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Identification of a Small Molecule Signaling Factor That Regulates the Biosynthesis of the Antifungal Polycyclic Tetramate Macrolactam HSAF in *Lysobacter enzymogenes*

Yong Han[1,2], **Yan Wang**[1,5], **Simon Tombosa**[1], **Stephen Wright**[1], **Justin Huffman**[1], **Gary Yuen**[3], **Guoliang Qian**[4], **Fengquan Liu**[4], **Yuemao Shen**[2,*], and **Liangcheng Du**[1,*]

[1]Department of Chemistry, University of Nebraska-Lincoln, NE 68588, USA

[2]Key Laboratory of Chemical Biology, School of Pharmaceutical Sciences, Shandong University, Jinan 250100, China

[3]Department of Plant Pathology, University of Nebraska-Lincoln, NE 68583, USA

[4]College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China

[5]College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China

Abstract

Lysobacter species are emerging as new sources of antibiotics. The regulation of these antibiotics is not well understood. Here, we identified a small molecule metabolite (*Le*DSF3) that regulates the biosynthesis of the antifungal antibiotic HSAF (heat-stable antifungal factor), a polycyclic tetramate macrolactam with a structure and mode of action distinct from the existing antifungal drugs. *Le*DSF3 was isolated from the culture broth of *L. enzymogenes*, and its chemical structure was established by NMR and MS. The purified compound induced green fluorescence in a reporter strain of *Xanthomonas campestris*, which contained *gfp* gene under the control of a DSF (diffusible signaling factor)-inducible promoter. Exogenous addition of *Le*DSF3 in *L. enzymogenes* cultures significantly increased the HSAF yield, the transcription of HSAF biosynthetic genes, and the antifungal activity of the organism. The *Le*DSF3-regulated HSAF production is dependent on the two-component regulatory system RpfC/RpfG. Moreover, *Le*DSF3 up-regulated the expression of the global regulator Clp (cAMP receptor-like protein). The disruption of *clp* led to no HSAF production. Together, the results show that *Le*DSF3 is a fatty acid-derived, diffusible signaling factor positively regulating HSAF biosynthesis and that the signaling is mediated by the RfpC/RpfG-Clp pathway. These findings may facilitate the antibiotic production through applied genetics and molecular biotechnology in *Lysobacter*, a group of ubiquitous yet underexplored microorganisms.

Keywords

Diffusible signaling factor; natural product biosynthesis; regulation; HSAF; *Lysobacter enzymogenes*

[*]Corresponding authors: Liangcheng Du, ldu3@unl.edu. Yuemao Shen, yshen@sdu.edu.cn.
YH and YW contributed equally to this work.

Introduction

Lysobacter is a genus of ubiquitous environmental bacteria that belong to the *Xanthomonadaceae* family within the gamma proteobacteria (Christensen and Cook 1978; Sullivan *et al.* 2003). The genus is emerging as a novel biocontrol agent against pathogens of crop plants (Yuen *et al*. 2001a; Yuen and Zhang 2001b; Zhang and Yuen 1999), including *Bipolaris sorokiniana*, *Uromyces appendiculatus*, and *Rhizoctonia solani*. We have been studying strains of *L. enzymogenes* as a new source of bioactive natural products (Li *et al.* 2012; Li *et al.* 2014; Lou *et al.* 2011; Lou *et al.* 2012; Wang *et al.* 2013a; Wang *et al.* 2013b; Yu *et al.* 2007; Zhang *et al.* 2011). We recently identified biosynthetic genes in strain OH11 for WAP-8294A, a group of cyclic lipodepsipeptides with very potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (Zhang *et al.* 2011). We also isolated HSAF (heat-stable antifungal factor), an antifungal metabolite from strain C3 (Lou *et al.* 2011; Yu *et al.* 2007). HSAF is a polycyclic tetramate macrolactam (PTM) that has a chemical structure distinct from any existing antifungal drug or fungicide and appears to involve a novel mode of action in inhibiting hyphal growth in fungi (Li *et al.* 2006). The biosynthetic gene cluster for HSAF consists of a core gene (*hsaf pks-nrps*) encoding a hybrid polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS), and tailoring genes for HSAF structural modifications (Li *et al.* 2014; Lou *et al.* 2011). Despite the research progress towards elucidating the molecular mechanism for HSAF biosynthesis, little is known about the regulation of HSAF production in *L. enzymogenes*.

Some endogenous or exogenous signal molecules can mediate cell-to-cell communications in microorganisms. One well-known signaling system is quorum sensing (QS) (Comella and Grossman 2005; Dusane *et al.* 2011; Galloway *et al.* 2011; Miller and Bassler 2001), which can regulate the expression of multiple genes through the accumulation and recognition of auto-induced signals, or autoinducers in the local environments. Signaling via autoinducers regulate virulence, biofilm formation and biosynthesis of secondary metabolites in microorganism. *N*-acyl-homoserine lactones (AHLs) are a major class of small molecule signaling factors that have been extensively studied (Case *et al.* 2008). Another class is the furanosyl borate diesters that exist in a number of Gram-positive and Gram-negative bacteria (Winzer *et al.* 2003). In *L. enzymogenes*, we recently found that two signaling pathways are involved in regulation of secondary metabolite biosynthesis and colony morphology (Qian *et al.* 2013a). One of the signaling pathways likely involves signal molecules belonging to hydroxylated benzoic acids (the so-called diffusible factors, or DFs) (He *et al.* 2011). The other is likely to involve analogs of the so-called diffusible signaling factor (DSF) initially reported in *Xanthomonas campestris* pv. *campestris (Xcc)* that is involved in the Rpf (regulation of pathogenicity factors) signaling system (Barber *et al.* 1997; Deng *et al.* 2011; Ryan and Dow 2011). DSF isolated from *X. campestris* was found to be (*Z*)-11-methyl-2-dodecenoic acid (Barber *et al.* 1997; Wang *et al.* 2004), and now DSF-like molecules have been reported in other microorganisms (Deng *et al.* 2009; Deng *et al.* 2010; He *et al.* 2010; Huang and Wong 2007; Newman *et al.* 2004). These signal molecules are fatty acids typically with a *cis* double bond at C2 position, and their chain lengths vary from 12 to 14 carbons. They regulate multiple behaviors of the cells in a

density-dependent manner (quorum sensing). Some DSF contain a methyl branch, such as (*Z*)-11-methyl-2-dodecenoic acid from *X. campestris*, (2*Z*,5*Z*)-11-methyl-2,5-dodecadienoic acid from *X. oryzae* pv. *oryzae* (He *et al.* 2010), and 12-methyltetradecanoic acid from a citrus strain of *X. fastidiosa* (Simionato *et al.* 2007). Some DSF contain a linear fatty acid chain, such as (*Z*)-2-dodecenoic acid from *Burkholderia cenocepacia* (Boon *et al.* 2008) and *Pseudomonas aeruginosa* (Amari *et al.* 2013) and (*Z*)-2-tetradecenoic acid from a grape strain of *Stenotrophomonas maltophilia* (Huang and Wong 2007).

The genes in the Rpf signaling system encode enzymes for DSF biosynthesis and proteins functioning as sensor and response regulators (Barber *et al.* 1997; Deng *et al.* 2011; Ryan and Dow 2011). Among them, *rpfF* encodes a bifunctional 3-hydroxyacyl-ACP dehydratase and thioesterase (Bi *et al.* 2012), and *rpfB* was predicted to encode an acyl CoA synthetase (Almeida *et al.* 2012). The *rpfC* and *rpfG* genes encode for proteins involved in a two-component of signal transduction system, RpfC/RpfG, which serve as the sensor/response regulator of DSF (Slater *et al.* 2000). RpfC is a membrane-bound histidine kinase sensor protein with dual functions. Its intracellular domain is associated with RpfF, which suppresses the activity of RpfF to synthesize DSF (Cheng *et al.* 2010; He *et al.* 2006; Slater *et al.* 2000). When the extracellular DSF concentration reaches a threshold, RpfC undergoes autophosphorylation at its active site histidine residue. This leads to the release of RpfF, which then becomes active and in turn synthesizes more DSF (thus auto-induction of DSF). At the same time, the kinase function of RpfC phosphorylates the partner intracellular response regulator RpfG, and this activates the cyclic di-GMP phosphodiesterase activity of RpfG (Barber *et al.* 1997; Deng *et al.* 2011; Ryan and Dow 2011). Cyclic di-GMP is a second messenger involved in numerous cellular processes, including those mediated by the global regulator Clp, cAMP receptor-like protein (Chin *et al.* 2010). The RpfC/RpfG-Clp-mediated DSF signaling has been observed in diverse bacteria and linked to virulence, motility, biofilm dispersal, extracellular enzyme and production of extracellular polymeric substances (EPS) (Barber *et al.* 1997; Deng *et al.* 2011; Ryan and Dow 2011). In *L. enzymogenes*, *clp* has been shown to regulate the production of lytic enzymes and other antifungal factors and to be critical in biological control activity (Kobayashi *et al.* 2005). However, neither the direct involvement of Clp in regulating HSAF biosynthesis nor the connection between Clp and RpfC/RpfG has been demonstrated. More importantly, the actual signal molecule has not been identified from any *Lysobacter* species.

We have found that the genome of *L. enzymogenes* OH11 contains the homologous genes of the *rpf* cluster (Qian *et al.* 2013a; Qian *et al.* 2013b). This suggests that a DSF-like molecule is very likely produced by *L. enzymogenes*. The objectives of this study were to isolate and identify the DSF-like molecule from *L. enzymogenes* OH11 and to verify that it is involved in RpfC/RpfG-Clp regulation of HSAF biosynthesis. In this report, we describe a new DSF-like molecule (*Le*DSF3) from *L. enzymogenes* and present evidence for the involvement of *Le*DSF3 in regulating HSAF production through the RpfC/RpfG-Clp signaling pathway.

Materials and methods

Instruments and analytical methods

NMR spectra were recorded on a Bruker Advance 400 spectrometer at 400/100 MHz (Bruker, Fällanden, Switzerland). Mass spectra were obtained on LCQ mass spectrometers (Thermo, West Palm Beach, FL, USA). An Agilent 1120 HPLC system (Agilent, Santa Clara, CA, USA), with RF C18 columns (10.0 × 250 mm, 5 μm, for preparative HPLC; 4.6 × 150 mm, 3.5 μm, for analytic HPLC), was used in the studies. For column chromatography, a silica gel 60 (Merck, Darmstadt, Germany) column and a Sephadex LH-20 (GE healthcare, Uppsala, Sweden) column were used. TLC analyses were performed with pre-coated silica gel 60 F254 plates (Merck, Darmstadt, Germany). The fluorescent microscope pictures were obtained on an Olympus IX 81 inverted confocal microscope (Olympus, Tokyo, Japan). All general chemical reagents were purchased from Sigma-Aldrich or Fisher Scientific.

Microorganism Strains

Escherichia coli DH5α was used for the general DNA manipulation. *E. coli* S17-1 was used for conjugation. *Lysobacter enzymogenes* OH11 (CGMCC No. 1978) and its mutants including OH11Δ*rpfB*, OH11Δ*rpfC*, OH11Δ*rpfF*, OH11Δ*rpfG*, and OH11 *DC* were from the Liu lab (Qian *et al*. 2013a; Kobayashi *et al.* 2005). The DSF reporter strain *X. campestris* 8523/pKLN55 was from the Lindow lab (Newman *et al.* 2004), and *Fusarium verticillioides* A0149 (FGSC No. 7600) was used as the test organism in the antifungal assays.

Isolation of *Le*DSF3

L. enzymogenes OH11Δ*rpfC* was grown in 1/10 Tryptic Soy Broth (TSB, 100 ml 3000 shake flask, total 300 liters) at 28 °C, at 200 rpm, for 2 days. The culture broth was extracted with the same volume ethyl acetate (EtOAc) until the filtrate was colorless. The combined extract, upon evaporation, yielded the crude extract, which was partitioned between methanol and petroleum ether (250 ml each, 3 times). The petroleum ether layer was concentrated under vacuum to afford a yellow syrupy extract (1.4 g). The extract was subjected to column chromatography (60 g silica gel 60; hexane - ethyl acetate, gradient elute; 250 ml for each gradient) to afford 20 fractions, Fr. 1–20. Fraction 2 (52 mg) was subjected to preparative HPLC (RF C18 column, 10.0 × 250 mm, 5 μm; 80% acetonitrile; flow rate 3 ml/min; detection wavelength 210 nm) to afford *Le*DSF3 (~5 mg).

Assay for the Green Fluorescent Activity in the DSF Reporter Strain

The promoterless *gfp* gene fused to the promoter from the DSF-inducible *engXCA* gene was cloned in a plasmid conferring spectinomycin and streptomycin resistance, generating construct pKLN55 (Newman *et al*. 2004). The construct was mated into the *rpfF* mutant *X. campestris* 8523 to generate the DSF reporter strain *X. campestris* 8523/pKLN55. To detect any DSF-like molecule in *L. enzymogenes*, we followed the protocol described by Newman et al (Newman *et al.* 2004). Briefly, *X. campestris* 8523/pKLN55 was inoculated into liquid NYGB medium (peptone 5.0 g, yeast extract 3.0 g, glycerol 20.0 g, water 1.0 L, pH 7.2) and incubated at 30 °C for 24 h. A 5 μl of the culture was spotted onto LB plates, and the

individual extracts from *L. enzymogenes* were added to sterile paper discs along the reporter strain. The *X. campestris* DSF, (*Z*)-11-methyl-2-dodecenoic acid (Cayman Chemical), was used as the positive control. For the test of the purified *Le*DSF3, 1 μg (in 5 μl DMSO) of the compound was spotted on a paper disc near the *X. campestris* reporter strain. The reporter strain was incubated at 28–30 °C for 48 h, and the green fluorescence was then visualized using a confocal microscopy. The excitation wavelength was 488 nm, and the emission wavelength was 509 nm.

Effect of the small molecule signal *Le*DSF3 on the antifungal HSAF Production

*Le*DSF3 (final concentration 0.2–10 μM) was added into 50 ml 1/10 TSB culture of various strains of *L. enzymogenes*. The cultures grew at 28 °C, 200 rpm for 2 days, and were extracted with the same volume of ethyl acetate. The organic phase was concentrated under vacuum, and the crude extract was dissolved with 0.5 ml methanol. A fraction (20 μl) of the methanol extract was injected in HPLC to analyze HSAF and analogs. For semi-quantification, the peak area of HSAF and analogs was measured to obtain the relative yield of the compounds, and each treatment was repeated at least twice. The HPLC method is shown in Table S1 in the Supplementary Material.

Test of the Antifungal Activity of *L. enzymogenes* strains treated with *Le*DSF3

Fusarium verticillioides was used as the test fungus. Various strains of *L. enzymogenes* and *F. verticillioides* were co-inoculated on 40% TSB plates, with or without *Le*DSF3. Half of the test plate was spread with the *L. enzymogenes* wild type or one of its mutants; the other half was point-inoculated with *F. verticillioides* mycelium. The plates were placed in a 28 °C incubator for 3 days.

RNA Extraction, Reverse-transcription PCR and Real Time-PCR

L. enzymogenes OH11 and its mutants were grown on 100 ml 1/10 strength TSB medium for 24 h. An aliquot of 3 ml cells was transferred to a sterile centrifuge tube and centrifuged for 3 min at 15,300 g. RNA was extracted from the strains using TRIZOL solution following the manufacturer's instruction. For DNA removing and reverse transcription PCR, PrimerScript RT reagent Kit with gDNA Eraser Kit (TaKaRa biocompany) was used in this study. For real time-PCR assay, iQ SYBR green supermix kit (BIO-RAD company) was used. The primers for realtime PCR were listed in Table S2.

Results

Isolation and structural determination of the signaling molecule *Le*DSF3

We used the DSF reporter strain *X. campestris* 8523/pKLN55, which contains *gfp* (green fluorescent protein) gene under the control of a DSF-inducible promoter (Newman *et al.* 2004) to isolate putative signal molecules produced by *L. enzymogenes* OH11. GFP expression occurs when the reporter strain is grown in the presence of a source of DSF or a DSF-like molecule. Initially, all efforts failed to isolate a sufficient amount of DSF for structural determination from fermenter broth cultures of the wild type OH11, although we could detect very weak green fluorescence using crude extract from such cultures. We subsequently shifted to the *rpfC* disruption mutant of OH11, with the hope that disruption of

this DSF sensor/repressor would lead to a higher yield of the DSF-like compound as observed in other organisms (Chatterjee *et al.* 2008; Slater *et al.* 2000). The estimated concentration of the main DSF in the wild type and the *rpfC* mutant was 13 and 71 μg/L, respectively. In addition, because *L. enzymogenes* tends to self-lyse in broth culture when the culture is older than 4–5 days, we chose to grow the cells in flasks with 100 ml medium so that the culture could reach a relatively high cell density ($OD_{600\ nm}$ ~1–1.5) in 2 days. With these modifications, we collected the ethyl acetate extracts from 3000 flasks of culture and followed fractions that induced GFP expression in the reporter strain (Fig. 1). The *X. campestris* DSF was used as a reference, which gave a retention time of 7.5 minute in HPLC. In the active fraction, three main peaks were detected by HPLC (Fig. 1). We focused on the mid-peak (*Le*DSF3), which had a retention time of 13.3 minute, because this peak showed the strongest activity in HSAF regulation. The purified *Le*DSF3 was tested for DSF-like activity using the reporter strain *X. campestris* 8523/pKLN55, which carries *gfp* gene under the control of a DSF-inducible promoter., green fluorescence was observed in the colonies (Fig. 2). Green fluorescence was observed in the colonies when 1 μg of *Le*DSF3 was spotted onto the solid media near the colonies of *X. campestris* 8523/pKLN55 (Fig. 2)

The *Le*DSF3 ^{1}H-NMR and the ^{13}C NMR spectra displayed 11 CH_2 (one connected with a carbonyl group), one CH, and two CH_3 groups (Table S3, Fig. S1–S2). ^{1}H-NMR signals at δ_H 0.88 (d, J = 6.6 Hz, 6H), 1.51–1.54 (m, 1H), and the ^{13}C-NMR signals at δ_C 22.9 indicated the presence of an isopropyl group. ^{1}H-NMR signals at δ_H 1.27–1.36 (m, 16 H) revealed the presence of an aliphatic carbon chain. The mass of the compound was determined by ESI-MS to be 242 Da ($[M-1]^-$ = 241; $[M+1]^+$=243) (Fig. S3). The FT-IR absorption at 1697 cm^{-1} indicated the presence of C=O carbonyl group, and the FT-IR absorption from 2952 to 2871 cm^{-1} indicated the presence of aliphatic chain (Figure S4). These data are consistent with those reported for a chemically synthesized fatty acid (Sarpe & Kulkarni 2011). Therefore, *Le*DSF3 was determined to be 13-methyltetradecanoic acid (Fig. 3).

The small molecule signal *Le*DSF3 induces the antifungal HSAF production

We tested the effect of *Le*DSF3 on the production of HSAF analogs by strain OH11. The strain mainly produced 3-deOH-HSAF (**5**) and 3-deOH-alteramide (**6**), with HSAF (**3**) being detected in relatively low amounts and alteramide being undetected (**4**) (Fig. 3 and 4). When *Le*DSF3 was added into the cultures, the relative amounts of the different HSAF analogs was unchanged but total HSAF production was increased in a dose-dependent manner. The yield of HSAF analogs began to increase at 0.2 μM *Le*DSF3 and continued to increase as the *Le*DSF3concentration increased (1.5–4.6 fold from 0.2 to 10 μM *Le*DSF3) (Fig. 4).

The small molecule signal *Le*DSF3 regulates the antifungal HSAF biosynthesis through the two-component regulators RpfC/RpfG

A gene cluster homologous to *X. campestris rpf* cluster is present in the genome of *L. enzymogenes* OH11 (Figure S5) (Qian *et al.* 2013a). The above results have demonstrated that *L. enzymogenes* indeed produces at least one DSF-like molecule. To test the hypothesis that *Le*DSF3-induced HSAF production is mediated by the *rpf* system, we examined the production of HSAF analogs in mutants of OH11 disrupted in individual *rpf* genes. As

shown in Figure S6, the disruption of any of *rpfB*, *C*, *F* and *G* nearly eliminated production of HSAF analogs, verifying that the *rpf* signaling system is involved in the regulating HSAF production. Then, we exogenously added *Le*DSF3 (5 μM) to cultures of the *rpf* mutants. The HSAF production was partly restored in the *rpfB* mutant (~50%) and the *rpfF* mutant (~75%), but not in mutants disrupted in *rpfC* or *rpfG* mutant (Fig. 5). The results are consistent with the expected involvement of *Le*DSF3 as a signaling compound in the *rpf* system. In the *rpfB* and *rpfF* mutants, which are inactive in the DSF biosynthesis enzymes RpfB and RpfF, respectively, the two-component sensor/response regulator proteins (RpfC/RpfG) are active, whereas the entire sensor/response regulator is inactive in the *rpfC* and *rpfG* mutants. Therefore, positive response of *rpfB* and *rpfF* mutants, but not *rpfC* and *rpfG* mutants to exogenously added *Le*DSF3, is evidence that this compound is the signal, or one of the signals, received by RpfC.

Next, we evaluated the effect of *Le*DSF3 on expression of HSAF *pks-nrps*, the key gene for HSAF biosynthesis (Li *et al.* 2014; Lou *et al.* 2011), first in the wild type OH11 and then in the *rpfB* mutant. In the wild type, the exogenous addition of *Le*DSF3 (10 μM) increased *pks-nrps* expression by ~ 3 fold, as determined by Q-RT-PCR (Fig. 6A). In the *rpfB* mutant, *pks-nrps* expression was elevated by ~ 4 fold by exogenous *Le*DSF3 (Fig. 6B). Because the *rpfB* mutant is expected to produce very little, if any, of its own *Le*DSF3, the exogenous addition of *Le*DSF3 would be expected to have a more significant impact on HSAF production in *rpfB* mutant than that in the wild type. The results from these two experiments are consistent with this model and are in agreement with the observed effects of the *Le*DSF3 on HSAF yield.

The small molecule signal *Le*DSF3 regulates the antifungal activity of *L. enzymogenes* through the two-component regulators RpfC/RpfG

L. enzymogenes is known for its biocontrol activity against fungal pathogens, and antifungal HSAF is known to be the main factor contributing to this antifungal activity (Li *et al.* 2006; Yu *et al.* 2007). To further understand the *Le*DSF signaling, we tested the activity of various *rpf* mutant strains, in the presence or absence of exogenous *Le*DSF3, against the growth of the plant pathogen *Fusarium verticillioides* (Fig. S7). The wild type *Le*OH11 exhibited a clear activity against this fungus, and this activity was markedly enhanced when *Le*DSF3 was added into the medium (Fig. S7A and F). Mutation of the DSF biosynthetic gene *rpfB* led to a decrease in the antifungal activity, which was restored by exogenous *Le*DSF3 (Fig. S7B and G). Mutation of the two-component sensor gene *rpfC* led to a complete loss of the antifungal activity, which could not be restored by exogenous *Le*DSF3 (Fig. S7C and H). Mutation of the other DSF biosynthetic gene *rpfF* led to a significant decrease in the antifungal activity, which was restored by exogenous *Le*DSF3 (Fig. S7D and I). Finally, mutation of the two-component response regulator gene *rpfG* led to a complete loss of the antifungal activity, which could not be restored by exogenous *Le*DSF3 (Fig. S7E and J). These results clearly support the above observed results, which showed that *Le*DSF3 regulates HSAF biosynthesis through the two-component regulatory system RpfC/RpfG.

The two-component regulators RpfC/RpfF and the small molecule signal *Le*DSF3 regulate antifungal HSAF biosynthesis through the global regulator Clp

To determine if the regulation of HSAF biosynthesis in *L. enzymogenes* by RpfC/RpfF and associated *Le*DSF3 acts through the global regulator Clp, we first analyzed the production of HSAF analogs in *clp* mutant. The production of HSAF analogs was completely eliminated in the *clp* mutant (Fig. 7A), clearly demonstrating the connection between the global regulator and HSAF biosynthesis. Next, we tested the effected of the *Le*DSFs on *clp* expression. Q-RT-PCR analysis showed that *Le*DSF3 increased *clp* expression by 5 fold in *rpfB* mutant (no endogenous *Le*DSFs) over the control (Fig. 7B). The results are consistent with the observed effect of *Le*DSF3 on HSAF production and *pks-nrps* gene expression. Together, the results showed that that the RfpC/RfpG-Clp pathway mediates *Le*DSF3 signaling towards antifungal HSAF biosynthesis in *L. enzymogenes*.

Discussion

Diffusible small molecule signals are well known in antibiotic-producing bacteria, such as the Gram positive bacteria *Streptomyces* which often use gamma-butyrolactones as specialized regulatory small molecules for the biosynthesis of natural products (Liu *et al*. 2013). The so-called A-factor, a diffusible extracellular molecule [2-(6′-methylheptanoyl)-3*R*-hydroxymethyl-4-butanolide], triggers both aerial mycelium formation and streptomycin biosynthesis in *S. griseus* (Khoklov *et al*. 1967; Horinouchi and Beppu 1994). A-factor like signals have been found in numerous *Streptomyces* species, and their biosynthetic genes are commonly associated with antibiotic biosynthetic gene clusters (Liu *et al*. 2013). In addition to gamma-butyrolactones, small furans were found to control antibiotics in some *Streptomyces*, such as the epoxycyclopentenone methylenomycin A in *S. coelicolor* (Corre et al. 2008). Another widely studied group of small molecule signals is *N*-acyl homoserine lactones (AHL). These signals involve in the regulation of luminescence, antibiotic production, biofilm formation, virulence, and motility in numerous bacteria including *Vibrio fischeri* and *Pseudomonas aeruginosa* (Fuqua and Greenberg EP 2002; Schuster *et al*. 2013).

Lysobacter species are emerging as prolific producers for bioactive natural products (Xie *et al.* 2012). The biosynthetic mechanisms for these natural products have been subjected for intensive research in the past several years (Hou *et al.* 2011; Li *et al.* 2012; Li *et al.* 2014; Lou *et al.* 2011; Lou *et al.* 2012; Wang *et al.* 2013a; Wang *et al.* 2013b; Zhang *et al.* 2011). However, the molecular mechanisms by which *Lysobacter* control the biosynthesis of these natural products are not well understood. In this study, we report the structure of a diffusible signal molecule *Le*DSF3 deployed by the valuable biocontrol organism *L. enzymogenes*, and demonstrate that the signal molecule is essential to the up-regulation and biosynthesis of HSAF analogs, the primary antifungal antibiotics in *L. enzymogenes*. The chemical structure of the signal molecule was elucidated by spectroscopic analyses, which showed to be 13-methyltetradecanoic acid. Although 14-carbon DSF had been isolated from other organisms (Deng *et al.* 2011), *Le*DSF3 is a new structure. In addition to *Le*DSF3, several fatty acid-derived molecules were produced by *L. enzymogenes* (Fig. 1A). The main function of these molecules did not seem to be HSAF-related, and we are currently investigating their roles.

DSF-like signal molecules are long-chain fatty acids and do not contain a lactone or a furan ring. These signal molecules have been isolated from several Gram negative bacteria, particularly plant pathogens in the class of gamma-proteobacteria (Deng *et al*. 2011). In plant pathogens such as *Xanthomonas campestris* and other Gram negative bacteria, DSF are involved in the regulation of several important processes, such as virulence, motility, biofilm dispersal, extracellular enzyme and production of extracellular polymeric substances, but to our knowledge DSF had not been shown to be involved in the regulation of a specific antibiotic. *Lysobacter*, which also belongs to gamma proteobacteria, produces several structurally very interesting antibiotics (Xie *et al*. 2012), but the regulation of these antibiotics is largely unknown. Our work represents the first DSF isolated from a *Lysobacter* species. It is also the first time to link a specific antibiotic to a specific DSF signal through the two-component regulators RpfG/C and the global regulator Clp. The induction of HSAF analogs by *Le*DSF3 became detectable when the concentration of exogenously added *Le*DSF3 was above 0.2 μM (Fig. 4). Although the detectable concentration (13 μg/L, or 53.7 nM) of *Le*DSF3 in the wild type is lower than the action concentration (0.2 μM) under our experimental conditions, we believe the detectable concentration does not fully reflect the actual concentration of *Le*DSF3 in vivo. DSFs are quorum sensing signal molecules, and their concentrations can change dramatically due to the auto-induction mechanism in the actual situations (Deng *et al*. 2011). Furthermore, due to the chemical nature of long-chain fatty acids, the extraction and isolation of *Le*DSF3 from the bacterial culture would not be very efficient. This is evident from the ~5 mg *Le*DSF3 isolated from 300 liters of culture of *rpfC* mutant (a *Le*DSF3 “high-yield strain”), although the total detectable *Le*DSF3 would have been ~21.3 mg in *rpfC* mutant (with a detectable concentration of 71 μg/L, or 293.4 nM, under the experimental conditions).

In summary, this is the first reported case connecting quorum sensing-related diffusible signaling factors to antibiotic production in the ubiquitous environmental *Lysobacter* species. Our data revealed a regulatory mechanism for HSAF production: a fatty acid-derived *Le*DSF3 functions as an extracellular signal, sensed and transducted by a two-component regulatory system (RfpC/RfpG), mediated by a global regulator (Clp), to control the production of antifungal natural products (HSAF analogs) in *L. enzymogenes*. We believe these findings will be useful in applied genetics and molecular biotechnology to improve antibiotic production in *Lysobacter*, a group of ubiquitous yet underexplored microorganisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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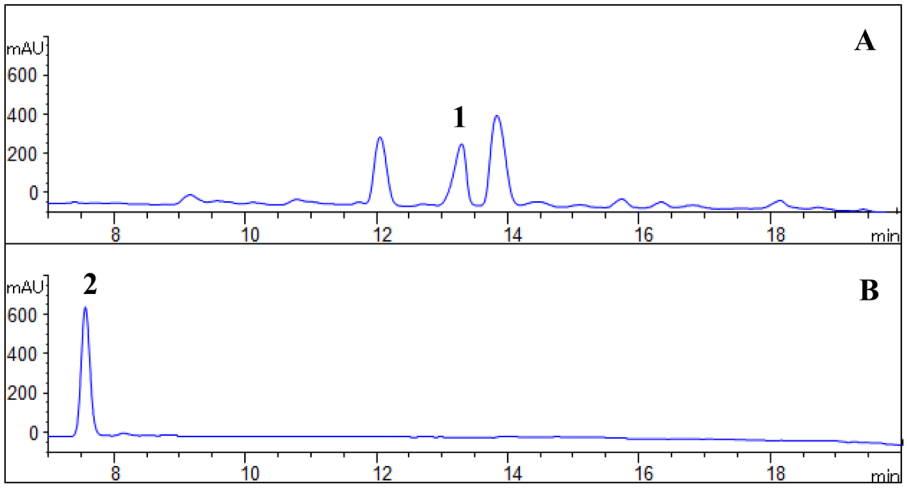

Fig. 1.

HPLC analysis of *Le*DSFs. A: Semi-purified extracts of *Le*DSF3 (**1**); B: *X. campestris* DSF (**2**) as reference.

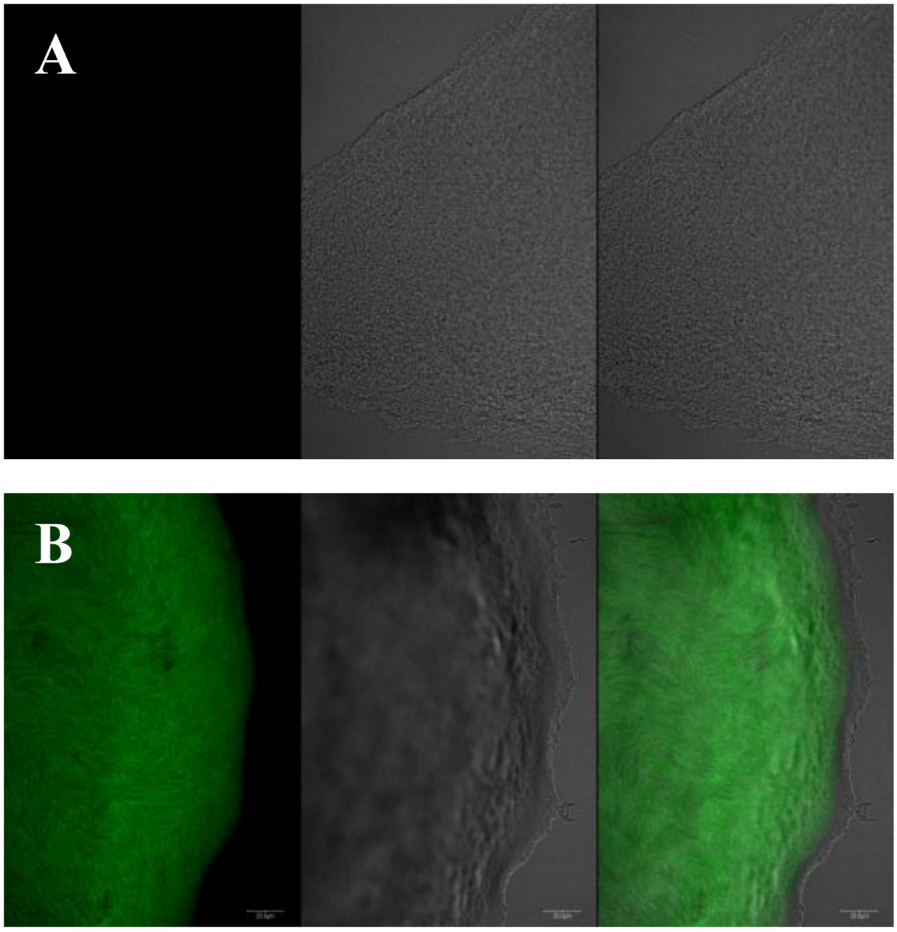

Fig. 2.

DSF-like activity of *Le*DSF3, shown as the green fluorescence induction activity in the DSF reporter strain *X. campestris* 8523/pKLN55. **A**: untreated strain *X. campestris* 8523/pKLN55; **B**: 8523/pKLN55 treated with 1 μg *Le*DSF3. For each of panels, a) picture of a 8523/pKLN55 colony taken at 488 nm excitation wavelength and 509 nm emission wavelength, b) picture of the same colony taken under transmitted light, and c) merged a & b.

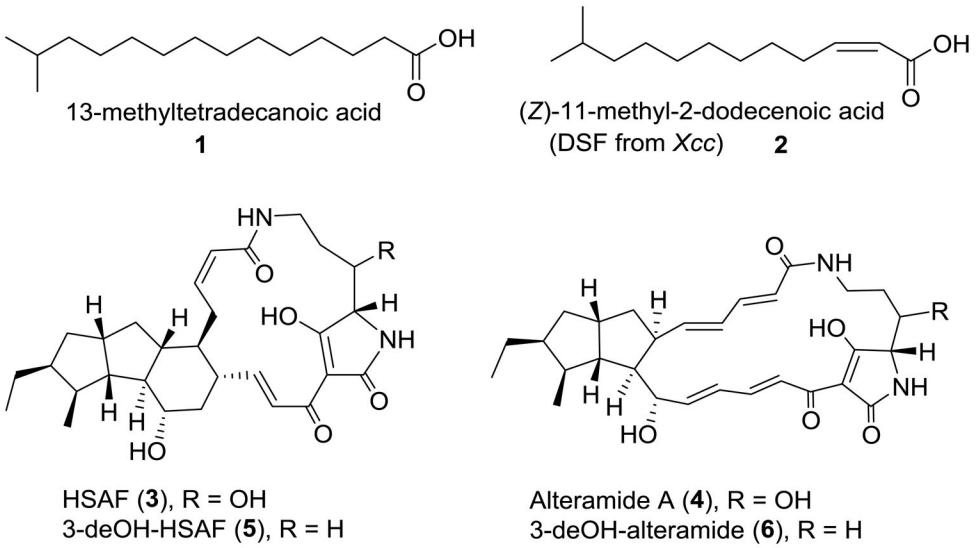

Fig. 3.

Chemical structures of *Le*DSF3 (**1**), *X. campestris* DSF (**2**), and HSAF analogs (**3**–**6**).

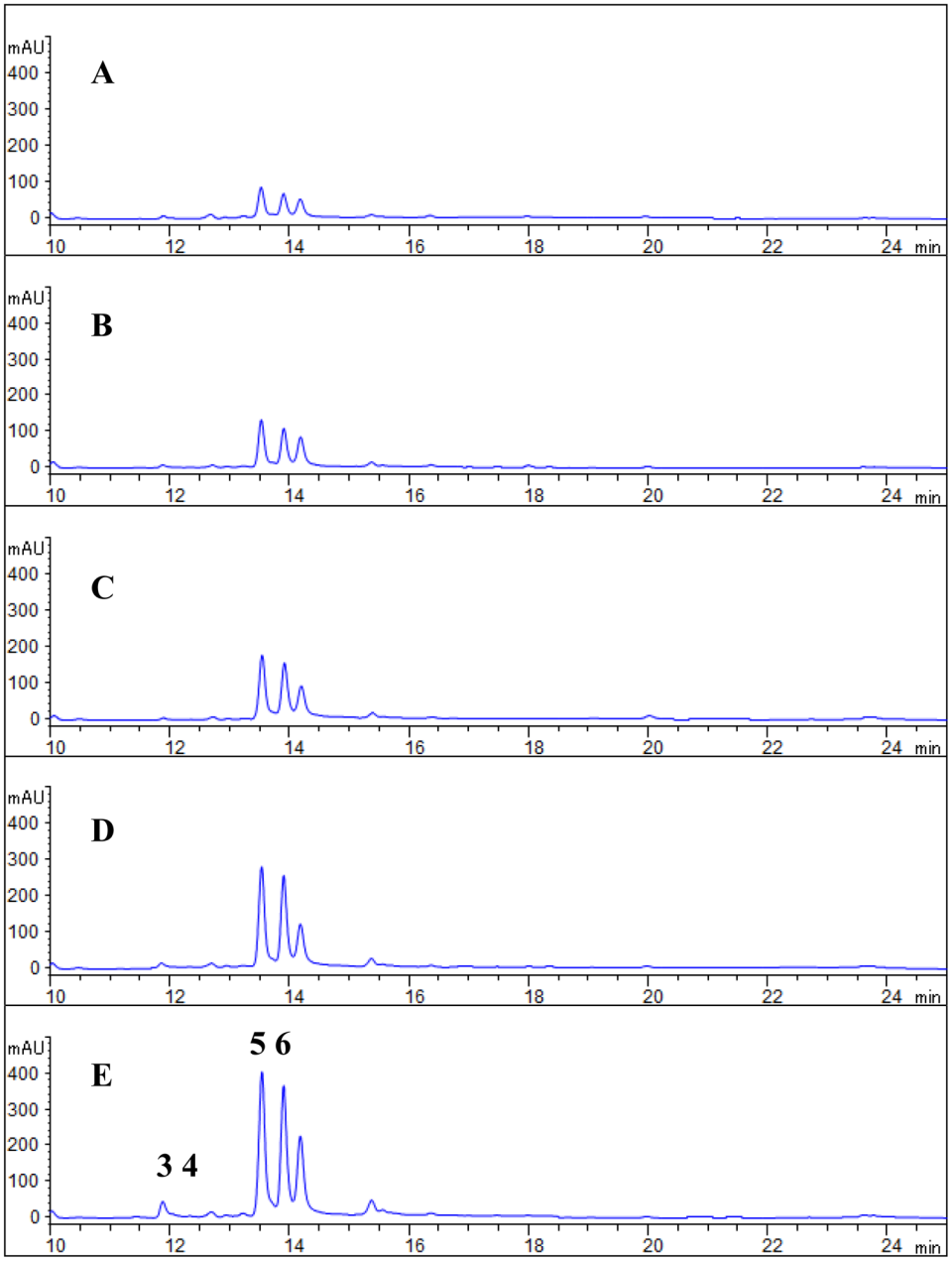

Fig. 4.

Induction of HSAF production by *Le*DSF3 in the wild type *Le*OH11. The *L. enzymogenes* OH11 cultures were grown in 10% TSB for 24 h, and HSAF was extracted from the individual cultures and analyzed by HPLC. **A:** No addition; **B:** 0.2 μM *Le*DSF3 added; **C:** 1.0 μM *Le*DSF3 added; **D:** 5.0 μM *Le*DSF3 added**, E:** 10.0 μM *Le*DSF3 added. For identity of the compounds, HSAF (**3**), alteramide A (**4**), 3-deOH-HSAF (**5**), and 3-deOH-altermide A (**6**).

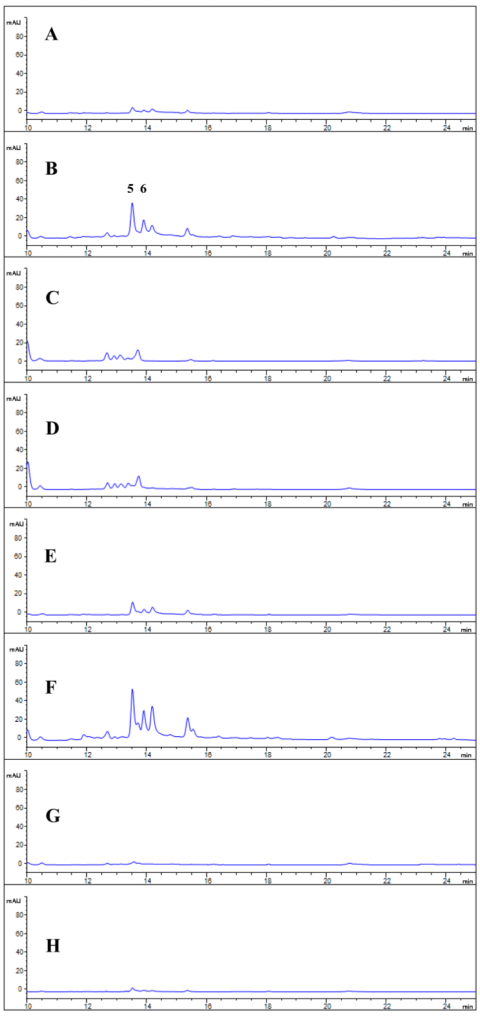

Fig. 5.

Effect of *Le*DSF3 on HSAF production in various *Le*OH11 mutants. **A:** *Le*OH11Δ*rpfB*; **B:** Δ*rpfB* treated with 5 μM *Le*DSF3; **C:** Δ*rpfC*; **D:** Δ*rpfC* treated with 5 μM *Le*DSF3; **E:** Δ*rpfF*; **F:** Δ*rpfF* treated with 5 μM *Le*DSF3; **G:** Δ*rpfG*; **H:** Δ*rpfG* treated with 5 μM *Le*DSF3.

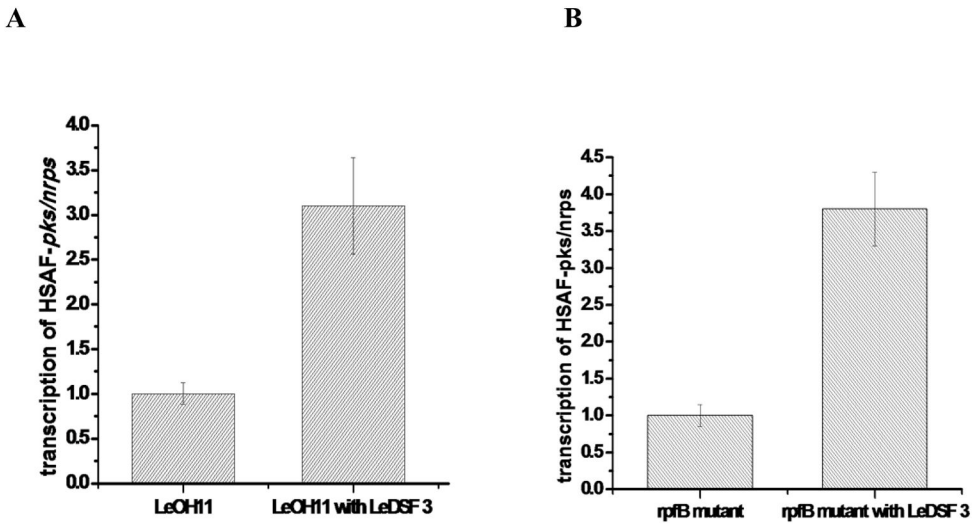

Fig. 6.

Effect of exogenous *Le*DSF3 on the transcription of HSAF *pks-nrps* gene in the wild type *Le*OH11 (A) and in *rpfB* mutant (B), as detected by Q-RT-PCR. The concentration of *Le*DSF3 was 10 μM. The relative transcription level of *pks/nrps* in the untreated *Le*OH11 was set as 1.

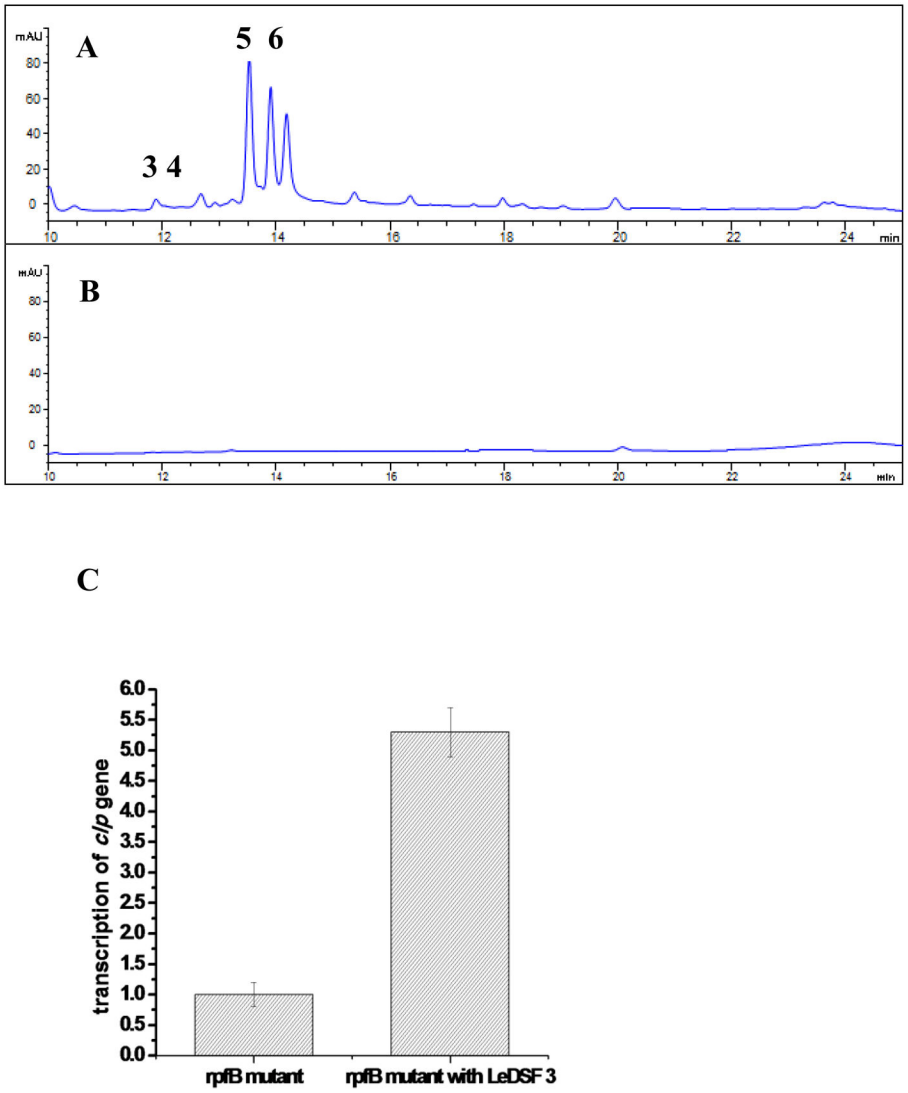

Fig. 7.

Involvement of the global regulator Clp in the RpfC/RpfG-mediated *Le*DSF signaling toward HSAF biosynthesis. A: HSAF production in the wild type *L. enzymogenes* OH11. B: HSAF production in the *clp* mutant. C: Effect of exogenous *Le*DSF3 on the transcription of *clp* gene in *rpfB* mutant, as detected by Q-RT-PCR. The concentration of *Le*DSF3 was 10 μM. The relative transcription level of *clp* gene in untreated *rpfB* mutant was set as 1.