Xanthomonas campestris cell–cell communication involves a putative nucleotide receptor protein Clp and a hierarchical signalling network

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Summary
The bacterial pathogen Xanthomonas campestris pv. campestris co-ordinates virulence factor production and biofilm dispersal through a diffusible signal factor (DSF)-mediated cell–cell communication mechanism. The RpfC/RpfG two-component system plays a key role in DSF signal transduction and appears to modulate downstream DSF regulon by changing intracellular content of cyclic dimeric GMP (c-di-GMP), an unusual nucleotide second messenger. Here we show that Clp, a conserved global regulator showing a strong homology to the cAMP nucleotide receptor protein Crp of Escherichia coli, is essential for DSF regulation of virulence factor production but not for biofilm dispersal. Deletion of cip in Xcc changed the transcriptional expression of 299 genes including a few encoding transcription factors. Further genetic and microarray analysis led to identification of a homologue of the transcriptional regulator Zur, and a novel TetR-type transcription factor FhrR. These two regulatory factors regulated different sets of genes within Clp regulon. These results outline a hierarchical signalling network by which DSF modulates different biological functions, and may also provide a clue on how the novel nucleotide signal can be coupled to its downstream regulatory networks.

Introduction
Xanthomonas campestris pv. campestris Pammel (Xcc) is a Gram-negative pathogen that infects all the members

of cabbage family and could cause severe economical losses (Onsando, 1992). Xcc produces a range of extracellular enzymes (including proteases, pectinases and cellulases) and extracellular polysaccharide (EPS), which are collectively essential for pathogenesis (Dow and Daniels, 1994). Production of these virulence factors is co-ordinated by a cell–cell communication mechanism through a small diffusible signal factor (DSF) (Barber et al., 1997; Slater et al., 2000), which has been characterized as cis-11-methyl-2-dodecenolic acid (Wang et al., 2004). DSF-like activity has been observed in a range of microbial species (Slater et al., 2000; Wang et al., 2004), suggesting that DSF may represent a family of widely conserved cell–cell communication signals.

Diffusible signal factor also modulates a range of other biological functions of Xcc, including biofilm dispersal (Dow et al., 2003; He et al., 2006a), toxin resistance and bacterial survival (He et al., 2006a). A recent microarray analysis has identified at least 165 genes whose expressions are significantly up- or downregulated by DSF signals. These genes encode proteins and enzymes belonging to 12 functional groups, including extracellular enzymes and polysaccharide production, iron uptake, multidrug resistance and detoxification, tricarboxylic acid (TCA) cycle, respiration, flagellar protein and hrp genes, and protein metabolism (He et al., 2006a), highlighting the critical role of cell–cell communication in Xcc physiology and ecology.

The production, detection and response to DSF signals are dependent on the products of several rpf genes (Barber et al., 1997). The rpfF gene encodes a putative enoyl-CoA hydratase that is involved in the synthesis of DSF, whereas the products of rpfC and rpfG constitute a two-component regulatory system. Addition of DSF can restore virulence factor production in rpfF mutants but not in the strains with mutations in rpfC or rpfG (Barber et al., 1997; Slater et al., 2000; Wang et al., 2004), suggesting that RpfC/RpfG two-component system plays a role in DSF signal perception and signal transduction. The essential role of RpfC, which is a hybrid sensor kinase, has been further confirmed by the findings that substitution of any conserved residues involved in phosphorelay, or deletion of any functional domain of RpfC, abrogates the Xcc response to exogenous addition of DSF (He et al.,

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RpfG belongs to a subclass of HD domain proteins which have an additional GYP signature motif (Galperin et al., 1999; Slater et al., 2000). A recent study has shown that the HD-GYP domain is a novel cyclic dimeric GMP (c-di-GMP) phosphodiesterase, which degrades c-di-GMP to generate GMP (Ryan et al., 2006). Taken together, these findings indicate that DSF cell–cell signals is coupled to c-di-GMP through RpfG and by extension that the downstream transcription factor(s) of DSF signalling pathway may be modulated by this novel second messenger or associated signals.

Although DSF regulon and the RpfC/RpfG two-component system involved in DSF signal perception and response have been characterized, many open questions remain. One intriguing issue is the DSF signalling pathway and how the downstream genes are regulated. In this study, we have attempted to determine the key components and the order of signalling pathway. Based on the finding discussed above that nucleotide signals may play a role as second messengers in DSF signalling pathways, we searched for potential downstream regulatory element(s) with a domain that may recognize the nucleotide messengers. We showed that the expression of clp, which encodes a homologue of cAMP receptor protein known previously for its role in regulation of EPS and extracellular enzyme production (de Crecy-Lagard et al., 1990; Hsiao and Tseng, 2002; Hsiao et al., 2005), was induced by DSF signals. Based on Clp regulon analysis, we identified additional two transcription factors, which regulate different subsets of the genes within Clp regulon. These data, together with others, depict a hierarchical DSF signalling network for gene regulation in the pathogenic bacterium Xcc.

**Results**

**Expression of global regulation factor Clp is mediated by DSF cell–cell communication signal**

Microarray analysis showed that expression of clp (Xcc0472), which encodes a global transcription regulator with a cNMP-binding domain (Fig. 1A), was decreased at different growth stages of the deletion mutants of rpfF, rpfC and rpfG respectively (Table 1). Exogenous addition of DSF to the rpfF deletion mutant ΔrpfF significantly enhanced the transcriptional expression level of clp, in particular, at the later stage (OD_{600} = 2.0) of bacterial growth (Table 1). These microarray data were further verified by reverse transcription polymerase chain reaction (RT-PCR) analysis. While at early growth stage (OD_{600} = 0.5) there was no significant difference among XC1 and its derivatives defective either in DSF biosynthesis (ΔrpfF) or in DSF signal transduction (ΔrpfC, ΔrpfG) (Fig. S1A), clp transcripts level was significantly lower in these DSF mutants than in XC1 (Fig. S1B). As expected, exogenous addition of DSF to mutant ΔrpfF restored the clp expression level (Fig. S1B), and further deletion of the DSF response regulator gene rpfG in the genetic background of ΔrpfF abrogated the DSF-dependent clp expression (Fig. S1C). Peptide sequence alignment of Clp with its homologues showed that Clp shared four and five of the six conserved residues implicated in cAMP binding with the Crp of Escherichia coli and the Vfr of Pseudomonas aeruginosa respectively (Fig. 1B). Crp and Vfr are the well-characterized cAMP-dependent transcription regulators but also seem to
process a cGMP binding activity (Gorshkova et al., 1995; Beatson et al., 2002).

**Clp is essential for DSF signalling regulation of EPS and extracellular enzyme production**

For determination of the role of Clp in DSF signalling pathway, deletion mutants of clp and several key genes in DSF signal synthesis and transduction were constructed using wild-type XC1 as the parental strain. The mutant Δclp of XC1 produced about 82% less EPS, and showed 65% and 82% decreases in cellulase and protease activity, respectively, than its parental strain XC1 (Fig. 2A–C). We also found that deletion of clp resulted in much more severe reduction in EPS production and extracellular enzyme activity than deletion of rpfF or rpfG (Fig. 2A–C), which encodes for DSF production and signal transduction respectively. Importantly, overexpression of clp in the mutants Δrpf and ΔrpfG restored the virulence factor production to the wild-type level (Fig. 2A–C), whereas deletion of clp in XC1 or further deletion of clp in the genetic background of ΔrpfF abolished the response of the mutants to exogenous DSF signals in restoration of EPS production and extracellular enzyme activity (Table S1).

**Clp is not involved in the regulation of DSF-dependent biofilm dispersal**

Another DSF-regulated biological function is biofilm dispersal, mutation of the genes involved in DSF synthesis (rpfF) or in signal transduction (rpfC/rpfG) results in formation of strain-dependent mild or extensive cell aggregates (Dow et al., 2003; He et al., 2006a). Unlike the control ΔrpfF, which did not produce DSF but produced visible cell aggregates under a microscope (Fig. 3) (He et al., 2006a), deletion of clp alone in XC1 did not affect DSF production (data not shown), nor did it cause the formation of cell aggregates (Fig. 3). Unexpectedly, deletion of both rpfF and clp in XC1 led to extensive cell aggregates formation, whereas addition of DSF to this double mutant dissolved the biofilm (Fig. 3). Albeit the mechanism accounting for the interesting phenotype of this double deletion mutant remains to be further investigated, the data suggest that DSF may use other regulatory mechanism(s) rather than Clp to modulate biofilm dispersal.

**Clp regulates the expression of a large set of genes in DSF regulon**

The results above showed that Clp regulates some but not all DSF-dependent functions. To understand to what extent Clp is involved in DSF signalling regulation, we conducted microarray analysis to identify the Clp-influenced genes. By using the same method described previously (He et al., 2006a), a total of 299 Clp-regulated genes were identified, in which 39 were downregulated by Clp and 260 upregulated. Based on the published gene list of Xcc strain ATCC33913 (Da Silva et al., 2002), except for the hypothetical proteins, the products of the remaining Clp-regulated genes could be grouped into the following 12 functional categories in addition to hypothetical proteins: (i) extracellular enzymes, (ii) EPS synthesis, (iii) multidrug resistance and detoxification, (iv) flagellar synthesis and motility, (v) hypersensitive reaction and pathogenicity system (Hrp), (vi) iron uptake, (vii) amino acid and protein metabolism, (viii) TCA cycle, (ix) aerobic and anaerobic respiration, (x) transcription regulators, (xi) membrane components and transporters, (xii) fatty acid metabolism and others and (xiii) hypothetical proteins (Table 2). Comparing these results with our previous data (He et al., 2006a), we found that 94 out of the 165 DSF-regulated genes were subjected to Clp regulation with similar expression patterns (Table 2). Consistent with the microarray data, which showed that deletion of clp caused 2.4- to 7.6-fold decreases in transcriptional expression of the genes coding for acriflavin (acrA, acrB, acrF) and hydrogen peroxide (katE, sodC2, sodM) resistance, and flagellar biosynthesis (flgCDLMP, flgABEFHKL), phenotype analysis showed that deletion of clp reduced the bacterial flagella-driven motility (Fig. 4A), and decreased the resistance to acriflavin, and to H2O2 (Fig. 4C).

Table 1. Expression of clp is dependent on RpfC/RpfG two-component regulatory system and promoted by DSF signal.

| OD600 | XC1 versus ΔrpfF | ΔrpfF + DSF versus ΔrpfF | XC1 versus ΔrpfC | XC1 versus ΔrpfG |
|-------|-----------------|--------------------------|-----------------|-----------------|
| 1.0   | 0.90 ± 0.2      | 2.1 ± 0.3                | 1.1 ± 0.2       | 1.0 ± 0.3       |
| 1.6   | 1.5 ± 0.2       | 3.2 ± 0.7                | 1.7 ± 0.2       | 1.8 ± 0.2       |
| 2.0   | 2.1 ± 0.2       | 6.4 ± 2.2                | 2.2 ± 0.3       | 2.5 ± 0.3       |

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same DNA binding specificity as Crp and recognizes the same conserved DNA binding site: TGTGA-N6-TCACA (Dong and Ebright, 1992). In an attempt to explore the genes that may be regulated directly by Clp, we designed a computational algorithm to search for the putative Clp-binding motif within the promoter regions (arbitrarily defined as the 500 bp upstream of translation start codon ATG or GTG) of the 299 Clp-regulated genes. By using a set of 24 E. coli genes that have been experimentally verified as being regulated by Crp as the reference of training (Zheng et al., 2004), we set the cut-off score at 0.6 (60% homology with the conserved DNA binding site) and found 86 genes with at least one putative Clp binding site in promoter region. These genes encode proteins or enzymes belonging to seven of the 12 Clp-regulated functional groups, including (i) extracellular enzymes, (ii) EPS synthesis, (vi) iron uptake, (vii) protein metabolism, (xi) membrane protein and transporter, (x) transcription regulators and (xii) others (Table 2) respectively.

Clp regulates the genes encoding flagellar, Hrp and ribosomal proteins through a novel transcription factor FhrR

The analysis shown in Table 2 also identified seven regulatory genes including clp, containing a putative Clp binding site in their corresponding promoter regions. To understand whether Clp regulates part of the genes within Clp regulon through these transcription factors, we generated a range of mutants by deleting separately the corresponding genes encoding these transcriptional regulatory proteins at the genetic background of XC1, and conducted phenotype analysis including EPS synthesis, extracellular enzyme activity, motility and resistance to chemical toxins. The analysis revealed two regulatory components important for DSF signalling. The first one is encoded by the annotated operon Xcc1215, whose expression level was decreased by 6.4-fold in the clp mutant. Deletion of Xcc1215, designated as fhrR according to the gene categories regulated by its product as described later, resulted in bacterial motility with a halo zone about 25% smaller than wild-type XC1 (Fig. 4A). Expression of the wild-type fhrR in either fhrR or clp deletion mutant restored the bacterial motility (Fig. 4A), supporting the role of FhrR regulator in modulation of motility and in Clp regulatory pathway. The gene contains a putative Clp binding site (CTTGACCGCCTC TACA, cut-off score = 0.6) at the promoter region (−183) (Table S2). It is located within the vicinity of the hrp operon (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genome&cmd=Retrieve&dopt=Protein+Table&list_uids=240). Its putative product is a peptide of 208 amino acids sharing less than 20% amino acid identity with other proteins in databases but contains a TetR family transcription factor domain (Fig. 4B). Microarray analysis showed that deletion of fhrR affected a total of 53 genes showing ≥2-fold changes in hybridization signal intensity (Table 2). Among them, 40 were FhrR-activated and 13 FhrR-repressed. Further comparison of Clp- and FhrR-
regulated genes led to identification of an overlapping of 45 genes, and most of them encode proteins belonging to the following three functional categories: flagellar proteins (12), hrp system (7) and ribosomal proteins (16) (Fig. 5). Importantly, the microarray data also showed that FhrR positively regulated transcription of the genes encoding flagellar and ribosomal proteins and negatively regulated the transcription of hrp genes, which is exactly the same as the regulation pattern of DSF (He et al., 2006a) and Clp (Table 2).

The Clp-dependent regulation of iron uptake, multidrug resistance and detoxification is mediated by a Fur family transcription factor

Deletion of Xcc2685, which encodes a homologue of Fur family transcription factor (Fig. 4B) sharing an identical peptide sequence as the zinc uptake regulator Zur encoded by the open reading frame Xc1430 of Xcc strain 8004 (Tang et al., 2005), also resulted in significant phenotype changes. As shown in Fig. 4C, the mutant was more sensitive to acriflavin and H₂O₂ than the wild-type strain. Furthermore, overexpression of the zur gene in clp deletion mutant restored the resistance to acriflavin and H₂O₂ (Fig. 4C), confirming that Zur functions at the downstream of Clp. The zur gene promoter of strain XC1 also contains a putative Clp binding site (TGGGCAAGGTG GCCCA, cut-off score = 0.6); deletion of clp caused a 4.7-fold decrease in zur transcriptional expression. Microarray analysis of the zur deletion mutant identified 94 genes showing ≥2-fold changes in hybridization signal (Table 2). Comparison of Clp regulon and Zur-regulated genes led to identification of an overlapping set of 90 genes with similar expression patterns (Table 2). The products of these overlapping genes belong to five of the Clp-influenced functional categories, i.e. (iii) multidrug resistance and detoxification, (vi) iron uptake, (viii) TCA cycle, (ix) aerobic respiration and (xii) others (Table 2, Fig. 5). Noticeably, there is not overlapping between FhrR- and Zur-regulated genes. This is agreeable with the findings of phenotype analysis that deletion of zur had no influence on the FhrR-modulated bacterial motility (Fig. 4A), and deletion of fhrR did not affect the Zur-dependent resistance to H₂O₂ and acriflavin (Fig. 4C).

Discussion

The RpfC/RpfG two-component regulatory system plays an essential role in signal perception and signal transduction for co-ordinating the production of a range of virulence factors and biofilm dispersal. In this study, we identified three transcriptional regulators, i.e. Clp, FhrR and Zur, which function at the downstream of RpfC/RpfG in DSF signalling pathway. This is supported by several lines of evidence. First, expression of clp is partly dependent on DSF, and RpfC/RpfG. Second, overexpression of Clp in the DSF signal generation mutant ΔrpfF and the signal transduction mutant ΔrpfG, respectively, restored the production of EPS and extracellular enzymes to the wild-type level. Third, microarray analysis showed that DSF regulon (He et al., 2006a) and Clp regulon were largely overlapped with similar expression patterns. Similarly, previous microarray data showed that the genes encoding FhrR and Zur were regulated by DSF; deletion of rpfF that encodes DSF biosynthesis resulted in 9.2- and 1.2-fold decrease in the transcriptional expression of Xcc1215 that encodes FhrR and Xcc2685 that encodes Zur respectively (He et al., 2006a). This study established that expression of fhrR and
Table 2. Functional groups of Clp-, FhrR- and Zur-regulated genes.

| Gene family | Gene name or ID | Fold change<sup>b</sup> | WT/Δclp | WT/ΔfhrR | WT/Δzur |
|-------------|----------------|------------------------|---------|----------|---------|
| (i) Extracellular enzymes | | | | | |
| Cellulases | egl<sup>1</sup>, egl<sup>2</sup>, celS<sup>1</sup>, engXCA<sup>1</sup>, Xcc0027 Xcc3535 | 3.5 to 10.7 | – | – | |
| Pectate lyases | pgl<sup>A</sup>, Xcc0645 | 4.4 to 4.7 | – | – | – |
| Proteases | Xcc0852, Xcc2269, Xcc0851, Xcc2669, Xcc2601, Xcc2602 | 3.3 to 4.4 | – | – | – |
| Lipase and amylase | Xcc0375, Xcc3359 | 4.6 to 5.0 | – | – | – |
| Carbohydrate metabolism | Xcc2891, Xcc3162, Xcc3163 | 3.5 to 4.7 | – | – | – |
| (ii) LPS and EPS synthesis and secretion | | | | | |
| Gum proteins | gumK<sup>1</sup>, gumM, gumM<sup>B</sup>, gumDHI, kds<sup>A</sup>, galU<sup>1</sup>, Xcc0134, Xcc0606, Xcc2011, Xcc3035 | 1.9 to 8.7 | – | – | |
| Sugar kinase | Xcc3471 | 3.7 | – | – | – |
| Sugar transaminase | vioA | 4.1 | – | – | – |
| (iii) Multidrug resistance and de to xification | | | | | |
| Drug resistance | ftsI, acrF, acrAB, mexAB, pmrA, Xcc3489, dacc, yrbF, tolR | 3.5 to 13.2 | – | 3.2 to 6.2 | – |
| Oxidative stress resistance | sodC2, sodM, katE, hhpC, Xcc0395 | 3.6 to 8.1 | – | 3.6 to 8.1 | – |
| (iv) Flagellum synthesis and motility | | | | | |
| Flagellar and pilus proteins | fliMP, flgABL, fliCI, flgFK, flh<sup>A</sup>, fliR, flgE | 1.5 to 3.8 | – | 2.1 to 6.2 | – |
| | fliL, flgHL | – | 2.1 to 4.3 | – | – |
| | fliD, fliN | 3.3 to 3.8 | – | – | – |
| (v) Hypersensitive reaction and pathogenicity | | | | | |
| Hrp related proteins | hpb5D5, hrcR, Xcc2100, hpaP, hpb2, hpb7 | –2.3 to –4.1 | –2.0 to –4.3 | – | – |
| Avirulence proteins | Vdr<sup>3</sup>, Vdr<sup>B</sup>, Xcc2153 | – | –2.7 to –3.4 | – | – |
| Virulence proteins | Xcc2109, Xcc21100 | – | 2.1 to 3.0 | – | – |
| (vi) Iron uptake | | | | | |
| TonB proteins | tonB<sup>1</sup>, bfeA<sup>1</sup>, lepA<sup>1</sup>, fyuA<sup>1</sup>, fecA<sup>1</sup>, bfu<sup>B</sup>, hha<sup>A</sup>, Xcc2944, Xcc3358 | 3.2 to 5.4 | – | 2.7 to 4.7 | – |
| Other proteins | exb<sup>B</sup>, exb<sup>D</sup>, exb<sup>D2</sup>, Xcc3216 | 3.2 to 11.5 | – | 3.2 to 7.1 | – |
| | Xcc1738<sup>1</sup> | 4.7 | – | – | – |
| (vii) Amino acid and protein metabolism | | | | | |
| Chaperone and peptidases | groEL, scoF, tld<sup>D</sup>, Xcc0094, Xcc2820, Xcc2821 Xcc0076<sup>1</sup>, pah, prf<sup>C</sup>, pth, ppi<sup>B</sup><sup>1</sup>, htk<sup>1</sup>, Xcc1105<sup>1</sup>, Xcc1116, flpA, hflX, hisD, prf<sup>B</sup>, inf<sup>A</sup>, Xcc2082<sup>1</sup>, sly<sup>D</sup>, dsbA, Xcc3506<sup>1</sup>, dnaK, csp<sup>A</sup>, htpG | 3.3 to 20 | – | – | – |
| Ribosomal proteins | dhaA<sup>1</sup>, murF<sup>1</sup>, pms, Xcc1420<sup>1</sup>, Xcc1535, Xcc3494, ccb<sup>A</sup> | −3.8 to −5.0 | – | – | – |
| | rpsC<sup>ULS</sup>, rplCPW, rpsM, Xcc0891, Xcc0906, Xcc0910, Xcc0911<sup>1</sup>, Xcc0912, Xcc1375, Xcc2460, Xcc3226, Xcc1370 | 2.1 to 5.7 | 2.5 to 7.2 | – | – |
| | Xcc0904, Xcc0908, rpsD, rplU | 4.0 to 5.4 | – | – | – |
| Ribosomal protein | Xcc0909, Xcc0914, Xcc1199 | – | 3.0 to 5.0 | – | – |
| (viii) TCA cycle | | | | | |
| Dehydrogenase | idp<sup>1</sup>, mdh<sup>A</sup>, icd<sup>A</sup>, othA | 4.1 to 16.4 | – | 3.7 to 7.8 | – |
| Succinate metabolism | succ<sup>B</sup>C<sup>D</sup>, sdh<sup>B</sup>C<sup>D</sup>, sdh<sup>A</sup> | 3.3 to 14.8 | – | 3.2 to 8.9 | – |
| Carbohydrate synthase | ppi<sup>C</sup>, acn<sup>A</sup>, acn<sup>B</sup>, gltA | 4.7 to 14.6 | – | 3.9 to 6.3 | – |
| Gene family | Gene name or ID | Fold change<sup>b</sup> | WT/Δclp | WT/ΔfrhR | WT/Δzur |
|-------------|----------------|-------------------------|---------|----------|---------|
| (ix) Aerobic and anaerobic respiration | CyoABD, petC, nuoCEF1KM, qxtAB | cydAB, nuoH | 3.5 to 7.6 | – | 2.9 to 6.4 |
| Oxidases and oxidoreductase | Dehydrogenase and | | | | |
| CoA-transferase | lactD, tktA | | 3.3 to 3.8 | – | – |
| | gctAB | | 2.6 to 2.7 | – | 2.6 to 2.7 |
| (x) Transcription regulators | Xcc1215<sup>[1]</sup>, Xcc1847, Xcc2464, Xcc2685<sup>[1]</sup>, Xcc2704<sup>[1]</sup>, Xcc2735, Xcc2748<sup>[1]</sup>, Xcc3880, Xcc0472<sup>[1]</sup> | Xcc2065<sup>[1]</sup>, Xcc3349 | – | – | – |
| Regulators to rs | Xcc2065<sup>[1]</sup>, Xcc3349 | – | 3.1 to 4.4 | – | 3.6 to 4.3 |
| RNA polymerase | Xcc2065, Xcc0381, Xcc3619<sup>[1]</sup> | – | – | – | – |
| | Xcc2065, Xcc0381, Xcc3619<sup>[1]</sup> | – | – | – | – |
| (xi) Membrane components and transporters | Xcc156, Xcc2532 | Xcc2098, Xcc1524<sup>[1]</sup>, Xcc2781<sup>[1]</sup>, Xcc0312, Xcc3277<sup>[1]</sup> | 3.3 to 11.5 | – | – |
| Membrane proteins | Xcc3194 | – | 8.7 | 4.3 | 3.2 |
| ATP-synthases | ydgJ | – | 4.7 | – | – |
| | atpBEF | 5 to 12.3 | – | – | 3.7 to 5.3 |
| | atpHAGD, yrbE, cysW, Xcc1381 | – | 3.3 to 12.3 | – | – |
| (xii) Fatty acid metabolism and others | Xcc0153, Xcc0739<sup>[1]</sup>, Xcc1016, Xcc1019<sup>[1]</sup>, Xcc1697, Xcc2094<sup>[1]</sup> | Xcc2166<sup>[1]</sup>, Xcc2199, Xcc2244, Xcc2770, Xcc2886<sup>[1]</sup>, Xcc2946, Xcc3194<sup>[1]</sup>, Xcc3249<sup>[1]</sup>, Xcc3696<sup>[1]</sup> | 3.3 to 11.5 | – | – |
| Fatty acid and others | Xcc3194 | – | 8.7 | 4.3 | 3.2 |
| | Xcc3194 | – | 4.7 | – | – |
| Phage-related | BioB, BioF, BioD | – | 5.7 | – | – |
| Biotin synthesis-related | BioB, BioF, BioD | – | 5.7 | – | – |
| | BioB, BioF, BioD | – | 5.7 | – | – |
| (xiii) Hypothetical proteins | BioB, BioF, BioD | – | 5.7 | – | – |
| Hypothetical proteins | Xcc0538<sup>[1]</sup>, Xcc1181, Xcc3531<sup>[1]</sup>, Xcc0274, Xcc6324<sup>[1]</sup>, Xcc0717, Xcc1246<sup>[1]</sup>, Xcc1325, Xcc2039, Xcc2134<sup>[1]</sup>, Xcc3041 | Xcc2783, Xcc3504, Xcc0202, Xcc0537, Xcc0573, Xcc0951, Xcc1718, Xcc2203, Xcc2390, Xcc2513 | 3.3 to 7.1 | – | – |
| | Xcc2098, Xcc1524<sup>[1]</sup>, Xcc2781<sup>[1]</sup> | – | 3.5 to 10.0 | – | 3.4 to 4.5 |
| | Xcc0312, Xcc3277<sup>[1]</sup> | – | – | – | – |
| | Xcc0312, Xcc3277<sup>[1]</sup> | – | – | – | – |
| Hypothetical proteins | Xcc3194 | – | – | – | – |
| | Xcc3194 | – | – | – | – |
| | Xcc3194 | – | – | – | – |
| Total gene number | Overlapping 94 genes in DSF regulon | 299 (-39) | 53 (-13) | 94 (-5) |

<sup>a</sup> The DSF-regulated genes (He et al., 2006a) were indicated by bold font with the negatively regulated being underlined. Symbol ‘¶’ indicates the gene with a putative Clp-binding motif in promoter region; the details were given in Table S2.

<sup>b</sup> The detailed fold changes were listed in Table S3.
zur were also influenced by Clp, and constitutive expression of the two genes, respectively, could rescue the clp deletion mutant on certain phenotypes. Furthermore, microarray analysis showed that FhrR and Zur regulate different subsets of the genes within the Clp regulon. Our data, together with others, thus outline a hierarchical DSF signalling network that consists of at least five key components, i.e. RpfC, RpfG, Clp, FhrR and Zur, with Clp regulating Fhr and Zur in parallel.

Clp is a member of the conserved global regulator family. Within this family, the Crp of E. coli directly regulates about 192 genes including a dozen encoding transcription factors such as the ferric uptake regulator Fur (Martinez-Antonio and Collado-Vides, 2003; Zheng et al., 2004), and a mutation in Vfr of P. aeruginosa affects production of at least 60 proteins (Suh et al., 2002). Consistent with its role as a conserved global regulator, microarray analysis showed that the Clp of Xcc influenced the expression of 299 genes. Similar to Crp, Clp may also regulate a large set of genes directly. Clp shares a conserved DNA-binding domain with Crp and Vfr, and previous studies showed that it could bind to Crp-specific promoter and substitute for Crp in transcriptional activation of a target gene (de Crecy-Lagard et al., 1990; Dong and Ebright, 1992). A recent study by using gel retardation showed that Clp protein could bind to the promoter region of engXCA (Hsiao et al., 2005). Highly consistently, we found that about 86 genes within the Clp regulon, including the engXCA gene encoding a cellulase, Xcc1215 encoding FhrR and Xcc2685 encoding Zur, contain at least one putative Crp-binding specific sequence at the promoter region. The genes within Clp regulon encode products belonging to 12 functional categories. In addition to the genes encoding extracellular enzymes and EPS synthesis, Clp also regulates a range of other genes encoding flagellum synthesis, protein metabolism, multidrug resistance, Hrp system, iron uptake, TCA cycle, respiration and membrane components, and fatty acid metabolism. The data are agreeable with the previous genetic studies that Clp regulates the transcriptional expression of varied genes in different Xcc strains, including the cellulase gene engXCA, the protease gene ptr1, the flagellin gene fliC and the heat-shock protein gene groESL (de Crecy-Lagard et al., 1990; Hsiao and Tseng, 2002; Lee et al., 2003; Chang et al., 2005; Hsiao et al., 2005).

We showed recently that the hybrid sensor kinase RpfC modulates DSF signal synthesis by utilization of a novel domain-specific protein–protein interaction mechanism and regulates virulence factor production through a conserved phosphorelay system (He et al., 2006b). The latter signalling pathway is dependent on its cognate response regulator RpfG, but the former is not. The further signal transduction from RpfG to downstream regulatory elements seems to rely on a novel second messenger c-di-

![Fig. 4. Characterization of two transcription factors that function at the downstream of Clp. A. Null mutation of Clp and FhrR, respectively, decreased Xcc bacterial cell motility. Constitutive expression of fhrR in the clp deletion mutant restored the wild-type phenotype. B. The predicted domain structure of transcription factors FhrR and Zur of Xcc by SMART program. C. Clp and Zur regulate resistance to acriflavin (AF) and hydrogen peroxide (HP). Constitutive expression of zur in the clp deletion mutant restored the wild-type phenotypes.](image-url)
GMP. It has been demonstrated that RpfG functions by degrading c-di-GMP (Ryan et al., 2006). Identification of Clp as the key global regulator in DSF signalling pathway may provide a clue on how the nucleotide signal influenced by RpfG is transduced to downstream genes. Similar to Crp and Vfr, Clp contains a conserved helix-turn–helix domain associated with DNA-binding and a conserved cNMP-binding domain. Crp functions as a dimer in the form of a Crp–cAMP complex (Weber and Steitz, 1987; Lee et al., 1994), and directly regulates transcription initiation by binding to a symmetrical DNA sequence (consensus sequence 5′-AAATGTGATCTA GATCACATTT-3′), located near or within the promoter regions (Zheng et al., 2004). At Crp-dependent promoters, Crp activates transcription by making direct protein–protein contacts with RNA polymerase. Crp also binds to cGMP, a structural analogue of cAMP, but the binding does not seem to activate the protein for specific DNA binding (Ebright et al., 1985; Gorshkova et al., 1995). However, one amino acid residue substitution (S128T) in Crp makes the protein responsive to cGMP (Lee et al., 1994), which suggests that Clp, which contains a threonine in the position corresponding to the S128 of Crp, may be able to bind to cGMP or c-di-GMP. In connection with this possibility, it is interesting to note that Vfr, which also has a threonine counterpart of Crp S128, appears to be capable of responding to either cAMP or cGMP signals and hence activating the expression of different sets of genes (Beaton et al., 2002). The detailed mechanism by which the RpfG and c-di-GMP interaction modulating clp expression remains to be investigated.

Xcc strain-dependent variation in virulence has been documented (Qian et al., 2005), but the underlying biochemical and genetic mechanism remains elusive. Identification of Clp as a key component in DSF-dependent signalling regulation of virulence and the observed strain-dependent variation in Clp-dependent functions seem to provide an explanation. Similar to the clp mutant of strain NRRLAB1459 (De Crecy-Lagard et al., 1990), our data showed that deletion of clp in strain XC1 resulted in reduced EPS production and decreased cellulase activity. However, in contrast to the former, which produces significantly more proteases than its parental strain (De Crecy-Lagard et al., 1990), the clp mutant of XC1 showed a decreased protease activity than the wild-type strain. We also found that deletion of clp resulted in much more severe reduction in EPS production and extracellular enzyme activity than the null mutants of RpfF or RpfG. This is consistent with the microarray data that deletion of any gene involved in DSF signal generation or sensing, i.e. rpfF, rpfC and rpfG, caused a mild 1.5- to 1.8-fold reduction in clp transcription (Table 1, OD600 = 1.6), whereas comparison of the clp deletion mutant and its wild-type strain at the same bacterial density indicated a more than 11-fold change in clp transcription (Table S3). Given that more than one gene encodes proteins containing a HD-GYP domain in Xcc genome (Da Silva et al., 2002; Qian et al., 2005), and other widely distributed protein domains such as GGDEF and EAL, are also implicated in c-di-GMP metabolism (Römling et al., 2005), it is highly possible that clp expression is also subject to modulation by other signalling pathway(s) other than the RpfC/RpfG-mediated DSF cell–cell communication mechanism.

The predicted peptide sequences of the transcriptional regulators FhrR and Zur of strain XC1 are identical with their counterparts encoded by Xcc1215 and Xcc2685 of strain ATCC33913 (Da Silva et al., 2002) and those encoded by Xc3027 and Xc1403 of strain 8004 (Qian et al., 2005) respectively. While the FhrR of Xcc shares only weak local homology at the N-terminal region with other homologues, such as the AcrR of E. coli that regulates the expression of a multidrug efflux pump AcrAB (Ma
et al., 1996), the Zur of Xcc strains shares 43% and 42% amino acid identity with the Zur of E. coli (GenBank Accession No. NP_418470) and that of Salmonella typhimurium (No. NP_463106) respectively. In E. coli, Zur regulates zinc uptake by modulating the expression of the ZncABC high-affinity zinc uptake system (Patzer and Hantke, 1998). The zur knockout mutant of S. typhimurium showed reduced virulence when mice were inoculated intraperitoneally (Campoy et al., 2002). The zur-disrupted mutant of strain 8004 showed reduced EPS production, attenuated zinc tolerance and decreased virulence on the host plant Chinese radish (Tang et al., 2005). Consistent with the general role of Zur in bacterial virulence, microarray analysis of the zur-deletion mutant of strain XC1 showed that Zur regulates more than 90 genes, encoding proteins and enzymes required for iron uptake, multidrug resistance and detoxification, which collectively could be important for the pathogen to establish infection and survival in host plant. However, in contrast to strain 8004 (Tang et al., 2005), deletion of zur in strain XC1 did not appear to influence the EPS gene expression based on the microarray analysis presented in this study and EPS production assay (data not shown), suggesting the existence of strain-dependent variations in Zur-modulated biological functions.

Except for FhrR, the roles in pathogenicity of other key members in DSF signalling pathway, including RpfF, RpfG, Clp and Zur, have been demonstrated using two Xcc strains (de Crecy-Lagard et al., 1990; Barber et al., 1997; Tang et al., 2005). In agreement with the previous studies as well as our microarray data, we found that null mutation of either rpfF or rpfG or clp in strain XC1 resulted in most significant reduction of the virulence on Chinese cabbages, whereas deletion of zur or fhrR decreased to a less extent the pathogenicity of the pathogen (Fig. S2).

Taken together, our results, along with others, suggest a signal regulatory pathway, which is essential for regulation of bacterial virulence, for sensing and responding to DSF cell–cell communication signals in Xcc. In this model (Fig. 6), the sensor kinase RpfC interacts with RpfF to modulate DSF signal generation (He et al., 2006b); upon reaching a threshold concentration at extracellular environment, DSF signal is transduced through RpfC sensor to RpfG response regulator via a conserved phosphorylation mechanism (He et al., 2006b); the activated RpfG degrades c-di-GMP second messenger (Ryan et al., 2006), and the event consequently activates Clp, which modulates gene expression directly by binding to the promoters of target genes or indirectly through the downstream transcription factors including FhrR and Zur. Independent from Clp, RpfG may also control another set of genes, including those implicated in biofilm dispersal (Dow et al., 2003; He et al., 2006a), which awaits further investigations.

**Experimental procedures**

**Bacterial strains and growth conditions**

Xcc wild-type strain XC1 has been described previously (Wang et al., 2004). 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 and complementation**

A spontaneous rifampicin-resistant derivative of strain XC1R was used as a parental strain for generation of deletion
mutants. In-frame deletion mutants of clp, zur and fhrR were generated using the primers listed in Table S4 following the methods described previously (He et al., 2006a). For complementation, the coding regions of these regulatory proteins were amplified by PCR using the primers listed in Table S4 and cloned under the control of lac promoter in expression vector pLAFR3 respectively. The resultant constructs were transferred into Xcc strains through triparental mating.

Quantitative determination of enzyme activity and EPS

The extracellular cellulase and protease activity in the culture supernatants of Xcc strains were analysed according to the methods described previously (Boyer et al., 1984; Swift et al., 1999). For analysing EPS production, 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 to the supernatants and the mixtures kept at −20°C for half an hour. The precipitated EPS molecules were spun down and dried at 55°C overnight before determination of dry weight.

Oligomicroarray analysis

Bacterial cells were collected at OD600 = 1.6 or otherwise indicated by centrifugation at 4°C for 5 min at 10 000 r.p.m. Total RNA samples were prepared by using RNeasy midi column following the manufacturer’s instruction (Qiagen). cDNA was generated by using random hexamers as primers for reverse transcription (Invitrogen). cDNA labelling and oligonucleotide microarray analysis were conducted as described previously (He et al., 2006a). Each treatment had at least three replicates and the genes showing ≥ 2-fold changes (wild type versus mutant in hybridization signal ratio) in at least two replicates were selected. The data presented were the means of two representative replicates.

Genome scale searching of Clp binding sites

A PERL script was used to parse and match the promoter sequences of the Clp-regulated genes using the putative Clp-binding motif ‘TGTGA-N6-TCACA’ (Dong and Ebright, 1992). The promoter region sequence, which was arbitrarily defined as a 500 bp region upstream of an open reading frame, was retrieved from the published genome sequence of Xcc strain ATCC33913 (Da Silva et al., 2002). A confidence of 60% match to the non-variable sequences of the putative Clp-binding motif is taken as the cut-off value. Only the best match was listed in the case when more than one putative motifs were located per promoter.

Motility assay

Bacterial cells were inoculated onto low-agar-concentration (0.3%) XLON plates (Lee et al., 2003). The plates were incubated at 28°C for 36 h, and motility was assessed qualitatively by examining the circular halo formed by the motile bacterial cells.

Acriflavin and H2O2 resistance assay

Bacterial strains were grown in YEB medium until the OD600 of the cultures reached about 1.0; acriflavin and H2O2 were added to a final concentration of 2.5 μg ml−1 and 0.03% respectively. Bacterial population density at 2, 4, 6 and 8 h after addition of the toxic chemicals were determined by plate-counting of colony-forming units (cfu) as described previously (Hu et al., 2003). Within this assay period, XC1 and mutants Δclp, Δzur, ΔfhrR, and other derivatives showed no difference in growth rate in the absence of toxic chemicals. The cfu data at 6 h after chemical treatment were presented.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. RT-PCR analysis of ctp, zur and fhrR transcript in Xcc wild-type and mutants.

Fig. S2. Virulence testing of XC1 and derived mutants.

Table S1. Ctp is essential for DSF-dependent virulence factors production.

Table S2. The putative genes directly regulated by Ctp.

Table S3. Functional groups of Ctp, FhrR and Zur regulated genes.

Table S4. Oligos used in this study.

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