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A bacterial cell–cell communication signal with cross-kingdom structural analogues

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Summary

Extracellular signals are the key components of microbial cell–cell communication systems. This report identified a diffusible signal factor (DSF), which regulates virulence in *Xanthomonas campestris* pv. *campestris*, as *cis*-11-methyl-2-dodecenoic acid, an α,β unsaturated fatty acid. Analysis of DSF derivatives established the double bond at the α,β positions as the most important structural feature for DSF biological activity. A range of bacterial pathogens, including several *Mycobacterium* species, also displayed DSF-like activity. Furthermore, DSF is structurally and functionally related to farnesoic acid (FA), which regulates morphological transition and virulence by *Candida albicans*, a fungal pathogen. Similar to FA, which is also an α,β unsaturated fatty acid, DSF inhibits the dimorphic transition of *C. albicans* at a physiologically relevant concentration. We conclude that α,β unsaturated fatty acids represent a new class of extracellular signals for bacterial and fungal cell–cell communications. As prokaryote–eukaryote interactions are ubiquitous, such cross-kingdom conservation in cell–cell communication systems might have significant ecological and economic importance.

Introduction

Xanthomonas campestris pv. *campestris* (Xcc) is a major bacterial pathogen of cruciferous plants worldwide. The pathogen produces polysaccharide and extracellular enzymes (including proteases, pectinases and endoglucanase) that are key virulence factors (Onsando, 1992; Chan and Goodwin, 1999). Transposon mutagenesis of *rpfF*, which encodes a putative enoyl CoA hydrotase, reduced polysaccharide production, extracellular enzyme production and virulence (Barber *et al*., 1997). Xcc culture extract restored the phenotypes of *rpfF* mutants, indicating the presence of a diffusible signal factor (DSF). A putative long-chain fatty acyl CoA ligase, encoded by *rpfB*, is also implicated in DSF biosynthesis (Barber *et al*., 1997). Expression of several virulence genes, e.g. the *engXCA* gene encoding endoglucanase and the genes encoding proteases, was shown to be inducible by DSF in Xcc strain 8004 (Barber *et al*., 1997; Slater *et al*., 2000; Vojnov *et al*., 2001). Partial characterization of DSF indicated that it might be a fatty acid derivative (Barber *et al*., 1997). Mutation of *rpfC*, which encodes a hybrid two-component regulatory protein, resulted in overproduction of DSF and attenuation of virulence (Slater *et al*., 2000). It was proposed that the RpfC–RpfG two-component regulatory system plays a key role in cell–cell signalling for regulation of virulence through sensing and transducing environmental cues, including DSF (Slater *et al*., 2000).

Cell–cell communication by means of small signal molecules is of vital importance to both prokaryotes and eukaryotes. In the prokaryotic kingdom, several families of cell–cell communication signals have been identified, which regulate a range of biological functions, e.g. bioluminescence, plasmid transfer, virulence and biofilm formation (Eberhard *et al*., 1981; Ji *et al*., 1995; Flavier *et al*., 1997; Fuqua *et al*., 2001; Miller and Bassler, 2001; Chen *et al*., 2002). Yeast and *Candida albicans*, single-celled eukaryotic organisms, exploit oligopeptide pheromones and farnesoate-like signals for the regulation of mating and cell morphological transition respectively (Herskowitz, 1995; Elion, 2000; Hornby *et al*., 2001; Oh *et al*., 2001). Genome comparison has shown extensive conservation among metabolic enzymes in prokaryotes and eukaryotes (Jardine *et al*., 2002). A recent finding indicates that cross-kingdom conservation might also exist in cell–cell communication systems (Gallio *et al*., 2002). Both the fly *Drosophila* and the bacterium *Providentia stuartii* rely on conserved serine proteases to mediate proteolytic activation of signal ligands in extracellular signalling, which reg-

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ulates animal epidermal growth factor-dependent cell fate specification in the former and population density-dependent gene expression in the latter (Gallio *et al*., 2002). The potential cross-kingdom cell–cell communication signals have yet to be identified. In this report, we present evidence that DSF, a cell–cell communication signal produced by bacterial pathogen Xcc, is a structural and functional homologue of farnesoic acid, a fungal extracellular signal (Oh *et al*., 2001).

Results

Identification of DSF overproduction mutants

To identify DSF overproduction mutants, we screened about 7000 Xcc strain XC1 mutants, generated by Tn*5* random insertion, with the DSF biosensor strain Xcc FE58. Two of the mutants, Xc1853 and Xc4199, produced significantly more DSF than the wild-type Xcc strain XC1. Sequence analysis showed that Xc1853 and Xc4199 contained Tn*5* insertions in *rpfC* and *rpfH* respectively (Fig. 1A and B). The two genes from the Asian strain XC1 encode identical peptide sequences to their counterparts from the European Xcc isolate ATCC33913 (Da Silva *et al*., 2002). The exact Tn*5* insertion was at a position corresponding to the 912th basepair of the 2181 bp coding sequence of *rpfC* and the 260th of the 630 bp coding sequence of *rpfH.* The *rpfC* gene encodes a hybrid two-component regulatory protein containing the sensor and regulator domains, whereas RpfH is structurally related to the sensory input domain of RpfC (Slater *et al*., 2000). Our data are consistent with the finding that RpfC acts negatively to regulate the synthesis of DSF (Slater *et al*., 2000). The *rpfH* mutant Xc4199 produced more DSF than the wild-type XC1 but less than the *rpfC* mutant Xc1853. This is consistent with the finding that *rpfG*, *rpfH* and *rpfC* are transcribed as an operon from a common promoter, but *rpfC* may also have its own weak promoter situated within *rpfH* (Slater *et al*., 2000). Apparently, the Tn*5* insertion in *rpfH* blocked the transcription of *rpfC* from the common promoter, but did not interrupt the activity of its weak promoter (Fig. 1A).

We compared the DSF production time course of the mutant Xc1853 with that of the wild-type strain XC1. Both strains grew at similar rates in YEB medium, and maximal DSF production occurred at about 36 h after inoculation; however, mutation in RpfC led to about a 16-fold increase in DSF production at the peak time (Fig. 1C and D).

DSF purification and structural characterization

We prepared active extracts from 30 l of culture supernatants of the *rpfC* mutant Xc1853 by ethyl acetate extraction and flash column chromatography. We also purified the collected active component, which was about 90% pure, using high-performance liquid chromatography (HPLC). We monitored DSF activity across the eluted fractions using the DSF biosensor FE58 and found a single peak at 11.2 min in HPLC elute showing a maxi-

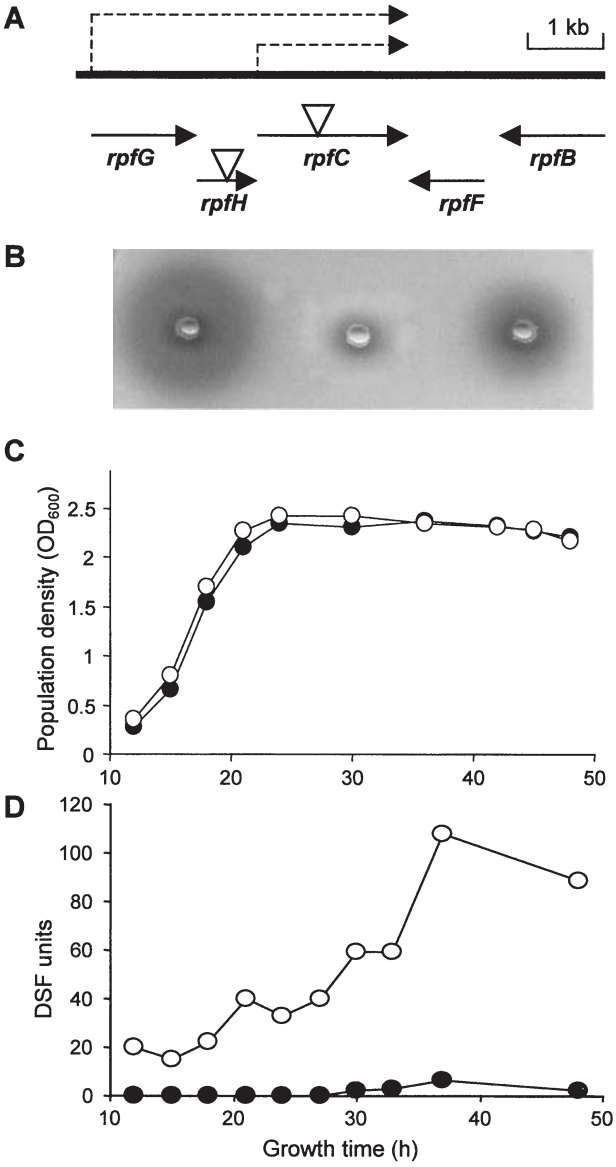


Fig. 1. DSF production in Xcc strain XC1 and its derivatives.
A. Transcriptional organization of the *rpfGHC* locus. The dashed arrows indicate the sites and direction of transcription (Slater *et al*., 2000). The solid arrows indicate the ORFs of the *rpf* genes. The open triangles mark the relative locations of Tn*5* insertions in *rpfH* and *rpfC*.
B. DSF bioassay with the supernatants of XC1 (middle), the *rpfC*::Tn*5* mutant Xc 1853 (left) and the *rpfH*::Tn*5* derivative Xc4199 (right).
C. The growth curves of XC1 (solid circle) and Xc 1853 (open circle).
D. DSF production by strains XC1 (solid circle) and Xc1853 (open circle). At each time point, 10 ml of bacterial culture was collected. The supernatant was extracted with the same volume of ethyl acetate. After evaporation, the residue was dissolved in methanol and diluted for bioassay. The data are the means of two repeats.

mum UV absorption at 210 nm and strong DSF activity (Fig. 2A). About 2 mg of pure DSF was obtained from the 30 l of culture supernatants after concentration and evaporation of the solvent.

High-resolution electrospray ionization mass spectrometry (ESI-MS) showed the *m/z* of DSF $(M\text{-}H)^{\bullet}$ to be 211.1691, suggesting a molecular formula of $C_{13}H_{23}O_2$ (211.1698). The ^{13}C nuclear magnetic resonance (NMR) spectrum shows that there are 12 peaks that represent 13 carbons as stated below (Fig. 2B). The nine ^{13}C peaks in the range of 20–40 p.p.m. were most likely from acyclic hydrocarbons. The ^{1}H-^{13}C correlated heteronuclear multiple quantum coherence (HMQC) data indicate that the peak at 22.62 p.p.m. is correlated with the six aliphatic protons at 0.80 p.p.m., probably representing two terminal CH_3 groups (C-12 and C-13). The peak at 27.94 p.p.m. is correlated to a single proton at 1.45 p.p.m., easily assignable to C-11. The remaining seven ^{13}C peaks in the 20–40 p.p.m. range represent seven CH_2 groups (C-4 to C-10) based on HMQC and distortionless enhancement by polarization transfer (DEPT) spectra. Among three carbon signals over 100 p.p.m., two at 118.61 and 153.46 p.p.m. are correlated with the two olefinic protons at 5.73 and 6.29 p.p.m., respectively, and easily assigned to the double bond by the ^{1}H-^{13}C correlated HMQC spectrum (C-2, C-3). These two olefinic proton signals couple to each other with a coupling constant of 11.1 Hz, thus establishing the *cis* configuration of the double bond. A quaternary carbon peak at 170.24 p.p.m. should be assigned to a carbonyl (C-1), which conjugates to the double bond based on the ^{1}H-^{13}C correlated heteronuclear multiple bond coherence (HMBC) spectrum. Analysis of the ^{1}H-^{1}H correlation spectroscopy (COSY) spectrum of DSF established the proton-coupling relationships among hydrocarbon groups and showed the presence of an aliphatic chain. These data indicate that DSF is *cis*-11-methyl-2-dodecenoic acid (Fig. 2C), a novel α,β unsaturated fatty acid.

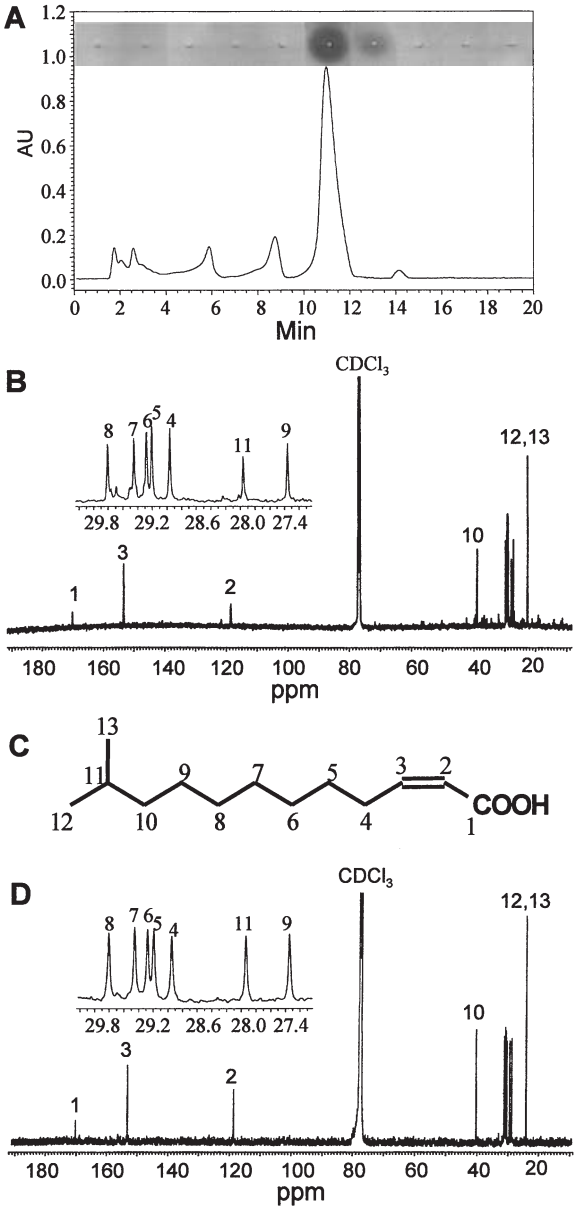


Fig. 2. Purification and NMR analysis of DSF.
A. HPLC analysis of the active fraction after flash column chromatography. Inset: bioassay of the HPLC fractions. The eluted fractions were collected every 2 min and bioassayed; the blue halo indicates the DSF activity.
B. The ^{13}C NMR spectra of the natural DSF.
C. The predicted chemical structure of DSF.
D. The ^{13}C NMR spectra of the synthetic *cis*-11-methyl-2-dodecenoic acid. The NMR analyses were conducted at room temperature ($CDCl_3$, 125 MHz).

To confirm the identity of DSF, we synthesized *cis*-11-methyl-2-dodecenoic acid, and found that its ^{13}C NMR spectrum (Fig. 2D) is virtually indistinguishable from that of natural DSF (Fig. 2B). Figure 3A shows that the synthetic and natural DSF samples had a similar activity in activating the *engXCA* promoter, which was fused to the GUS reporter gene; the maximal enzyme activity was detected about 4 h after the addition of DSF (Fig. 3A). We then tested whether natural and synthetic DSF could restore protease production in the *rpfF::*Tn*5* mutant FM3, which is deficient in DSF production. Figure 3B shows that DSF at a final concentration of 1 μM, regardless of whether the inducer was natural or synthetic, stimulated protease production at a similar level.

DSF inhibition of Candida albicans *germ tube germination*

DSF is structurally related to 3,7,11-trimethyl-2,6,10-dodecatrienoic acid (farnesoic acid) (Fig. 4), which is also an α,β unsaturated fatty acid, known as autoregulatory substance (ARS) in *Candida albicans. C. albicans* normally exists as a yeast but can form germ tubes (mycelia)

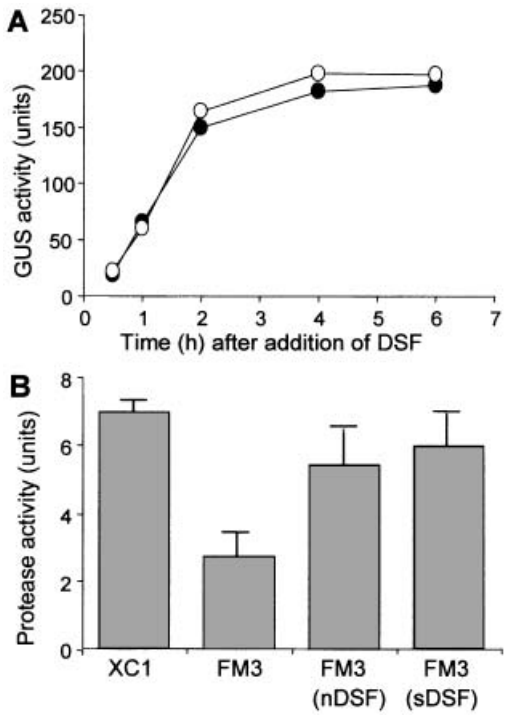


Fig. 3. DSF induction of endoglucanase and protease expression in Xcc strains.
A. To determine the DSF-dependent expression pattern of the endoglucanase encoded by *engXCA*, the DSF biosensor strain FE58, in which the GUS reporter gene was fused to the *engXCA* promoter, was cultured and induced by DSF. The bacterial cultures were collected at different time points after the addition of purified (solid circle) or synthetic DSF (open circle), and the GUS activities were determined as described.
B. Synthetic (sDSF) and purified DSF (nDSF) samples complemented the production of extracellular protease by the *rpfF::*Tn*5* mutant FM3 derived from XC1.

in response to unknown environmental cues. The germ tubes can invade human cells to enter the bloodstream and cause disease. Farnesoic acid (FA) inhibits *C. albicans* germ tube formation and plays a key role in the regulation of the morphological transition in *C. albicans* (Oh *et al*., 2001). We found that FA induced the *engXCA* promoter-directed expression of *gusA* but was about 2000-fold less active than DSF (Fig. 4). We then tested the effect of DSF on *C. albicans* and found that it had a slightly weaker activity than FA in the prevention of the yeast-to-mycelium conversion (Fig. 5). Further titration analysis showed that the minimum concentrations of FA and DSF for inhibiting germ tube growth were 2 and 20 μM respectively.

Biological activity of DSF derivatives

To identify the structural features of DSF that are important for biological activity, we synthesized a range of DSF derivatives (Fig. 4). In the plate bioassay, the minimum concentration of DSF required for induction of the DSF biosensor was about 0.5 μM, which is 200-fold lower than that of the DSF conformational isomer (*trans*-11-methyl-2-dodecenoic acid) and 20 000-fold lower than that of the corresponding saturated fatty acid (11-methyldodecanoic acid) (Fig. 4). These data demonstrated that the double bond at the α,β positions and *cis* configuration of the fatty acid play a critical role in the biological activity. Furthermore, the methyl group at the C-11 position also plays a certain role in the activity, as *cis*-2-tridecenoic acid and *cis*-2-dodecenoic acid, the close derivatives of DSF lacking the methyl substitution, were 60-fold and 120-fold less active than DSF respectively (Fig. 4). To determine the role of fatty acid chain length, we tested a range of DSF derivatives, including saturated fatty acids and *cis* and *trans* α,β unsaturated fatty acids. In general, the DSF derivatives that have the same fatty chain length as DSF exhibited higher activities than those with longer or shorter carbon chains (Fig. 4).

DSF might be a widely conserved signal

Previous studies have shown that 12 strains of *X. campestris* and one *Erwinia herbicola* isolate produced detectable DSF-like activity (Barber *et al*., 1997). A database search found that several other bacterial species, including *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* (Cole *et al*., 1998; Stover *et al*., 2000), contain RpfF homologues. We then randomly assayed 31 bacterial strains from 13 bacterial species. Except for *Agrobacterium tumefaciens*, *Salmonella enterica* and *Mycobacterium marinum*, the DSF-like activity was detected in the 25 strains of the other 10 bacterial species, i.e. Xcc, *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas oryzae* pv. *oryzicola*, *Xanthomonas albilineans*, *Pseudomonas aeruginosa*, *Mycobacterium avium*, *Mycobacterium chelonae*, *Mycobacterium smegmatis*, *Mycobacterium intracellulare* and *Mycobacterium kansasii* (Table 1). As stringent structural features are required for DSF activity (Fig. 4), it is rational to predict that these bacterial species might also produce α,β unsaturated fatty acids structurally similar to the DSF of Xcc.

Discussion

DSF, a cell–cell communication signal involved in the regulation of virulence gene expression, was initially reported in five of the six tested European Xcc isolates (Barber *et al*., 1997). The genetic and biochemical data presented here indicate that the pathogenic Xcc isolate XC1 also produces DSF (Fig. 1). Transposon disruption of RpfC, a hybrid two-component regulatory protein, resulted in about a 16-fold increase in DSF production, which significantly facilitated DSF purification. The NMR and mass

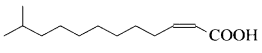
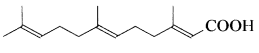
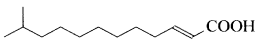
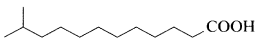
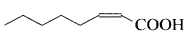
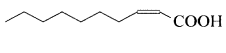
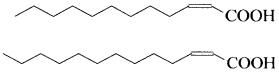
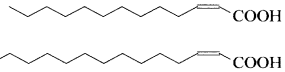
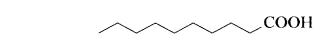
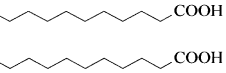
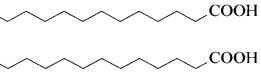
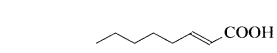
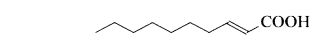
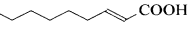
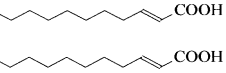
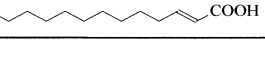
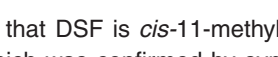
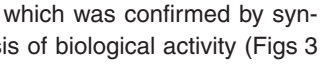
DSF and derivatives	Structure	Minimum concentration required for activity (μM)
Cis-11-methyl-2-dodecenoic acid (DSF)†		0.5
3,7,11-trimethyl-2,6,10-dodecatrienoate (FA)‡		1,000
Trans-11-methyl-2-dodecenoic acid‡		100
11-methyldodecanoic acid		10,000
Cis-2-octenoic acid‡		-
Cis-2-decenoic acid‡		3,000
Cis-2-dodecenoic acid‡		60
Cis-2-tridecenoic acid‡		30
Cis-2-pentadecenoic acid‡		5,000
Decanoic acid		-
Lauric acid		20,000
Tridecanoic acid		30,000
Pentadecanoic acid		-
Trans-2-octenoic acid		-
Trans-2-decenoic acid		10,000
Trans-2-dodecenoic acid‡		2,000
Trans-2-tridecenoic acid‡		800
Trans-2-tetradecenoic acid‡		10,000

Fig. 4. The biological activity of DSF and its derivatives. Samples were diluted, and the minimum concentration of each chemical required to induce the formation of visible blue halo on the bioassay plate were presented. The data were the means of two repeats. †, Purified and synthetic DSF showed identical biological activity; ‡, the compounds were synthesized in the laboratory, identified by ESI-MS and ^{1}H NMR spectra; –, no activity in the concentration range from 0.1 to 30 000 μM.

spectrometry data suggested that DSF is *cis*-11-methyl-2-dodecenoic acid (Fig. 2), which was confirmed by synthetic chemistry and analysis of biological activity (Figs 3 and 4).

Comparison of the biological activities of a range of DSF derivatives identified a few key structural features that determine DSF activity. The most important feature is the unsaturated double bond at the α,β position of DSF; the saturated derivative was about 20 000 times less active than DSF (Fig. 4). The other features, including the *cis* 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 (Fig. 4). Interestingly, two groups of bacterial cell–cell communication signals, i.e. acyl homoserine lactones (AHLs) produced by many Gram-negative bacterial species (Moré *et al*., 1996; Schaefer *et al*., 1996) and the 3-hydroxypalmitic acid methyl ester from *Ralstonia solanacearum* (Flavier *et al*., 1997), are also derived from fatty acid but do not contain α,β unsaturated fatty acid moieties. Our data suggest that DSF-like α,β unsaturated fatty acid signals represent a new family of bacterial cell–cell communication signals.

Several well-characterized bacterial cell–cell communication signals are confined in certain groups of bacterial species only, e.g. AHLs were found in the Proteobacteria family of Gram-negative bacteria (Eberhard *et al*., 1981; Zhang *et al*., 1993; Pearson *et al*., 1994), whereas oligopeptide signals were produced by Gram-positive bacteria (Ji *et al*., 1995). In contrast, DSF-like activity has been detected in a range of Gram-negative and Gram-positive bacterial species, including 14 strains from four *Xanthomonas* species, four *P. aeruginosa* isolates and five *Mycobacterium* species (Table 1). The list could be expanded further as database searches found RpfF homologues in other bacterial species, such as *Xylella fastidiosa* (GenBank: AAF83925.1), *Desulfitobacterium hafniense* (GenBank: ZP 00101463.1), *Xanthomonas axonopodis* pv. *citri* (GenBank: AAM36741.1), *Burkholderia fungorum* (GenBank: ZP 00034710.1) and *Vibrio vulnificus* (GenBank: AAO07442.1). Moreover, DSF is structurally similar to 3,7,11-trimethyl-2,6,10-dodecatrienoic acid (farnesoic acid), an α,β unsaturated fatty acid involved in the regulation of morphological transition in *C. albicans* (Oh *et al*., 2001). These findings are highly

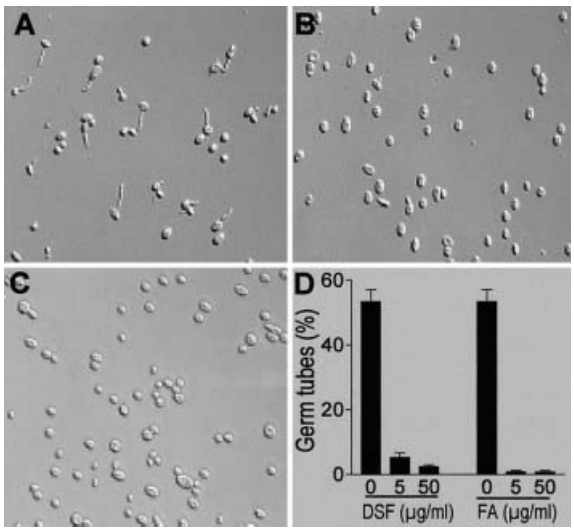


Fig. 5. Effect of DSF and FA on germ tube growth of *C. albicans* strain CAI4 (ATCC MYA-682). Yeast cells were incubated at 37°C for 4 h in GS medium (Oh *et al*., 2001) supplemented with uridine at 0.1 mg ml^{-1} without (A) or with 10 μg ml^{-1} of DSF (B) and farnesoic acid (FA) (C). The dosage (0, 5, 50 μg ml^{-1}) effect of DSF and FA on *C. albicans* germ tube formation (D) was determined microscopically on about 300 yeast cells in each treatment; the mean and standard error from three repeats are presented.

intriguing because Xanthomonads belong to the Gram-negative bacterial group, whereas Mycobacteria are Gram-positive species, and *C. albicans* is a fungal pathogen. To our knowledge, DSF-like signals represent the first family of the characterized cell–cell communication signals that are conserved in both prokaryotes and eukaryotes. Cross-kingdom signalling could be much more common than we think. Recent studies showed that certain legume plants secrete a number of compounds capable of stimulating the AHL-mediated bacterial quorum-sensing systems (Teplitski *et al*., 2000; Gao *et al*., 2003), although the chemical nature of these AHL mimic compounds remains to be characterized.

The similarity in cell–cell communication signals of different organisms might pose a problem of cross-communication. As interactions between prokaryotes and eukaryotes are common, the potential impact of such cross-communications should be investigated. It is known that *P. aeruginosa* and *C. albicans* interact with each other in the human body, and the interaction might affect the ecology and virulence of microbial communities (Burns *et al*., 1999; Hermann *et al*., 1999; Hogan and Kolter, 2002). The data presented here suggest that cross-kingdom communication via DSF-like signals is possible (Figs 4 and 5). Farnesoic acid might not be able to replace DSF to induce the expression of the DSF-dependent genes in *Xanthomonas*, as its minimum concentration required for activity is about 2000 times higher than DSF (Fig. 4). However, DSF signal at a physiologically relevant concentration could be recognized by the potential receptor of farnesoic acid in *C. albicans* and result in termination of filamentous growth of the fungus (Fig. 5). It appears that the DSF-dependent cell–cell communication system of Xcc has a stringent structural requirement on signal molecules, whereas the unidentified receptor of farnesoic acid in *C. albicans* is less rigorous in the selection of its ligand.

Similar to the acyl homoserine lactones, which regulate a range of biological activities (Fuqua *et al*., 2001; Miller and Bassler, 2001), DSF-like signals may also regulate different biological functions. The *rpfF* and *rpfB* homologues have recently been characterized in *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *Sinorhizobium meliloti* respectively. The *rpfF* mutants of Xoo exhibited a growth deficiency under low iron conditions but were unaffected in the production of extracellular polysaccharide and xylanase, suggesting that the gene product may be involved in the regulation of an iron uptake system (Chatterjee and Sonti, 2002). The *fadD* of *S. meliloti*, which encodes an RpfB homologue, appears to be involved in controlling the motility of the bacterium and *nod* gene expression. The transposon insertional mutant of *fadD* showed conditional swarming and was impaired in establishing an association with its host plant (Soto *et al*., 2002). Database searches showed that *S. meliloti* contains an RpfF homologue, Sma1408, which shares about 31% identity at the peptide level with the RpfF of Xcc (Galibert *et al*., 2001).

Besides DSF, another extracellular signalling molecule, known as diffusible factor (DF), is also implicated in the regulation of the virulence-related traits in Xcc. DF production, which is controlled by the *pigB* gene product (Poplawsky and Chun, 1997; 1998), is essential for xanthomonadin pigment and extracellular polysaccharide (EPS) biosynthesis (Chun *et al*., 1997; Poplawsky and Chun, 1997). Mutation of *pigB* abolished DF production and reduced the ability of Xcc to infect the host plant via hydathodes (Poplawsky and Chun, 1998). It was shown that DSF and DF represent two separate cell–cell signalling pathways but have overlapping regulatory roles in EPS production (Poplawsky *et al*., 1998). Final elucidation of the chemical structure of DF, which was tentatively characterized as a butyrolactone derivative containing an oxidized alkane side-chain (Chun *et al*., 1997), would further facilitate the investigation of the molecular mechanisms of the multiple cell–cell signalling processes in Xcc.

Cell–cell communication in bacteria, also known as quorum sensing in many cases, plays an important role in functional co-ordination among family members in a range of biological activities, including expression of virulence genes and biofilm formation. Elucidation of the DSF structure would significantly facilitate characterization of DSF-like signals in other microbial species and investigation of the genetic regulation of bacterial activities at both

Table 1. Bacterial strains and production of DSF-like activity.[a]

Species	Strain	Source	Medium[b]	Reaction[c]
Xanthomonas campestris pv. *campestris*	XC1	OC	NYG/YEB	++
	XV	OC	NYG/YEB	–/+
	AS1654	OC	NYG/YEB	+
	Xc1853	OC	NYG/YEB	++++
	Xc4199	OC	NYG/YEB	+++
Xanthomonas oryzae pv. *oryzae*	XooC1	G. L. Wang	NYG	++
	XooC2	G. L. Wang	NYG	+
	XooC3	G. L. Wang	NYG	+
	XooC4	G. L. Wang	NYG	++
	XooC5	G. L. Wang	NYG	+
	XooC6	G. L. Wang	NYG	++
	XooC7	G. L. Wang	NYG	++
	XooAc	C. Dong	NYG	–
	XooC8	G. L. Wang	NYG	++
Xanthomonas oryzae pv. *oryzicola*	XooL8	C. Dong	NYG	++
	Xoo812	C. Dong	NYG	++
Xanthomonas albilineans	XA3	OC	NYG/SMG3	++
	XA13	OC	NYG/SMG3	++
Agrobacterium tumefaciens	A6	A. Kerr	NYG	–
	C58C1	S. Farrand	NYG	–
Pseudomonas aeruginosa	PAO1	K. Mathee	LB/NYG	++
	PA14	L. G. Rahme	NYG	+
	AS1204	OC	NYG	+
	AS1512	OC	NYG	+
Salmonella enteritica	SE1	J. Kwang	NYG	–
Mycobacterium avium	ATCC35717	ATCC	NYG	+
Mycobacterium smegmatis	ATCC19420	ATCC	NYG	+
Mycobacterium kansasii	ATCC35775	ATCC	NYG	+
Mycobacterium chelonei	ATCC35751	ATCC	NYG	+/++
Mycobacterium marinum	CAP E1-2001	OC	NYG	–
Mycobacterium intracellulare	ATCC13950	ATCC	NYG	+

a. OC, our collection; ATCC, American Type Culture Collection.
b. When two media are shown, the strain was tested in both.
c. Reaction: assessed by Petri dish plate assay as described in *Experimental procedures*, except the slow-growing *Mycobacterium* species, which were spotted and grown on NYG plates for about 1 week before adding the DSF biosensor strain. The assay was repeated twice. –, no detectable zone of diffusing blue colour in the overlay surrounding the colony or culture supernatant being tested; +, very narrow zone (1–3 mm diameter); ++, narrow (3–6 mm diameter); +++, wide (6–9 mm diameter); ++++, extra wide (9 mm diameter or more).

cellular and community levels. This might also lead to the development of new strategies for disease control. It has been shown recently that quorum-sensing signals are promising molecular targets for the control of bacterial infections (Dong *et al*., 2000; 2001; Manefield *et al*., 2000; Zhang, 2003). Although the biological functions of DSF-like signals in *Xanthomonas* and *C. albicans* have been uncovered, their molecular mechanisms of action, their distribution and roles in other organisms and the impact of cross-kingdom communications promise to be highly intriguing.

Experimental procedures

Bacterial strains, growth conditions and Tn5 mutagenesis

Xcc strain XC1, isolated from oilseed rape in China, was used in this study. Xcc strains were grown at 28°C in NYG (per litre contains 5 g of peptone, 3 g of yeast extract, 20 g of glycerol, pH 7.0) or YEB media (Zhang *et al*., 2002). *Xanthomonas albilineans* was maintained at 28°C in SMG3 medium (Zhang *et al*., 1998). *Escherichia coli* strains were grown at 37°C in LB medium. The mini-Tn*5* transposon carried by the suicide plasmid pTGN in *E. coli* strain S17.1(λ*pir*) was introduced into the genome of the rifampicin-resistant XC1 as described previously (Tang *et al*., 1999). Antibiotics were added at the following concentrations when required: kanamycin, 50 μg ml^{-1}; gentamicin, 50 μg ml^{-1}; rifampicin, 50 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; tetracycline, 5 μg ml^{-1}. Xgluc (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.

Construction of DSF biosensor

To construct a DSF biosensor, we first generated a *rpfF* knock-out mutant of Xcc. A 216 bp fragment from the *rpfF* coding region was amplified by polymerase chain reaction (PCR; forward primer 5′-CTGGTCGACGACATCACTGGCTA; reverse primer 5′-GACGTCGACCG ACATGAAAGGCATG CACGCC) from XC1 genomic DNA and ligated into the *Sal*I-digested suicide vector pJP5603. The resulting plasmid pJP-F was transferred into *E. coli* strain S17-1(λ*pir*) and then mobilized into a rifampicin-resistant variant of strain XC1. The transconjugants were selected on a YEB agar plate contain-

ing 100 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin. Mutation of *rpfF* was confirmed by PCR analysis and DSF deficiency phenotype. The mutant was designated FM3. We then cloned the promoter of the DSF-inducible *engXCA* gene, which encodes an endoglucanase (Slater *et al*., 2000), from strain XC1. The open reading frame of β-glucuronidase of *E. coli* (*gusA*) was fused to the *engXCA* promoter, which was amplified by PCR (forward primer: 5′-GGAGTCGACA AGCTTCCGGGGATCACAAACGACGCGA; reverse primer: 5′-ATGGGATCCGTTGTTGATGGAATAGCTGAAGG). The promoter–*gusA* cassette was cloned in the broad-host-range vector pLAFR3; the resulting plasmid pEG was mobilized to the *rpfF* mutant FM3 to generate the DSF biosensor FE58.

Bioassay of DSF

Unless otherwise stated, DSF production by bacterial strains was assayed according to the bioassay plate method (Slater *et al*., 2000), using the DSF biosensor strain FE58. The same method was used to assay liquid samples, except that wells (4 mm in diameter) were prepared on the bioassay plate, and 25 μl of sample solution was added to each well. The samples were diluted with methanol–phosphate buffer (0.02 M, pH 7.2; 50:50 v/v). The bioassay plates containing Xgluc were incubated at 28°C for 24 h. DSF activity was indicated by the presence of a blue halo around the well. To quantify DSF production in Xcc strains, blue halo zone widths in the bioassay were converted to DSF units using the formula: DSF (unit ml^{-1}) = 0.1341 $e^{(1.9919W)}$, where W is the width in centimetres of the blue halo zone surrounding each well. One unit of DSF was defined as the amount of DSF inducing the formation of a 1 cm blue halo zone on an FE58 bioassay plate containing 20 ml of NYG medium. The formula was derived from a dose–response plot of the biosensor strain to DSF, with a correlation coefficiency (R^2) of 0.98.

Purification of DSF

Xc1853 was cultured in YEB medium for 36 h. Thirty litres of bacterial supernatant were collected by centrifugation and extracted with an equal volume of ethyl acetate. The organic phase was evaporated, and the residue was dissolved in 20 ml of methanol. The crude extract, divided into four batches, was subjected to flash column chromatography using a silica gel column (12 × 150 mm, Biotage Flash 12M cartridge), eluted with ethyl acetate–hexane (4:96 v/v). The collected active component was then applied to HPLC on a C18 reverse-phase column (7.8 × 150 mm, Waters), eluted with methanol–water (80:20 v/v) at a flow rate of 4 ml min^{-1}.

Synthesis of DSF and derivatives

DSF and its *cis* derivatives were synthesized by Favorsky rearrangement of the corresponding 1,3-dibromo-2-ones prepared from methyl ketones or fatty acids in alkaline solution at room temperature (Rappe and Adeström, 1965). *Trans*-11-methyl-2-dodecenoic acid (DSF isomer) and its *trans* derivatives were synthesized through elimination of 2-bromo fatty acids prepared by bromination of the corresponding fatty acid, using potassium *tert*-butoxide as a base in 2-methyl-2-propanol solution at 55°C. Farnesoic acid (FA; 3,7,11-trimethyl-2,6,10-dodecatrienoic acid) was prepared by alkaline hydrolysis of methyl farnesoate, which was obtained from oxidation of *trans*,*trans*-farnesol as described previously (Corey *et al*., 1968). All the chemicals and reagents were purchased from Sigma-Aldrich unless otherwise stated.

Structure analysis

^{1}H, ^{13}C, ^{1}H-^{1}H COSY and DEPT NMR spectra were recorded on a Bruker AMX500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer using $CDCl_3$ solution with tetramethylsilane as an internal standard at room temperature. ^{1}H-^{13}C correlated HMBC and HMQC spectra were performed by Bruker AVANCE DRX500 spectrometer. ESI-MS and tandem mass spectrometry were performed on a Finnigan LCQ DECA ion trap mass spectrometer. High-resolution ESI-MS was performed with a Finnigan/MAT MAT 95XL-T mass spectrometer. The sample dissolved in methanol–water (50:50 v/v) was introduced into the mass spectrometer by loop injection.

Quantitative analysis of the enzyme activities after DSF induction

The extracellular protease activity in the culture supernatants of Xcc and the *rpfF*::Tn*5* mutant of Xcc was analysed according to the method described previously (Swift *et al*., 1999). The DSF-dependent expression pattern of the *engXCA* gene, which encodes endoglucanase, was determined by analysing the activity of GUS encoded by *gusA* under the control of the *engXCA* promoter. Strain FE58 was grown at 30°C in YEB medium until the OD_{600} reached about 0.6. DSF was then added to a final concentration of 1 μM. Aliquots of bacterial cell culture were taken, and total soluble protein samples were prepared by sonification. The GUS activity was determined as described previously (Jefferson *et al*., 1987) and defined as pmol of methyl umbelliferone produced min^{-1} μg^{-1} total soluble protein.

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