Genome scale analysis of diffusible signal factor regulon in Xanthomonas campestris pv. campestris: identification of novel cell–cell communication-dependent genes and functions

Ya-Wen He,¹ Min Xu,¹ Kui Lin,² Yu-Jin Alvin Ng,¹ Chao-Ming Wen,¹ Lian-Hui Wang,¹ Zi-Duo Liu,³ Hai-Bao Zhang,⁴ Yi-Hu Dong,¹ J. Maxwell Dow² and Lian-Hui Zhang⁴*
¹Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673.
²Institute of Ecology, Beijing Normal University, Beijing 100875, China.
³BIOMERIT Research Centre, Department of Microbiology, BioScience Institute, National University of Ireland, Cork, Ireland.

Summary
The bacterial pathogen Xanthomonas campestris pv. campestris (Xcc) recruits a diffusible signal factor (DSF), which has recently been structurally characterized as cis-11-methyl-2-dodecenoic acid, as a cell–cell communication signal to synchronize virulence gene expression and biofilm dispersal. In this study, we showed that despite the existence of phenotypic variations in different Xcc isolates, the DSF-mediated functions were in general conserved. To investigate the genomic profiles of DSF regulation, we designed and conducted oligomicroarray analysis by comparison of the gene expression patterns of wild-type strain XC1 and its DSF-deficient mutant XC1dF, as well as those of XC1dF in the presence or absence of DSF signals. The analyses led to identification of 165 genes, whose expression was significantly influenced by DSF signals. These genes encode proteins and enzymes belonging to at least 12 functional groups. In addition to those previously known DSF-dependent activities such as production of extracellular enzymes and extracellular polysaccharides, microarray analyses also revealed new functions mediated by DSF, such as flagellum synthesis, resistance to toxins and oxidative stress, and aerobic respiration. Phenotype analyses confirmed that DSF signalling contributed to resistance to toxin acriflavin and hydrogen peroxide, and to the survival of bacterial cells at different temperatures. We conclude that DSF cell–cell signalling is not only essential for co-ordinating the expression of virulence genes but also plays a vital role in keeping up the general competence of the pathogen in ecosystems.

Introduction
Xanthomonas campestris pv. campestris (Xcc), the causal agent of black rot of crucifers, is a bacterial pathogen of significant economic importance (Onsando, 1992). The disease occurs in many countries and regions. Xcc produces a range of extracellular enzymes (including proteases, pectinases and cellulases) and extracellular polysaccharide (EPS), which collectively play essential roles in pathogenesis (Dow and Daniels, 1994). The production of these factors is regulated by the products of the rpfABFGCH cluster of genes. Two of them, rpfF encoding a putative enoyl CoA hydratase and rpfB encoding a long-chain fatty acyl CoA ligase, have been implicated in synthesis of a small diffusible signal factor (DSF) (Barber et al., 1997; Slater et al., 2000). The rpfF transposon mutant, which is DSF-deficient, produces less extracellular enzymes and polysaccharide than the wild-type strains, and the phenotypes could be restored by exogenous addition of DSF (Barber et al., 1997). Besides controlling production of extracellular enzymes and EPS, DSF has also been shown recently to provide a regulatory role in biofilm dispersal of Xcc. External addition of DSF or endo-β-1,4-mannanase encoded by manA, which is believed to be partially induced by DSF, disperses the cell aggregates formed by rpfF mutants (Dow et al., 2003). These findings suggest that DSF could be a global signal which may regulate various genes and functions.

We have recently purified DSF from the Xcc strain XC1 and characterized it as cis-11-methyl-2-dodecenoic acid, which is an α,β unsaturated fatty acid (Wang et al., 2004). It is structurally different from other known quorum sensing and cell–cell communication signals (for a review, see Zhang and Dong, 2004). The homologues of RpfF, the
enzyme implicated in DSF biosynthesis, have been found in a range of bacterial genomes. In keeping with the in silico findings, DSF-like activity has been detected in more than 20 bacterial strains belonging to 10 bacterial species (Wang et al., 2004). Intriguingly, DSF is structurally and functionally related to farnesolic acid, a eukaryotic signal regulating the morphological transition and virulence by the fungal pathogen *Candida albicans*. Similar to farnesolic acid, which is also an α,β unsaturated fatty acid, DSF inhibits the dimorphic transition of *C. albicans* at a physiologically relevant concentration (Wang et al., 2004). These data suggest that DSF represents a new type of cell–cell communication signals conserved in both prokaryotic and eukaryotic kingdoms. However, little information is available on the scope and expression profiles of DSF-mediated genes and the regulatory networks.

Recently, the genome sequences of two *Xcc* strains, i.e. ATCC33913 and *Xcc* 8004, have been reported (da Silva et al., 2002; Qian et al., 2005). While the general homology between the two genomes is very high, there are certain genetic variations between the two genomes. The most prominent ones are the genomic-scale rearrangement across the replication axis, and loss and gain of gene clusters (Qian et al., 2005). It is not clear how such variations may affect the DSF-dependent gene expressions. In this study, we have compared the role of DSF in regulation of virulence factor production using two *Xcc* strains of different origins. We designed and prepared *Xcc* oligonucleotide microarray chips for transcriptome analysis with an objective to identify the genes and new functions regulated by DSF. Based on the microarray results, we further demonstrated experimentally several new functions of *Xcc*, which were co-ordinated by DSF cell–cell communication signals.

**Results**

*Xcc* strains of different origins produced variable levels of virulence factors, but appear to be subjected to the same regulation by DSF cell–cell communication signals

Most DSF-dependent phenotypes, including biofilm dispersal (Dow et al., 2003), EPS and extracellular enzyme production (Barber et al., 1997; Slater et al., 2000), were documented based on the studies of the *Xcc* strain 8004. Given that different *Xcc* isolates may contain considerable genetic variations (Qian et al., 2005), we decided to verify the roles of DSF signals in the Asian *Xcc* isolate XC1, from which we have recently purified and determined the chemical structure of DSF (Wang et al., 2004). The *rpfF* in-frame deletion mutants of strains XC1 and 8004, designated as XC1dF and 8004dF, respectively, were generated by double cross-over homologous recombination. Deletion was confirmed by sequence analysis of the corresponding polymerase chain reaction (PCR) product and DSF-deficient phenotype (data not shown). Similar to strain 8004, null mutation of *rpfF* in XC1 resulted in significant decreases in the production of extracellular protease (Fig. 1A), cellulase (Fig. 1B) and EPS (Fig. 1C). In agreement with the results of biochemical analysis, both mutants also showed significant reduction in virulence when challenged against host plant Chinese cabbage (data not shown). Addition of DSF to the mutants XC1dF and 8004dF, respectively, restored the production of these
Diffusible signal factor modulated the expression of a wide range of genes encoding various functions

Considering that timing and concentration of DSF signal might play a critical role in modulation of gene expression, we used two experimental approaches to identify the ‘core genes’ which are under DSF regulation. We first compared the global gene expression patterns of the wild-type Xc1 and the mutant Xc1dF at optical density at 600 nm (OD$_{600}$) = 1.0, 1.6 and 2.0, corresponding to middle exponential, late exponential and early stationary phase (Wang et al., 2004), respectively, and selected the genes showing ≥2-fold changes in hybridization signals intensity in at least two time points. We then determined the gene expression patterns of Xc1dF with or without 1 μM DSF at the three growth stages, and picked up the DSF-dependent genes using the same criteria as the first set of experiments. By comparing the data of X1 versus Xc1dF and those of Xc1dF with and without DSF, we identified 183 and 421 DSF-dependent genes respectively. Further comparison of the two sets of data led to identification of an overlapping set of 165 DSF-dependent genes. Among them, 133 were DSF-activated, and 32 DSF-repressed (Table 1). Based on the published gene list of Xcc strain ATCC33493 (da Silva et al., 2002), except for the 10 genes encoding hypothetical proteins, the products of the remaining 155 DSF-regulated genes could be grouped into the following 12 functional categories: (i) extracellular enzymes, (ii) lipopolysaccharide (LPS) and EPS synthesis and secretion, (iii) multidrug resistance and detoxification, (iv) flagellar synthesis, motility and chemotaxis, (v) hypersensitive response and pathogenicity (Hrp) system, (vi) iron uptake, (vii) protein metabolism, (viii) tricarboxylic acid (TCA) cycle, (ix) aerobic and anaerobic respiration, (x) transcription regulators, (xi) membrane components and transporters and (xii) fatty acid metabolism and others (Table 1).

Identification of a range of genes encoding extracellular enzymes, EPS synthesis and hypersensitive reactions in microarray analyses, which were mostly upregulated by DSF (Table 1), is highly consistent with the previous findings that the bacterial virulence, production of endoglucanase, polygalacturonate lyase and EPS are activated by DSF signals (Barber et al., 1997; Poplawsky and Chun, 1997; Slater et al., 2000). Most interestingly, microarray analysis also revealed that DSF cell–cell communication signals might control other biological functions than what we had known previously. For example, expression of a range of genes, which encode multidrug resistance, flagellar synthesis and iron uptake, were shown to be upregulated by DSF signals in Xcc for the first time.

Genome organization of DSF-regulated genes

Bacterial pathogens could acquire virulence genes through lateral gene transfer. In this case, the virulence genes are normally clustered and thence ‘pathogenic islands’ become obvious. For example, comparison of the genomes of the non-pathogenic Enterobacter cloacae strain K12 with the pathogenic strain O157 revealed nine O157-specific gene blocks that carry the virulence-related genes (Perna et al., 2001). We were curious how these DSF-
dependent genes are located in the bacterial genome. The mapping analysis showed that there is no obvious chromosomal clustering. The genes identified are in general randomly distributed throughout the bacterial genome (Fig. 2), suggesting that DSF cell–cell signalling regulation mechanism is unlikely to be the late event of the bacterial species adaptation and evolution.

Of the DSF-dependent genes listed in Table 1, we observed strings of genes with similar functions. These strings often represent known or suspected operons. For example, the 12 gum genes (Xcc2443 to Xcc2454) are known to exist in one operon (Katzen et al., 1998). To facilitate characterization of the DSF regulatory networks, we conducted in silico analysis to determine how many operons were influenced by DSF cell–cell signals. Operon organization was predicted based on the following criteria: (i) every gene within a gene cluster is in the same orientation, (ii) there is less than 250 bp between two
adjacent open reading frames (ORFs), (iii) the functions of genes within an operon are correlated and (iv) the genes within an operon showed a similar pattern of response to DSF. By using these criteria, we identified a total of 34 operons; among them 29 were activated by DSF (Fig. 3A), and five were repressed (Fig. 3B).

**Phenotype analysis of new functions regulated by DSF signals**

Production of bactericidal substance, superoxide and its derivatives is the common plant defence mechanism triggered when host cells encounter pathogens. The findings that the genes encoding multidrug resistance, acriflavin resistance, and superoxide dismutase, and catalase, were activated by DSF suggest that DSF signal could also play important roles in adaptation to hostile stresses in plant and in environment (Table 1). To verify these new functions, we first determined the resistance of XC1 and XC1dF to acriflavin and hydrogen peroxide (H₂O₂). However, preliminary results showed that the growth of both XC1 and the DSF-deficient mutant XC1dF were significantly inhibited by the two toxic chemicals, respectively, if they were included in the medium at the beginning of culture. Analysis of the microarray data revealed clear growth phase-dependent transcriptional expression patterns of several genes encoding detoxification functions, including acrD and acrF, which encode acriflavin resistance proteins, and mexA and mexB that encode multidrug efflux transporter and resistance protein respectively (Fig. 4A). Among the three time points determined, significant transcriptional changes (≥ 2-fold) were observed only when bacterial cultures reached late exponential growth phase (OD₆₀₀ = 1.6). These data suggest that these detoxification functions could be not only subject to DSF regulation, but also under the influence of growth phase-dependent mechanisms.

We hence modified the experimental procedures by growing the rpfF mutant in liquid YEB medium with or without DSF till OD₆₀₀ reached about 1.0 (14 h after inoculation) before addition of the toxic chemicals. Figure 4B...
shows that deletion of rpfF significantly decreased the resistance to acriflav, especially at the late growth stages. As expected, addition of DSF to the mutant restored the resistance to the toxin. Similarly, deletion of rpfF also decreased the resistance to H2O2, which could be rescued by addition of DSF (Fig. 4C).

While in the process of characterizing the rpfF mutant, we noted that the DSF-deficient mutant was prone to die when maintained in medium. This finding appeared to be consistent with the microarray analysis data that DSF was required for expression of a range of genes implicated in aerobic respiration, protein synthesis and detoxification (Table 1), which are important in keeping up energy regeneration and adaptation to oxidative stress. To analyse the overall role of DSF signalling system in regulation of Xcc adaptation to environmental stresses, we determined the survival time of the wild-type Xcc and the rpfF mutant in the presence or absence of DSF. The results showed that the survival time of the wild-type strain at room temperature (25°C) or 4°C was 37 or 25 days; however, deletion of rpfF sharply reduced that to 23 or 13 days respectively (Fig. 5). Addition of 3 µM DSF restored the general fitness of the rpfF mutant by increasing its survival time to 32 days at room temperature and to 25 days at 4°C (Fig. 5).

**Xcc strains of different origins produced cell aggregates of different scales, whose dispersal was DSF-dependent but not solely relied on ManA**

It was reported previously that mutation of rpfF whose product is implicated in DSF biosynthesis (Barber et al., 1997) resulted in formation of matrix-enclosed cell aggregates (biofilm) (Dow et al., 2003). Mutation of the rpfG and rpfC genes, which encode key components in DSF signalling pathway, also produced the same phenotype. Addition of endo-β-1,4-mannanase, which is an extracellular enzyme encoded by the manA gene of Xcc, could disperse the cell aggregates produced by all rpf mutants (Dow et al., 2003). However, microarray analysis showed that manA (Xcc1728) expression was only slightly upregulated by DSF signals at three different growth stages.

![Fig. 4. DSF cell–cell communication signal modulates resistance to toxin and oxidative stress. Microarray analysis showed that the transcriptional expression of DSF-controlled genes implicated in acriflav and oxidative stress resistance was also subjected to growth phase-dependent regulation (A). Deletion of rpfF reduced acriflav resistance (B) and H2O2 tolerance (C), which were rescued by external addition of DSF. In assay of acriflav (AF) and H2O2 (HS) resistance, DSF was added as indicated to YEB medium before inoculation, while the two toxic chemicals were separately added 14 h (OD600 = 1.0) after inoculation of fresh bacterial cultures in YEB medium.](image)

![Fig. 5. DSF cell–cell communication signal was important for Xcc long-term survival. After growth for 2 days at 30°C on YEB plates, the bacterial cultures were maintained at 4°C or room temperature (RT). The experiment was repeated twice, each with three replicates. DSF was added as indicated to the medium before inoculation.](image)
(1.1- to 1.7-folds); hence it was not included in the DSF regulon (Table 1). Given that addition of DSF could effectively disperse the cell aggregates of the rpf mutants within a short period of 3 h (Dow et al., 2003), we doubted whether ManA was the sole enzyme responsible for dispersal of biofilms. To test this matter, we first compared the effect of DSF on biofilm dispersal in these two Xcc strains. As reported previously (Dow et al., 2003), mutation of the rpfF gene in 8004 (8004dF) caused the bacterial cells to form visible aggregates. However, the visible cell aggregation phenotype was not observed in the rpfF mutant of XC1 (XC1dF). Instead, we noticed a new phenotype of XC1dF, i.e. a layer of gum-like substance accumulated on the top of cell pellets after centrifugation, which was not observed in mutant 8004dF (Fig. 6A). However, microscopy analysis showed that both rpfF mutants of different origins could form cell aggregates but their intensity of aggregation was highly different. While 8004dF formed extensive biofilm with many bacterial cells aggregated together as described (Dow et al., 2003), the aggregates of XC1dF consisted of only a few dozens bacterial cells (Fig. 6B), suggesting that the
gum-like substance was merely a light version of cell aggregates. As expected, addition of DSF to the bacterial cultures stopped formation of cell aggregates by strain 8004dF and the gum-like substances by mutant XC1dF (Fig. 6A and B).

To test the role of manA in dispersal of cell aggregates, we generated a series of deletion mutants using 8004 and XC1 as parental strains. Null mutation of manA alone in both 8004 and XC1 (8004dM and XC1dM respectively) did not cause noticeable phenotype changes (Fig. 6C). Double deletion of rpfF and manA in 8004 (8004dFdM) showed more severe cell aggregations than mutation of rpfF alone (Fig. 6A and C), which is consistent with the role of endo-β-1,4-mannase in biofilm dispersal (Dow et al., 2003). XC1dFdM, the double rpfF and manA deletion mutant of XC1, produced a similar gum-like substance phenotype as Xc1dF. Inclusion of DSF in bacterial cultures of 8004dFdM and XC1dFdM double mutants, respectively, however, could still stop cell aggregation or formation of gum-like substance (Fig. 6C). Even when cell aggregates were formed, addition of DSF effectively dispersed the cells within 3–4 h (data not shown). These data strongly suggest the involvement of a DSF-dependent factor, other than the endo-β-1,4-mannase encoded by manA, in biofilm dispersal.

Discussion

Diffusible signal factor, as the cell–cell communication signal of the bacterial pathogen Xcc, plays an important role in regulation of virulence factor production and biofilm dispersal. In this study, by developing an oligomicroarray gene chip assay, we have identified 165 genes as the core members of DSF regulon. These DSF-dependent genes were selected because their expression levels were significantly changed (≥2-fold in hybridization signal ratio) not only by endogenous DSF (based on comparison of wild-type XC1 and DSF-minus mutant XC1dF) but also by exogenous signals (based on comparison of XC1dF cultured in the presence or absence of DSF). Highly consistent with the previous reports (Barber et al., 1997; Slater et al., 2000; Vojnov et al., 2001), the identified DSF regulon includes the genes implicated in production of extracellular proteases, cellulase and EPSs. Moreover, several new DSF-dependent functions predicted by microarray analysis, including acriflavin resistance and hydrogen peroxide detoxification, have been experimentally demonstrated. These findings, taking together, verified the reliability of the oligomicroarray gene chips developed in this study for investigation of global gene expression profiles of Xcc.

We should indicate that the core members of DSF regulon listed in Table 1 represent only a very conservative estimate of the DSF-controlled regulon in Xcc. DSF is a newly characterized cell–cell communication signal (Wang et al., 2004), and little information is available regarding its biosynthesis profile and the regulatory mechanism. However, similar to other cell–cell communication systems, such as the well-characterized acylhomoserine lactone (AHL) quorum-sensing systems (Schuster et al., 2003; Wagner et al., 2003), we expect that many factors, such as timing, culture and environmental conditions, could affect the DSF-controlled gene expression. In addition, the microarray chips designed in this study is based on the genome sequence of Xcc strain ATCC33913 (da Silva et al., 2002). Although the general suitability of these chips for analysing different Xcc strains is evident from the recent paper on genome sequence analysis of Xcc strain 8004 (Qian et al., 2005), which shows that majority of Xcc genes in strains ATCC33913 and 8004 are conserved and identical at the nucleotide sequence level, it is also noted that each strain contains a small percentage of strain-specific genes (less than 2.5%) and single-nucleotide polymorphisms (Qian et al., 2005). We hence shall not expect 100% accurate in detection of all the potential DSF-dependent genes. As the objective of this study was to elucidate the general scope of DSF-regulated genetic pathways and biological functions, we have only used one exogenous DSF concentration and one set of culture conditions in microarray analysis. To minimize the problems of identifying genes with relative unstable transcripts, we deliberately eliminated the genes whose expression levels were above or below the selection threshold (≥2-fold versus control) at only one occasion out of the three time points we have determined. Nevertheless, the identified genes provide rich information on the potential genetic pathways and biological functions controlled by DSF-mediated cell–cell communication mechanisms. Besides the previously identified DSF-dependent functions, including production of EPS and extracellular enzymes, microarray analyses showed that DSF could also control a range of other metabolic pathways and functions, such as multidrug resistance and detoxification, flagellum synthesis, Hrp, iron uptake and cell respirations (Table 1).

We have experimentally confirmed that DSF plays important regulatory roles in modulation of acriflavin resistance and detoxification of hydrogen dioxide in Xcc bacterial cells. Addition of DSF to the rpfF mutant of Xcc, which is deficient in DSF production, substantially increased resistance to acriflavin and hydrogen peroxide (Fig. 4A and B). Both functions could be important for Xcc bacterial cells in adaptation to the biotic stresses of host plant. Recognition of pathogen invasion by host plant normally accompanies activation of an array of defence mechanisms; for example, the LPSs produced by Xcc are potent inducers of oxidative burst, including hydrogen peroxide production, in plant (Meyer et al., 2001). Acriflavin resistance proteins encoded by acrABDEF,
functioning as multidrug efflux pumps, confer resistance not only to acriflavin but also to a broad range of noxious dyes, detergents, antibiotics and phytoalexins of plant origin (Rosenberg et al., 2000; Nishino and Yamaguchi, 2001; Burse et al., 2004). These proteins appear to be functionally complementary; expression of acrEF in the aceB mutant of E. coli rescued its hypersensitive phenotype to organic solvents (Kobayashi et al., 2001).

In addition to its role in modulating the expression of the genes implicated in resistance to toxic chemicals and oxidative stresses, DSF also controls other biological activities which contribute to the general fitness of bacterial cells in stressed environment. These functions could at least include LPS and EPS synthesis, iron uptake, protein metabolism, TCA cycle, aerobic respiration and production of member components (Table 1). Considering their respective roles in maintaining cell vigour and protective membrane matrix, we were tempted to speculate that the downregulation of these activities in the absence of DSF could collectively be responsible for loss of vitality of the DSF-deficient mutant cells accompanying prolonged culture (Fig. 4C). It is interesting to note that DSF co-ordinates the expression of a range of genes encoding a string of related functions. For example, deletion of rpfF resulted in decreased expression of genes implicated in TCA cycle, aerobic respiratory enzymes (cyoABCD), membrane-associated proton receptors and antiporters (nuoCEFJKLIM), and membrane-associated energy generators (atpBEF) (Table 1), which are the key components responsible for sequential reactions in carbon metabolism and energy generation (Calhoun et al., 1994; Poole and Cook, 2000). These data suggest that DSF-mediated cell–cell communication system, similar to AHL quorum-sensing systems (Withers et al., 2001), is also an integrated cellular genetic regulatory mechanisms. Although the molecular mechanisms are unknown, the downregulation of aerobic respiratory systems in the absence of DSF seems to match well with the biofilm formation phenotype of the DSF-deficient mutant, as the bacterial cells in biofilm predominately rely on anaerobic respiratory systems (Yoon et al., 2002).

Previous study showed that addition of DSF or purified endo-β-1,4-mannanase encoded by manA could disperse the cell aggregates produced by all rpf mutants (Dow et al., 2003). However, it has not been established whether DSF triggers biofilm dispersal through induction of manA expression. Microarray data appeared to preclude such a possibility as manA had not been significantly induced and there were high basal level of manA expression in the rpfF mutant (data not shown). Deletion of manA at the genetic background of rpfF deletion mutant seemed to facilitate cell aggregate formation, confirming that ManA did play a role in controlling biofilm formation. If ManA is the sole enzyme responsible for biofilm dispersal, we speculated that deletion of manA should show similar cell aggregation phenotype as the rpfF deletion mutant. However, deletion of manA alone in Xcc did not cause formation of cell aggregates (Fig. 6A). Most importantly, addition of exogenous DSF to the rpfF and manA double deletion mutants effectively stopped biofilm formation (Fig. 6). These results strongly indicate that it is not ManA but another DSF-inducible component(s) that is responsible for DSF-dependent biofilm dispersal. Because mutation of rpfC, which encodes a putative DSF receptor, resulted in not only formation of cell aggregations (Dow et al., 2003) but also DSF overproduction (Slater et al., 2000; Wang et al., 2004), we can preclude any direct role of DSF in biofilm dispersal.

Of the 165 genes listed in DSF regulon, the majority (133) was activated by the signal. This ‘gene-inducer’ nature of DSF is also the feature of AHL-type quorum-sensing systems with most genes in quorum-sensing regulon being upregulated by AHL signals (Schuster et al., 2003; Wagner et al., 2003). While the DSF-activated genes belong to many functional groups, as discussed above, most of the genes repressed by the signal are located in three functional groups, i.e. chemotaxis, hypersensitive reaction and pathogenicity (hrp), and certain antiporter and efflux protein homologues (phaDEF). Among these, what is particular interesting could be that how DSF co-ordinates the production of the type III secretion system-dependent Hrp proteins and other virulence factors such as extracellular enzymes when encountering host plants. Although less characterized in Xcc, the hrp gene clusters of other closely related Xanthomonas species are known to encode type III secretion system, which injects bacterial effector proteins into plant cells (Zhu et al., 2000; Büttner et al., 2004; Sugio et al., 2005). Most Gram-negative plant bacterial pathogens contain Hrp systems, which control the ability to establish infection in host plants and to elicit specific defence responses in resistant plants (Büttner and Bonas, 2002). The hrp gene expression is usually activated when bacterial cell senses plant metabolic signals and by physical contact with host cell wall components (Büttner and Bonas, 2002). The findings that the hrp gene expression was downregulated by DSF under in vitro culture conditions suggest that Xcc might have subtle signal-sensing mechanisms, which enable the pathogen to balance DSF signal and host stimuli in timing the expression of hrp and other virulence genes.

How does DSF regulate the diverse genes encoding different biological functions? It was suggested that RpfC and RpfG, which are two-component sensor and response regulator, respectively, form a phosphorelay system to transduce DSF cell–cell communication signals (Slater et al., 2000). Our deletion and microarray analyses confirmed this hypothesis (Y.-W. He et al., in preparation).
However, it remains unclear how the signal from RpfG is conveyed and converted to activate or repress the expression of various sets of genes in DSF regulon. Identification of DSF-dependent transcription factors (Table 1) and operons (Fig. 3) would significantly facilitate the dissection of the DSF signalling network.

It is highly interesting to note that certain putative DSF-dependent functions predicted by the microarray analysis in the current study, such as iron uptake and survival under biotic stress, have been demonstrated previously in bacterial species closely related to Xcc. Transposon mutation of rpfF in Xanthomonas oryzae pv. oryzae resulted in growth deficiency phenotype under low iron conditions and attenuated virulence (Chatterjee and Sonti, 2002). The DSF-minus mutant of Xylella fastidiosa, generated by deleting rpfF via allelic exchange mutagenesis, showed a substantial reduction in the frequency of colonization in its insect host leafhoppers (Newman et al., 2004). It appears that not only the DSF signal production (Wang et al., 2004), but also the DSF-dependent functions, are conserved in these important bacterial pathogens. These fascinating findings could hence underpin the possibility and feasibility of developing new generic approaches, such as quorum quenching (Dong et al., 2001; Zhang, 2003), to prevent and control these economically important pathogens.

**Experimental procedures**

**Bacterial strains and growth conditions**

* Xanthomonas campestris pv. campestris strains XC1 (Asian isolate) and 8004 (European isolate) described previously (Tang et al., 1991; Wang et al., 2004) were used in this study. Xcc strains were grown at 30°C in YEB medium (Zhang et al., 2002), unless otherwise stated. E. coli strains were grown at 37°C in LB medium. Antibiotics were added at the following concentrations when required: kanamycin, 100 μg ml−1; rifampicin, 50 μg ml−1; X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside) was included in medium at 60 μg ml−1 for detection of GUS (β-glucuronidase) activity. Synthesis and detection of DSF were described previously (Wang et al., 2004); the signal was added to medium in a final concentration of 3 μM when necessary.

**Generation of in-frame deletion mutants**

The wild-type Xcc strain XC1 was described previously (Wang et al., 2004). A spontaneous rifampicin-resistant derivative of strain XC1 was used as a parental strain to generate deletion mutants. In-frame deletion of rpfF was generated following the methods described previously (Slater et al., 2000). Briefly, two rpfF-flanking fragments were generated by PCR using primer pairs rpfF-1F (5′-CGATGC GATGGCCGAAGGG) and rpfF-1R (5′-GCTCTAGACAA CAATTGCGGAAATTGACC), and rpfF-2F (5′-GCTCTA GAAAGCGCCGATATGAACAC and rpfF-2R (5′-CTATGG GAAGAGCGCCGAGAC) respectively. These two prod-

DSC regalon 619

ucts were cleaved with XbaI and ligated. Primers rpfF-1F and rpfF-2R were then used to obtain the fused PCR fragment, which was cloned into pK18mobsacB vector. This recombinant construct was transformed into E. coli strain S17-1 (λpir) and subsequently mobilized into the strain XC1 by conjugation. Transconjugants were selected on LB medium supplemented with rifampicin (50 μg ml−1) and kanamycin (100 μg ml−1). Positive colonies were plated on YEB medium containing 5% (w/v) sucrose and rifampicin to select for resolution of the construct by a second cross-over event. The resultant mutant XC1ΔF containing the rpfF in-frame deletion was confirmed by DNA sequencing.

To generate manA in-frame deletion mutants, two PCR fragments that contain the flanking regions of manA were amplified using the following two pairs of primers: Xcc1778-F1 (5′-CGACGCGGGAATTGACCAAT) and Xcc1778-R1 (5′-GGAATTCCGCGATGAAGCCAGCAACC); and Xcc1778-F2 (5′-GGAATTCCGACCTTTCCGACCAAAAT) and Xcc1778-R2 (5′-GTTGATACACGCGCGTTCGTC) respectively. The PCR products were digested by EcoRI and fused by ligation. Primers Xcc1778-F1 and Xcc1778-R2 were then used to obtain the fused PCR fragment, which was cloned to pK18mobsacB vector and transformed into Xcc strains as described above. Same protocols were used to obtain the manA and rpfF double deletion mutants of strains XC1 and 8004 by consecutively deleting manA and then rpfF.

**Quantitative determination of enzyme activities and EPS production**

The extracellular cellulase and protease activity in the culture supernatants of Xcc strains was analysed according to the method described previously (Boyer et al., 1984; Swift et al., 1999). To analyse the production of EPS, the supernatants of overnight bacterial culture (10 ml, OD600 ≈ 2.3) were collected by centrifugation at 14 000 r.p.m. for 10 min. Two volumes of absolute ethanol were added the supernatants and the mixtures were kept at −20°C for 30 min. The precipitated EPS were spun down and dried at 55°C oven for overnight before determination of dry weights.

**Diffusible signal factor bioassay and virulence test**

Diffusible signal factor bioassay was performed as described previously (Wang et al., 2004). Chinese cabbage was used for inoculation. Seedlings were individually planted in pots containing sterilized soil. After 6 weeks of growth at 25°C and with illumination for 16 h per day, scissors-clipping method was used to inoculate fresh cell cultures (OD600 = 0.2) onto the cut edges of the mature leaves. Symptom development was scored 2 weeks after inoculation. Ten plants were inoculated per bacterial strain, and the experiments were repeated twice.

**Design and synthesis of Xcc ORF-specific oligonucleotides**

Based on the annotated genome sequence of Xcc strain ATCC33913 (da Silva et al., 2002), we used an ORF-specific oligonucleotide selection algorithm (Lin et al., 2004) to select

© 2005 The Authors
two unique 50-mer sequences for each ORF. In brief, 50-mer sequences were first selected from ORF sequences based on a set of criteria that included GC content of 45–65%, no inverted repeat of more than five consecutive nucleotides, no single-nucleotide run of >11 consecutive nucleotides, and no more than 50% of the same nucleotide. ORF-specific oligonucleotides were then selected to have no more than 68% similarity to other ORF sequences in the genome to ensure ORF specificity. The oligos representing the genes of transposases or insertion elements were excluded in the final oligo list. For those ORFs with more than one qualified oligo, only the oligo located in the middle region was selected. As specificity controls, 50-mer oligonucleotides were also designed based on the sinat5 (NCBI No.: AF480944) and nac1 (NCBI No.: AF198054) genes of Arabidopsis thaliana, and the rag1 (NCBI No.: NM_131389) of zebrafish and the olf1 (NCBI No.: U56420) genes of human. Thus, a total of 8092 ORF-specific oligonucleotides representing 4042 annotated ORFs, and eight specificity controls were selected. Oligonucleotides were synthesized at a 50 nmol scale by Operon Technologies (Alameda, CA, USA).

Preparation of Xcc oligonucleotide microarray chips

All oligos were dissolved in saline sodium citrate buffer (3x SSC) to make a final concentration of 40 µM and transferred into 384-well plates. Oligonucleotide samples were arrayed from 384-well plates with Pixsys 5500XL Arrayer (Cartesian) loaded with 32-pins (TeleChem International) to poly-L-lysine-coated microscope slides. Upon completion of arraying, DNA samples were fixed by rehydration over a 37°C water bath for 30 s, snap-dried on a heating block (100°C) for 10 s, followed by UV cross-linking at 65 mJ. The remaining poly-L-lysine on the slides was rendered non-reactive by treatment with blocking solution (150 mM succinic anhydride in 1-methyl-2-pyrrolidinone, buffered with 85 mM sodium borate, pH 8.0) for 30 min. After washing with water, the array plates were rinsed with 95% ethanol and then dried by centrifugation (5 min, 500 r.p.m.). The prepared oligo chips were kept at room temperature under desiccation conditions till further use.

RNA purification

Cells were grown as described above. Culture samples were collected at OD_{600} = 1.0, 1.6 and 2.0 respectively. Bacterial cells were pelleted by centrifugation at 4°C for 4 min at 10 000 r.p.m., and the supernatants removed. RNA was purified by using RNeasy midi column (Qiagen) following the manufacturer’s instruction. The eluted RNA samples were treated for 30 min at 37°C with DNase I (0.1 unit per µg of RNA) in the presence of 4 mM MgCl₂, 20 mM Tris-HCl (pH 8.0), and RNase inhibitors (1 unit per µg of RNA). DNase I was removed by phenol/chloroform extraction. RNA integrity was confirmed by electrophoresis using 1.3% formaldehyde-agrose gel. RNA quality was monitored by reverse transcription polymerase chain reaction (RT-PCR) analysis of at least two known genes.

Oligonucleoarray analysis cDNA was generated by using random hexamers as primers for reverse transcription (Invitrogen). The primers were annealed (65°C for 10 min, followed by 25°C for 10 min) to total RNA (50 µg) and extended with Superscript II reverse transcriptase (Invitrogen) and labelling mixture (final concentration: 1x Superscript II buffer, 10 mM DTT, 500 µM each of dATP, dCTP and dGTP, 200 µM dTTP, 100 µM Cy3- or Cy5-dUTP, 1 µl of RNasin, 2 µl of Superscript II reverse transcriptase) for 2 h at 42°C. Cy3- and Cy5-dUTP were products from Amersham Pharmacia, and RNasin was purchased from Promega respectively. Residual RNA was removed by alkaline treatment followed by neutralization, and Cy3- and Cy5-labelled cDNAs were purified using a QIAquick PCR purification kit (Qiagen). The dye-labelled cDNAs were resuspended in hybridization solution (60 µl) and the final concentration of each component in the mixture were: 2.6× SSC, 0.2% SDS, and 3 µg of Cy3-labelled cDNAs and 3 µg of Cy5-labelled cDNAs, 30% of formamide. The hybridization mixture was denatured for 3 min at 90°C, cooled down to 42°C and applied to the array. After covering the array with a 22 × 50 mm LiferSlips™ coverslip, the slide was placed in a sealed, humidified chamber (TeleChem International). Hybridization was carried out in a 42°C water bath. After approximately 16 h, the slides were washed three times using the following buffers: 2× SSC containing 0.1% SDS, 0.2× SSC and 0.05× SSC, respectively, each time for 5 min at room temperature. The slides were dried immediately by centrifugation (5 min at 500 r.p.m.). Using a ScanArray 5000 laser scanner, hybridized arrays were first scanned with ‘quickscan’ to determine the array area as well as the appropriate laser power and PMT value. Upon determining these parameters, the slides were scanned sequentially for Cy5 and Cy3 signals. The signal intensities were quantified by using the software ImaGene 5 (BioDiscovery). Hybridization signals were normalized using the scale normalization procedure previously described (Yang et al., 2002). The statistical significance of the expression difference was calculated following the published method (Yang et al., 2003). The fold changes were then calculated from the normalized log ratios. Each treatment had at least two replicates and the data presented are means of at least four replicates as only those genes that appeared twice above the selection bar in three time points determined were selected (see text).

Acriflavin and H₂O₂ resistance assay and determination of survival time

Bacterial strains were grown in YEB medium with or without DSF until the OD_{600} of the cultures reached about 1.0; acriflavin and H₂O₂ was added to a final concentration of 2.5 µg ml⁻¹ and 0.03% respectively. Bacterial population density at different time points after addition of the toxic chemicals was determined by plate-counting of colony-forming units (cfu) as described (Hu et al., 2003).

For survival testing, the wild-type and rpfF mutant strains were subcultured on YEB agar plate with or without DSF as indicated. After growth at 30°C for 2 days, the agar plates were maintained at room temperature or 4°C. From the seventh day onward, the viability of cells was tested every day by randomly selecting and subculturing three colonies from each treatment to fresh YEB plate. The growth of bacterial cells was monitored for 2 days at 30°C. Loss of viability was further confirmed by growing five colonies before treatment in liquid YEB medium, separately.
Acknowledgements

We thank Dr Chaozu He of the Institute of Microbiology, Chinese Academy of Sciences, for sharing information on the genome sequence of Xcc strain 8004. Ji Ren Wang of the Bioinformatics Institute for providing excellent technique assistance in bioinformatics analysis. Funding was from the Agency of Science, Technology and Research (A*Star), Singapore. J.M.D. is supported by the Science Foundation of Ireland through an investigator award (03/IN3/B373).

References


Supplementary material
The following supplementary material is available for this article online:

Table S1. DSF-regulated genes.

This material is available as part of the online article from http://www.blackwell-synergy.com