REVIEW ARTICLE

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Abstract

It is now clear that cell–cell communication, often referred to as quorum sensing (QS), is the norm in the prokaryotic kingdom and this community-wide genetic regulatory mechanism has been adopted for regulation of many important biological functions. Since the 1980s, several types of QS signals have been identified, which are associated commonly with different types of QS mechanisms. Among them, the diffusible signal factor (DSF)-dependent QS system, originally discovered from bacterial pathogen pv. , is a relatively new regulatory mechanism. The rapid research progress over the last few years has identified the chemical structure of the QS signal DSF, established the DSF regulon, and unveiled the general signaling pathways and mechanisms. Particular noteworthy are that DSF biosynthesis is modulated by a novel posttranslational autoinduction mechanism involving protein–protein interaction between the DSF synthase RpfF and the sensor RpfC, and that QS signal sensing is coupled to intracellular regulatory networks through a second messenger cyclic-di-GMP and a global regulator Clp. Genomic and genetic analyses show that the DSF QS-signaling pathway regulates diverse biological functions including virulence, biofilm dispersal, and ecological competence. Moreover, evidence is emerging that the DSF QS system is conserved in a range of plant and human bacterial pathogens.

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The genus is one of the most ubiquitous groups of plant-associated bacterial pathogens. Members of this genus have been shown to infect at least 124 monocotyledonous and 268 dicotyledonous plant species (Leyns *l.*, 1984). Among them, is the most dominant species with at least 141 pathovars, which infect a wide range of plants including many of agricultural interest, e.g. cabbage, cauliflower, broccoli (. . pv. -), tomato and pepper (. . pv.), cotton (. . pv. *l*), soybean (. . pv. *l*), and walnut (. . pv. *l*) (Hayward, 1993).

pv. (Pammel) Dowson (hereafter) is the causal agent of black rot of crucifers, which is possibly the most important disease of crucifers worldwide (Williams, 1980). is a small, rod-shaped, aerobic gram-negative, nonspore-forming bacterium (Onsando, 1992). The bacterium has a single polar flagellum, is positive in catalase activity and hydrogen sulfide reaction, oxidase negative, and does not produce nitrate or indole (Onsando, 1992). According to the Commonwealth Mycological Institute (CMI) distribution map, the disease has been recorded in over 90 countries representing all the five continents (Anon, 1978). The most important hosts of are the members of *B* *l* , including cabbage, cauliflower, broccoli, Brussel sprouts, and kale. The bacterial cells infect cabbage through hydathodes at the leaf margins, causing V-shaped lesions, or through stomata, causing round lesions (Lopes & Quezado-Soares, 1997). Another study shows that normally gains entry into plants via hydathodes (Hugouvieux *l.*, 1998). Once inside the plant, colonizes the vascular system where it produces an extracellular polysaccharide (EPS) called xanthan, which can obstruct the xylem vessels, causing tissue necrosis and severe leaf wilting (Williams, 1980; Onsando, 1992). Evidence is accumulating that xanthan may also play other roles in infection. Treatment of plants with xanthan suppresses the accumulation of callose and enhances plant susceptibility to (Yun *l.*, 2006).

Xanthan is also associated with the formation of biofilms, which was proposed to play a protective role for the bacterial cells inside the biofilm structure against plant defense responses (Torres *l.*, 2007). In addition, is known to encode the biosynthesis of other virulence determinants, including extracellular enzymes, such as endoglucanase and polygalacturonase, and a type III secretion system (also known as the Hrp secretion system), which contribute to the pathogenecity of by providing nutrients through degradation of the xylem walls and facilitating the passage of bacteria via degradation of the bordered pit membranes in the vascular system, or through interference with plant defenses, respectively (Onsando, 1992; Dow & Daniels, 1994). Furthermore, produces a yellow pigment xanthomonadin, which appears to contribute to the bacterial epiphytic survival (Poplawsky *l.*, 2000).

In addition to its roles in virulence, xanthan has had valuable industry and biotechnology applications. It was known long time ago that is capable of producing substantial quantities of xanthan during the fermentation of carbohydrate substrates (Lilly *l.*, 1958). Xanthan is a heteropolysaccharide with a primary structure consisting of repeated pentasaccharide units formed by two glucose units, two mannose units, and one glucuronic acid unit (García-Ochoa *l.*, 2000). Xanthan has distinctive rheological properties in aqueous solutions (Morris *l.*, 1977). Xanthan is nontoxic, nonsensitizing, and does not cause skin or eye irritation (Kennedy & Bradshaw, 1984). The gum also has unusual stability to temperature, pH, shear, enzyme degradation, and excellent compatibility with salts over a wide pH range (Leela & Sharma, 2000). Owing to its unique properties, xanthan has utility in a wide range of applications. In the food industry, it has been used in a wide variety of foods as a suspending, stabilizing, and thickening agent (Leela & Sharma, 2000). Because of its properties in thickening aqueous solutions, as a dispersing agent, and stabilizer of emulsions and suspensions, xanthan is used in pharmaceutical formulations, cosmetics, and agricultural products. It is used in textile printing pastes, glazes, slurry explosive formulations, and rust removers. Furthermore, the high viscosity of solutions and the water solubility of the polymer have also created important applications for xanthan in the petroleum industry where it is commonly used in drilling fluids and in enhancing oil recovery processes (García-Ochoa *l.*, 2000). The widespread utility of xanthan has made it an important industrial biopolymer and its global demand continues to increase each year (Sutherland, 1998).

Research progress in the last few years shows that has evolved a unique quorum-sensing (QS) system, which plays a key role in the regulation of xanthan (EPS) biosynthesis and bacterial virulence. The QS system of differs from other known QS systems in various aspects, including the signal chemistry, autoregulation of signal production, and ways to couple to the bacterial central regulatory networks. This review will focus on these fascinating features, as well as the latest findings on QS regulon and signaling networks. The potential implications of these findings on other bacterial pathogens will also be discussed.

Xcc's escape from engagement of engage

It is increasingly evident that bacteria are not isolated solitary organisms, but actively 'speak' to one another using small chemical signaling molecules as languages. QS is one of the sophisticated mechanisms of cell–cell communications, by which bacteria count their own number by producing, detecting, and responding to the accumulation of signaling molecules that they export into their environment (for reviews, see Whitehead *l.*, 2001; Fuqua & Greenberg, 2002; Bassler & Losick, 2006; Williams *l.*, 2007). Booming research on QS since the 1980s has led to identification of several types of bacterial QS signals (Fig. 1). Among them, *N*-acyl homoserine lactones (AHLs) seem to be one family of the most widely conserved QS signals. The chemical structure of the first AHL signal from marine bacterium

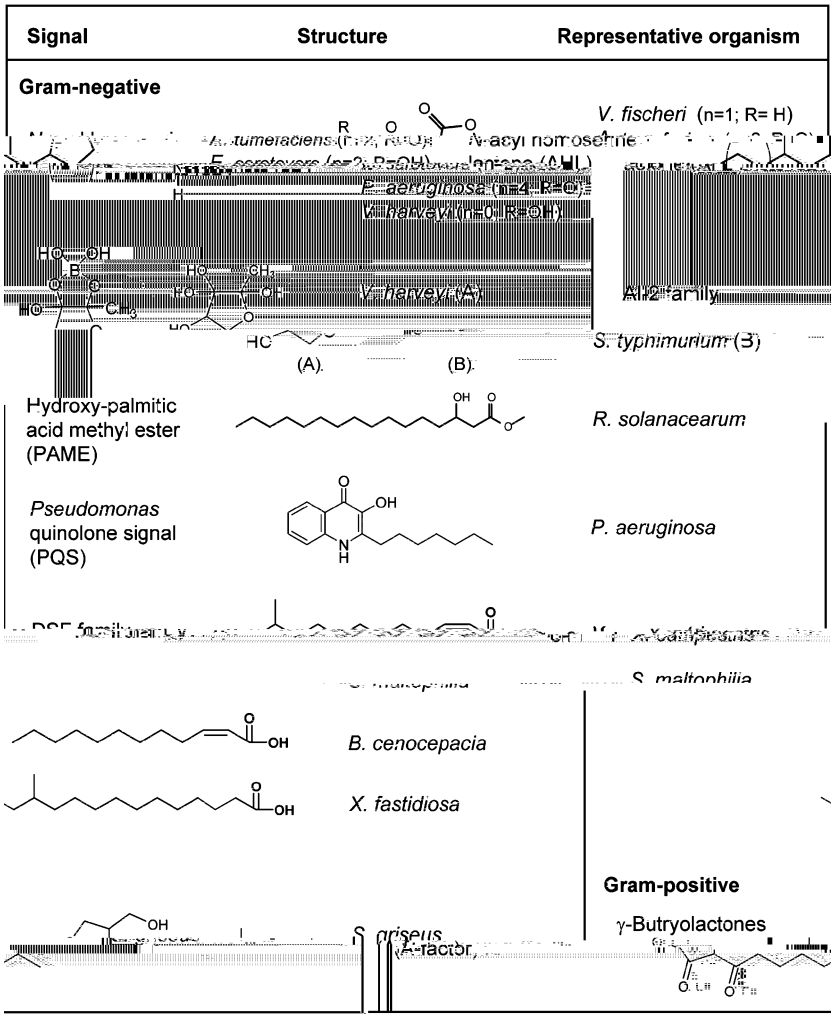

Fig. 1. Chemical structures of representative microbial QS signals. The information was summarized from the following references: Bassler & Losick (2006); Dong *et al*. (2007); Boon *et al*. (2008); Colnaghi Simionato *et al*. (2007); and Huang & Wong (2007a).

level. An early transposon mutagenesis analysis unveiled that a cluster of genes, designated *ABCDEFG* (for regulation of pathogenicity factors), are involved in the production of EPS and extracellular enzymes (Tang *l*., 1991). Subsequently, it was found that the protease production in the mutant of the *F* gene, which encodes a putative enoyl CoA hydratase, was restored by cocultivation in proximity to wild-type strains, suggesting that wild-type strains could produce a diffusible signal factor (DSF) (Barber *l*., 1997). To detect DSF, the original bioassay is based on the ability of DSF to restore endoglucanase production in the *F* mutant (Barber *l*., 1997). The detection system was later improved by fusing the promoter of an endoglucanase gene to the β-glucuronidase (GUS) reporter gene (Slater *l*., 2000; Wang *l*., 2004). This improved DSF sensor, and the finding that mutation of the *C* gene that encodes a hybrid sensor implicated in DSF signaling increases DSF production up to 16-fold (Slater *l*., 2000; Wang *l*., 2004), have greatly facilitated the purification and characterization of the DSF signal as -11-methyl-2-dodecenoic acid (Fig. 1) (Wang *l*., 2004).

Comparison of the biological activities of a range of DSF derivatives has led to identification of a few key structural features that determine the signaling activity. The most important feature is the unsaturated double bond at the α,β position of DSF; the saturated derivative is about 20 000 times less active than DSF (Wang *l*., 2004). The other features, including the configuration of the α,β double bond, the chain length, and the methyl substitution at the C-11 position, also contribute to the biological activity of DSF (Wang *l*., 2004). Interestingly, two groups of Gram-negative bacterial QS signals, i.e., AHLs produced by many Gram-negative bacterial species and the 3-hydroxypalmitic acid methyl ester from *l l* (Flavier *l*., 1997), are also derived from fatty acids but do not contain an α,β unsaturated bond at the fatty acid moieties (Fig. 1). The chemical structures of another two groups of QS signals produced by Gram-negative bacteria, i.e., PQS from and AI-2 from *V.* and

, are clearly different from DSF (Fig. 1). The lipoic-type QS signal A factor produced by gram-positive is also structurally different from DSF (Fig. 1). Collectively, these data indicate that DSF represents a new family of bacterial QS signals.

However, DSF may not be the sole QS-signaling molecule used by . A diffusible factor (DF) is implicated in the regulation of the production of xanthomonadins and EPS in (Poplawsky & Chun, 1997; Poplawsky *l*., 1998). The nonpigmented *B* mutants of cannot synthesize DF, make fourfold less EPS, cause fewer lesions on cabbage, and are impaired in epiphytic survival (Poplawsky & Chun, 1998). The *B* mutant can be restored extracellularly for xanthomonadin and EPS production by a DF-producing strain, or via addition of partially purified exogenous DF. Similarly, application of the DF extracts on cauliflower plants restores the virulence of the DF-deficient mutant to a level comparable to its wild-type parental strain (Chun *l*., 1997). A subsequent study has shown that *B* and DF are needed for epiphytic survival because normal xanthomonadin production is critical for protection of the bacterial pathogen against UV light (Poplawsky *l*., 2000). MS analysis of DF predicted a butyrolactone structure (Chun *l*., 1997), which remains to be validated by nuclear magnetic resonance (NMR) analysis or synthetic chemistry. Similar to DSF, DF is also likely a conserved signal. Most species and some strains produce DF (Chun *l*., 1997; Poplawsky *l*., 2005). Further analysis of the *B* DNA sequence reveals the presence of two genes and only one of them (*4014*), which encodes a pteridine-dependent deoxygenase-like protein, is needed for production of the DF signal (Poplawsky *l*., 2005). DF and DSF are clearly distinct signals because they differ in biological activity, chromatographic mobility, chemical structure, and the genes encoding biosynthesis (Barber *l*., 1997; Poplawsky *l*., 1998, 2005; Wang *l*., 2004), The fact that EPS production is influenced by both DF and DSF suggests that the two QS systems may interact with each other at certain regulatory junctions. Utilization of two QS signal molecules to regulate different sets of virulence genes may provide the plasticity in response to different environments. Further characterization of the chemical structure of DF, and its signaling mechanisms in regulation of xanthomonadin and EPS production will be essential for a clear understanding of the roles of the two sophisticated QS systems (DSF and DF) in modulation of physiology and virulence.

DSF eg a d DSF-de e de b g ca f

DSF-dependent biological functions were initially determined from the phenotype analysis of the DSF-deficient mutant *F*::Tn*5l* of strain 8004 and *F* deletion mutant ΔrpfF of strain XC1. Null mutation of *F* in both strains leads to reduced production of EPS and extracellular enzymes, including proteases and cellulases (Tang *l*., 1991; Barber *l*., 1997; He *l*., 2006b). Protease production of the *F* mutant strain (*F*::Tn*5l*) could be restored by cultivation in proximity to wild-type strains (Barber *l*., 1997). DSF activity could be extracted with ethyl acetate from supernatants of overnight cultures of , and addition of the extracted DSF could restore extracellular enzyme production of the DSF-deficient mutants (Barber *l*., 1997; Wang *l*., 2004). Similarly, addition of purified or synthetic DSF at a physiologically relevant concentration (1 μM) to the *F* deletion mutants of strain XC1 restores the production of EPS and extracellular enzymes (He *l*., 2006b), suggesting that DSF positively regulates the production of EPS and extracellular enzymes. Another important DSF-dependent biological function in is biofilm formation. Mutation of the *F* gene in both strains 8004 and XC1 causes the formation of bacterial cell aggregates, but whose size varies depending on the strains (Dow *l*., 2003; He *l*., 2006b). Addition of DSF to the bacterial cultures stops formation of cell aggregates by the *F* deletion mutants, suggesting a critical role of DSF signal in modulation of switching between planktonic and biofilm growth forms.

The availability of the genome sequence and advancement of microarray technology have greatly facilitated the determination of the scope of genes and biological functions regulated by the DSF signal. Genome annotation of strains ATCC33913 and 8004 leads to prediction of 4181 and 4273 genes, respectively (da Silva *l*., 2002; Qian *l*., 2005). The two genomes are highly similar, sharing over 92% identical or almost identical genes with merely about 2% strain-specific genes (Qian *l*., 2005). Measurement of gene expression levels has been performed using an oligonucleotide microarray based on the genome sequence of strain ATCC33913 (He *l*., 2006b). Comparison of the global gene expression profiles of wild-type strain XC1 and its DSF-deficient mutant XC1ΔrpfF at three growth stages (middle exponential, late exponential, and early stationary phase) identifies 183 genes showing significant changes in expression level (greater than and equal to twofold), whereas altered expression of 421 genes is evident when comparison was made between XC1ΔrpfF with and without DSF addition of the DSF signal (He *l*., 2006b). Further comparison of the two sets of data establishes an overlapping set of 165 DSF-dependent genes, known as the 'core' members of the DSF regulon (He *l*., 2006b). Among them, $> 80\%$ are DSF-activated, and $< 20\%$ are DSF-repressed.

Interestingly, the DSF-dependent genes are in general randomly distributed throughout the bacterial genome (He *l*., 2006b). The pattern is similar to the .

genes regulated by AHL-type QS signals, which are also randomly scattered in the bacterial genome (Schuster *l.*, 2003). These data suggest that both AHL-type and DSF-type QS systems are unlikely to have been acquired recently by these microorganisms.

Based on the published gene list of strain ATCC33913 (da Silva *l.*, 2002), except for the 10 genes encoding hypothetical proteins, the products of the remaining 155 DSF-regulated genes are grouped into 12 functional categories: (1) extracellular enzymes, (2) lipopolysaccharide and EPS synthesis and secretion, (3) multidrug resistance and detoxification, (4) flagellum biosynthesis, motility, and chemotaxis, (5) hypersensitive response and pathogenicity (Hrp) system, (6) iron uptake, (7) protein metabolism, (8) tricarboxylic acid (TCA) cycle, (9) aerobic and anaerobic respiration, (10) transcription regulators, (11) membrane components and transporters, and (12) fatty acid metabolism and others (He *l.*, 2006b). Thus, in addition to these previously known DSF-dependent activities such as production of EPS and extracellular enzymes (Barber *l.*, 1997), microarray analysis unveils several new functions mediated by DSF, such as flagellum synthesis, resistance to toxin and oxidative stress, iron uptake, and aerobic respiration. Some of these newly identified DSF-mediated phenotypes, including resistance to toxin acriflavin and hydrogen peroxide and bacterial survival at different temperatures, have been verified by genetic and phenotype analysis (He *l.*, 2006b). Taken together, the available genetic and genomic data suggest that the DSF-signaling system is not only essential for coordinating the expression of virulence genes at the community level but also appears to be of critical importance for maintenance of ecological competence.

Intriguingly, in contrast to the positive-regulation pattern of the DSF signal on most virulence genes, microarray analysis reveals that in YEB medium mutation of *F* encoding DSF biosynthesis significantly increases the expression of the genes encoding the type III secretion system (T3SS, also known as Hrp) (He *l.*, 2006b). However, the other report shows that when grown in MME medium, mutation of *G* encoding DSF signal transduction has no effect on the expression of and *G*, which encode the master regulators of the operon (Ryan *l.*, 2007). While the impact of DSF on the T3SS remains to be further verified by genetic and genomic analysis under different conditions favorable to the expression of type III secretion genes, it is interesting to note that the AHL-type QS system in also negatively controls the expression of the T3SS regulon (Bleves *l.*, 2005). Given that T3SS is one set of key bacterial virulence determinants in many bacterial pathogens (Hueck, 1998; Ghosh, 2004), thorough investigation of the regulatory mechanisms of T3SS by QS and other signaling mechanisms would be of key importance for designing and developing effective new treatments against bacterial virulence and infections.

DSF- g a g e *Xcc*

How is the DSF signal detected, transduced, and connected to the intracellular regulatory networks? This intriguing puzzle has been resolved recently by the collective works of several laboratories. Although many details remain to be added on, the general outline of the DSF-signaling network has been established, which could be a useful platform for further in-depth investigations.

RpfC and RpfG constitute a two-component system to sense and to transduce the DSF signal

In the genome, *G*, *H*, and *C* are transcribed as the same operon although *C* has its own weak promoter, suggesting that the three genes could be functionally related (Slater *l.*, 2000). RpfC is a hybrid sensor kinase consisting of five transmembrane domains (TM), a histidine kinase (HK) domain, a receiver (REC) domain, and a histidine phosphotransfer (HPT) domain. RpfG protein contains two major domains: a receiver domain and an HD-GYP domain with conserved HD and GYP motifs (Fig. 2b). Deletion of either *C* or *G* decreases the production of EPS and extracellular enzymes (Slater *l.*, 2000), which is reminiscent of the DSF-deficient mutants (Barber *l.*, 1997; He *l.*, 2006b). Based on these results, it was proposed that the RpfC/RpfG is a signal transduction system that couples the synthesis of pathogenic factors to sensing of environmental signals that may include DSF itself (Slater *l.*, 2000).

This speculation was later confirmed by site-directed mutagenesis and domain deletion analysis. Peptide alignment analysis with other well-characterized two-component sensors and regulators reveals four conserved residues, i.e., His^{198} in the HK domain, Asp^{512} in the receiver domain, His^{657} in the HPT domain of RpfC, and Asp^{80} in the receiver domain of RpfG (He, 2006; He *l.*, 2006a). Substitution of these residues with unrelated amino acids or deletion of any one of the domains containing these conserved residues results in decreased virulence factor production (He, 2006; He *l.*, 2006a), suggesting that RpfC/RpfG use the conserved phosphorelay mechanisms to transduce the DSF signal for modulation of virulence factor production. In addition, while exogenous addition of DSF to the DSF-deficient mutant ΔrpfF restores the production of EPS and extracellular enzymes to the wild-type levels, the same treatment has no effect on mutant ΔrpfG and the double-deletion mutant ΔrpfF ΔrpfC (He *l.*, 2006b). Consistent with the above genetic analysis data, microarray analysis shows that RpfG and RpfC regulons are highly similar to the DSF regulon (He, 2006; He *l.*, 2006b). Cumulatively,

these findings suggest that RpfC and RpfG constitute a two-component system essential for sensing and transducing the DSF signal (Fig. 2b).

DSF signaling is mediated by the second messenger cyclic-di-GMP

Different from the orthodox response regulator that typically contains a receiver domain and a DNA-binding domain (for a review, see Stock *l*., 2000), RpfG contains a receiver domain and an HD domain (Slater *l*., 2000). Proteins containing the HD domain comprise a superfamily of metal-dependent phosphohydrolases (Aravind & Koonin, 1998). RpfG belongs to a subgroup of the HD superfamily that contains an additional GYP signature motif (Slater *l*., 2000). *I* expression of the HD-GYP domain of RpfG alone in the null mutants of RpfG restores the production of EPS and extracellular enzymes to a level close to the wild-type strain, whereas expression of the receiver domain alone has no effect (He, 2006; Ryan *l*., 2006), indicating that the regulatory activity of RpfG is associated with the HD-GYP domain. Bioinformatic analysis reveals that in general the number of proteins with the HD-GYP domain in bacterial genomes is positively proportional to the number of GGDEF domain proteins that are implicated in synthesis of cyclic-di-GMP, and is negatively proportional to that of EAL domain proteins, which are implicated in the degradation of cyclic-di-GMP (Römling *l*., 2005). These findings, together with the observation revealing that the proteins with HD domains share the conserved motifs with eukaryotic cyclic-nucleotide phosphodiesterases (Aravind & Koonin, 1998), led to an insightful prediction that the proteins containing HD-GYP domains may have phosphodiesterase activity and could be involved in cyclic diguanylate signaling (Galperin *l*., 1999).

A recent study presents several lines of evidence that demonstrate that the HD-GYP domain of RpfG is a cyclic-di-GMP phosphodiesterase (Ryan *l*., 2006). Firstly, the purified HD-GYP domain protein is able to degrade the model substrate bis(-nitrophenyl) phosphate but has no activity against -nitrophenyl phosphate, indicating that it is a phosphodiesterase but not phosphomonoesterase. Secondly, the purified HD-GYP domain can degrade cyclic-di-GMP to generate GMP. Thirdly, in contrast to the effect on cyclic-di-GMP, the protein has no detectable activity against ATP, GTP, GMP, cGMP, or cAMP. Fourthly, substitution of the H and D residues of the HD-GYP domain abrogates both the enzymatic activity against cyclic-di-GMP and the regulatory activity on virulence factor production. These biochemical and genetic data, together with the findings that the HD-GYP domain alone can substitute RpfG in regula-

a nucleotide-binding domain and a DNA-binding domain, is likely to be a potential candidate (He *l*., 2007). The *l* (CAP-like protein) gene was named due to the observation that its product shares a high similarity to the catabolite activation factor (CAP, also widely known as Crp derived from its function as a cAMP receptor protein) of *E* *l* (de Crecy-Lagard *l*., 1990). However, the null mutation of *l* in strain NRRLB1459 does not affect the utilization of various carbon sources but instead decreases the biosynthesis of EPS, extracellular cellulase, and polygalacturonate lyase (de Crecy-Lagard *l*., 1990), the phenotypes similar to those of the DSF-deficient mutants (Barber *l*., 1997; Slater *l*., 2000; Wang *l*., 2004). In addition, the transcriptional expression of several DSF-regulated genes (He *l*., 2006a), including the cellulase gene *CA*, the protease gene *1*, the flagellin gene *C*, and the heat-shock protein gene *E L*, is also positively regulated by Clp in different strains (de Crecy-Lagard *l*., 1990; Hsiao & Tseng, 2002; Lee *l*., 2003; Chang *l*., 2005; Hsiao *l*., 2005). These original findings, as well as the observation that the transcriptional expression of *l* is influenced by DSF and dependent on the RpfC/RpfG two-component system (He *l*., 2006b, 2007), strongly suggest a key role of Clp in the DSF regulatory pathway. The speculation has been validated by the findings that deletion of *F* or *G* decreases the transcriptional expression of *l* and overexpression of *l* in the mutant ΔrpfF and ΔrpfG, respectively, restores the production of EPS and extracellular enzymes to the wild-type level (He *l*., 2007). Moreover, microarray analysis shows that the DSF regulon and the Clp regulon are largely overlapping with similar expression patterns (He *l*., 2006b, 2007). Even though it is not clear how Clp detects and responds to the signal input from RpfG, the available lines of evidence have unequivocally established the important status of Clp in the DSF-signaling network (Fig. 3).

Clp mediates DSF signaling through a hierarchical regulatory network

Microarray analysis reveals that null mutation of Clp affects 299 genes at the transcriptional level (He *l*., 2007). The available data suggest that Clp modulates the transcriptional expression of this large set of genes by either direct activation or indirect regulation via other transcriptional regulators. Clp is homologous to the members of the Crp-FNR superfamily that are implicated in responding to a broad spectrum of intracellular and exogenous signals such as

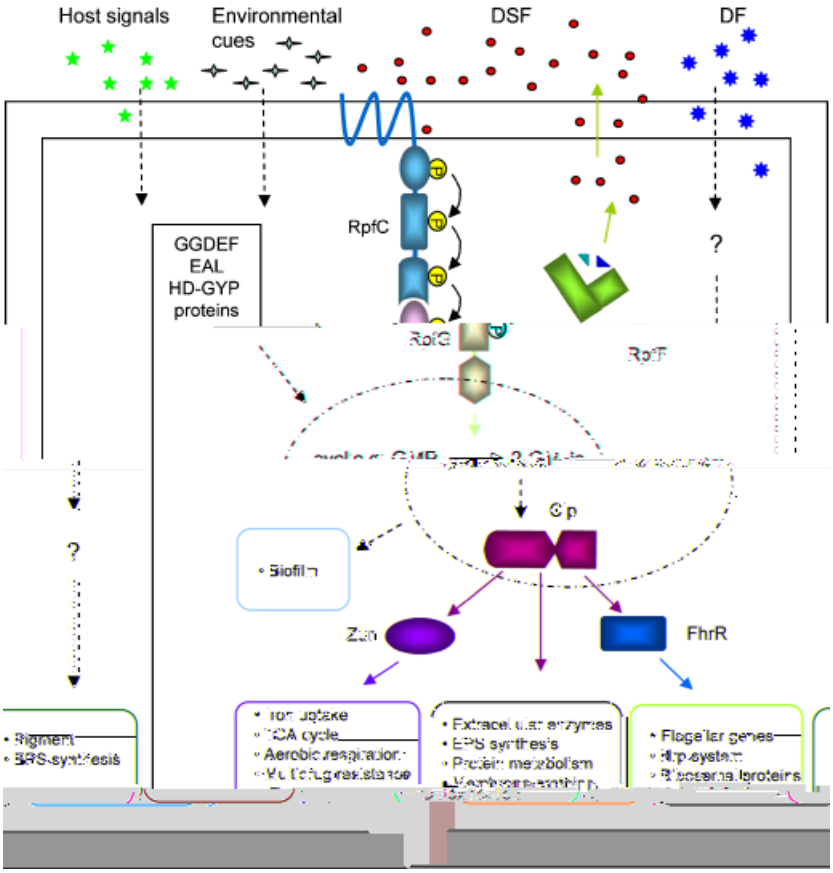

Fig. 3. Schematic representation of the QS-signaling network in *Xcc*. The solid arrow indicates the demonstrated or predicted directed protein–protein interaction or directed signal modulation. The dashed arrows suggest a potential signal regulation pathway. As discussed in the text, it is likely that only a portion of the proteins with cyclic-di-GMP metabolic domains (GGDEF, EAL, and HD-GYP) may contribute to the change in the central pool of cyclic-di-GMP, with a subsequent effect on the transcriptional expression of the genes in the DSF regulon.

cAMP, anoxia, the redox state, oxidative and nitrosative stress, nitric oxide, carbon monoxide, 2-oxoglutarate, or temperature (Körner *l*., 2003). Within this family, the Crp of *E. l* and the Vfr of . have been well studied as global regulators affecting transcription of many genes directly or indirectly (Suh *l*., 2002; Martinez-Antonio & Collado-Vides 2003; Zheng *l*., 2004). Clp shares about 45% identity and 73% similarity to Crp (Dong & Ebright, 1992), which functions as a dimer in the form of a Crp–cAMP complex (Weber & Steitz, 1987; Lee *l*., 1994). The Clp dimer regulates transcriptional initiation by binding to a symmetrical DNA sequence (consensus sequence 5′-AAATGTGATCTAGATCACATTT-3′), located near or within the promoter regions (Zheng *l*., 2004). Similar to Crp and Vfr, Clp contains a conserved helix–-turn–helix domain associated with DNA binding at the C-terminal and a putative cNMP-binding domain at the N-terminal (He *l*., 2007). It was shown that Clp has the same DNA-binding specificity as Crp at positions 5, 6, and 7 of the DNA half site (Dong & Ebright, 1992), and a consensus Clp-binding sequence (TGTGA-N6-TCACA) was thus suggested (Lee *l*., 2003). Using a computational algorithm with a confidence of 60% match to the nonvariable sequences of the putative Clp-binding motif, a total of 86 genes with at least one putative Clp-binding site at the promoter regions (arbitrarily defined as the 500 bp upstream of translation start codon ATG or GTG) were predicted out from the 299 Clp-regulated genes (He *l*., 2007). Most proteins or enzymes encoded by these 86 genes belong to the following functional groups: (1) extracellular enzymes, (2) EPS biosynthesis, (3) protein metabolism, and (4) membrane protein and transporter. These findings suggest that Clp may directly regulate the transcriptional expression of these genes (Fig. 3). Consistent with these findings, electrophoretic mobility shift assays and point mutation analysis confirm that Clp binds to the consensus Clp-binding sites at the promoter of *CA* gene, which encodes an extracellular cellulase (Hsiao *l*., 2005).

Further genetic and microarray analysis led to identification of a novel transcription factor FhrR, which shares < 20% amino acid identity with homologues in databases but contains a TetR family transcription factor domain, and a homologue of the Fur family transcription factor, designated as Zur for its function in the regulation of zinc uptake (Tang *l*., 2005; He *l*., 2007). The expression of both and is regulated by DSF and Clp and these two regulatory factors regulated different sets of genes within the Clp regulon (He *l*., 2007). These data suggest that Clp regulates the genes encoding flagella, Hrp, and ribosomal proteins through FhrR and the Clp-dependent regulation of iron uptake, multidrug resistance, and detoxification is mediated by Zur (Fig. 3). It is worth noting that DSF and Clp up- and downregulate more than a dozen genes encoding various types of transcriptional factors (He *l*., 2006b, 2007). We could expect that further genetic and phenotypic analysis will reveal more regulatory elements within the DSF-Clp-regulatory networks.

Xcc b f 'f a' f e ced b c e 'e d' a d e DSF g a

One of the DSF-regulated biological functions is biofilm dispersal. In rich medium, mutation of the DSF synthase gene . *F*, or . *C*/. *G* encoding DSF sensing and signal transduction, results in formation of strain-dependent mild or extensive cell aggregates (biofilm) whereas the wild-type does not (Dow *l*., 2003; He *l*., 2006b). Consistently, exogenous addition of DSF at a physically relevant concentration to the . *F* deletion mutants grown in rich medium restores the wild-type planktonic growth mode (He *l*., 2006b). Interestingly, however, evidence is emerging that wild-type could also form a biofilm under favorable conditions. A recent study showed that in static cultures in minimum medium, wild-type form structured biofilms, whereas the . *F* and . *C* mutants (DSF overproducer and defective in DSF sensing) produce an unstructured biofilm (Torres *l*., 2007). Coinoculation of the wild-type strain 8004 with the . *C* mutant, which overproduces DSF, at a 1 : 1 ratio (but not at 4 : 1 ratio) abolishes the ability of 8004 to form a wild-type biofilm structure (Torres *l*., 2007). These findings suggest that may form different types of biofilms depending on the culture conditions and DSF signals.

In wild-type strain XC1, deletion of . *F* results in formation of cell aggregates comprising a few dozens to a few hundred cells, whereas deletion of . *l* alone does not affect DSF production, not does it cause the formation of cell aggregates (He *l*., 2007). However, deletion of . *l* in the genetic background of the . *F* mutant leads to formation of much larger cell aggregates than the . *F* single deletion mutant, and exogenous addition of DSF to this double mutant disperses cell aggregations (He *l*., 2007). These phenotypes are puzzling, given that the transcriptional expression of . *l* is upregulated by DSF. Identification of other genes encoding for biofilm formation/dispersal and further analysis of the role of Clp in biofilm formation would facilitate characterization of these seemingly complicated regulatory mechanisms.

B e f e DSF g a a' eg a ed b a' e ' a' a eg a' ec a

QS signals, such as AHL signals, are also widely known as autoinducers (Eberhard *l*., 1981; Fuqua & Greenberg, 2002), which are, in general, capable of autoregulating their own biosynthesis. In the QS system of *V*. , which is

keeping the DSF synthesis at a basal level. Along with bacteria proliferation, the accumulated DSF signal interacts with and triggers autophosphorylation of RpfC, which results in a conformational change allowing release of RpfF, which leads to boosted DSF biosynthesis, and facilitating the four-step phosphorelay that activates RpfG and the downstream DSF regulon (He *l*., 2006a). In this manner, autoinduction of DSF biosynthesis is achievable without substantial elevation of *F* transcription.

Interestingly, a recent study shows that in another species, pv. , which contains conserved genes encoding RpfF, RpfC/RpfG, similar protein–protein interactions exist (Andrade *l*., 2006). The yeast two-hybrid and direct assays show that RpfC interacts with both RpfF and RpfG. The study also shows that the HD-GYP domain of RpfG interacts directly with several proteins containing the GGDEF domain. The finding suggests that this bacterial pathogen may also use the same posttranslational regulatory mechanism as for autoregulation of DSF production, and that RpfC and RpfG may regulate subsets of genes through protein–protein interactions. (Importantly, this need not involve alteration in gene expression.) Interestingly, a recent study shows that null mutation of RpfG results in decreased expression of three genes encoding the proteins containing GGDEF or EAL or both domains (Ryan *l*., 2007), although the mechanism of regulation remains unknown.

O e' e' g' e e a e ?

It becomes clear that the RpfC/RpfG two-component system couples the bacterial cell–cell communication to the bacterial intracellular regulatory networks through the global regulator Clp and the novel second messenger cyclic-di-GMP (Fig. 3). The intracellular level of cyclic-di-GMP is controlled through synthesis, catalyzed by the GGDEF protein domain, and degraded by the EAL or the HD-GYP domain (Tal *l*., 1998; D'Argenio *l*., 2004; Paul *l*., 2004; Christen *l*., 2005; Schmidt *l*., 2005; Ryan *l*., 2006). The finding that the HD-GYP domain of RpfG is a cyclic-di-GMP phosphodiesterase and that the functionality of RpfG is dependent on this enzyme activity (He, 2006; Ryan *l*., 2006) suggests that the intracellular level of cyclic-di-GMP is the key variable component that influences virulence and physiology. Intriguingly, besides RpfG, the genome of also encodes additional 36 proteins with the GGDEF, EAL, or HD-GYP domain (Ryan *l*., 2007). These proteins commonly contain other domains implicated in signaling sensing and receiving, such as receiver domain of a two-component response regulator (Stock *l*., 2000), GAF (Aravind & Ponting, 1997), HAMP (Aravind & Ponting, 1999), MHYT (Galperin *l*., 2001), and PAS domains (Taylor *l*., 2001; Wolanin *l*., 2002; Imamoto & Kataoka, 2007). The contribution of each of these proteins to virulence, extracellular enzyme synthesis, and biofilm formation has been investigated using a panel of mutants (Ryan *l*., 2007). A total of 13 genes, including *G*, are found with an effect on virulence on Chinese radish but only five of them significantly influence the production of extracellular enzyme virulence factors under conditions. It is similarly intriguing that only *G* and *A* seem to regulate biofilm formation and only *G* controls motility under the conditions tested (Ryan *l*., 2007).

The above findings in are agreeable with the general patterns of cyclic-di-GMP signaling systems in other organisms (Jenal & Malone, 2006; Ryan *l*., 2007; Tamayo *l*., 2007), which have been summarized as follows: first, it is likely that not all the proteins with the GGDEF, EAL, or HD-GYP domains are involved in cyclic-di-GMP metabolism. Some of these proteins contain variations in the conserved GGDEF signature motifs (Ryan *l*., 2007). Evidence is accumulating that the proteins with noncanonical GGDEF domains have roles other than being directly associated with cyclic-di-GMP biosynthesis (Christen *l*., 2005; Kazmierczak *l*., 2006; Suzuki *l*., 2006). Second, specific signaling systems may modulate the intracellular levels of cyclic-di-GMP in a highly localized fashion, thus affecting the function of the proteins in the vicinity and allowing the pathogen to show distinct responses to different environmental cues. Alternatively, the difference in the relative enzyme activity and the expression level of the cyclic-di-GMP metabolic enzymes in various signaling systems could result in different changes in the level of cyclic-di-GMP. As the sensitivity of downstream regulatory systems to the change of cyclic-di-GMP (or related nucleotides) level may differ, these subtle differences would be translated to distinct bacterial responses to different environmental cues. Third, may be able to sense various cues of the environment and integrate the signal inputs by changing the cellular content of cyclic-di-GMP, and thus to modulate the bacterial physiology and virulence. A strong piece of supporting evidence is that several proteins with cyclic-di-GMP metabolic domains are implicated in the regulation of extracellular enzyme production in the presence of low oxygen tension and glutamine, respectively, which are the environmental conditions likely to be encountered by a vascular pathogen such as in the process of infection (Ryan *l*., 2007). For a clear understanding of the roles of these putative cyclic-di-GMP metabolic proteins in physiology and virulence, much work remains to be done.

Another key variable component in this virulence regulatory network is the global regulator Clp, whose intracellular level is influenced by DSF (He *l*., 2007). It is rather curious to note however, that, the *l* transcripts, although decreased, still maintain a moderate level in the DSF null mutant ΔrpfF; this accompanies a moderate 28–40%

reduction in the production of EPS, protease, and cellulase, respectively. In contrast, the *l* deletion mutant of XC1 produces about 65–82% less EPS, protease, and cellulase, respectively, than its parent strain XC1 (He *l*., 2007). Given that DSF may most likely influence the *l* transcription via the enzyme activity of RpfG, as the deletion mutants ΔrpfF and ΔrpfG show a similar decrease in transcriptional expression of *l* (He *l*., 2007), the factors that could change the intracellular level of cyclic-di-GMP may likely, but not necessarily, affect *l* expression or influence its activity.

These finding suggest that Clp and cyclic-di-GMP may serve as the core of the regulatory network that modulates virulence, whose intracellular levels may change in response to different environmental cues, including those only found in host plants, and hence alter the expression patterns of downstream genes, including those coding for virulence factor production. In addition, as discussed above, RpfC and RpfG could also modulate subsets of genes through protein–protein interactions independent of Clp (Andrade *l*., 2006; He *l*., 2006a).

Intriguingly, the story does not seem to end here. A recent study shows that initiates expression of the gene encoding a proline iminopeptidase upon sensing an unknown signal(s) from the host plant, and mutation of results in a substantial decrease in bacterial virulence on host plant cabbage (Zhang *l*., 2007). Expression of requires a novel LuxR homologue designated as XccR that acts as a positive regulator. Mutation of also resulted in significantly attenuated virulence of . Regardless of whether XccR/Pip modulates virulence through cyclic-di-GMP or Clp or other regulatory elements, this novel regulation mechanism clearly adds a new puzzle to the existing complexity of the virulence regulatory systems.

Genome analysis of the DSF-QS system in bacteria

The availability of complete genomic sequences of over 300 bacterial species facilitates evaluation of the conservation of the DSF-signaling system among these bacteria. Using the key components of the DSF-signaling network (RpfF, RpfC, RpfG and Clp) as templates to BLAST the bacterial genome database in NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1), the counterparts of the four proteins showing high similarities (54–100% amino acid identity) are found from . pv. , . pv. , pv. , *l ll* , and *l* . The homologues of RpfC, RpfG, and RpfF (34–51% amino acid identity) are also identified from the genomes of *M l ll l ll* -, *ll* , and *L ll* sp. The genes encoding the homologues of RpfF, RpfC, and RpfG show a similar organization in their respective genomes; the only difference is the *C* homologue UBAL79310316 of *L ll* sp. that shares the same orientation as UBAL79310317 (*F*) (Fig. 4). The DSF synthase RpfF homologues with 28–37% amino acid identity are also found in the genomes of more than 60 species, for example, *M* (ZP_01451794), *l* sp. (YP_001357875), *B l* (YP_837187), *E* sp. (ZP_01039845), *ll* (ZP_00825829), *E* (YP_001437677), - *l* (YP_001480964), and sp. (ZP_01305181). However, no obvious homologues of the RpfC/RpfG two-component system could be identified from these genomes. These data suggest that the DSF-RpfC/RpfG-signaling system may very likely be conserved in -, *l ll*, , *M l ll*, *ll*, and *L ll* .

Consistent with the above bioinformatics information, DSF activity has been detected in a number of spp., including . pv. (Chatterjee & Sonti, 2002), . pv. (Jacques *l*., 2005), . (Scarpari *l*., 2003; Newman *l*., 2004), . *l l* (Fouhy *l*., 2007), and *B.* (Boon *l*., 2008). The DSF extracts from . , . *l l*, and *B.* restore the cellulase and EPS production in the *F* mutant of , and vice versa, the DSF from restore the *F*-dependent phenotypes in . *l l* and . (Scarpari *l*., 2003; Fouhy *l*., 2007; Boon *l*., 2008), suggesting a conserved DSF structure among these bacterial pathogens. Recently, the DSF-like signaling molecule (BDSF) from *B.* has been purified and characterized as -dodecenoic acid (Boon *l*., 2008), which is highly similar to the DSF of but lacks only a methyl substitution. The structure of the DSF-like signal from . has also been characterized tentatively as 12-methyl-tetradecanoic acid by MS analysis (Colnaghi Simionato *l*., 2007). Moreover, DSF (-Δ2-11-methyl-dodecenoic acid) and several fatty acids or related structures have been identified from . *l l* (Huang & Wong, 2007a).

The general role of the DSF-signaling system in the modulation of virulence seems to be conserved in other bacterial species but the regulatory mechanisms and DSF-dependent traits may differ among taxa. In . pv. , transposon insertional mutants of *F* are deficient in virulence and exhibit an unusual tetracycline susceptibility phenotype and a growth deficiency under low iron conditions (Chatterjee & Sonti, 2002). The *C* mutants of are defective in EPS production and attenuated in virulence on rice, but the mutation has no effect on extracellular enzyme biosynthesis (Tang *l*., 1996). As discussed in the previous section, . pv. seems to use similar mechanisms as to control DSF

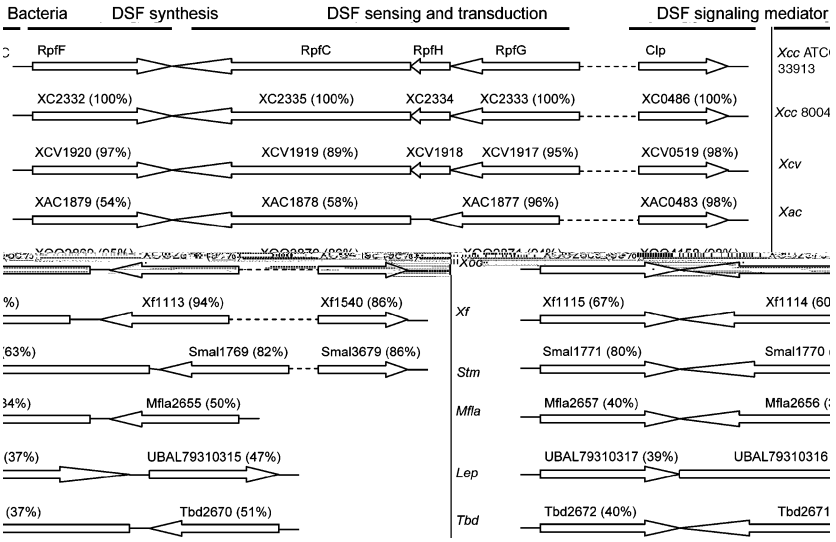

Fig. 4. Conservation of the key genes involved in DSF synthesis, DSF perception, and DSF signal transduction in the genomes of different bacteria. RpfH is a protein showing significant peptide sequence similarity to the N-terminal part of RpfC. Its function in the DSF-signaling system remains unclear. Deletion of *rpfH* does not affect the virulence factor production. The numbers in the brackets indicate the percentages of identical amino acid compared with those in *Xcc* strain ATCC 33913. *Xcv*, *Xanthomonas campestris* pv. *vesicatoria*; *Xac*, *Xanthomonas axonopodis* pv. *citri*; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*; *Xf*, *Xylella* f*astidiosa*; *Stm*, *Stenotrophomonas maltophilia*; *Mfl*, *Methylobacillus flagellatus*; *Tbd*, *Thiobacillus denitrificans*; and *Lep*, *Leptospirillum* sp Group II UBA. All sequences are retrieved from NCBI Microbial Genomes Resources (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html).

production and DSF-dependent functions, because RpfC interacts with both RpfG and RpfF (Andrade *l*., 2006). In . , DSF signaling has been found to influence the interactions of the pathogen with its insect vector and host plant. A more recent study shows that the DSF sensor RpfC is required for virulence and insect transmission of . (Chatterjee *l*., 2008). Similar to , RpfC is involved in negative regulation of DSF biosynthesis by RpfF; *C* mutants are deficient in virulence to grape. However, unlike in , mutation of *F* increases virulence when the bacteria are mechanically inoculated into plants (Newman *l*., 2004). Reverse transcriptase (RT)-PCR analysis also reveals the contrasting effects of the *C* and *F* mutation on the expression pattern of a range of downstream genes including those encoding adhesins (Chatterjee *l*., 2008). Mutation of *F* in . *l* generates pleiotropic effects. The *F* mutants show severely reduced motility, decreased production of extracellular protease, decreased tolerance to a range of antibiotics and heavy metals, and form cell aggregates (Fouhy *l*., 2007). In another report, deletion of *F* or *B* in . *l* WR-C results in reduced transcription of *A*, which encodes a ferric citrate receptor that transports exogenous siderophore-ferric citrate from the environment into the bacterial periplasm, and addition of synthetic DSF restores the *F A* expression (Huang & Wong, 2007b). Transposon inactivation of , which encodes a Clp homologue of , results in reduced *A* and *F* transcriptional expression (Huang & Wong, 2007b).

Conclusions and perspectives

QS, in which bacterial cells communicate by means of small signal molecules, plays essential roles in the synchronization of gene expression and functional coordination among bacterial communities. The DSF signal, originally identified in , represents a novel class of the QS signal, that appears to be widely conserved in a range of bacterial species. In comparison with the classic AHL-type QS systems, the DSF-type QS-signaling system has evolved a unique auto-induction mechanism that modulates DSF biosynthesis, including a novel second messenger cyclic-di-GMP that facilities coupling of QS signal sensing to the bacterial intracellular regulatory networks. Accumulating evidence also suggests that cyclic-di-GMP and the global regulator Clp, which contains a cNMP-binding domain, are the two key components that seem to serve as a core linking the responses to QS, environmental cues, and perhaps plant signals to the downstream genetic regulatory networks.

These exciting findings have laid down valuable frameworks for designing and developing novel strategies against bacterial infections, such as the quorum quenching or antipathogenic method against AHL-type QS systems (for reviews, see Williams, 2002; Zhang, 2003; Bjarnsholt & Givskov, 2007; Dong *l*., 2007). Similar to AHL-type signaling systems, DSF signaling is normally finely balanced during the disease process and disruption of such a fine balance could be a promising way for control of the disease.

Encouragingly, a recent study has identified a variety of bacteria isolates, belonging to the genera *B ll* , - *ll* , *M* , *l* , and , that show an obvious ability to inactivate the DSF signal molecules from (Newman *l.*, 2008). Mutation of the

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