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**Title: The DSF Family of Quorum Sensing Signals: Diversity, Biosynthesis and Turnover**

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**Abstract**

The diffusible signaling factor (DSF)-based quorum sensing (QS) system has emerged as a widely conserved cell-cell communication mechanism in Gram-negative bacteria. Typically, signals from the DSF family are *cis*-2-unsaturated fatty acids which regulate diverse biological functions. Recently, substantial progress has been made on the characterization of new members of this family of signals. There have also been new developments in the understanding of the biosynthesis of these molecules where dual enzymatic activities of the DSF synthase and the use of various substrates have been described. The recent discovery of a naturally occurring DSF turnover mechanism and its regulation provides a new dimension in our understanding of how DSF-dependent microorganisms modulate virulence gene expression in response to changes in the surrounding environment.

**DSF-Dependent QS Signaling System in Diverse Gram-Negative Bacteria**

Bacterial cells are capable of sensing and responding to changes in their populations through communication using small signal molecules, a mechanism known as quorum sensing (QS). Over the past few decades, several groups of QS signals have been identified, paving the way for the dissection of signaling networks and significantly advancing our understanding on the remarkable ability of microorganisms to modulate a wide range of biological functions [1,2]. The diffusible signal factor (DSF) family represents an intriguing type of QS signal molecules found in diverse Gram-negative bacterial pathogens [3-5]. DSF type-based QS systems can be generally grouped into three categories according to their genomic context. The first category, represented by the crucifer pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*), typically shows colocalization of the genes encoding key signaling components such as RpfF, RpfC, and RpfG in the *rpf* gene cluster [3,4]. RpfF encodes a key enzyme required for DSF biosynthesis whereas RpfC and RpfG constitute a two-component system involved in signal perception and transduction [6, 7]. The activated HD-GYP domain of RpfG has phosphodiesterase activity and is able to degrade cyclic di-GMP (c-di-GMP), an inhibitory ligand of the global transcription factor Clp. Consequently, derepressed Clp drives the expression of several hundred of genes including those encoding virulence factor production [8-10]. This type of QS system has been functionally verified in *Xanthomonas* sp., *Xylella fastidiosa*, *Lysobacter enzymogenes*, and *Stenotrophomonas maltophilia* [3, 11]. The second category, represented by the opportunistic pathogens *Burkholderia cenocepacia* and *Cronobacter turicensis*, does not contain a typical *rpf* cluster, having only *rpfF* and a novel sensor gene *rpfR* in the same locus [12, 13]. Similarly the RpfF/RpfR system modulates intracellular c-di-GMP level in *B. cenocepacia*. The third category is represented by the opportunistic human pathogen *Pseudomonas aeruginosa*. In this organism the biosynthesis of the DSF type molecule *cis*-2-decenoic acid has been attributed to the putative enoyl-coenzyme A hydratase DspI although the mechanism of perception of this molecule remains to be elucidated [14, 15]. Recently, a cluster of five genes (PA4978 - PA4983) has also been proposed to be involved in *cis*-2-decenoic acid synthesis and perception in *P. aeruginosa* [16].

60

With the improvement of DSF detection methods, significant progress has been made in our understanding of the QS systems driven by the DSF family of signal. This includes the discovery of several new members of the DSF family of signals as well as the elucidation of some new DSF-dependent biological functions. Biochemical and genetic analyses have also unveiled the biosynthetic pathways and the various substrates for these signal molecules. Furthermore, a naturally occurring DSF turnover mechanism has recently been identified in *Xcc* and the rice bacterial blight pathogen, *X. oryzae* pv. *oryzae* (*Xoo*). Through this system, DSF signaling in the post-quorum growth phase can be effectively terminated. These findings together with previous research, have placed the DSF-type signaling system as one of the best-studied QS systems in bacteria. This review will provide an update on these new developments with the aim to build a more comprehensive picture of the QS systems driven by the DSF family of signals. More detailed background on the DSF family signals can be found in previous reviews [3-5, 17].

**Diversity of the DSF Signal Family and DSF-Regulated Biological Functions**

Previously, *cis*-11-methyl-dodecenoic acid (DSF), *cis*-2-dodecenoic acid (BDSF), and *cis,cis*-11-methyldodeca-2,5-dienoic acid (CDSF) were identified in cultures of *Xcc*, *Xoo* and the *B. cepacia* complex (Figure 1) [3,18,19]. Similarly, *cis*-2-decenoic acid and *trans*-2-decenoic acid (SDSF) were found to be produced by *P. aeruginosa* and *Streptococcus mutans* respectively (Figure 1) [14, 20]. Recently, three biologically active new members of the DSF family of signals, *cis*-10-methyl-2-dodecenoic acid (IDSF or DSF-II), *cis*-9-methyl-2-decenoic acid, and *cis*-2-undecenoic acid have been characterized in *Xcc* (Figure 1) [21, 22]. A variety of both saturated and unsaturated free fatty acids were identified in the cultures of the phytopathogen *X. fastidiosa*, with 2-*cis*-unsaturated fatty acids XfDSF1 (2-tetradecenoic acid) and XfDSF2 (2-*cis*-hexadecanoic acid) being biologically active (Figure 1) [23,24]. Furthermore, a DSF-like signal (LeDSF3) was characterized as 13-methyltetradecanoic acid in the biocontrol agent strain *Lysobacter enzymogenes* (Figure 1) [25]. Surprisingly, LeDSF3, unlike other members of the DSF family, does not contain the *cis* double bond, which has been shown to be essential for its

90    biological activity in *Xcc* [19]. Whether LeDSF3 is the true QS signal produced by *L.*

91    *enzymogenes* remains to be determined. These findings show a much broader spread of the

92    DSF family of signals amongst bacteria than initially anticipated.

93

94    RpfF-dependent signaling has been associated with the regulation of motility, biofilm

95    formation, iron uptake, EPS and extracellular enzyme production, and virulence [3].

96    Recent evidence indicates that the DSF signal family provides a fitness advantage to *Xcc*

97    during interspecies competition in mixed cultures. DSF type signals from *Xcc* interfered

98    with morphological transition and sporulation in *Bacillus thuringiensis* through modulation

99    of the expression of *ftsZ*, which encodes a key protein involved in bacteria cell division

100   [21].  DSF also elicited innate immunity in plants, an effect that was suppressed through

101   the secretion of xanthan, the main exopolysaccharide component in *Xcc* [26]. In *L.*

102   *enzymogenes* OH11, LeDSF3 positively regulates the biosynthesis of an antifungal

103   antibiotic known as the heat-stable antifungal factor [25]. Recently, BDSF from

104   *Burkholderia* species has been shown to cause biofilm dispersion, increased levels of *relA*

105   and (p)ppGpp production and an upregulation of iron uptake mechanisms through

106   induction of siderophore production in *Francisella novicida*, a model organism for

107   *Francisella tularensis* [27]. The XfDSF synthase gene *rpfF* from *X. fastidiosa* was

108   expressed ectopically in 'Freedom' grape which is susceptible to Pierce's disease caused by

109   *X. fastidiosa*. DSF activity could be detected in xylem sap of transgenic grape

110   overexpressing *rpfF* [28]. Production of DSF family signals in transgenic grape may cause

111   pathogen confusion, thereby reducing the severity of Pierce's Disease in grape [28]. These

112   new findings illustrate the increasing expansion of the spectrum of the biological functions

113   attributed to the DSF signal family, particularly in the areas of interspecies and

114   inter-kingdom communication.

115

116   **Biosynthetic Pathways Leading to the Production of the DSF Family of Signals**

117   Biosynthesis of DSF family of signals in *Xcc* is dependent on the synthase RpfF [29]. RpfC

118   negatively controls DSF biosynthesis via a post-translational mechanism involving

119   RpfC-RpfF interactions [30]. Recently, the enzymatic activity of RpfF, corresponding

120  substrates, reaction products and biosynthetic pathway of DSF family of signals, have been

121  elucidated in *Xcc*. These genes have been identified in diverse bacterial species, suggesting

122  that biosynthesis of DSF family of signals appears to be widely conserved.

123  ***RpfF Has Both Dehydratase and Thioesterase Activities***

124  The DSF synthase RpfF is the key enzyme involved in the synthesis of signals from the

125  DSF family in a wide range of bacterial species. Bcam0581 shares about 37% identity with

126  *Xcc* RpfF and is responsible for BDSF biosynthesis in *B. cenocepacia* [31]. Bcam0581 is a

127  bifunctional enzyme that has been shown not only to dehydrate 3-hydroxydodecanoyl-acyl

128  carrier protein (ACP) to yield *cis*-2-dodecenoyl-ACP, and but also cleaves its thioester

129  bond to generate the final product *cis*-2-dodecenoyl acid (BDSF) [32]. The dehydratase

130  and thioesterase activities of the *Xcc* DSF synthase RpfF have also been experimentally

131  verified recently [22]. This RpfF firstly cleaves the thioester bonds of acyl-ACPs,

132  including 3-hydroxydodecanoyl-ACP to release holo-ACP, indicating the presence of

133  thioesterase activity. Then, RpfF converts 3-hydroxyacyl-ACP substrates into

134  *cis*-2-acyl-ACP, supporting a further activity for this enzyme as dehydratase. BDSF was

135  detected in *in vitro* reaction mixtures containing 3-hydroxydodecanoyl-ACP and RpfF [22].

136  *In vivo* these two enzymatic activities from RpfF and Bcam0581 may be coupled, although

137  the underlying mechanistic details remain unclear.

138

139  Using *in vitro* assays, RpfF from *Xcc* showed thioesterase activity towards acyl-ACP

140  substrates with carbon chains ranging from 8 to 14, suggesting a broad substrate specificity

141  for this enzyme. This probably explains why a single bacterial species is able to produce

142  multiple DSF family signals in rich medium [21, 22]. However, among all of the five

143  acyl-ACP substrates tested, RpfF showed the highest activity on decanoyl-ACP,

144  dodecanoyl-ACP and 3-hydroxydodecanoyl-ACP, suggesting that RpfF might have a

145  preference for substrates with 10-12 carbons.

146

147  ***The Biosynthetic Pathway of the DSF Family of Signals Probably Branches from the***

148  ***Classic Fatty Acid Synthesis Pathway***

149  In bacteria, fatty acid synthesis is catalyzed via a set of distinct monofunctional enzymes

150    (type II) [33]. Fatty acid synthesis is best understood in *Escherichia coli* where acetyl

151    coenzyme A (acetyl-CoA) is the primer and malonyl-CoA is the chain extender. A range of

152    enzymes, including ACC (acetyl-CoA carboxylase), FabD (malonyl-CoA:ACP

153    transacylase), FabH (β-ketoacyl-ACP synthase III), FabG(β-ketoacyl-ACP reductase),

154    FabA/FabZ (β-hydroxyacyl-ACP dehydratase), FabI (enoyl-ACP reductase), and

155    FabB/FabF (β-ketoacyl-ACP synthase I or II) are involved in fatty acid synthesis [33]. The

156    *Xcc* genome contains almost all the genes required for bacterial fatty acid synthesis,

157    including the gene cluster *Xcc0581-Xcc0582* (encoding FabB and FabA), a *fab* cluster

158    (*Xcc1016-Xcc1020* encoding FabH, FabD, FabG, AcpP and FabF), *Xcc1362* (FabZ) and

159    *Xcc0115* which encodes a newly identified enoyl-ACP reductase (FabV) [22,34]. Analysis

160    of deletion mutants showed that *Xcc0581-Xcc0582* and the *fab* clusters are essential for

161    bacterial growth in *Xcc* [22]. The *Xcc* biosynthetic pathway for the DSF family of signals

162    probably branches off from the classic fatty acid synthesis pathway. First, intermediate

163    3-hydroxyacyl ACPs are usually generated during elongation, and β-ketoacyl-ACP

164    reductase (FabG) is directly responsible for 3-hydroxyacyl ACPs production in bacteria. In

165    an Δ*rpfC* mutant strain, overexpression of *Xcc1018,* which encodes FabG, led to a

166    significant increase in the production of DSF, BDSF, CDSF and IDSF [22]. Second, the

167    addition of cerulenin, an antibiotic that binds to long chain 3-keto-acyl-ACP synthases

168    (FabF and FabB) and blocks fatty acid synthesis [35], to cultures of the *Xcc* Δ*rpfC* mutant

169    had only a slight effect on bacterial growth but significantly inhibited the biosynthesis of

170    DSF family signals [22]. Finally, FabH encoded by *Xcc1016* was shown to be required for

171    the biosynthesis of DSF family of signals in *Xcc* [36].

172

173    ***Carbohydrates and Non-Branched Amino Acids Promote BDSF Biosynthesis***

174    The composition and ratio of the diverse DSF type signals produced by cultures of *Xcc* and

175    *Xoo* are influenced by the composition of the growth media [18, 22]. In rich media, DSF is

176    the main signal being produced. In contrast, in nutrient limiting media, BDSF appears to be

177    the dominant signal [18, 22]. To gain a further insight on how medium composition

178    influences the production of DSF type signals in *Xcc*, media XY containing XOLN salts

179    and 0.2 g/L of yeast extract was developed as a base medium [22]. Since carbohydrates and

180   amino acids are two major nutrients present in the xylem fluids of plants [37,38], the effect

181   of sucrose, glucose, starch and fructose as well as and non-branched amino acids on the

182   biosynthesis of different types of DSF signal molecules was tested. In XY medium with

183   these carbon sources, BDSF represented more than 80% of the DSF type signals produced

184   [22]. Deng *et al.* [39] showed that exogenous addition of host plant juice or ethanol extract

185   to the growth medium of *Xcc* could significantly boost the biosynthesis of DSF type

186   molecules. Further $^{13}$C-labeling experiments demonstrated that glucose acts as a substrate

187   providing the carbon element for the biosynthesis of the DSF family of signals.

188

189   ***Methyl Substitutions in DSF and IDSF Originate from Branched-Chain Amino Acids***

190     In bacteria, branched-chain fatty acids are synthesized from branched-chain acyl-CoA

191   primers with malonyl-CoA as the chain extender [40]. The branched-chain acyl-CoA

192   primer can be synthesized from the α-ketoacids, α-ketoisocaproic acid, α-ketoisovaleric

193   acid, and α-keto-b-methylvaleric acid. These α-ketoacids are derived from the catabolism

194   of the branched-chain amino acids leucine, valine, and isoleucine [41]. *Xanthomonas*

195   typically has many branched and hydroxyl-branched fatty acids [42]. Using XYS medium

196   (XY supplemented with 2.0 g/L sucrose) as a base medium, the effect of branched-chained

197   amino acids on the production of different DSF type signals was investigated. The addition

198   of leucine significantly promoted DSF biosynthesis, suggesting that the 11-methyl

199   substitution is derived from leucine [22]. Although valine has one carbon less than leucine,

200   the addition of high concentrations of valine to cultures of *Xcc ΔrpfC* mutant also resulted

201   in an increase in DSF biosynthesis [22]. This is probably because *in vivo* valine is

202   converted into α-ketoisovalerate, which can be further used for leucine biosynthesis [35].

203   The addition of isoleucine significantly promoted IDSF biosynthesis, suggesting that the

204   10-methyl substitution is derived from isoleucine. The metabolic origin of different

205   members of the DSF family of signals explains why *Xcc* and *Xoo* produce multiple DSF

206   type of signals in rich media. These media contain sucrose and a high concentration of

207   tryptone, peptone or yeast extract, which provide a rich source of amino acids including

208   branched-chain amino acids [22].

209

Considering all of the above, a general biosynthetic pathway for DSF, BDSF and IDSF is shown in Figure 2 [22]. The relative concentrations of the acyl-ACP intermediates and their affinities for RpfF lead to differential production of DSF, BDSF and IDSF [22].

*Control of DSF Biosynthesis Through RpfF and RpfC Interactions*

One of the remarkable features of QS systems is that the QS signals are capable of autoregulating their own biosynthesis. This simple yet sophisticated QS signal autoinduction mechanism enables bacteria to sense their population density and effectively synchronize the expression of QS-regulon within the community [43]. The mechanism also allows resetting of the whole QS circuit when a portion of bacterial cells are transferred to a new environment [44]. Increasing evidence suggests that *Xcc* is able to autoregulate the biosynthesis of the DSF family of QS signals [3-5]. Previous results revealed that RpfC, a DSF sensor, can also bind to RpfF via its REC domain to negatively control DSF biosynthesis [3-5]. This was further verified with the resolution of the crystal structure of a complex containing RpfF and the REC domain of RpfC [45]. Recent work with *X. fastidiosa* has provided further insights into the role of the RpfF–RpfC interactions [46]. XfDSF-dependent signaling in *Xylella* requires both RpfC and RpfF. RpfF represses RpfC signaling activity, which in turn is derepressed by XfDSF. Enzymatically inactive variants of RpfF can also support DSF signal transduction. Intriguingly, two populations of RpfF (RpfF-1 and RpfF-2) and RpfC (RpfC-1 and RpfC-2) with differences in their amino acid sequences were found in a panel of clinical isolates of *S. maltophilia*. Each RpfF variant was associated with a specific RpfC variant (RpfF-1 with RpfC-1 and RpfF-2 with RpfC-2) [47]. These findings further support the role of RpfC–RpfF interactions in the control of DSF biosynthesis. However, the detailed mechanism behind this control remains to be elucidated.

**Turnover of the DSF Family of Signals**

It is now widely accepted that bacterial cells need to exit the highly energy-demanding QS maximal activation phase during the post-quorum phase. The QS signal turnover systems are one of the QS exit mechanisms most frequently identified in bacteria [48]. Several

240 bacterial strains belonging to the genera *Bacillus*, *Paenibacillus*, *Microbacterium*,

241 *Staphylococcus*, and *Pseudomonas* are capable of rapidly breaking down DSF [49]. The

242 genes *carAB*, which encodes enzymes responsible for the synthesis of carbamoylphosphate

243 in *Pseudomonas* spp strain G, were identified to be required for DSF inactivation [49].

244 However, the mechanism by which bacteria degrade or inactivate DSF remains unclear.

245 The naturally occurring turnover systems have been less studied for the DSF family of

246 signaling molecules [50].

247

248 ***RpfB is a Fatty Acyl-CoA Ligase Involved in the Turnover of the DSF Family of Signals***

249 ***in Xanthomonas***

250 Previous results in *Xcc* and *Xoo* showed that the DSF family of signals accumulate in the

251 early stationary phase of growth, and their levels subsequently decline sharply [18, 19, 29].

252 This suggested the existence of a naturally occurring DSF signal turnover system which

253 might be responsible for this decline in DSF signal levels during the stationary phase of

254 growth. In *Xcc*, the *rpfB* gene located immediately upstream of *rpfF* was initially predicted

255 to be involved in DSF biosynthesis [29]. However, the defects in DSF production observed

256 in *rpfB* mutants in the *Xcc* 8004 strain were caused by a polar effect on the downstream

257 *rpfF* gene [51] despite the fact that a previous finding revealed that *rpfF* also has its own

258 promoter which would enable its expression independently of *rpfB* [6]. Hence, instead of

259 participating in DSF biosynthesis, it was suggested that *rpfB* may be involved in DSF

260 processing in *Xcc* and *X. fastidiosa*, affecting the profile of DSF-like fatty acids as

261 observed by thin-layer chromatography in an *rpfB* mutant [51]. Subsequent detailed

262 biochemical and genetic analysis revealed that in *Xcc* RpfB could functionally replace the

263 archetypal bacterial fatty acyl-CoA ligase (FCL) FadD, a key enzyme involved in the

264 β-oxidation pathway in *E. coli* [52]. *In vitr*o, RpfB was found to activate a wide range of

265 fatty acids to their CoA esters [52]. The authors suggested that these fatty acyl-CoAs

266 activated by RpfB could be further catabolized by the fatty acid β-oxidation pathway.

267 Alternatively, they could also be utilized to restore membrane lipid synthesis *in vivo* [52].

268 Surprisingly, although RpfB utilizes different fatty acids of variable chain lengths, *in vitro*

269 enzymatic activity assays have shown that RpfB has little apparent effect on the QS signals

DSF and BDSF [52]. Therefore, the authors proposed that RpfB plays a more important role in pathogenesis by counteracting the thioesterase activity of the DSF synthase RpfF [52].

To improve the detection sensitivity of the DSF family of signals, a quantitative detection method using liquid chromatography-mass spectrometry (LC-MS) was developed [53]. This resulted a reduction of the threshold levels of detection of DSF and BDSF to 1µM, enabling a fast and more accurate determination of the levels of these molecules in *Xcc* cultures and reaction mixtures [53]. The *in vitro* assay as described by Bi *et al.* [52] was then repeated to test the effect of purified RpfB on DSF and BDSF levels. The purified RpfB was shown to have little effect on BDSF and DSF *in vitro*, but to rapidly inactivate sodium oleate. Deletion of *rpfB* in *Xcc* or *Xoo* significantly boosted DSF and BDSF production during growth, while over-expression of *rpfB* or its homolog *fadD* completely abolished DSF signal production. In addition, expression of *rpfB* in *E. coli* also efficiently scavenged exogenous BDSF and DSF [53]. Finally, RpfB functionally complemented the *E. coli* Δ*fadD* mutant strain for growth on fatty acids as a sole carbon source, and the key residue E-365, required for the enzymatic activity, was shown to be critical for the catalytic activity of the RpfB FCL, suggesting that FCL activity is required for signal turnover in *Xcc* [52, 53].

The reasons behind the different activity of RpfB on DSF type signals under *in vitro* and *in vivo* conditions remain unknown. However, there are two potential explanations that may explain this discrepancy. One is that RpfB-dependent DSF and BDSF turnover may require additional factors such as co-factors, metals, or salts, which are only present *in vivo*. Another possibility is that RpfB may adopt different conformations *in vivo* and *in vitro*. Nevertheless, further research is required to explain these differences.

### *Regulation of rpfB Expression in Xanthomonas*

*rpfB* expression is growth phase-dependent in *Xcc* and *Xoo* [53, 54]. RpfB transcript levels are low in mid-exponential stage, slightly increase during the late exponential stage, are

maximal at early stationary phase, and subsequently decline [53]. This very much matches the pattern of DSF production during growth [18, 19, 29], further supporting the idea that RpfB might be responsible for DSF turnover. Analysis of *rpfB* expression in an Δ*rpfF* mutant strain in the presence of different concentrations of DSF also showed that RpfB expression is regulated by the DSF signal in a concentration-dependent manner. Exogenous addition of DSF (0.5 μM to 2.5 μM) maintained *rpfB* expression at wild-type levels, whereas further increases of DSF concentrations (10.0 μM to 50.0 μM) significantly enhanced *rpfB* expression [53].

As outlined above, DSF signaling in *Xanthomonas* involves the two-component system RpfC/RpfG, the second messenger c-di-GMP, and the global regulator Clp [3, 4]. Previously, S1 nuclease protection assays revealed that *rpfB* expression was upregulated by RpfC [6]. Recent findings demonstrated that mutation of *rpfC*, *rpfG*, or *clp* in *Xcc* and *Xoo* led to an increase in expression of *rpfB* at the transcriptional and translational levels [53, 54]. Furthermore, *in vitro* studies showed that the global transcriptional factor Clp represses *rpfB* expression through direct interaction with the conserved DNA motif AATGC-tgctgc-GCATC on the *rpfB* promoters of *Xcc* and *Xoo* [50]. The second messenger c-di-GMP, which is the ligand of Clp, effectively reverses the interaction between Clp and the *rpfB* promoters [53].

Taken together, these findings clearly show that RpfB represents a naturally occurring DSF-family QS signal turnover system in the phytopathogen *Xanthomonas*. Although more detailed regulatory mechanisms remain to be experimentally verified, a general working model for the regulation of the RpfB-dependent DSF type signal turnover in *Xanthomonas* is proposed (Figure 3).

***Biological Significance of the Turnover System for DSF Type Signals in Xanthomonas***

In *Xcc* strains XC1 and 8004, the DSF family of signals positively regulate EPS and extracellular enzyme production, but negatively regulate biofilm formation in [3-5]. In line with this observation, deletions of *rpfB* in *Xcc* strains marginally increased the production

11

of extracellular protease, amylase, cellulase, and EPS, and consequently led to enhanced virulence on Chinese radish in a leaf clipping virulence assay [53]. On the other hand, over-expression of *rpfB* in *Xcc* significantly reduced the production of extracellular enzymes and EPS, and attenuated bacterial virulence on plants [53]. In contrast to what was found in *Xcc*, *rpfB* deletion in *Xoo* strain PXO99A significantly reduced EPS and extracellular amylase production, and resulted in reduced virulence on rice cultivars IRBB3 and IR24 [54]. The *rpfB* deletion mutant of PXO99A also displayed reduced EPS production [54]. Further analysis showed that simply deleting *rpfB* in PXO99A did not affect xanthomonadin production, however, a double deletion of *rpfB* and *rpfC* affected the level of xanthomonadin (yellow pigment) production in *Xoo* PXO99A [54].

The discrepancies in bacterial virulence-associated traits between the *rpfB* mutants of *Xcc* and *Xoo* are proposed to be at least partially due to the different levels of the DSF family of signals produced by these two *Xanthomonas* species [54]. The *Xoo* wild-type strain PXO99A produces approximately 10 times more DSF and BDSF than the *Xcc* strain XC1 [52, 53]. The biosynthesis of the DSF family of signals, EPS and xanthomonadin demands a high level of common metabolic precursors, carbohydrates and amino acids in *Xanthomonas* [22, 39]. Over-production of the DSF type signals by PXO99A Δ*rpfB*Δ*rpfC* probably drains the pool of carbohydrates and amino acids needed for EPS and xanthomonadin biosynthesis, which in turn affects EPS production.

***The RpfB-Dependent Signal Turnover System Is Present in a Wide Range of Bacterial Species***

Searches against the Nr database in NCBI revealed that *rpfB* homologs are widely present in all the bacterial species containing the three categories of DSF-based QS systems. In the first category, all the bacterial species habour homologs of *rpfB*, *rpfF*, *rpfC*, *rpfG* and *clp* [4]. The putative Clp binding site was also found in the promoter regions of the *rpfB* homologs in some of these bacteria such as *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas fuscans* subsp. *fuscans* and *Xanthomonas hortorum* pv. *carotae* [53]. Thus, it is likely that these bacteria also rely on RpfB to turnover DSF type QS signals. In the other

360 two categories of DSF-based QS systems represented by *P. aeruginosa* and *B. cenocepacia*,

361 RpfB homologs are also present, however, their roles in signal degradation and their

362 regulation remain to be investigated.

363

364 **Concluding Remarks and Future Perspectives**

365 We have provided an update on current state of knowledge for the DSF family of signaling

366 systems including the increasing diversity of the DSF family of signals, the functions they

367 regulate, their biosynthetic pathway and a naturally occurring turnover system for these

368 signal in *Xanthomonas*. These exciting findings have shown that the signaling cascade and

369 signal turnover system for the DSF family of signals play an important role in the

370 regulation of virulence in a wide range of *Xanthomonas* species. However, many questions

371 on the regulation of these systems remain to be answered (see Outstanding Questions).

372 First, the mechanism underlying the broad substrate specificity of RpfF and how both

373 dehydratase and thioesterase activities found in RpfF are coupled deserves further

374 investigation. The roles of branched-chain amino acid aminotransferase and α-keto acid

375 dehydrogenase in the proposed biosynthetic pathways of the DSF family of signal

376 molecules also needs to be studied. Second, in the *in vitro* enzymatic assay, RpfB

377 efficiently activates a group of free fatty acids exclusive to DSF and BDSF. The

378 mechanism behind this phenomenon and the existence of any potential cofactors working

379 together with RpfB *in vivo* needs to be elucidated. Moreover, it will be interesting to

380 understand how the inactivated DSF signals are recycled by *Xanthomonas* and whether

381 RpfB is required for β-oxidation of other fatty acids in *Xanthomonas*. Whether other

382 signaling pathways or c-di-GMP effectors have a role in regulating *rpfB* expression or

383 other Clp-regulated functions, which may be involved in controlling DSF turnover, deserve

384 further investigation. Finally, *cis*-2-decenoic acid synthesis and perception in *P*. *aeruginosa*

385 and BDSF signaling in *B*. *cenocepacia* deserve further investigation. Addressing these

386 questions will be key to gain a more detailed understanding on the signaling and regulatory

387 mechanisms of this family of cell-cell communication signals. These findings could pave

388 the way to develop new tools to fight against crop losses resulting from diseases caused by

389 pathogens using these signaling systems to control the production of virulence traits.

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**Figure legends**

**Figure 1.** The Chemical Structures of the DSF-Family of Quorum Sensing Signals. This family comprises *cis*-2-unsaturated fatty acids of different chain lengths and branching. The archetype *cis*-11-methyl–dodecenoic acid designated DSF was first described in *Xanthomonas campestris*. DSF, BDSF, CDSF, IDSF, cis-9-methyl-2-decenoic acid, cis-2-undecenoic acid were then identified from *X. campestris and X. oryzae*. These family

of molecules were also found to be produced by *Burkholderia cenocepacia* (BDSF, CDSF, DSF), *Pseudomonas aeruginosa* (cis-2-decenoic acid), and *Xylella fastidiosa* (XfDSF1, XfDSF2). The related molecules are produced by *Lysobacter enzymogenes* (LeDSF3) and *Streptococcus mutans* (SDSF).

**Figure 2.** Schematic Model for the Biosynthesis of DSF, BDSF and IDSF [22]. When there are carbohydrates, acetyl-CoA is produced and converted into malonyl-CoA by acetyl-CoA carboxylase (ACC). FabD synthesizes malonyl-ACP from ACP and malonyl-CoA, and malonyl-ACP is condensed with acetyl-CoA by FabH to form 3-keto-butyl-ACP for the initial step of the fatty acid synthesis elongation cycle. The elongation cycle results in the intermediate 3-hydroxydodecanoyl-ACP. RpfF catalyzes the synthesis of BDSF using 3-hydroxydodecanoyl-ACP. In the presence of carbohydrates, leucine and isoleucine, the branched-chain amino acid aminotransferase IlvE catalyzes the deamination of leucine and isoleucine to form 2-keto-isocaproic acid (KIC) and 2-keto-β-methylvaleric acid (KMV) respectively, which the α-ketoacid dehydrogenase (BCKA) uses to form *iso*-butyryl-CoA and 2-methylbutyryl-CoA respectively. Malonyl-ACP is then condensed with these acyl-CoAs to form 3-keto-butyl-ACP, *iso*-3-keto-hexanoyl-ACP and *anteiso*-3-keto-hexanoyl-ACP for the initial step of the fatty acid synthesis cycle. The intermediates 3-hydroxydodecanoyl-ACP, 11-methyl-3-hydroxydodecanoyl-ACP and 10-methyl-3-hydroxydodecanoyl-ACP are formed via the fatty acid elongation cycle. RpfF converts these acyl-ACP intermediates to DSF (11-methy-*cis*-2-dodecenoic acid), BDSF (*cis*-2-dodecenoic acid) and IDSF/DSF-II (10-methy-*cis*-2-dodecenoic acid).

**Figure 3.** Proposed Model for Cell Density-Dependent Turnover of DSF Type Signals in *Xanthomonas* [53]. At the pre-quorum sensing (QS) phase, the DSF sensor RpfC forms a complex with the DSF synthase RpfF through its receiver domain, which limits DSF biosynthesis at a basal level. High intracellular levels of c-di-GMP bind to the transcription factor Clp. The Clp complex then binds to *rpfB* promoter region to inhibit its transcription. The bound Clp fails to bind to the promoter region of the virulence genes *engXCA*. At the QS phase, RpfC undergoes autophosphorylation upon sensing high levels of extracellular DSF signals. Through the conserved phosphorelay mechanism, RpfG is then phosphorylated leading to activation of its c-di-GMP phosphodiesterase activity. Clp is

570     freed from c-di-GMP and can then bind to the promoter region of the virulence genes

571     *engXCA* to initiate their transcription. Clp is also released from the promoter region of *rpfB*

572     enabling its transcription. At the post-QS phase, the extracellular levels of the DSF family

573     of signal molecules returns to a low level and the dephosphorylated RpfC and RpfF

574     reforms a complex. Dephosphorylation of RpfG leads to inactivation of its c-di-GMP

575     phosphodiesterase activity. The intracellular levels of c-di-GMP return to a high level

576     enabling c-di-GMP-bound Clp to bind to the promoter region of *rpfB* therefore repressing

577     the transcription of this gene.

578
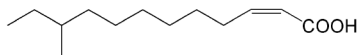
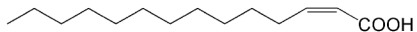*cis*-11-methy-dodecenoic acid (DSF)
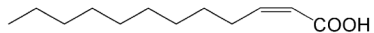
*cis*-2-dodecenoic acid (BDSF)
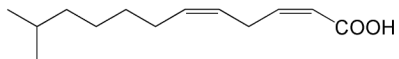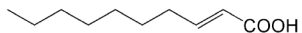
*cis,cis*-11-methyldodeca-2,5-dienoic acid (CDSF)
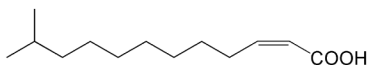
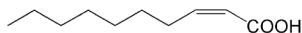*cis*-10-methyl-2-dodecenoic acid (IDSF/DSF-II)
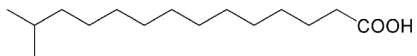
13-methyltetradecanoic acid (*Le*DSF3)

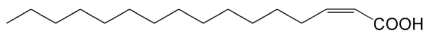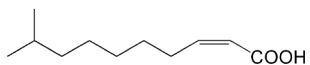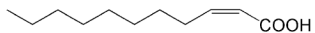*cis*-2-tetradecenoic acid (*Xf*DSF1)
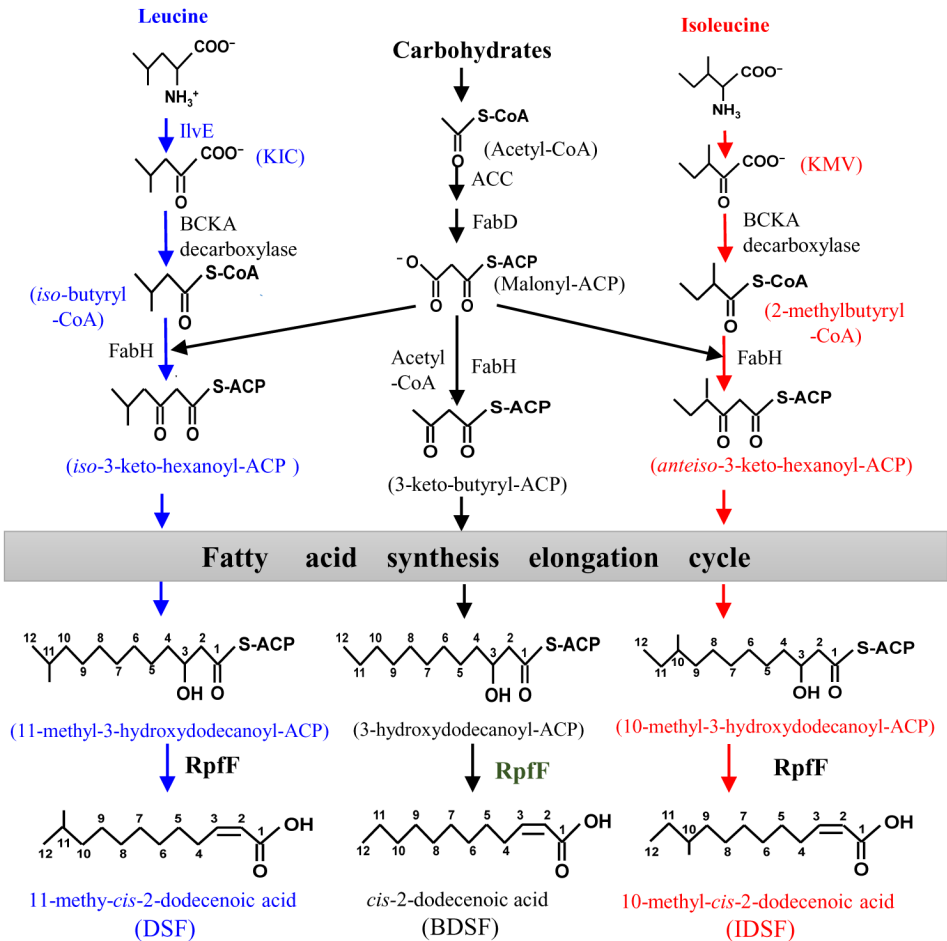
Trans-2-decenoic acid (SDSF)
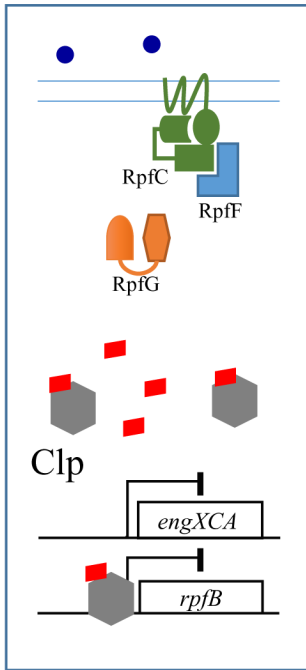
*cis*-2-decenoic acid

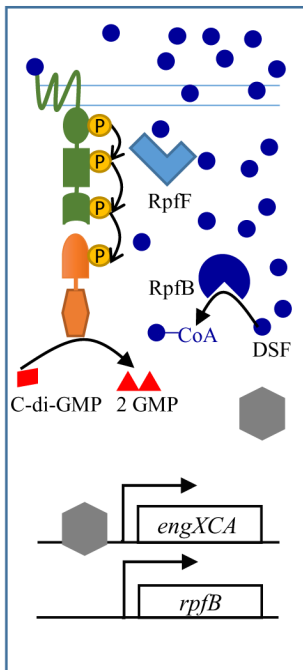2-*cis*-hexadecenoic acid (XfDSF2)

cis-9-methyl-2-decenoic acid

cis-2-undecenoic acid

**Leucine**

(KIC)

IlvE

BCKA
decarboxylase

(*iso*-butyryl
-CoA)

FabH

(*iso*-3-keto-hexanoyl-ACP )

**Carbohydrates**

(Acetyl-CoA)

ACC

FabD

(Malonyl-ACP)

Acetyl
-CoA    FabH

(3-keto-butyryl-ACP)

**Isoleucine**

(KMV)

BCKA
decarboxylase

(2-methylbutyryl
-CoA)

FabH

(*anteiso*-3-keto-hexanoyl-ACP)

**Fatty   acid   synthesis   elongation   cycle**

(11-methyl-3-hydroxydodecanoyl-ACP)

(3-hydroxydodecanoyl-ACP)

(10-methyl-3-hydroxydodecanoyl-ACP)

**RpfF**

**RpfF**

**RpfF**

11-methy-*cis*-2-dodecenoic acid
(DSF)

*cis*-2-dodecenoic acid
(BDSF)

10-methyl-*cis*-2-dodecenoic acid
(IDSF)

Pre-QS phase

QS phase

Post-QS phase

RpfC

RpfF

RpfG

Clp

*engXCA*

*rpfB*

P

P

P

P

RpfF

RpfB

—CoA

DSF

C-di-GMP    2 GMP

*engXCA*

*rpfB*

RpfC

RpfF

RpfG

Clp

*engXCA*

*rpfB*