family signal[28,29,36] (Table 1). A new nomenclature system has recently been proposed in an attempt to cover all the possible DSF-family signals.[28] For example, *cis*-11-methyl-2-dodecenoic acid (DSF) is designated as 11-Me-C_{12}:Δ^2 and *cis*-2-dodecenoic acid (BDSF) is designated as C_{12}:Δ^2 (Table 1). This nomenclature is based on one of the fatty acid nomenclature systems where substitution and its position are indicated first (for example, 11-Me indicates a methyl group on C-11 of the fatty acid carbon chain), followed by the length of the fatty acid carbon chain (C_{12} represents a 12-carbon fatty acid chain), and then the position of the double bond in the fatty acid chain (Δ^2 indicates a double bond in *cis*-configuration at site 2, i.e., between C-2 and C-3 of the fatty acid carbon chain). Similarly, DSF molecules with *trans*-configuration can also be named following the same rules by inserting a "t" next to the position of the double bond. For example, Δ^2 means a double bond at site 2 with a *cis*-configuration, while Δ^{2t} suggests a double bond at site 2 with a *trans*-configuration. Following this nomenclature system, all the reported DSF-family molecules have been renamed and listed in Table 1. It should be noted that the biological activities of some DSF derivatives have not yet been verified (Table 1). At this stage, it is premature to regard them as functional QS signals. In addition, the naming of several molecules from *S. maltophilia* is not complete due to a lack of information about the double-bond configuration (Table 1); whether these double bonds are in *trans*- or *cis*-configuration requires further characterization.

2.5. Structural Features and Biological Activity

DSF-family signals share a fatty acid carbon chain with variations in chain length, double-bond configuration, and side-chain (Table 1). Evidence is emerging that these structural features may contribute to their biological activity and signaling specificity in intraspecies signaling and interspecies communication.

One of the key features that could affect intraspecies signaling activity is the presence or absence of a double bond and its configuration. By synthesizing a range of structural derivatives of DSF, Wang et al analyzed the structural features of fatty acids that are important for QS-dependent regulation of virulence in *Xcc*.[24] DSF (11-Me-C_{12}:Δ^2), which contains a *cis*-double bond in α,β-position, showed about 200- and 20 000-fold stronger activity than its *trans*-isomer and saturated isomer, respectively, in the induction of virulence gene expression. These data have demonstrated that the α,β-double bond and *cis*-configuration of fatty acid are critical for QS signaling activity in *Xcc*. However, these findings in *Xcc* may not translate to other bacterial species. DSF and C_{12}:Δ^2 showed similar activities in the induction of xylanase activity and exopolysaccharide production when assayed using the *rpfF* mutant of *X. oryzae* pv *oryzae*, indicating that methyl substitution at C11 has no obvious effect on the biological activity of DSF signals in this bacterial pathogen.[29] Similarly, *cis*-Δ2-11-methyldodecenoic acid (11-Me-C_{12}:Δ^2) and its saturated derivative 11-methyldodecanoic acid (11-Me-C_{12}) appeared to be equally effective in the stimulation of flagella-independent translocation by *S. maltophilia*,[36] suggesting that the double bond at α,β-position does not affect this biological activity in *S. maltophilia*.

Variations in methyl group substitution and chain length can also substantially affect the biological activity of DSF-family signals. DSF, which differs from C_{12}:Δ^2 by containing a methyl group substitution at C11, showed ~120-fold higher potency in induction of virulence gene expression in *Xcc*.[24] The same study also showed that increasing one carbon or decreasing two carbons in the fatty acid chain could compromise signaling activity by 2- to 5-fold. In addition, comparison of DSF with 11-Me-C_{12}:$\Delta^{2,5}$ showed that one more double bond causes ~20% reduction of signaling activity in the induction of *X. oryzae* pv *oryzae* EPS and xylanase production.[29]

The structural features of DSF-family signals also affect their biological activity in interspecies and cross-kingdom communications. It has been reported that among DSF, C_{12}:Δ^2, and 11-Me-C_{12}:$\Delta^{2,5}$, C_{12}:Δ^2 is the strongest inhibitor of germ tube formation by *Candida albicans*, followed by 11-Me-C_{12}:$\Delta^{2,5}$ and DSF. The findings suggest that the methyl group at C11-site of DSF reduces the inhibitory activity on *C. albicans* germ tube formation, but an additional double bond at C5−C6 of 11-Me-C_{12}:$\Delta^{2,5}$ has the opposite effect.[27,28] Similar variations have also been found in bacterial interspecies communications. A recent study showed that C_{12}:Δ^2 was ~10% less active than DSF and 11-Me-C_{12}:$\Delta^{2,5}$ in the induction of *Xcc* EPS production, whereas in *B. cenocepacia* it is DSF that was inferior to the other two signals in the induction of virulence gene expression.[28] Taken together, the above findings indicate that the biological activity of DSF-family signals depends on not only their structural features but also the bacterial species on which they act.

3. DSF Signaling Mechanisms in Xanthomonas campestris pv campestris

X. campestris pv *campestris* (*Xcc*) is the causal agent of black rot of cruciferous plants, which is possibly the most important disease of crucifers worldwide.[41] It belongs to the γ-subdivision of proteobacteria and is a bacterial pathogen with significant economic importance.[41] As a model organism of a new QS system, *Xcc* has been well characterized using an array of approaches, including genetic, genomic, biochemical, and cell biology. The research progress over the past decade has unveiled a range of enzymes and regulators that are of critical importance in DSF-dependent QS signaling pathways. The roles and molecular mechanisms of the key components in DSF signaling system will be discussed separately in this section.

3.1. Enzymes Associated with DSF Biosynthesis

An early transposon mutagenesis analysis of the genes involved in the synthesis of extracellular enzymes led to identification of a gene cluster designated as *rpf* in *Xcc*.[42] On the basis of the findings that decreased production of extracellular enzymes of *rpfF* mutant could be restored by a diffusible extracellular factor (DSF) produced by various *Xcc* strains, except for the *rpfF* and *rpfB* mutants, it was therefore speculated that the enzymes encoded by *rpfF* and *rpfB* were involved in DSF biosynthesis.[23] Null mutation of *rpfF* or *rpfB* abolished DSF production, suggesting that they are essential for DSF production in *Xcc*.[23,26] In silico analysis predicted that *rpfF* and *rpfB* encode a putative enoyl-CoA hydratase and a putative long-chain fatty acyl CoA ligase, respectively.[23,26] However, their catalytic mechanisms, corresponding substrates, and reaction products require further investigation. A recent crystal structural analysis of RpfF has shown that it is structurally similar to the members of the crotonase superfamily containing an N-terminal α/β-spiral core domain and a C-terminal α-helical region.[43] Sequence alignment and structural analysis resulted in the identification of two putative catalytic glutamate residues (Glu141 and Glu161), which are conserved in the enoyl-CoA hydratases/dehydratases. Substitution of these two residues in RpfF completely abolished DSF production, underscoring their critical role in DSF biosynthesis.[43] In addition, structural analysis of RpfF also unveiled a hydrophobic pocket composed of several hydrophobic residues, including Leu136 from βB3, Gly137 and Gly138 from βB3-α3 loop, Gly85 from βB2-αB2 loop, Leu276 from α10, Met170 from βB4-αB4 loop, and Trp258 from α9.[43] The structural features and residue properties of this hydrophobic pocket suggest that it is likely the putative substrate-binding pocket for DSF biosynthesis.[43] Consistent with this speculation, a single-point mutation in any of these residues resulted in either no DSF production or dramatically reduced enzymatic activity of RpfF in DSF biosynthesis.[43]

Microarray analysis of the *rpfF* mutant in the presence and absence of DSF led to the identification of >165 genes that are regulated by the DSF-based QS system.[44] Significantly, within the DSF-regulon, 133 genes are positively regulated and 32 are negatively modulated by DSF, which is consistent with the regulation pattern for other QS systems.

3.2. Dual Roles of the DSF Sensor Kinase RpfC

Within the *rpf* gene cluster, the *rpfC* gene encodes a two-component-type hybrid, histidine sensor kinase consisting of multidomains, i.e., five transmembrane (TM) domains, a histidine kinase (HK) domain, a receiver (REC) domain, and a histidine phosphotransfer (HPT) domain. The findings that the *rpfC* and *rpfF* mutants displayed similar phenotypes such as decreased production of exopolysaccharides (EPS) and extracellular enzymes,[26] and that the *rpfF* mutant phenotype could be fully restored by exogenous addition of DSF but the *rpfC* mutant failed to respond to DSF, argued strongly that RpfC is a DSF sensor.[26,45] Further genetic analysis showed that substitution of the conserved residues associated with two-component-type phosphorelays, i.e., His198 in the HK domain, Asp512 in the REC domain, and His657 in the HPT domain of RpfC, abolished the DSF-dependent functions such as virulence factor production and biofilm dispersal.[44,45] These results have demonstrated that RpfC uses a conserved phosphorelay mechanism to transduce the DSF signal when modulating virulence factor production (Figure 1). However, the mechanism by which RpfC detects DSF and triggers the phosphorelay process remains undetermined.

Unusually, apart from functioning as a DSF sensor to positively regulate virulence factor production, RpfC also acts negatively to regulate the biosynthesis of DSF.[26] DSF biosensor-based diffusion plate assays revealed that the *rpfC* mutant produced a larger DSF diffusion zone than its parental wild-type strain.[26] Subsequent quantification analysis showed that mutation in *rpfC* caused ~16-fold increase in DSF production at the peak time.[24] Detailed genetic and biochemical analyses have demonstrated that RpfC regulation of DSF production is independent of the conserved phosphorelay mechanism, and presented several lines of evidence indicating that RpfC negatively controls DSF production via a novel post-translational mechanism involving domain-

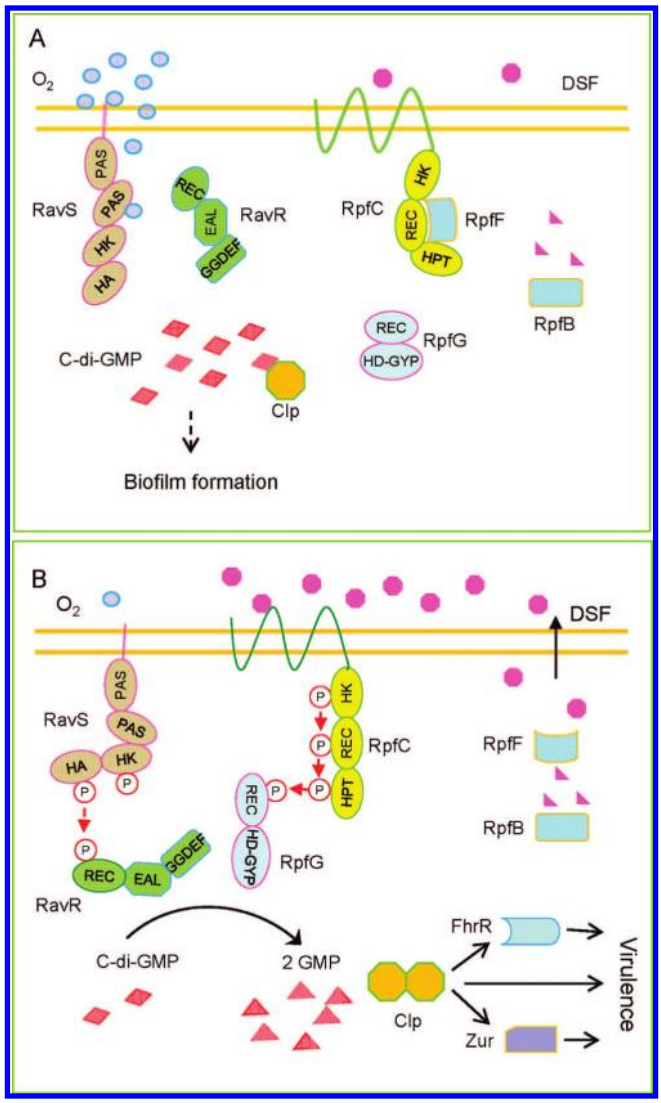


Figure 1. Schematic representation of the QS signaling network in *Xcc*. (A) At low cell density and under normal oxygen level conditions, the DSF sensor RpfC forms a complex with the DSF synthase RpfF through its receiver domain, which limits DSF biosynthesis at a basal level. Similarly, interaction between oxygen molecules with RavS keeps the RavS/RavR two-component system at an inert state. High intracellular levels of c-di-GMP promotes biofilm formation and inhibits the Clp transcription factor activity by formation of a protein−ligand complex. (B) At high cell density, RpfC undergoes autophosphorylation upon sensing accumulated extracellular DSF signals. Through the conserved phosphorelay mechanism, RpfG is phosphorylated, and that leads to activation of its c-di-GMP phosphodiesterase activity. Degradation of c-di-GMP releases Clp, which regulates subsets of virulence genes directly or through downstream transcription factors FhrR and Zur, respectively. In addition, perception of low-oxygen tension by the sensor RavS activates the c-di-GMP phosphodiesterase activity of RavR, which degrades c-di-GMP and results in enhanced virulence gene expression through Clp. The solid arrow indicates the signal tranduction or regulation pathway. The dashed arrow indicates an unknown mechanism.

specific protein−protein interactions.[45] First, the deletion variants of RpfC lacking the REC domain were unable to repress DSF biosynthesis. Second, coimmunoprecipitation analysis revealed that RpfF, the DSF synthase, interacted with the REC domain of RpfC under in vivo conditions. Third, purified REC domain bound specifically to RpfF with high affinity as seen through far Western blot analysis. Furthermore, overexpression of the REC domain abolished DSF production in *Xcc*. These findings suggest a novel post-translational autoinduction mechanism of DSF biosynthesis (Figure 1), totally unlike the transcriptional autoinduction mechanism of AHL-type QS systems.[2,4] According to this model, at a lower cell density, the unphosphorylated RpfC maintains a conformation that facilitates formation of a complex between RpfF and RpfC, limiting DSF production at a basal level (Figure 1A). At high cell density, the accumulated DSF molecules trigger the autophosphorylation of RpfC and release of RpfF, resulting in increased DSF signal production (Figure 1B).

The above model is strongly supported by crystal structural analysis of the RpfF monomer and RpfF−REC complex,[43] which showed that interaction between RpfF and REC involved both hydrophilic and hydrophobic contacts. When RpfF is bound to the REC domain, the entrance of the substrate binding pocket is blocked, locking it into an inactive form and thereby negatively regulating DSF biosynthesis. Although these findings provide a structural model for the RpfC−RpfF interaction-mediated QS autoinduction mechanism, the mechanisms by which RpfC detects DSF and consequently releases RpfF remain unclear.

3.3. Response Regulator RpfG is a Novel DSF-Modulated c-di-GMP Degradation Enzyme

Several lines of evidence indicate that RpfC and RpfG constitute a two-component regulatory system responsible for the detection and transduction of the QS signal DSF. In *Xcc* genome, *rpfG*, *rpfH*, and *rpfC* are transcribed as an operon, suggesting that these three genes might be functionally related.[26] In particular, RpfC is a sensor kinase, as discussed above, and RpfG is a response regulator that contains a typical receiver domain and a HD-GYP domain. In addition, the *rpfG* and *rpfC* mutants display similar phenotypic changes, including decreased virulence factor production and, especially, insensitivity to DSF.[26,46] Moreover, *in trans* expression of the RpfC−RpfG two-component system in *P. aeruginosa* enabled the pathogen to respond to exogenous addition of DSF, consequently affecting bacterial motility.[47] The role of RpfG in DSF signal transduction has been further confirmed by site-directed mutagenesis and deletion analysis. Substitution of Asp80 in the receiver domain of RpfG, which is the conserved residue implicated in two-component-type phosphorelays, decreased *Xcc* virulence factor production and abolished the bacterial response to DSF signal.[48] Importantly, *in trans* expression of the HD-GYP domain of RpfG in the *rpfG* mutant was sufficient to restore virulence factor production,[47,48] suggesting that this domain plays a key role in DSF signal transduction.

In silico and biochemical analysis of the HD-GYP domain has revealed its novel function in c-di-GMP metabolism. c-di-GMP is a global second messenger implicated in the regulation of various biological functions in bacteria, including cellulose biosynthesis,[49] bacterial motility,[50] biofilm formation,[49,51] and virulence factor production.[47,52] The intracellular concentration of c-di-GMP was initially found to be modulated by the proteins containing GGDEF or EAL domains.[53] Typically, proteins containing a GGDEF domain are diguanylate cyclases, which catalyze the synthesis of c-di-GMP from two molecules of GTP,[53,54] whereas proteins containing the EAL domain are c-di-GMP phosphodiesterases that hydrolyze c-di-GMP to generate linear nucleotide pGpG.[55,56] HD-GYP was predicted by in silico analysis as a novel domain involved in signal transduction and nucleotide metabolism as a putative diguanylate phosphodi-

esterase.[57,58] It is a subgroup of the HD superfamily of metal-dependent phosphohydrolases containing an additional GYP motif. On the basis of the findings that the proteins consisting of the HD-GYP domain and a CheY-like two-component receiver (REC) domain are present in many bacterial species, it was predicted that this novel HD-GYP domain may have important roles in bacterial signaling and response to environmental cues.[57,58] Subsequent biochemical analysis confirmed that the HD-GYP domain degraded c-di-GMP to generate two molecules of GMP.[47] Point mutation of the conserved residues H or D of the HD-GYP domain abrogated the RpfG enzymatic activity against c-di-GMP and regulatory activity on virulence factor production,[47] whereas substitution of the GYP motif had no effect on the activity of RpfG to degrade c-di-GMP.[59] Consistent with the predicted role of HD-GYP domain in RpfG-dependent signal transduction, *in trans* expression of the HD-GYP domain alone in the *rpfG* mutant was sufficient to restore the mutant phenotypes including virulence factor production and biofilm dispersion.[47]

Taken together, these findings indicate that the HD-GYP of RpfG domain is a novel c-di-GMP degradation enzyme, which is activated by the DSF signaling system to perform its regulatory activity by enzymatic degradation of intracellular c-di-GMP (Figure 1B). Interestingly, a subsequent study found that RpfG controlled the expression of a subset of proteins with GGDEF or EAL domains, which possibly function to synthesize or degrade c-di-GMP.[60] To fully understand the RpfG signaling pathways and mechanisms, it will be essential to determine the biological roles of these GGDEF and EAL proteins and their target processes.

3.4. Clp is a Novel c-di-GMP Effector

The *clp* gene of *Xcc* was identified in the early 1990s through screening of *Xcc* genomic DNA library in complementation of the adenylate cyclase (*cya*)-deficient mutant of *E. coli*.[61] DNA sequencing analysis of the *Xcc* DNA fragment that partially restored the ability of the *cya* mutant of *E. coli* to catabolize carbohydrate did not find a *cya* homologue, but instead revealed an open reading frame (ORF) that encodes a homologue of the *E. coli* cyclic AMP (cAMP) receptor protein (CRP). The *Xcc* homologue was accordingly named as Clp for CAP-like protein.[61] Interestingly, however, null mutation of Clp in *Xcc* did not affect carbohydrate catabolism but decreased the biosynthesis of EPS, extracellular cellulase, and polygalacturonate lyase,[61] which are reminiscent of the phenotypes of the DSF-deficient mutants.[23,24,26] The role of Clp in DSF-based QS system was not established until genetic and genomic analyses showing that Clp regulated a set of genes within the DSF regulon and that *in trans* expression of *clp* restored the phenotypes of *rpf* mutants.[62] These findings provide confirmation that Clp is a key component of the DSF regulatory network in *Xcc* (Figure 1).

Similar to the Crp of *E. coli*, Clp contains a cNMP binding domain at N-terminal and a DNA-binding domain at C-terminal. This structural similarity prompted two studies investigating the effect of c-di-GMP on the functionality of Clp. The results showed that the Clp proteins from *Xcc* and *X. axonopodis* pv *citri* were allosterically inhibited by c-di-GMP.[63,64] In *Xcc*, Clp acts as a global regulator that positively regulates ~260 genes and negatively regulates ~39 genes.[62] Clp has the same DNA-binding specificity as Crp and recognizes the same conserved DNA-binding site (TGTGA-N6-TCACA).[65] In silico analysis of the promoters of Clp-dependent genes showed that >80 genes contains a putative Clp binding site,[62] including the previously characterized gene *engXCA*, which encodes an extracellular endoglucanase.[66] Isothermal titration calorimetry, far-UV circular dichroism, and electrophoretic mobility shift assay analyses found that c-di-GMP specifically bound to Clp with high affinity and induced allosteric conformational changes that abolished the interaction between Clp and the promoter of *engXCA* in *Xcc*.[63] The same study also showed that Clp did not interact with cAMP and other nucleotides. Site-directed mutagenesis and structural analyses showed that the cNMP binding domain and the region between cNMP and DNA-binding domains of Clp in *Xcc* contain several residues implicated in c-di-GMP binding. Substitution of these residues with alanine or serine resulted in decreased sensitivity to changes in the intracellular c-di-GMP level and attenuated bacterial virulence.[63,67] The results of structural analysis suggest that Clp adopts an intrinsically active conformation for DNA binding, and binding with c-di-GMP may influence the helix αD of Clp, affecting its DNA-binding activity.[67]

The identification of Clp as a novel effector of c-di-GMP in *Xcc* presents a new member to the list of known c-di-GMP effectors identified in other bacterial pathogens, including PilZ domain "adaptor" protein,[68] transcriptional regulators such as FleQ[69] and Vpst,[70] proteins with inactive GGDEF and/or EAL domains such as LapD[71] and FimX,[72] and proteins with binding sites similar to GGDEF domains such as PelD.[73] These findings have also shown for the first time that the widely conserved cNMP domain can be grouped into two categories, with one being cAMP receptors such as the Crp of *E. coli* and the other being c-di-GMP effectors represented by the Clp of *Xcc*.[63]

3.5. Networking the DSF Signaling Pathways in Virulence Regulation

The identification of the key components of the DSF-based QS system and advances in microarray and biochemical technologies have facilitated the understanding of DSF signaling pathways. Upon characterization of Clp as one of the key members in the DSF-based QS system, two Clp-modulated transcription factors, i.e., FhrR and Zur, have been identified through microarray analysis and genetic verification.[62] Genomic analysis and phenotype characterization have demonstrated that Clp regulated the genes encoding flagellar, hypersensitive reaction and pathogenicity system, and ribosomal proteins through a TetR-family transcription factor FhrR as well as the genes encoding iron uptake, multidrug resistance, and detoxification via a Fur-family transcription factor Zur.[62] Interestingly, a recent study showed that physical interaction between RpfG and two GGDEF-domain proteins, i.e., XC_0249 and XC_0420, regulated DSF-dependent bacterial cell motility but had no effect on other DSF-dependent functions such as extracellular enzyme production and biofilm formation.[59] Substitution of the conserved GYP motif in the HD-GYP domain of RpfG abolished this interaction, indicating that the GYP motif is essential for the interaction between RpfG and these GGDEF-domain proteins.[59] It is noteworthy that substitution of the HD motif within the HD-GYP domain of RpfG had no effect on RpfG-GGDEF interaction but affected the ability of RpfG to regulate motility and extracellular enzyme production.[59] Given that the HD motif, but not the GYP motif, is essential

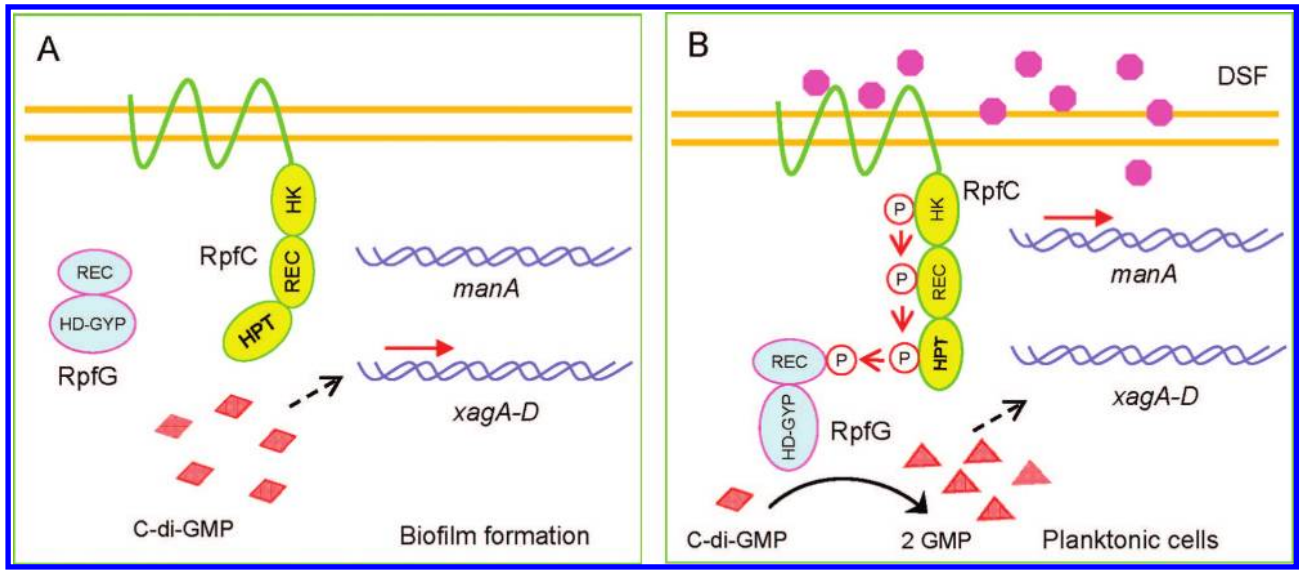


Figure 2. Schematic representation of DSF-dependent biofilm dispersal. (A) In the absence of DSF, in particular in the *rpf* mutants of *Xcc*, expression of the *xagABCD* gene cluster implicated in polysaccharide biosynthesis causes biofilm formation. (B) At high cell density, accumulation of DSF signals leads to increased expression of *manA* encoding biofilm degradation and suppression of the *xagABC* transcriptional expression. Both processes require the involvement of RpfC/RpfG two-component system. Consequently, *Xcc* grows in planktonic form.

for the enzymatic activity of RpfG,[47,59] the above findings seem to suggest that RpfG may modulate *Xcc* motility through two mechanisms, i.e., the HD motif-dependent c-di-GMP degradation and the GYP motif-dependent RpfG-GGDEF interaction. Consistent with the former putative mechanism, deletion of the c-di-GMP effector Clp or its downstream regulator FhrR resulted in decreased *Xcc* cell motility.[62] How the observed RpfG-GGDEF interaction influences bacterial motility remains unclear.

The above findings, together with the current understanding of the conserved molecular mechanisms of two-component regulatory systems,[74] have outlined the DSF-based QS networks and related regulatory mechanisms in *Xcc* (Figure 1). Similar to the QS systems in Gram-positive bacteria,[75] the DSF-dependent cell−cell communication system in *Xcc* employs a two-component system consisting of RpfC/RpfG in signal perception and transduction.[23,26,45] Upon detection of DSF signal when *Xcc* reaches a threshold population, autophosphorylation of RpfC triggers phosphorelay through its HK to REC to HPT domains, and subsequently to the REC domain of RpfG. Phosphorylation of RpfG is believed to induce protein conformational changes and activate its phosphodiesterase activity. Degradation of c-di-GMP molecules reduces the intracellular level of this second messenger, resulting in increased intracellular level of free Clp, which regulates the target genes expression directly or indirectly through its downstream transcription factors including Zur and FhrR[63] (Figure 1B). Additionally, RpfG could also regulate a subset of functions through interaction with certain GGDEF proteins.[59]

3.6. DSF-Dependent Biofilm Dispersal

Apart from regulation of virulence, another important biological function modulated by the DSF-based QS system in *Xcc* is biofilm dispersal. Mutation of *rpfF* in both *Xcc* strains 8004 and XC1 caused the formation of bacterial cell aggregates, and addition of DSF suppressed cell aggregation,[44,46] suggesting a critical role of DSF signal in modulation of *Xcc* switching between planktonic and biofilm forms. An early study found that a DSF-inducible enzyme, endo-β-1,4-mannanase (ManA), was able to disperse *Xcc* biofilms.[46] Interestingly, a subsequent study showed that addition of DSF in culture medium enabled the QS- and ManA-deficient double-deletion mutant ΔrpfFΔmanA to grow in planktonic form.[44] These findings suggest that DSF could modulate another mechanism(s) associated with biofilm development in addition to upregulating *manA* expression. This intriguing mechanism has been unveiled by a recent investigation, which showed that DSF inhibited biofilm formation by reducing the biosynthesis of extracellular matrix through a novel gene locus *xagABC*.[76] Within the locus, *xagA* encodes a hypothetical protein with a 19 aa signal peptide, *xagB* encodes a GSPII_E_N (general secretory system II protein E) domain at the N-terminus followed by a glycosyl transferase family 2 domain surrounded by two transmembrane regions on each side, and *xagC* encodes a peptide with nine transmembrane regions. The XagABC systems are conserved in other bacterial species, including *X. campestris* pv *vesicatoria*, *X. axonopodis* pv *citri*, *X. oryzae* pv *oryzicola*, and *Gluconacetobacter diazotrophicus*, with 31−70% amino acid identity.[76] Like the well-studied PgaABCD system of *E. coli*[77] and HmsHFRS of *Yersinia pestis*,[78] XagABC also contains a putative glycosyl transferase (XagB), which shares the conserved catalytic residues of glycosyl transferases and plays a role in EPS biosynthesis. Mutation of the *xag* genes resulted in decreased extracellular polysaccharide production, reduced *Xcc* biofilm formation, and attenuated bacterial resistance to oxidative stress.[76] The biofilms formed by *in trans* expression of *xagABC* in *Xcc* wild-type were dispersed by ManA, suggesting ManA and the XagABC system are two key mechanisms associated with *Xcc* biofilm formation and dispersion, respectively (Figure 2). Genetic analysis revealed that the transcriptional expression of *xagABC* was under stringent negative regulation by DSF through the RpfC/RpfG two-component system[76] (Figure 2). In contrast to XagABC, the production of ManA is positively regulated by the Rpf/DSF system and Clp.[46,76,79] These findings suggest that *xagABC* and *manA* are differentially regulated by DSF during the switch between biofilm and planktonic lifestyles, but the regulatory mechanisms remain largely undiscovered.

4. DSF-Based QS Mechanisms in Other Organisms

4.1. *Xylella fastidiosa*

Xy. fastidiosa is an important plant pathogen that causes disease in many important economic crops and plants, such as citrus, grape, coffee, peach, and other important horticultural species.[80−82] The pathogen belongs to γ-proteobacteria, is restricted to the xylem vessels of plants, and is transmitted by xylem sap feeding insects such as the sharpshooter species.[83,84] The cell−cell signaling in *Xy. fastidiosa* is mediated by an analogue of DSF, tentatively identified as 12-methyl-tetradecanoic acid (12-Me-C_{14}) through GC-MS analysis[32] (Table 1). Although the chemical structure of this putative signal and its role in bacterial physiology awaits further verification and investigation, there is no doubt that *Xy. fastidiosa* utilizes a DSF-based QS system in the regulation of virulence. In silico analysis showed that the pathogen contains a conserved *rpfGCF* gene locus, and their peptide products share >60% identity to their corresponding counterparts from *Xcc*.[25] Importantly, mutation of *rpfF* in *Xy. fastidiosa* abolished DSF signal production and gave rise to enhanced virulence, impaired insect transmission, and disabled biofilm formation in host insects.[33] In contrast, the *rpfC* mutants overproduced DSF; were deficient in virulence, migration along xylem vessels, and insect transmission; and showed a hyperattachment phenotype.[34] It was speculated that these divergent expression patterns of the genes encoding biofilm formation and virulence might indicate two possible distinct pathways for DSF perception in *Xy. fastidiosa*.[34] In one pathway, RpfC, which is likely membrane-bound, acts as the sensor and DSF perception leads to activation of the genes involved in biofilm formation through RpfG. DSF perception also influences the interactions among RpfC with RpfF and a putative intracellular repressor. In the absence of RpfF, the putative repressor is sequestered by excessive RpfC, leading to expression of the downstream virulence genes, such as *tolC*, *pglA*, and *PD0279*, which are required for colonization and movement of *Xy. fastidiosa* in host plant.[34] In another pathway, in the absence of RpfC, DSF is overproduced and the excessive signals may be sensed by a low-affinity intracellular DSF sensor, leading to expression of the genes required for attachment and biofilm formation.[34] The response regulator RpfG was also proposed to be involved in this signaling pathway. Genetic analysis showed that RpfC regulated the expression of RpfG, RpfB, and a GGDEF domain protein that may be involved in intracellular signaling by modulating the levels of c-di-GMP.[34] Much work is needed to elucidate the seemingly complex DSF-based QS signaling pathways and regulatory mechanisms in this important bacterial pathogen.

4.2. *Xanthomonas axonopodis* pv *citri*

X. axonopodis pv *citri* causes bacterial citrus canker, a serious disease affecting most citrus species.[85] It belongs to γ-proteobacteria and shares >80% similarity with *Xcc* in genome sequence.[86] *X. axonopodis* pv *citri* genome has an *rpf* locus containing *rpfF*, *rpfC*, and *rpfG*,[86] which share ~54−96% amino acid identity with their homologues in *Xcc*.[25] Like *Xcc*, the RpfF is the key enzyme for DSF signal generation, as deletion of *rpfF* abolished DSF biosynthesis.[31] Interestingly, while the RpfC homologue of *X. axonopodis* pv *citri* shares only ~58% identical amino acids with the RpfC of *Xcc*, its putative cognate response regulator RpfG contains >96% identical amino acids with its counterpart of *Xcc*.[39] Additionally, it is worth noting that the RpfC/RpfG proteins of *X. axonopodis* pv *citri* carry identical domain architecture as the RpfC/RpfG of *Xcc*.[39]

As could be expected from their high levels of genome sequence similarity, the DSF signaling system of *X. axonopodis* pv *citri* appears to play similar roles as the DSF-based QS system of *Xcc*. Crude DSF extracts from *X. axonopodis* pv *citri* restored the phenotypes of the *Xcc rpfF* deletion mutant, and deletion of *rpfF* or *rpfC* attenuated the ability of *X. axonopodis* pv *citri* to cause canker symptoms in lemon leaves.[31] However, the putative DSF-like signal from *X. axonopodis* pv *citri* has not yet been purified and characterized. While information is still limited and fragmented, several lines of evidence indicate that *X. axonopodis* pv *citri* and *Xcc* may share very similar DSF signaling mechanisms. First, yeast two-hybrid and in vitro biochemical analysis of *X. axonopodis* pv *citri* proteins show that RpfC interacted with RpfF,[39] and deletion of *rpfC* caused DSF overproduction in *X. axonopodis* pv *citri*,[31] reminiscent of their counterparts in *Xcc*.[45] The findings suggest that DSF biosynthesis in *X. axonopodis* pv *citri* could also be regulated post-translationally through protein−protein interaction. Second, it was found that the DNA-binding activities of the Clp from *X. axonopodis* pv *citri* and that from *Xcc* were both inhibited by c-di-GMP at its physiological concentrations.[63,64] Interestingly, the yeast two-hybrid analysis of *X. axonopodis* pv *citri* proteins revealed that RpfC can also interact with RpfG, and the HD-GYP domain of RpfG could interact with a number of GGDEF domain-containing proteins.[39] Further investigation on the roles and mechanisms of these protein−protein interactions may provide new insight on the sophisticated DSF-dependent QS regulatory mechanisms.

4.3. *Xanthomonas oryzae* pv *oryzae*

X. oryzae pv *oryzae* is a member of γ-proteobacteria and the causal agent of rice bacterial blight disease, which is the most serious bacterial disease of rice worldwide.[87] The DSF signaling system in *X. oryzae* pv *oryzae* positively regulates virulence and shares the same key components of DSF biosynthesis and signaling system of *Xcc*.[29,88] Null mutation of the *rpfF* homologue of *X. oryzae* pv *oryzae* abolished the production of DSF-family molecules and reduced the bacterial virulence.[29,89] Interestingly, while the *rpfF* mutant of a *X. oryzae* pv *oryzae* Korea strain KACC10331 showed the decreased production of EPS and extracellular enzymes,[29] the same gene mutation in an India isolate BXO1 caused proficient production of these virulence factors.[89] This different regulation pattern seems to suggest a possible strain-specific variation in the nature of DSF regulation in *X. oryzae* pv *oryzae,* which awaits further investigation. Structural characterization revealed that *X. oryzae* pv *oryzae* produced three members of DSF-family signals, i.e., DSF, *cis*-2-dodecenoic acid (C_{12}:Δ^2), and *cis*,*cis*-11-methyldodeca-2,5-dienoic acid (11-Me-C_{12}:$\Delta^{2,5}$) (Table 1), with DSF being the major signal.[29] These three DSF-family signals were biologically active and could effectively restore the EPS production and extracellular xylanase activity in the *rpfF* deletion mutant of *X. oryzae* pv *oryzae*.[29] Apart from the regulation of virulence, DSF-based QS mechanisms are also implicated in regulation of other biological activities. The *rpfF* mutant of *X. oryzae* pv *oryzae* exhibited an unusual tetracycline susceptibility phenotype, overproduction of siderophores, and

growth deficiency under low iron conditions.[89] Intriguingly, while the *rpfC* gene of *X. oryzae* pv *oryzae* could complement the *rpfC* mutant of *Xcc*, mutation of *rpfC* in *X. oryzae* pv *oryzae* had no effect on production of extracellular enzymes.[88] The findings highlight functional variations of RpfC homologues in different bacterial species. Nevertheless, the role of RpfC in regulation of *X. oryzae* pv *oryzae* virulence is well conserved as its null mutants show reduced EPS biosynthesis and virulence.[88]

4.4. *Xanthomonas axonopodis* pv *glycines*

X. axonopodis pv *glycines* belongs to γ-proteobacteria and is the causal agent of bacterial pustule of soybean, a serious disease that results in reduced yield and deteriorated crop quality in several major soybean-producing countries.[90] Genomic sequencing analysis revealed that *X. axonopodis* pv *glycines* contains an *rpfF* homologue sharing >95% identity with the *rpfF* of *Xcc*.[35] Such a high level of sequence similarity suggests that it could encode for the biosynthesis of putative DSF-family signals. Mutation of *rpfF* in *X. axonopodis* pv *glycines* resulted in decreased production of extracellular polysaccharides and extracellular enzymes, and reduced virulence on soybean.[35] Consistent with the phenotype analysis, transcriptional expression of the genes encoding extracellular enzymes were downregulated in the *rpfF* mutant,[35] suggesting that the DSF signaling system plays a key role in regulation of exoenzyme production and virulence in *X. axonopodis* pv *glycines*.

4.5. *Stenotrophomonas maltophilia*

S. maltophilia belongs to γ-proteobacteria and is widespread in natural environments such as soil, water, and plants.[91] The organism has lately become widely known as an emerging opportunistic pathogen associated with nosocomial colonization and infection.[91−93] The *rpf* gene cluster is also well conserved in *S. maltophilia*, sharing ~62−82% similarity at the peptide sequence level with their counterparts in *Xcc*.[38] Interestingly, apart from DSF, seven other structural derivatives have also been identified in the supernatants of *S. maltophilia* with ESI-MS and GC-MS analysis[36] (Table 1). Two of them, 11-methyldodecanoic acid (11-Me-C_{12}) and *cis*-Δ2-11-methyldodecenoic acid (11-Me-C_{12}:Δ^2), were verified for their biological activity to promote flagella-independent translocation with the corresponding synthetic compounds.[36] Genetic analysis showed that the DSF signaling system in *S. maltophilia* plays an important role in regulation of various biological functions. Mutation of *rpfF* in *S. maltophilia* led to reduced motility, decreased production of extracellular proteases, decreased tolerance to a range of antibiotics and heavy metals, altered lipopolysaccharide (LPS) structure, formation of cell aggregates, and attenuated bacterial virulence on the model organism nematode.[38] In another study,[94] it was shown that inactivation of *rpfF* or *rpfB* in *S. maltophilia* strain WR-C decreased the transcription of *fecA*, which encodes a ferric citrate receptor that transports exogenous siderophore-ferric citrate from environment into the bacterial periplasm. Exogenous addition of synthetic DSF to the mutant ΔrpfF restored *fecA* expression, establishing a link between the DSF-based QS system and ferric citrate uptake in this bacterial pathogen.

Interestingly, in contrast to *Xcc*, where DSF positively modulates the transcriptional expression of the c-di-GMP effector Clp,[44,62,63] the cNMP binding domain protein encoded by *crp* from *S. maltophilia* acts to positively regulate the *rpfF* expression, possibly by binding the upstream regions of the *rpfF* promoter.[94] The Crp from *S. maltophilia* and Clp shares >86% identical amino acids, and not surprisingly, both proteins show similar ligand specificity. A recent study showed that, similar to the Clp of *Xcc*, the Crp from S. *maltophilia* did not interact with cAMP but bound strongly with c-di-GMP.[63] These indirect lines of evidence suggest the association of Crp and c-di-GMP with the DSF signaling pathways in *S. maltophilia*.

4.6. *Burkholderia cepacia* complex

B. cepacia complex is a group of closely related and phenotypically diverse bacterial organisms, which currently is composed of 17 formally named bacterial species.[95,96] Taxonomically, *B. cepacia* complex belongs to β-proteobacteria group. Strains of *B. cepacia* complex are ubiquitously distributed in nature, and some of them have emerged as problematic opportunistic pathogens in patients with cystic fibrosis or chronic granulomatous disease and immunocompromised individuals.[96−101] A DSF functional analogue designated BDSF from *B. cenocepacia* has recently been identified and characterized as *cis*-2-dodecenoic acid (C_{12}:Δ^2).[27] The chemical structure of C_{12}:Δ^2 is similar to that of DSF of *Xcc* but differs in methyl substitution at C11-site. Genetic analysis showed that the ORF *Bcam0581*, which encodes an enzyme sharing 37% identity at the peptide sequence level with the RpfF of *Xcc*, was essential for C_{12}:Δ^2 production.[27] *Bcam0581* could genetically substitute for the DSF synthase gene *rpfF* in *Xcc*.[27] However, in contrast to the *rpfF* of *Xcc*, which is located within the same locus as *rpfC* and *rpfG*, no *rpfC* or *rpfG* homologue is found in the vicinity of *Bcam0581*. It was suggested that the QS system in *B. cenocepacia* could have a different origin of evolution.[27]

While the genetic organization of the *rpf* genes differs, the biological functions of C_{12}:Δ^2 in *B. cenocepacia* are reminiscent of those regulated by DSF in the model organism *Xcc.* Deletion of *Bcam0581* caused retarded bacterial growth in minimal medium, reduced bacterial motility, and compromised biofilm attachment and virulence.[28,102−104] Genetic and biochemical analyses suggested that certain biological functions, including motility, biofilm formation, and virulence, were coregulated by a C_{12}:Δ^2 signaling system and the AHL-dependent QS system in *B. cenocepacia*[28,102−104] (Figure 3). More recently, a mutational screen identified Bcam0227 as a potential C_{12}:Δ^2 sensor, sharing 35.6% identity with RpfC of *Xcc*.[104] Bcam0227 contains four domains, including a histidine kinase phosphoacceptor domain, a histidine kinase domain, a CheY-like receiver domain, and a C-terminal histidine phosphotransfer domain.[104] Transcriptome profile analysis revealed that the sensor controls the expression of a subset of target genes belonging to the C_{12}:Δ^2 regulon.[104] Deletion of *Bcam0227* attenuated bacterial virulence,[104] suggesting that the C_{12}:Δ^2-based QS signaling system plays an important role in the physiology of *B. cenocepacia*.

In addition to *B. cenocepacia*, a subsequent study showed that other *B. cepacia* complex members also produced the QS signal C_{12}:Δ^2.[28] In this study, DSF signal production was determined in nine *B. cepacia* complex members. The results showed that like *B. cenocepacia*, four *B. cepacia* complex species, namely, *B. lata*, *B. vietnamiensis*, *B. dolosa*, and *B. ambifaria*, only produced C_{12}:Δ^2, whereas *B. multivorans*,

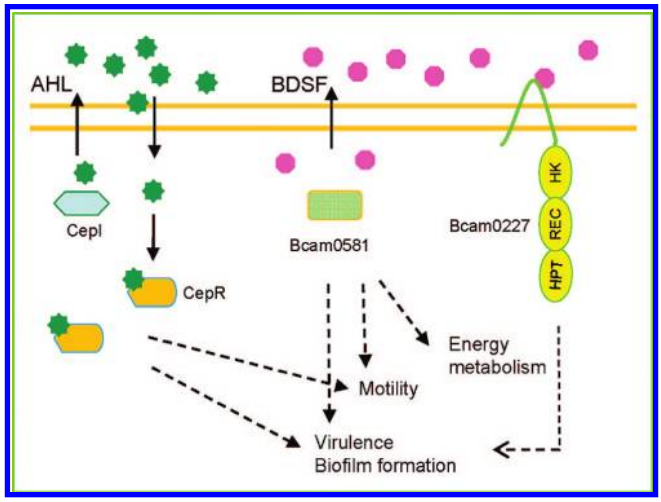


Figure 3. Schematic representation of the QS signaling networks in *B. cenocepacia*. The synthesis of BDSF signal requires the RpfF homologue Bcam0581. Apart from BDSF biosynthesis, Bcam0581 is also involved in energy metabolism. Bcam0227 is a sensor of BDSF and involved in regulation of biofilm attachment and virulence. In addition, BDSF signal positively regulates motility, which does not involve Bcam0227. The AHL-mediated QS system CepIR is also implicated in regulation of motility, biofilm formation, and virulence. Solid and dashed arrows indicate the signaling direction and unknown mechanism, respectively.

B. stabilis, *B. anthina*, and *B. pyrrocinia* also synthesized *cis*,*cis*-11-methyldodeca-2,5-dienoic acid (11-Me-C_{12}:$\Delta^{2,5}$) in addition to C_{12}:Δ^2. The unique exception was *B. multivorans*, which produced three DSF-family signals, i.e., DSF, C_{12}:Δ^2, and 11-Me-C_{12}:$\Delta^{2,5}$.[28] Although the role of DSF signaling systems in these *B. cepacia* complex species has not been characterized, DSF and 11-Me-C_{12}:$\Delta^{2,5}$ have been shown to be the functional analogues of C_{12}:Δ^2 in regulation of biofilm dispersal and virulence gene expression in *B. cenocepacia*. Intriguingly, while the C_{12}:Δ^2 synthase Bcam0581 is highly conserved (>94%),[28] a Blast search revealed a moderate similarity ~36% among the C_{12}:Δ^2 sensor Bcam0277 homologues from different *B. cepacia* complex members, This more variable pattern of conservation profiles of C_{12}:Δ^2 synthase and sensor in *B. cepacia* complex differs clearly from that of their well-conserved homologues in *Xanthomonas* species.[25]

4.7. *Pseudomonas aeruginosa*

P. aeruginosa belongs to γ-proteobacteria and is a major agent of opportunistic infections in immunocompromised individuals and cystic fibrosis patients.[105,106] It produces an organic compound that was recently identified as *cis*-2-decenoic acid (C_{10}:Δ^2)[37] (Table 1). This molecule is capable of inducing the dispersion of established biofilms and inhibiting biofilm development. Exogenous addition of this molecule at the nanomolar level to *P. aeruginosa* PAO1 biofilms effectively dispersed the biofilm microcolonies.[37] The role of C_{10}:Δ^2 in *P. aeruginosa* physiology and its corresponding signaling pathways deserves to be further characterized. In silico analysis does not reveal the presence of an *rpf* gene cluster or an obvious RpfF homologue in *P. aeruginosa*. The PAO1 genome encodes 12 enoyl-CoA hydratases sharing ~27% homology with the RpfF of *Xcc*, but it is unclear which one is the response for the biosynthesis of *cis*-2-decenoic acid.[37] Similarly, a number of putative two-component systems share moderate homologies to the RpfC/RpfG two-component system of *Xcc*, making the identification and characterization of the putative DSF signaling system in *P. aeruginosa* a tough challenge.

5. Interaction with Other Signaling Mechanisms

One of the interesting features of the DSF-based QS is that QS signal perception is coupled to intracellular regulatory networks through a second messenger c-di-GMP and its effector Clp.[25,63] Bacterial pathogens may produce many c-di-GMP metabolic enzymes. For example, the genome of *Xcc* encodes at least 37 proteins with GGDEF, EAL, or HD-GYP domains, which are known to be involved in c-di-GMP biosynthesis and degradation, respectively.[60] The annotated genome of *P. aeruginosa* strain PAO1 encodes 17 proteins containing a single GGDEF domain, 5 with a single EAL domain, and 16 that carry both domains.[107] These findings appear to indicate that these c-di-GMP metabolic enzymes, which are commonly fused to other domains implicated in signaling sensing and receiving,[25] may influence DSF-based QS by changing the intracellular level of c-di-GMP. Consistent with this notion, a recent study has revealed a novel two-component system, RavS/RavR, that is involved in coregulation of *Xcc* virulence with the DSF-based QS system[108] (Figure 1). Null mutation of either RavS or RavR resulted in decreased virulence factor production.[108] Genetic and biochemical analyses showed that the functionality of RavR was dependent on its EAL domain-associated c-di-GMP phosphodiesterase activity.[108] RavS is the sensor kinase of this two-component system with its PAS domain being implicated in sensing low-oxygen tension. Deletion of the PAS domain or substitution of the key residues associated with hypoxia sensing abrogated the sensor activity in regulation of virulence.[108] Similar to DSF-dependent signaling systems, RavS/RavR also positively regulates the transcriptional expression of the global regulator Clp.[108] These findings present an example of how QS and other environmental cue sensing systems could interact by influencing c-di-GMP and its cognate effector Clp in modulating bacterial physiology (Figure 1).

B. cenocepacia may have evolved another type of interaction between two different QS signaling systems. In addition to the BDSF-based QS system as discussed in section 4.7, an AHL-type QS system is also presented in Bcc species including *B. cenocepacia*.[109,110] Recent studies showed that some of the BDSF-regulated functions were also controlled by the AHL-dependent QS system.[102−104] Although the detailed mechanisms are not yet clear, the above findings suggest that the AHL- and BDSF-dependent QS systems regulate a similar set of virulence genes in parallel (Figure 3).

Adding to the diversity of interaction between signaling pathways, a physical interaction was found between the RpfC/RpfG two-component regulatory system and another two-component system consisting of NtrB/NtrC in *X. axonopodis* pv *citri*.[39] As discussed in section 4.2, RpfC and RpfG are the putative DSF sensor and cognate response regulator, respectively. The NtrB/NtrC system has been shown to regulate the σ^{54}-dependent promoters in some bacterial species,[111−113] putatively in response to a signal or environmental cue yet to be identified in *X. axonopodis* pv *citri*. Biochemical analysis showed that RpfC interacted with the HK domain of NtrB, whereas the HD-GYP domain of RpfG interacted with the σ^{54}-activating and DNA-binding domains of NtrC.[39] However, how these interactions influ-

ence the DSF signaling system in *X. axonopodis* pv *citri* is not yet clear.

6. Roles of DSF-Family Signals in Interspecies and Interkingdom Communication

It has been well established that DSF-family signals control a range of biological functions through intraspecies signaling. However, they may potentially play important roles in microbial ecology through interspecies and cross-kingdom communications.[24,27,28,114] Among different *Xanthomonads*, it is possible that interspecies signaling might occur frequently because these closely related bacterial pathogens produce common or similar DSF signals[24] (Table 1). DSF-dependent interspecies communication is not restricted to *Xanthomonads*. It was reported recently that the DSF signal produced by *S. maltophilia* influenced the biofilm formation and stress tolerance of *P. aeruginosa*.[114] Another study showed that the DSF-family signal C_{10}:Δ^2 produced by *P. aeruginosa* not only dispersed its own biofilm formation but also induced dispersion of the biofilms of *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Bacillus subtilis*, and *Staphylococcus aureus*.[37] In addition, BDSF from *B. cenocepacia* was found to be the functional homologue of DSF in the regulation of biofilm formation and virulence factor production by *Xcc*.[28] New evidence of this cross-species communication comes from the findings that a newly identified signal 11-Me-C_{12}:$\Delta^{2,5}$ from *B. multivorans* could functionally substitute DSF and C_{12}:Δ^2 to regulate the biofilm dispersal and virulence factor production in *Xcc* and *B. cenocepacia*, respectively.[28]

Besides involvement in interspecies cross-talk, DSF-family signals, including DSF, C_{12}:Δ^2, and 11-Me-C_{12}:Δ,^{2,5} have been shown to inhibit the morphological transition of *Candida albicans* through interkingdom interference.[24,27,28] Similarly, a fatty acid molecule *trans*-2-decenoic acid (C_{10}:Δ^{2t}, designated SDSF) from Gram-positive bacterium *S. mutans* is also a potent inhibitor of *C. albicans* morphological transition.[40] In addition, the signal C_{10}:Δ^2 from *P. aeruginosa* was found to be able to induce biofilm dispersion in *C. albicans*, as well as diverse bacteria.[37] *C. albicans* is an eukaryotic yeast and a causal agent of opportunistic oral and genital infections in humans. This fungal pathogen is a dimorphic organism and can grow as yeast- or hyphal-form depending on environmental conditions. This ability to switch between yeast and hyphae is an important aspect of its pathogenesis. DSF signals are structurally similar to farnesoic acid, which is an autoregulatory substance in *C. albicans*. Farnesoic acid inhibits *C. albicans* germ tube formation and plays a key role in the regulation of morphological transition in *C. albicans*.[115] These findings seem to suggest that DSF-family signals may use similar mechanisms as farnesoic acid in the modulation of *C. albicans* morphological transition.

7. Conclusions and Future Prospective

The DSF-based QS system, which was initially identified in a plant bacterial pathogen *Xcc*, has emerged as another widely conserved bacterial cell−cell communication mechanism in Gram-negative bacteria. The work on the DSF system in the model organism *Xcc* has unveiled several unique features that distinguish it from other known QS systems, including DSF autoinduction through RpfC/RpfF protein−protein interaction and QS coupling to bacterial intracellular regulatory network via a novel second messenger c-di-GMP and its effector Clp. Rapid research progress over the past few years has uncovered the presence of DSF-family signals in a variety of bacterial species belonging to γ-proteobacteria and β-proteobacteria groups, including both plant and human bacterial pathogens. Similar to the well-characterized AHL-based QS systems, DSF signaling systems are known to regulate a range of important biological functions such as biofilm formation, cell growth, and virulence factor production, which are critical for bacterial survival and pathogenesis. Identification of this widely conserved DSF-based signaling mechanism presents further evidence that argues strongly that QS is an indispensible community genetic regulation mechanism essential for bacterial physiology and virulence.

DSF-based QS systems can be grouped into two categories according to their genomic evolutionary origins. The first category, represented by *Xcc*, is distinguished by colocalization of the genes encoding key components of the QS system such as RpfF, RpfC, and RpfG. Other DSF-producing γ-proteobacteria species, except *P. aeruginosa*, also keep a similar genome organization to *Xcc* and share high levels of homology among corresponding *rpf* homologues. In contrast, the *rpfF* homologue *Bcam0581* and the *rpfC* homologue *Bcam0227* are located in different places within the genome of *B. cenocepacia*, which is a β-proteobacteria species. Significantly, while the Rpf proteins of *B. cenocepacia* do not share a high level of similarity to their counterparts in *Xcc*, the DSF-dependent biological functions, including virulence factor production and biofilm dispersion, are conserved in both γ-proteobacteria and β-proteobacteria.

The identification of DSF-family signals as a widely conserved mechanism in Gram-negative bacteria presents numerous challenges for further investigation. Recognition of the important roles of DSF-based QS systems in bacterial physiology might motivate further screening and characterization of putative DSF-family signals and corresponding signaling systems in other bacterial pathogens. It is intriguing to determine whether, and if so, how other key factors of the DSF-based QS system identified in *Xcc* and other γ-proteobacteria species, such as c-di-GMP and its cognate effector Clp, play roles in the DSF signaling pathways of β-proteobacteria species. Even in the model organism *Xcc*, although the framework of DSF signaling pathway has been well established, little is known how the sensor RpfC may perceive DSF signals and how the sensor−ligand interaction could induce the proposed conformational changes in RpfC. In addition, the DSF biosynthetic pathway and catalytic mechanisms remain to be a mystery. Furthermore, much work is needed to elucidate how DSF controls the transcriptional and translational expression of the XagABC system associated with biofilm formation. Moreover, given that *trans*-2-decenoic acid is produced by *S. mutans*,[40] it is intriguing to determine whether DSF-family signals play a role as QS signals in Gram-positive bacterial pathogens.

8. References

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