The predatory soil bacterium *Lysobacter* reprograms quorum sensing system to regulate antifungal antibiotic production in a cyclic-di-GMP-independent manner

Kaihuai Li1,2, Gaoge Xu1, Bo Wang1, Guichun Wu1, Rongxian Hou1,2 & Fengquan Liu1,2✉

Soil bacteria often harbour various toxins to against eukaryotic or prokaryotic. Diffusible signal factors (DSFs) represent a unique group of quorum sensing (QS) chemicals that modulate interspecies competition in bacteria that do not produce antibiotic-like molecules. However, the molecular mechanism by which DSF-mediated QS systems regulate antibiotic production for interspecies competition remains largely unknown in soil biocontrol bacteria. In this study, we find that the necessary QS system component protein RpfG from *Lysobacter*, in addition to being a cyclic dimeric GMP (c-di-GMP) phosphodiesterase (PDE), regulates the biosynthesis of an antifungal factor (heat-stable antifungal factor, HSAF), which does not appear to depend on the enzymatic activity. Interestingly, we show that RpfG interacts with three hybrid two-component system (HyTCS) proteins, HtsH1, HtsH2, and HtsH3, to regulate HSAF production in *Lysobacter*. In vitro studies show that each of these proteins interacted with RpfG, which reduced the PDE activity of RpfG. Finally, we show that the cytoplasmic proportions of these proteins depended on their phosphorylation activity and binding to the promoter controlling the genes implicated in HSAF synthesis. These findings reveal a previously uncharacterized mechanism of DSF signalling in antibiotic production in soil bacteria.

1 Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base, Ministry of Science and Technology, 210014 Nanjing, China. 2 College of Plant Protection, Nanjing Agricultural University, 210095 Nanjing, China. ✉email: fqliu20011@sina.com
nterspecies competition plays a key role in shaping microbial populations and determining the bacterial species that are dominant in a given niche. To fend off these competitors, bacteria deploy various toxins against eukaryotic or prokaryotic. The diffusible signal factor (DSF) family signals are important for maintaining the interspecies competitive fitness of bacteria. The DSF family signals interfere with the morphological transition of Candida albicans through inter-kingdom communication. DSFs represent a class of widely conserved QS signals with a fatty acid moiety that regulate various biological functions in pathogenic and beneficial environmental bacteria. The Rpf gene cluster is important for the DSF signaling network in bacteria, and the role of the RpfF and RpfC/RpfG two-component system (TCS) in this gene cluster in DSF production and signal transduction has been well documented. Several lines of evidence indicate that RpfC and RpfG constitute a TCS responsible for the detection and transduction of the QS signal DSF. RpfC undergoes autophosphorylation upon sensing high levels of extracellular DSF signals. A previous study revealed that RpfG contains both an N-terminal response regulator domain and a C-terminal HD-GYP domain. The activated HD-GYP domain of RpfG has cyclic dimeric GMP (c-di-GMP) phosphodiesterase (PDE) activity that can degrade c-di-GMP, an inhibitory ligand of the global transcription factor Clp. Consequently, derepressed Clp drives the expression of several hundred genes, including those encoding virulence factor production in the plant pathogen Xanthomonas. The DSF signal family is a structural class of QS signals with the cis-2-unsaturated fatty acid moiety. Surprisingly, a DSF-like signal (LeDSF3), unlike other members of the DSF family, does not contain the cis double bond, and has been characterized as a QS signal in the biocontrol agent strain Lysobacter enzymogenes. However, the regulatory mechanism of the DSF-mediated QS system remains unknown in bacteria that are beneficial to plants.

L. enzymogenes is a nonpathogenic strain that was used to control crop fungal diseases known for the synthesis of an antifungal factor (heat-stable antifungal factor, HSAF) that exhibits inhibitory activity against a wide range of fungal species. Our previous work revealed that RpfG affects production of the antifungal factor HSAF in L. enzymogenes. However, the molecular mechanism by which RpfG regulates the biosynthesis of HSAF remains unknown.

In the present study, we found that unlike the Xanthomonas RpfC/RpfG-Clp signaling pathway, the L. enzymogenes RpfG protein interacts with three hybrid two-component system (HyTCS) proteins (HtsH1, HtsH2, and HtsH3) to regulate the production of the antifungal factor HSAF and describe their regulatory functions in soil bacteria. The HtsH1, HtsH2, and HtsH3 functions likely represent a common mechanism that helps establish signaling specificity in bacteria for interspecies competition.

Results

The HD-GYP domain of RpfG has PDE activity and can degrade c-di-GMP. Sequence analysis revealed that the HD-GYP domain contains all residues essential for PDE activities, thus suggesting that RpfG may be a PDE enzyme. HD-GYP domain-containing proteins can degrade the c-di-GMP to GMP and 5’-pGpG. However, the in vitro enzyme activity of RpfG homologs has not been studied and identified. To obtain direct evidence for the biochemical function of RpfG, recombinant N-terminal maltose binding protein (MBP) RpfG (designated RpfG-MBP) was produced. The proteins had a monomeric molecular weight of 71 kDa, as observed by SDS gel electrophoresis, and were purified by Dextrin Sepharose High Performance to obtain the preparations (Fig. 1a and Supplementary Fig. 10). The RpfG protein was fused with the MBP tag, leading to the presence of some impurities. This RpfG-MBP protein was able to degrade the model substrate c-di-GMP to 5’-pGpG, consistent with its PDE activity (Fig. 1b). Quantitative analysis revealed that RpfG-MBP exhibited a high level of activity for the degradation of c-di-GMP with 100% degraded at 5 min after initiation of the reaction in comparison to the MBP enzyme as a control (Fig. 1c). To better understand the roles of the HD-GYP domain in RpfG function, we substituted the RpfG residues His-190, Asp-191, Gly-253, Tyr-254, and Pro-255 of the HD-GYP signature motif with alanine (Ala) by site-directed mutagenesis into constructs expressing the RpfG-H190A-MBP, RpfG-D191A-MBP, RpfG-G253A-MBP, and RpfG-P255A-MBP proteins. We tested the c-di-GMP PDE activity of these mutant proteins. Our results showed that point mutations of the H190, D191, G253, and P255 residues in RpfG almost abolished PDE activity (Fig. 1b, c). These data suggest that RpfG has PDE activity and that the HD-GYP individual residues are required for full PDE activity of RpfG in vivo.

HSAF production does not depend on RpfG PDE enzymatic activity. Previous studies found that the QS signal LeDSF3 positively regulates HSAF biosynthesis. To ultimately determine whether DSF type-based QS systems are critical for regulating the synthesis of HSAF, we quantified the HSAF production in the ΔrpfF and ΔrpfG mutant strains grown in 10% TSB medium or 10% TSB medium supplemented with 10 μM canonical DSF. As shown in Supplementary Fig. 1, HSAF production in the ΔrpfF and ΔrpfG mutant strains was completely suppressed, and DSF significantly rescued HSAF production in the ΔrpfF mutant strain, but did not rescued that by the ΔrpfG mutant strain. These findings suggested that DSF type-based QS systems are critical for regulating the biosynthesis of HSAF in L. enzymogenes. Our previous work also revealed that RpfG affects HSAF production in L. enzymogenes. However, the molecular mechanism by which RpfG regulates HSAF synthesis remains unknown. Since RpfG, as a PDE enzyme, was able to degrade the substrate c-di-GMP to 5’-pGpG, we investigated whether RpfG PDE activity played a major role on controlling the production of HSAF in L. enzymogenes. We quantified HSAF production in the ΔrpfG mutant and complementary strain (ΔrpfG/rpfG) by HPLC. We found that HSAF production in the ΔrpfG mutant strain was completely suppressed (Fig. 2a). The complementary strain ΔrpfG/rpfG yielded HSAF at the level of the wild-type strain (Fig. 2a). These results were similar to those of the above research. To examine the relationship between the regulatory and enzymatic activities of RpfG, mutations at the conserved His-190, Asp-191, Gly-253, Tyr-254, and Pro-255 of the HD-GYP signature motif with alanine (Ala) were examined by site-directed mutagenesis. We tested HSAF production in the ΔrpfG mutant strain carrying plasmids encoding these mutant proteins. The strains expressing the RpfG H190A, D191A, G253A, Y254A, and P255A mutant proteins showed increased HSAF production compared with the ΔrpfG mutant strain. These results were superior to those of the complementary strain ΔrpfG/rpfG. Importantly, the ΔrpfG mutant strain and complemented strains (ΔrpfG/rpfG, ΔrpfG/rpfG H190A, ΔrpfG/rpfG D191A, ΔrpfG/rpfG G253A, ΔrpfG/rpfG Y254A, and ΔrpfG/rpfG P255A) did not impair bacterial growth (Fig. 2b, c). As described above, His-190, Asp-191, Gly-253, Tyr-254, and Pro-255 were found to be critical for the PDE activity of RpfG (Fig. 1), implying that HSAF is regulated in a PDE independent manner. To test this prediction, we compared intracellular c-di-GMP concentrations in the ΔrpfG mutant and the wild type and in the HSAF-production medium.
We found that the concentration in the ΔrpfG mutant did not significantly change c-di-GMP production compared with the wild-type strain (Fig. 2d). These findings indicated that the regulatory activity of RpfG does not depend on its PDE enzymatic activity against c-di-GMPs.

RpfG binds directly to the HyTCS protein HtsHs. The above findings confirmed that RpfG does not regulate HSAF biosynthesis through the c-di-GMP signaling pathway, indicating that RpfG might regulate HSAF biosynthesis through interactions with other proteins in L. enzymogenes. To further explore the mechanisms underlying the contribution of RpfG to HSAF production, we used a bioinformatic tool (STRING) to identify potential interactors for RpfG; these represent interactions that possibly lead to alterations in HSAF synthesis. We discovered through bioinformatics predictions that RpfG interacts with three HyTCS proteins (Supplementary Fig. 2a). We designated the HyTCS protein HtsH (hybrid two-component signaling system regulating HSAF production) based on the findings of this study.
analyses, a series of RT-PCR primers (Supplementary Table 2 and Supplementary Fig. 2b) were designed to determine whether there are intergenic transcripts crossing the adjacent genes. As shown in Supplementary Fig. 2c, Le3071 (htsH1), Le3072 (htsH2), and Le3073 (htsH3) likely constitute a single transcription unit because the corresponding intergenic transcripts were successfully amplified. Le3071 (htsH1), Le3072 (htsH2), and Le3073 (htsH3) encode a group of typical HyTCS proteins with pfam Reg_prop, pfam Y-Y-Y, HisKA, HATPase_c, and Response_reg domains. All three HyTCS proteins contain one predicted transmembrane region (Supplementary Fig. 2d). We examined the alignments of three HyTCS proteins (HtsH1, HtsH2, and HtsH3), and the results showed that the HtsH1 protein shares 50% and 53% identity with HtsH2 and HtsH3, respectively. We also aligned HtsH2 with HtsH3, and the identity values were 50% (Supplementary Fig. 2c).

To examine whether RpfG could directly bind to the HyTCS proteins (HtsH1, HtsH2 and HtsH3), we used a pull-down assay using E. coli-expressed proteins in vitro. We purified recombinant RpfG-MBP and the cytoplasmic fragments of HtsH1, HtsH2, and HtsH3 (HtsH1C-Flag-His, HtsH2C-HA-His, and HtsH3C-Myc-His, respectively) from E. coli (Fig. 1a and Supplementary Figure 3). We measured the possible binding events between the RpfG and HtsH1C, HtsH2C, or HtsH3C proteins. The RpfG-MBP sensor physically bound HtsH1C-Flag-His with a binding constant ($K_D$) of 0.06675 μM (Fig. 3e), suggesting an intermediate level of protein–protein interaction. We also confirmed direct binding between the RpfG-MBP and HtsH2C-HA-His or HtsH3C-Myc-His proteins by SPR ($K_D = 0.2998$ μM or $K_D = 0.1678$ μM, respectively) (Fig. 3f, g). Additionally, the SPR assay revealed that HtsH1C-Flag-His bound to HtsH2C-HA-His or HtsH3C-Myc-His with reasonably high affinity ($K_D = 0.09619$ μM or $K_D = 0.1597$ μM, respectively), and revealed that HtsH2C-HA-His bound HtsH3C-Myc-His with a $K_D$ value of 0.1782 μM (Supplementary Fig. 5). Taken together, these experiments demonstrate that RpfG directly interacts with HtsH1, HtsH2, or HtsH3 proteins in vitro.

HtsHs inhibits the PDE enzymatic activity of RpfG. We wondered whether RpfG and HtsH1, HtsH2, or HtsH3 interactions affect the PDE activity of RpfG. To test this hypothesis, we used a biochemical assay in which c-di-GMP hydrolysis by RpfG-MBP was assayed in the absence or presence of HtsH1, HtsH2, or HtsH3 at concentrations ranging from 0 to 32 μM. The results of the assay showed that the PDE activity of RpfG-MBP was lower in the presence than in the absence of HtsH1C-Flag-His, HtsH2C-HA-His, or HtsH3C-Myc-His (Fig. 4). Therefore, the results of the assays suggested that HtsH1, HtsH2, and HtsH3 inhibited the PDE enzymatic activity of RpfG. This result further confirms that the ability of RpfG to regulate HSAF production does not depend on its PDE enzymatic activity against c-di-GMP in L. enzymogenes.

Deletion of htsH1, htsH2, and htsH3 resulted in decreased HSAF production. The above studies indicated that RpfG might regulate HSAF biosynthesis by interacting with HtsHs (HtsH1, HtsH2, and HtsH3) in L. enzymogenes. To identify the physiological functions of HtsH1, HtsH2, and HtsH3 in HSAF production, the genes htsH1 (Le3071), htsH2 (Le3072), and htsH3 (Le3073) were deleted using a two-step homologous recombination approach to construct the single knockout strains ΔhtsH1, ΔhtsH2, and ΔhtsH3; the double mutant knockout strains ΔhtsH12, ΔhtsH13, and ΔhtsH23; and the triple knockout strain ΔhtsH123. Using quantitative RT-PCR (qRT-PCR), we measured the mRNA abundance of htsH1, htsH2, htsH3, Le3074 and...
Le3075 in the htsH mutants (ΔhtsH1, ΔhtsH2, ΔhtsH3 and ΔhtsH123), and found that the genes downstream of the htsH gene deletion mutants were expressed (Supplementary Fig. 6). Subsequently, we quantified HASF production in all the above mutant strains described by HPLC. We found that ΔhtsH1, ΔhtsH2, and ΔhtsH3 exhibited slightly decreased HASF levels. However, the double-mutant strains ΔhtsH12, ΔhtsH13, ΔhtsH23, and triple-mutant strain ΔhtsH123 exhibited a significant decrease in HASF levels compared with the wild-type levels (Fig. 5a). To determine the role of HtsH1, HtsH2, and HtsH3 in the regulation of HASF biosynthesis, we complemented ΔhtsH1, ΔhtsH2, ΔhtsH3, the double-mutant strains ΔhtsH12, ΔhtsH13, ΔhtsH23, and the triple-mutant strain ΔhtsH123 with plasmid-borne htsH1, htsH2, htsH3, htsH12, htsH13, htsH23, and htsH123. HASF production in the complemented strains (ΔhtsH1/htsH1, ΔhtsH2/htsH2, ΔhtsH3/htsH3, ΔhtsH12/htsH12, ΔhtsH13/htsH13, ΔhtsH23/htsH23, and ΔhtsH123/htsH123) restored HASF biosynthesis compared with the wild-type levels (Fig. 5a). Importantly, ΔhtsH1, ΔhtsH2, and ΔhtsH3; the double-mutant strains ΔhtsH12, ΔhtsH13, and ΔhtsH23; and the triple-mutant strain ΔhtsH123 did not exhibit impaired bacterial growth (Fig. 5b, c), implying that HtsH1, HtsH2, and HtsH3 play a specific role in regulating HASF production.

HtsH1, HtsH2, and HtsH3 positively regulate HASF biosynthesis gene expression. Earlier, we found that deletion of htsH1, htsH2, and htsH3 resulted in decreased HASF production. Thus, we wondered whether HtsH1, HtsH2, and HtsH3 might directly target HASF biosynthesis gene promoters. To test this hypothesis, we performed an E. coli-based one-hybrid assay. As

Fig. 3 RpfG interaction with HtsH1, HtsH2, and HtsH3. a An MBP pull-down assay confirming interactions between RpfG-MBP and the cytoplasmic fragment of HtsH1, HtsH2, and HtsH3 (HtsH1C-Flag-His, HtsH2C-HA-His and HtsH3C-Myc-His, respectively). The pull-down assay was carried out using anti-MBP antibody. Western blotting was performed using anti-MBP, anti-Flag, anti-HA, and anti-Myc antibodies. b A His pull-down assay confirming interactions between the cytoplasmic fragment of HtsH1 (HtsH1C-Flag-His) and RpfG-MBP. The pull-down assay was carried out using Ni-NTA agarose. Western blotting was performed using anti-Flag and anti-MBP antibodies. c A His pull-down assay confirming interactions between the cytoplasmic fragment of HtsH2 (HtsH2C-HA-His) and RpfG-MBP. The pull-down assay was carried out using Ni-NTA agarose. Western blotting was performed using anti-HA and anti-MBP antibodies. d A His pull-down assay confirming interactions between the cytoplasmic fragment of HtsH3 (HtsH3C-Myc-His) and RpfG-MBP. The pull-down assay was carried out using Ni-NTA agarose. Western blotting was performed using anti-Myc and anti-MBP antibodies. e SPR showing that HtsH1C-Flag-His forms a complex with RpfG-MBP with $K_D = 0.06675 \mu M$. f SPR showing that HtsH2C-HA-His forms a complex with RpfG-MBP with $K_D = 0.2998 \mu M$. g SPR showing that HtsH3C-Myc-His forms a complex with RpfG-MBP with $K_D = 0.1678 \mu M$.
shown in Fig. 6a. HtsH1, HtsH2, and HtsH3 directly bound to the upstream region of the HSAF biosynthesis operon (plaB).

Next, we tested the ability of HtsH1, HtsH2, and HtsH3 to bind to the lafB promoter, using an electrophoretic mobility shift assay (EMSA). A PCR-amplified 590 bp DNA fragment from the plaB probe in a dose-dependent manner (Fig. 6b–d and Supplementary Fig. 12). We quantified the binding affinity of HtsH1, HtsH2, and HtsH3 to the HSAF operon promoter. In an SPR analysis, HtsH1C-Flag-His directly bound to the promoter of the HSAF biosynthesis gene (plaB) with high affinity ($K_0 = 0.5356 \mu M$) (Fig. 6e). In addition, HtsH2C-HA-His and HtsH3C-Myc-His bound to plaB with $K_0$ values of 1.379 $\mu M$ and 0.2491 $\mu M$, respectively (Fig. 6f, g). The results demonstrated that HtsH1, HtsH2, and HtsH3 could directly target the promoters of the HSAF biosynthesis gene.

Based on the above results, we compared the transcriptome profiles of the wild-type strain and the htsH mutants ($\Delta$htsH1, $\Delta$htsH2, $\Delta$htsH3, $\Delta$htsH12, $\Delta$htsH13, $\Delta$htsH23, and $\Delta$htsH123) by RNA-Seq and observed changes in the expression levels of several hundred genes (Supplementary Data 1). We then performed trend analysis of the differential gene expression and found that the amounts of HSAF biosynthesis gene cluster mRNA were constitutively decreased in the htsH mutants ($\Delta$htsH1, $\Delta$htsH2, $\Delta$htsH12, $\Delta$htsH13, $\Delta$htsH23, and $\Delta$htsH123) by 590 bp DNA fragment from the p

**Discussion**

Quorum sensing (QS) allows populations of bacteria to communicate via the exchange of chemical signals, resulting in...
coordinated gene expression in response to cell density. \(^{26}\) AHL signaling was first discovered in the marine bacterium *Vibrio fischeri*. In *V. fischeri*, LuxI and LuxR produce and respond to 3OC6-HSL, respectively. \(^{27,28}\) In addition to *V. fischeri*, *Pseudomonas aeruginosa* has emerged as an important model organism for QS research. \(^{27}\) In *P. aeruginosa*, the LasIR system produces and responds to 3OC12-HSL, and the RhlR system produces and responds to C4-HSL. \(^{26,27}\) QscR is an orphan LuxR receptor that is not linked to a luxI synthase gene. QscR responds to 3OC12-HSL produced by LasI. \(^{27,29}\) The quorum-sensing transcriptional activator TraR of *Agrobacterium tumefaciens*, which controls the replication and conjugal transfer of the tumor-inducing (Ti) virulence plasmid, is inhibited by the TraM antiactivator. \(^{30,31}\) In the DSF-mediated quorum sensing system, RpfC undergoes autophosphorylation upon sensing accumulated extracellular DSF signals. Through the conserved phosphorelay mechanism, RpfG is phosphorylated, which leads to activation of its c-di-GMP phosphodiesterase activity. Degradation of c-di-GMP releases Clp, which regulates subsets of virulence genes directly or through the downstream transcription factors FhrR and Zur. \(^{5,11}\)

Fig. 6 HtsH1, HtsH2, and HtsH3 directly bound the promoter of the HSAF biosynthesis gene (*plaf*B). a Direct physical interaction between HtsH1, HtsH2, and HtsH3 and the *plaf*B promoter region was detected in *E. coli*. Experiments were performed according to the procedures described in the "Methods" section. +, cotransformant containing pBX-R2031 and pTRG-R3133, used as a positive control; −, cotransformant containing pBXcmT and the empty pTRG, used as a negative control; HtsH1C and *plaf*B, cotransformant harboring pTRG-HtsH1C and pBXcmT-*plaf*B; HtsH2C and *plaf*B, cotransformant harboring pTRG-HtsH2C and pBXcmT-*plaf*B; HtsH3C and *plaf*B, cotransformant harboring pTRG-HtsH3C and pBXcmT-*plaf*B. -3AT-Str, plate with no selective medium (3AT 3-amino-1,2,4-triazole and Str streptomycin) and +3AT + Str, plate with M9-based selective medium. b–d Gel shift assay showing that HtsH1, HtsH2, and HtsH3 directly regulate an HSAF biosynthesis gene. HtsH1C-Flag-His, HtsH2C-HA-His, or HtsH3C-Myc-His protein (0, 2, 4, 8 or 16 μg) was added to reaction mixtures containing 50 ng of probe DNA, and the reaction mixtures were separated on polyacrylamide gels. e,f SPR showing that HtsH1C-Flag-His forms a complex with *plaf*B with \(K_D = 0.5356\) μM. f SPR showing that HtsH2C-HA-His forms a complex with *plaf*B with \(K_D = 1.379\) μM. g SPR showing that HtsH3C-Myc-His forms a complex with *plaf*B with \(K_D = 0.2491\) μM.
In this study, we revealed a signal pathway by which the DSF type-based QS system component protein RpfG to interacts with HtsH1, HtsH2, and HtsH3 to regulate the biosynthesis of HSAF in *L. enzymogenes*.

In previous studies, we and our collaborators have shown that QS was employed by *L. enzymogenes* to affect production of the antifungal factor HSAF. However, the mechanism through which QS coordinates the synthesis of HSAF remains unknown. RpfG, as a necessary component protein of the DSF mediated QS signal transduction system, contains a C-terminal HD-GYP domain that can affect production of the antifungal factor HSAF in *L. enzymogenes*. However, how RpfG regulates the synthesis of HSAF remains incompletely studied. The results of the present study provide biochemical, genetic, and biophysical evidence to demonstrate that *L. enzymogenes* reprograms the QS signal system that RpfG interacts with HtsHs to regulate the biosynthesis of the antifungal antibiotic HSAF. HtsH1, HtsH2, and HtsH3 regulate the expression of the synthetic genes of the antifungal factor HSAF depending on their phosphorylation (Fig. 8).

HD-GYP domain proteins are c-di-GMP PDEs that can degrade c-di-GMP. However, the role of the HD-GYP domain of RpfG has remained unelucidated. Therefore, we tested the PDE activity of RpfG and successfully showed that it was able to degrade the model substrate c-di-GMP to 5′-pGpG. To test whether the HD-GYP motif was important for catalytic activity in RpfG, we constructed RpfG mutant proteins (RpfG-H190A-MBP, RpfG-D191A-MBP, RpfG-G253A-MBP, and RpfG-P255A-MBP). We tested the c-di-GMP PDE activity of these mutant proteins and suggested that the HD-GYP domain was required for full PDE activity of RpfG in vivo. It is generally speculated that the PDE activity of HD-GYP domain proteins degrades c-di-GMP to GMP. However, we showed that the activity of the HD-GYP domain of RpfG is involved in the degradation of c-di-GMP to 5′-pGpG.

Intriguingly, we found that the strains expressing the RpfG H190A, D191A, G253A, Y254A, and P255A mutant proteins resulted in increased HSAF production compared with the ΔrpfG mutant strain. Importantly, we found that the ΔrpfG mutant did not significantly change c-di-GMP production compared with of the wild-type strain in the antifungal factor HSAF-production medium (10% TSB). These data demonstrated that the regulatory activity of RpfG does not depend on its PDE enzymatic activity. This is the first report showing that a PDE does not depend on its c-di-GMP-degrading activity to regulate a downstream pathway. Thus, we wondered whether RpfG regulates HSAF synthesis through interactions with other proteins in *L. enzymogenes*.

Bioinformatics predictions have shown that RpfG may interact with three HyTCS proteins (HtsH1, HtsH2, and HtsH3). Then, we used pull-down and SPR to demonstrate the binding events between the RpfG and HtsH1, HtsH2, or HtsH3 proteins. Notably, RpfG and HtsH1, HtsH2, or HtsH3 interactions affect the PDE activity of RpfG. However, how RpfG affects HtsH1, HtsH2, or HtsH3 remains unknown. We speculate that RpfG may affect HtsH1, HtsH2, or HtsH3 autophosphorylation. However,
we could not obtain the full-length HtsH1, HtsH2, and HtsH3 proteins, so further clarification of these possible mechanisms will help elucidate the mechanism underlying the RpfG interaction with HtsH1, HtsH2, or HtsH3. Moreover, we found that htsH1, htsH2, and htsH3 likely constitute a single transcription unit. HyTCS-based regulation may be crucial for responding to environmental changes and finely tuning gene expression. However, the biological function of these consecutive HyTCS proteins has not been reported in bacteria.

In this study, we found that the in-frame deletion of the htsH1, htsH2, and htsH3 coding sequences significantly decreased HSAF production. Thus, we speculate that RpfG may interact with three HyTCS proteins to coordinate HSAF production in L. enzymogenes. To test this hypothesis, we performed an E. coli-based one-hybrid assay and EMSA. The results demonstrated that HtsH1, HtsH2, and HtsH3 could directly target the promoters of HSAF biosynthesis genes. We further analysed the transcription level of HSAF biosynthesis-related genes in htsH1, htsH2, and htsH3 mutants. Knockout of htsH1, htsH2, and htsH3 significantly reduced the transcription level of the antifungal factor HSAF biosynthesis genes. These results suggest that HtsH1, HtsH2, and HtsH3 can directly regulate HSAF biosynthesis gene expression and increase production of the antifungal factor HSAF in L. enzymogenes. In this study, we found that RpfG interacts with the HtsH1, HtsH2, or HtsH3 protein and that HtsH1, HtsH2, and HtsH3 can interact with each other. We also found that knockout of rpfG or htsH1, htsH2, and htsH3 significantly reduced HSAF production. These results suggest that RpfG forms a complex with HtsH1, HtsH2 and HtsH3 to regulate HSAF biosynthesis. However, the mechanism needs to be further elucidated.

Phosphorylation of TCS is critical for regulating the expression of downstream genes. Phosphorylated HtsH1, HtsH2, and HtsH3 positively regulate HSAF biosynthesis and argue with the existing literature concerning whether this is a common route of gene expression regulation. We used Mn2+ -Phos-tag SDS-PAGE and EMSA to show that HtsH1, HtsH2, and HtsH3 target plaF depending on their phosphorylation activity. In this study, we report the biological functions of the three HyTCS proteins HtsH1, HtsH2, and HtsH3 in the regulation of antibiotic biosynthesis.

One of the notable results of this study is that RpfG, HtsH1, HtsH2, and HtsH3 regulatory patterns seem to be conserved mechanisms in Lysobacter and Xanthomonas. To our knowledge, RpfG represents a unique example of a c-di-GMP metabolic enzyme that directly interacts with three HyTCS proteins (HtsH1, HtsH2, and HtsH3) to regulate HSAF biosynthesis.

Methods

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are shown in Supplementary Table 1. E. coli strains were grown in Luria-Bertani medium (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, pH 7.0) at 37°C. L. enzymogenes strains were grown at 28°C in Luria-Bertani medium and 10% TSB. For the preparation of culture media, tryptone, peptone, beef extract, and yeast extract were purchased from Sangon Biotech (Shanghai, China). When required, antibiotics were added (30 μg/mL of kanamycin sulfate, 50 μg/mL of gentamicin) to the E. coli or L. enzymogenes cultures. The bacterial growth in liquid medium was determined by measuring the optical density at 600 nm (OD600) using a Bioscreen-C Automated Growth Curves Analysis System (OY Growth Curves FP-1100-C, Helsinki, Finland).

Site-directed mutagenesis. Site-directed mutagenesis and essentiality testing were performed as follows. To obtain the RpfG mutant proteins and rpfG site-directed mutant strains, plasmids harboring mutations in rpfG were constructed. For example, to obtain the H190A mutation in RpfG, approximately 500-bp DNA fragments flanking the rpfG gene were amplified with Pfu DNA polymerase using L. enzymogenes genomic DNA as template and either MBP-rpfG P1 and rpfG H190A P1 (for the Up rpfG H190A mutant), or rpfG H190A P1 and MBP-rpfG P2 (for the Down rpfG H190A mutant) as the primers (Supplementary Table 2). The fragments were connected by overlap PCR using the primers MBP-rpfG P1 and MBP-rpfG P2. The fused fragment was digested with BamH I and HindIII and inserted into pMAL-p2X to obtain the plasmid pMAL-rpfG H190A. The other four site-directed mutant plasmids (D191A, G253A, Y254A, and P255A) were constructed using a similar method.

Protein expression and purification. Protein expression and purification were performed as follows. To clone the rpfG gene, genomic DNA extracted from L. enzymogenes was used for PCR amplification using Pfu DNA polymerase, and the primers are listed in Supplementary Table 2. The PCR products were inserted into pMAL-p2X to produce the plasmids pMAL-rpfG. The rpfG gene was verified by nucleotide sequencing by Genscript (Nanjing, Jiangsu, China). Le rpfG and rpfG site-directed mutants with a vector-encoded maltose binding protein in the
N-terminus were expressed in *E. coli* BL21 (DE3) and purified with Dextrin Sepharose High Performance (Qagen, Chatsworth, CA, USA) using an affinity column. The protein purity was measured by SDS-PAGE. His-tagged protein expression and purification were performed as described previously [41,42].

**PDE activity assays in vitro.** The PDE activity assay was performed essentially as follows [25,41]. Briefly, 2 μM MBP-RPF or its derivatives were tested in buffer containing 60 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM MnCl₂, and 10 mM MgCl₂. The reaction was started by the addition of 100 nM c-di-GMP. All reaction mixtures were incubated at 28 °C for 5–60 min, followed by boiling for 10 min to stop the reaction. The samples were filtered through a 0.2 μm pore size cellulose-acetate filter, and 20 μL of each sample was loaded onto a reverse-phase C8 column and separated by HPLC. The separation protocol involved two mobile phases, 100 mM KH₂PO₄ plus 4 mM tetrabutylammonium sulfate (A) and 75% (A + 25% methanol (B)).

**C-di-GMP extraction and quantification.** C-di-GMP extraction and quantification were performed as follows [25,42]. Cultures were in 10% TSB at 28 °C until the OD₆₀₀ reached 1.5 based on the growth curve. Cells from 2 mL of the culture were harvested for protein quantification by BCA (TransGen, China). Cells from 8 mL of culture were used for c-di-GMP extraction using 0.6 M HClO₄, and 2.5 M K₂CO₃. The samples were subjected to 0.22 μm Mini-Star filtration, and the filtrate was concentrated to 100 μL for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis on an AB SCIEX QTRAP 6500 LC-MS/MS system (AB SCIEX, USA). The separation protocol involved two mobile phases, buffer A: 100% water plus 0.1% acetic acid, buffer B: 100% methanol (B). The gradient system was from 90% buffer A and 10% buffer B to 20% buffer A and 80% buffer B. The running time was 9 min, and the flow rate was 0.3 mL/min.

**Gene deletion and complementation.** The in-frame deletions in *L. enzymogenes* OH11 were generated via double-crossover homologous recombination using Gene deletion and complementation technology Co., Ltd (Guangzhou, Guangdong, China). To analyse the DEGs influence of different gene lengths and amounts of sequencing data corrected using <0.05 as the threshold.

**Statistics and reproducibility.** The experimental datasets were subjected to analyses of variance using GraphPad Prism 7.0. The significance of the treatment...
effects was determined by the F value ($F = 0.05$). If a significant $F$ value was obtained, separation of means was accomplished by Fisher’s protected least significant difference at $P = 0.05$.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/nuccore/, accession numbers RCTY01000033 (Lysobacter enzymogenes strain OH11 scffold34, whole genome shotgun sequence; Le4727/Le RpfG, locus tag = D9T7\_13845), RCTY01000055 (Lysobacter enzymogenes strain OH11 scffold56, whole genome shotgun sequence; Le3071/Le HtsH1, locus tag = D9T7\_21400), RCTY01000054 (Lysobacter enzymogenes strain OH11 scffold55, whole genome shotgun sequence; Le3072/Le HtsH2, locus tag = D9T7\_21390), RCTY01000055 (Lysobacter enzymogenes strain OH11 scffold55, whole genome shotgun sequence; Le3073/Le HtsH3, locus tag = D9T7\_21385). RNA-sequencing raw data are deposited into the NCBI’s Sequence Read Archive (SRA) and are accessible through BioProject series accession PRJNA758119. All source data to generate graphs have been combined in Supplementary Data 2. Any remaining data are available from the corresponding author on reasonable request.

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**References**

1. McCarthy, R. R. et al. Cyclic di-GMP inactivates T6SS and T4SS activity in Agrobacterium tumefaciens. Mol. Microbiol. 112, 632–648 (2019).

2. Deng, Y. et al. Diffusible signal factor family signals provide a fitness advantage to Xanthomonas campestris pv. campesiris in interspecies competition. Environ. Microbiol. 18, 1534–1545 (2016).

3. Boon, C. et al. A novel DSF-like signal from Lysobacter enzymogenes strain OH11 scffold55, whole genome shotgun sequence; Le4727/Le RpfG, https://www.ncbi.nlm.nih.gov/nuccore/, accession numbers RCTY01000033 (Lysobacter enzymogenes, strain OH11 scffold34, whole genome shotgun sequence; Le3073/Le HtsH3, locus tag = RCTY01000054 (Lysobacter enzymogenes strain OH11 scffold54, whole genome shotgun sequence; Le3071/Le HtsH1, locus tag = D9T7\_21400), RCTY01000055 (Lysobacter enzymogenes strain OH11 scffold55, whole genome shotgun sequence; Le3072/Le HtsH2, locus tag = D9T7\_21390), RCTY01000055 (Lysobacter enzymogenes strain OH11 scffold55, whole genome shotgun sequence; Le3073/Le HtsH3, locus tag = D9T7\_21385)). RNA-sequencing raw data are deposited into the NCBI’s Sequence Read Archive (SRA) and are accessible through BioProject series accession PRJNA758119. All source data to generate graphs have been combined in Supplementary Data 2. Any remaining data are available from the corresponding author on reasonable request.

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Author contributions

K.L. and F.L. conceived and designed the experiments. K.L., G.X., B.W., R.H., and G.W. performed the experiments. K.L. analysed the data and prepared the figures. K.L. wrote the manuscript draft. F.L. revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Fengquan Liu.

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