

A predatory soil bacterium reprograms a quorum sensing signal system to regulate antifungal weapon production in a cyclic-di-GMP-independent manner

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Article

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Abstract

Soil bacteria often provide multiple weapons for eukaryotes or prokaryotes to use against predators. 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 weapons production for interspecies competition remains largely unknown in soil biocontrol bacteria. In this study, we found 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 weapon (heat-stable antifungal factor, HSAF), which does not appear to depend on the enzymatic activity. Interestingly, we showed for the first time that RpfG interacts with three hybrid two-component system (HyTCS) proteins, HtsH1, HtsH2, and HtsH3, to regulate HSAF production in *Lysobacter*. In vitro studies showed that each of these proteins interacted with RpfG, which reduced the PDE activity of RpfG. Finally, we showed 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 new mechanism of DSF signalling in antifungal weapon production in soil bacteria.

Introduction

Interspecies competition plays a key role in shaping microbial populations and determining the bacterial species that are dominant in a given niche ¹. To fend off these competitors, bacteria deploy various toxins as weapons against eukaryotic or prokaryotic predators. The diffusible signal factor (DSF) family signals are important for maintaining the interspecies competitive fitness of bacteria ². The DSF-family signals interfere with the morphological transition of *Candida albicans* through inter-kingdom communication ^{3,4}.

DSFs represent a new class of widely conserved quorum sensing (QS) signals with a fatty acid moiety that regulate various biological functions in pathogenic and beneficial environmental bacteria ^{5,6}. The Rpf gene cluster is important for the DSF signalling 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 ^{5,7-10}. A previous study revealed that RpfG contains both an N-terminal response regulator domain and a C-terminal HD-GYP domain ¹¹. 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* ¹²⁻¹⁵. However, the regulatory mechanism of the DSF-mediated QS system remains unknown in bacteria that are beneficial to plants.

Lysobacter enzymogenes is a nonpathogenic strain that was used to control crop fungal diseases known for the synthesis of an antifungal weapon (heat-stable antifungal factor, HSAF) that exhibits inhibitory

activity against a wide range of fungal species¹⁶⁻²³. Our previous work revealed that RpfG affects production of the antifungal weapon HSAF in *L. enzymogenes*²⁴. 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 signalling pathway, the *L. enzymogenes* RpfG protein interacts with three hybrid two-component system (HyTCS) proteins (HtsH1, HtsH2, HtsH3) to regulate the production of the antifungal weapon HSAF and describe for the first time their regulatory functions in soil bacteria. The HtsH1, HtsH2, and HtsH3 functions likely represent a common mechanism that helps establish signalling 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. 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 and were purified by dextrin sepharose high performance to obtain preparations that exhibited by SDS-gel electrophoresis (Figure 1A). This RpfG-MBP protein was able to degrade the model substrate c-di-GMP to 5'-pGpG, consistent with its PDE activity (Figure 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 (Figure 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 His-190, Asp-191, Gly-253, and Pro-255 residues in RpfG almost abolished PDE activity (Figure 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

Our earlier work revealed that RpfG affects HSAF production in *L. enzymogenes*²⁴. 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 in *L. enzymogenes*. We quantified HSAF production in the $\Delta rpfG$ mutant and complementary strain ($\Delta rpfG/rpfG$) by HPLC. We found that HSAF production in the $\Delta rpfG$ mutant strain was completely suppressed (Figure 2A). The complementary strain $\Delta rpfG/rpfG$ yielded HSAF at the level of the wild-type strain (Figure 2A). These results were similar to those of the above research²⁴. 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 $\Delta rpfG$ 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 $\Delta rpfG$ mutant strain. These results were superior to those of the complementary strain $\Delta rpfG/rpfG$. Importantly, the $\Delta rpfG$ mutant strain and complemented strains ($\Delta rpfG/rpfG$, $\Delta rpfG/rpfG$ H190A, $\Delta rpfG/rpfG$ D191A, $\Delta rpfG/rpfG$ G253A, $\Delta rpfG/rpfG$ Y254A, and $\Delta rpfG/rpfG$ P255A) did not impair bacterial growth (Figure 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 (Figure 1), implying that HSAF is regulated in a PDE independent manner. To test this prediction, we compared intracellular c-di-GMP concentrations in the $\Delta rpfG$ mutant and the wild type and in the HSAF-production medium (10% TSB). We found that the concentration in the $\Delta rpfG$ mutant did not significantly change c-di-GMP production compared with the wild-type strain (Figure 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 signalling 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 that RpfG interacts with three HyTCS proteins through bioinformatics predictions (Figure S1A). We designated the HyTCS protein HtsH (hybrid two-component signalling system regulating HSAF production) based on the findings of this study. To verify the operon structure of *htsHs* for in-depth genetic analyses, a series of RT-PCR primers (Table S2 and Figure S1B) were designed to determine whether there are intergenic transcripts crossing the adjacent genes. As shown in Figure S1C, 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 (Figure S1D). 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% (Figure S2).

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* (Figure 1A and Figure S3). First, we tested the ability of RpfG-MBP to pull down HtsH1C-Flag-His, HtsH2C-HA-His, and HtsH3C-Myc-His. Finally, we confirmed the interaction between RpfG and HtsH1, HtsH2 or HtsH3 using pull down assays (Figure 3A). Conversely, we examined

the ability of HtsH1C-Flag-His, HtsH2C-HA-His, and HtsH3C-Myc-His to pull down RpfG-MBP and observed a positive signal (Figure 3B-D). Second, we used surface plasmon resonance (SPR) to measure the possible binding events between the RpfG and HtsH1, HtsH2, or HtsH3 proteins. The RpfG-MBP sensor physically bound HtsH1C-Flag-His with a binding constant (K_D) of 0.06675 μM (Figure 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 ($K_D = 0.2998 \mu\text{M}$ or $K_D = 0.1678 \mu\text{M}$, respectively) (Figure 3F-G). Additionally, the SPR assay revealed that HtsH1C-Flag-His bound to HtsH2C-HA-His or HtsH3C-Myc-His with reasonably high affinity ($K_D = 0.09619 \mu\text{M}$ or $K_D = 0.1597 \mu\text{M}$, respectively) and revealed that HtsH2C-HA-His bound HtsH3C-Myc-His with a K_D value of 0.1782 μM (Figure S4). 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, ranging from 0 μM 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 (Figure 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 $\Delta htsH1$, $\Delta htsH2$, and $\Delta htsH3$; the double mutant knockout strains $\Delta htsH12$, $\Delta htsH13$, and $\Delta htsH23$; and the triple knockout strain $\Delta htsH123$. Subsequently, we quantified HSAF production in all the above mutant strains described by HPLC. We found that $\Delta htsH1$, $\Delta htsH2$, and $\Delta htsH3$ exhibited slightly decreased HSAF levels. However, the double-mutant strains $\Delta htsH12$, $\Delta htsH13$, $\Delta htsH23$, and triple-mutant strain $\Delta htsH123$ exhibited a significant decrease in HSAF levels compared with the wild-type levels (Figure 5A). To determine the role of HtsH1, HtsH2, and HtsH3 in the regulation of HSAF biosynthesis, we complemented $\Delta htsH1$, $\Delta htsH2$, $\Delta htsH3$, the double-mutant strains $\Delta htsH12$, $\Delta htsH13$, $\Delta htsH23$, and the triple-mutant strain $\Delta htsH123$ with plasmid-borne *htsH1*, *htsH2*, *htsH3*, *htsH12*, *htsH13*, *htsH23*, and *htsH123*. HSAF production in the complemented strains ($\Delta htsH1/htsH1$, $\Delta htsH2/htsH2$, $\Delta htsH3/htsH3$, $\Delta htsH12/htsH12$, $\Delta htsH13/htsH13$, $\Delta htsH23/htsH23$, and $\Delta htsH123/htsH123$) restored HSAF biosynthesis compared with the wild-type levels (Figure 5A). Importantly, $\Delta htsH1$, $\Delta htsH2$ and $\Delta htsH3$; the double-mutant strains $\Delta htsH12$, $\Delta htsH13$, and $\Delta htsH23$; and the triple-mutant strain

$\Delta htsH123$ did not impair bacterial growth (Figure 5B, C), implying that HtsH1, HtsH2 and HtsH3 play a specific role in regulating HSAF production.

HtsH1, HtsH2, and HtsH3 positively regulate HSAF biosynthesis gene expression

Earlier, we found that deletion of *htsH1*, *htsH2* and *htsH3* resulted in decreased HSAF production. Thus, we wondered whether HtsH1, HtsH2, and HtsH3 might directly target HSAF biosynthesis gene promoters. To test this hypothesis, we performed an *E. coli*-based one-hybrid assay. As shown in Figure 6A, HtsH1, HtsH2, and HtsH3 directly bound to the promoter of the first gene *lafB* in the HSAF biosynthesis operon (*plafB*).

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 *plafB* was used as a probe. The addition of purified HtsH1C-Flag-His, HtsH2C-HA-His, and HtsH3C-Myc-His protein, ranging from 0 μM to 10 μM , to the reaction mixtures (20 μL and at 28°C, 25 min) caused a shift in the mobility of the *plafB* DNA fragment, and the EMSA revealed strong HtsH1, HtsH2, and HtsH3 binding with the *plafB* probe in a dose-dependent manner (Figure 6B-D). 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 (*plafB*) with high affinity ($K_D = 0.5356 \mu\text{M}$) (Figure 6E). In addition, HtsH2C-HA-His and HtsH3C-Myc-His bound to *plafB* with K_D values of 1.379 μM and 0.2491 μM , respectively (Figure 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 *htsHs* 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 (Table S3). 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 *htsHs* mutants ($\Delta htsH1$, $\Delta htsH2$, $\Delta htsH3$, $\Delta htsH12$, $\Delta htsH13$, $\Delta htsH23$ and $\Delta htsH123$) (Figure S5). Using quantitative RT-PCR (qRT-PCR), We measured the mRNA abundance of *lafB* in the *htsHs* mutants ($\Delta htsH1$, $\Delta htsH2$, $\Delta htsH3$, $\Delta htsH12$, $\Delta htsH13$, $\Delta htsH23$ and $\Delta htsH123$) and found that it was reduced compared to that in the wild type (Figure S6).

Taken together, these results suggested that HtsH1, HtsH2, and HtsH3 can directly target the promoters of the HSAF biosynthesis genes to increase their expression and HSAF production by *L. enzymogenes*.

Phosphorylated HtsH1, HtsH2, and HtsH3 positively regulate HSAF biosynthesis

Since HtsH1, HtsH2 and HtsH3 function as a group of HyTCS proteins, we explored whether they could directly target *plafB* depending on its phosphorylation activity in *L. enzymogenes*. To achieve this goal, we assessed the phosphorylation levels of these proteins with calf intestine alkaline phosphatase (CIAP) in the reaction mixtures (20 μL , 28°C, 60 min). Using Mn^{2+} -Phos-tag SDS-PAGE, we showed that the phosphorylation levels of HtsH1C-Flag-His, HtsH2C-HA-His, and HtsH3C-Myc-His decreased upon CIAP

treatment (Figure 7A-C). To test the effect of HtsH1-, HtsH2-, or HtsH3-mediated phosphorylation on the function of the target *plafB*, EMSAs were performed with the same experimental conditions. The EMSAs revealed that the amount of probes bound to HtsH1C-Flag-His, HtsH2C-HA-His, and HtsH3C-Myc-His decreased with increasing amounts of CIAP (Figure 7D-F). The above findings indicated that HtsH1, HtsH2 and HtsH3 could directly regulate HSAF biosynthesis gene expression depending on their phosphorylation activity in *L. enzymogenes*.

RpfG- and HtsH1-, HtsH2-, and HtsH3-dependent regulatory patterns are present in a wide range of bacterial species

By BLAST analysis of the nonredundant protein sequence (Nr) database of the National Center for Biotechnology Information (NCBI), we found that RpfG, the *rpf* cluster, and HtsH1, HtsH2, and HtsH3 were present not only in the genomes of *Lysobacter* species, but also *Xanthomonas* species (Figure S7). These findings suggest that RpfG-, HtsH1-, HtsH2-, and HtsH3-dependent regulatory patterns are conserved mechanisms in *Lysobacter* and *Xanthomonas*.

Discussion

In previous studies, we and our collaborators have shown that quorum sensing (QS) was employed by *L. enzymogenes* to affect production of the antifungal weapon HSAF^{6,25}. 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 weapon HSAF in *L. enzymogenes*^{11,25}. 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 for the first time 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 weapon HSAF depending on their phosphorylation (Figure 8).

HD-GYP domain proteins are c-di-GMP PDEs that can degrade c-di-GMP¹². However, the role of the HD-GYP domain of RpfG in the degradation of c-di-GMP in *L. enzymogenes* 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 is to degrade c-di-GMP to GMP^{11,26,27}. 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 $\Delta rpfG$ mutant strain. Importantly, we found that the concentration of the $\Delta rpfG$ mutant did not significantly change c-di-GMP production compared with of the wild-type strain in the antifungal weapon 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 expression²⁸⁻³¹. However, the biological function of three 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 weapon HSAF biosynthesis genes. These results suggest that HtsH1, HtsH2, and HtsH3 can directly regulate HSAF biosynthesis gene expression and increase production of the antifungal weapon HSAF in *L. enzymogenes*.

Phosphorylation of TCS is critical for regulating the expression of downstream genes³²⁻³⁴. 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 *plafB* depending on their phosphorylation activity. In this study, we report for the first time 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, and 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.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are shown in Table S1. *E. coli* strains were grown in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L 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 kanamycin sulphate, 50 µg/mL gentamycin) 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 described previously³⁵. To obtain the RpfG mutant proteins and *rpfG* site-directed mutant strains, plasmids harbouring 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 (Table S2). The fragments were connected by overlap PCR using the primers MBP-*rpfG* P1 and MBP-*rpfG* P2. The fused fragment was digested with *Bam*H I and *Hind*III 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 described previously³⁶. 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 Table S2. 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 (Qiagen, Chatsworth, CA, USA) using an affinity column (Qiagen). The protein purity was monitored by SDS-PAGE. His₆-tagged protein expression and purification were performed as described previously³⁵⁻³⁷.

PDE activity assays *in vitro*

The PDE activity assay was performed essentially as described earlier²⁴. Briefly, 2 µM MBP-RpfG 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 μM c-di-GMP. All reaction mixtures were incubated at 28°C for 5 to 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 C18 column and separated by HPLC. The separation protocol involved two mobile phases, 100 mM KH₂PO₄ plus 4 mM tetrabutylammonium sulphate (A) and 75% A + 25% methanol (B).

C-di-GMP extraction and quantification

C-di-GMP extraction and quantification were performed as described previously²⁴. Cultures were grown 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 mM ammonium acetate plus 0.1% acetic acid; buffer B: 100% methanol. 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 as described previously^{24,35} using the primers listed in Table S2. In brief, the flanking regions of each gene were PCR-amplified and cloned into the suicide vector pEX18Gm (Table S1). The deletion constructs were transformed into the wild-type strain by electroporation, and gentamycin was used to select for integration of the nonreplicating plasmid into the recipient chromosome. A single-crossover integrant colony was spread on LB medium without gentamycin and incubated at 28°C for 3 days, and after appropriate dilution, the culture was spread on LB plates containing 15% sucrose. Colonies sensitive to gentamycin were screened by PCR using the primers listed in Table S2, and the gene deletion strains were obtained.

For gene complementation constructs, DNA fragments containing the full-length genes along with their promoters were PCR amplified and cloned into the versatile plasmid pBBR1MCS5³⁸. The resulting plasmids were transferred into the *L. enzymogenes* strain by electroporation, and the transformants were selected on LB plates containing Gm.

RNA-Seq

The RNA-Seq assay was performed as described previously^{39,40}. Briefly, the wild-type, $\Delta htsH1$, $\Delta htsH2$, $\Delta htsH3$, $\Delta htsH12$, $\Delta htsH13$, $\Delta htsH23$, and $\Delta htsH123$ mutant strains were grown in 10% TSB medium at 28°C, and their cells were collected when the OD₆₀₀ reached 1.0 based on the growth curve. The collected cells were used for RNA extraction by the TRIzol-based method (Life Technologies, CA, USA), and RNA degradation and contamination were monitored on 1% agarose gels. Then, clustering and sequencing

were performed by Genedenovo Biotechnology Co., Ltd (Guangzhou, Guangdong, China). To analyse the DEGs between the wild-type, $\Delta htsH1$, $\Delta htsH2$, $\Delta htsH3$, $\Delta htsH12$, $\Delta htsH13$, $\Delta htsH23$, and $\Delta htsH123$ mutant strains, the gene expression levels were further normalized using the fragments per kilobase of transcript per million (FPKM) mapped reads method to eliminate the influence of different gene lengths and amounts of sequencing data on the calculation of gene expression. The edgeR package (<http://www.r-project.org/>) was used to determine DEGs across samples with fold changes ≥ 2 and a false discovery rate-adjusted P (q value) < 0.05 . DEGs were then subjected to enrichment analysis of GO functions and KEGG pathways, and q values were corrected using < 0.05 as the threshold.

Quantitative real-time PCR

Quantitative real-time PCR was carried out according to previous studies⁴¹. The bacterial cells were collected when the cellular optical density (OD₆₀₀) reached 1.0 in 10% TSB. Total RNA was extracted using a TRIzol-based method (Life Technologies, CA, USA). RNA quality control was performed via several steps: (1) the degree of RNA degradation and potential contamination were monitored on 1% agarose gels; (2) the RNA purity (OD₂₆₀/OD₂₈₀, OD₂₆₀/OD₂₃₀) was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA); and (3) the RNA integrity was measured using a Bioanalyser 2100 (Agilent, Santa Clara, CA, USA). The primers used in this assay are listed in Table S2. cDNA was then synthesized from each RNA sample (400 ng) using the TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. qRT-PCR was performed using TransStart Top Green qPCR SuperMix (TransGen Biotech) on a QuantStudio TM 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling parameters: denaturation at 94°C for 30 s, followed by 40 cycles of 94°C for 5 s and 60°C for 34 s. Gene expression analyses were performed using the $2^{-\Delta\Delta CT}$ method with 16S rRNA as the endogenous control, and the expression level in the wild type was set to a value of 1. The experiments were performed three times, and three replicates were examined in each run.

Pull-down assay

The assay was performed as described previously²⁴. Briefly, the purified proteins were used to perform the pull-down assay in a reaction system comprising 800 μ L PBS buffer, 5 μ M (final concentration) MBP-RpfG and HtsH1C-Flag-His, HtsH2C-HA-His or HtsH3C-Myc-His proteins, and 50 μ L Dextrin Sepharose High Performance agarose (Sigma-Aldrich, St. Louis, MO, USA). All samples were incubated at 4°C overnight. The agarose was collected by centrifugation and washed 10 times with PBS containing 1% Triton X-100 to remove non-specifically bound proteins. The MBP-bead-captured proteins were eluted by boiling in 6 \times SDS loading dye for 10 min. These samples were subjected to SDS-PAGE and Western blotting. Protein detection involved the use of MBP- (ab49923), Flag- (ab1162), HA- (ab187915), Myc- (ab32072), and His- (ab18184) specific antibodies obtained from Abcam, UK.

Phosphorylation analysis through Phos-tag gel

The purified HtsH1C-Flag-His, HtsH2C-HA-His, or HtsH3C-Myc-His proteins (100 ng) were incubated with CIAP (Solarbio, Beijing, China) at 28°C for 60 min and resolved by 8% SDS-PAGE prepared with 50 µM acrylamide-dependent Phos-tag ligand and 100 µM MnCl₂ as previously described⁴². Gel electrophoresis was performed with a constant voltage of 80 V for 3-6 h. Before transfer, the gels were equilibrated in transfer buffer with 5 mM EDTA for 20 min two times, followed by transfer buffer without EDTA for another 20 min. Protein transfer from the Mn²⁺ phos-tag acrylamide (APEX-BIO, Houston, USA) gel to the PVDF membrane (Millipore, Massachusetts, USA) was performed for ~24 h at 80 V at 4°C, and then the membrane was analysed by Western blotting using the anti-His antibody.

Bacterial one-hybrid assays

Bacterial one-hybrid assays were performed as previously described^{43,44}. In brief, the bacterial one-hybrid reporter system contains three components: the plasmids pBXcmT and pTRG, which are used to clone the target DNA and to express the target protein, respectively, and the *E. coli* XL1-Blue MRF⁺ kan strain, which is the host strain for the propagation of the pBXcmT and pTRG recombinants⁴⁵. In this study, the promoter of the HSAF biosynthesis gene (*p/afB*) was cloned into pBXcmT to generate the recombinant vector pBXcmT-*p/afB*. Similarly, the coding regions of Le *htsH1*, Le *htsH2*, and Le *htsH3* were cloned into pTRG to create the final constructs pTRG-*htsH1*, pTRG-*htsH2*, and pTRG-*htsH3*, respectively. The two recombinant vectors were transformed into the XL1-Blue MRF⁺ kan strain. If direct physical binding occurred between HtsH1, HtsH2, or HtsH3 and *p/afB*, the positive-transformant *E. coli* strain containing both pBXcmT-*p/afB* and pTRG-HtsHs would grow well on selective medium, that is, minimal medium containing 5 mM 3-amino-1,2,4-triazole, 8 µg/mL streptomycin, 12.5 µg/mL tetracycline, 34 µg/mL chloramphenicol, and 30 µg/mL kanamycin. Furthermore, cotransformants containing pBX-R2031/pTRG-R3133 served as a positive control⁴⁵, and cotransformants containing either empty pTRG or pBXcmT-*p/afB* were used as negative controls. All cotransformants were spotted onto selective medium, grown at 28°C for 3-4 days, and then photographed.

Electrophoretic mobility gel shift assays (EMSAs)

EMSA was performed as previously described^{46,47}. For HtsH1, HtsH2, or HtsH3 gel shift assays, we used DNA fragments that included *p/afB* as a probe. The probe DNA (50 ng) was mixed with protein in a 20 µL reaction mixture containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, and 0.4% glycerol. After incubation for 30 min at 28°C, samples were electrophoresed on a 5% nondenaturing acrylamide gel in 0.5× TBE buffer at 4°C. The gel was soaked in 10,000-fold-diluted SYBR Green I nucleic acid dye (Sangon Biotech, Shanghai, China), and the DNA was visualized at 300 nm.

HSAF extraction and quantification

HSAF was extracted from 50 mL *L. enzymogenes* cultures grown in 10% TSB for 48 h at 28°C with shaking (at 180 rpm). HSAF was detected via HPLC and quantified per unit of OD600 as described

previously^{6,24,48}. Three biological replicates were used, and each was examined with three technical replicates.

Statistical analyses

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 ($P = 0.05$). If a significant F value was obtained, separation of means was accomplished by Fisher's protected least significant difference at $P \leq 0.05$.

Declarations

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Author contributions

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

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Conflict of interest

The authors declare no competing financial interests.

Data availability statement

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 = D9T17_13845), RCTY01000055 (*Lysobacter enzymogenes* strain OH11 scffold56, whole genome shotgun sequence, Le3071/Le HtsH1, locus tag = D9T17_21400), RCTY01000054 (*Lysobacter enzymogenes* strain OH11 scffold55, whole genome shotgun sequence, Le3072/Le HtsH2, locus tag = D9T17_21390), RCTY01000054 (*Lysobacter enzymogenes* strain OH11 scffold55, whole genome shotgun sequence, Le3073/Le HtsH3, locus tag = D9T17_21385).

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Figures

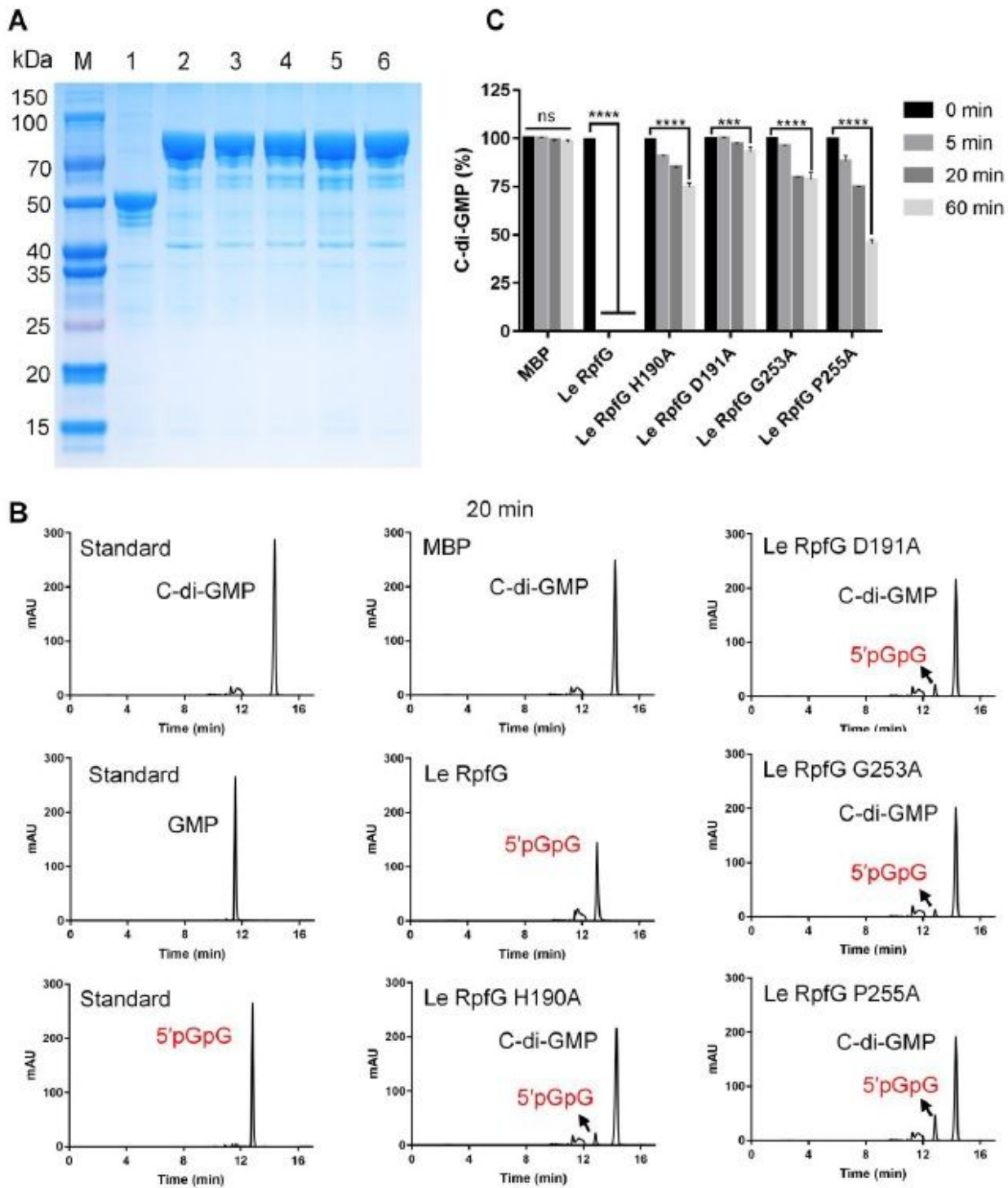


Figure 1

RpfG is a PDE enzyme. (A) The purified protein was analysed by 12% SDS-PAGE. Lane M, molecular mass markers; lane 1, MBP protein; lane 2, RpfG-MBP protein; lane 3, RpfG-MBP H190A protein; lane 4, RpfG-MBP D191A protein; lane 5, RpfG-MBP G253A protein; lane 6, RpfG-MBP P255A protein. (B) The purified protein has enzymatic activity against c-di-GMP. HPLC analysis of aliquots of reaction mixtures boiled immediately after addition of the enzyme and after 20 min of incubation shows the degradation of

c-di-GMP to a compound with the same mobility as the 5'pGpG standard. (C) The purified RpfG protein has PDE enzymatic activity. For convenience of comparison, the peak of c-di-GMP in the RpfG solution at 0 min was defined as 100% and used to normalize the c-di-GMP level ratios at different time points. Error bars, means \pm standard deviations (n = 3). *** P < 0.001, **** P < 0.0001, assessed by one-way ANOVA. All experiments were repeated three times with similar results.

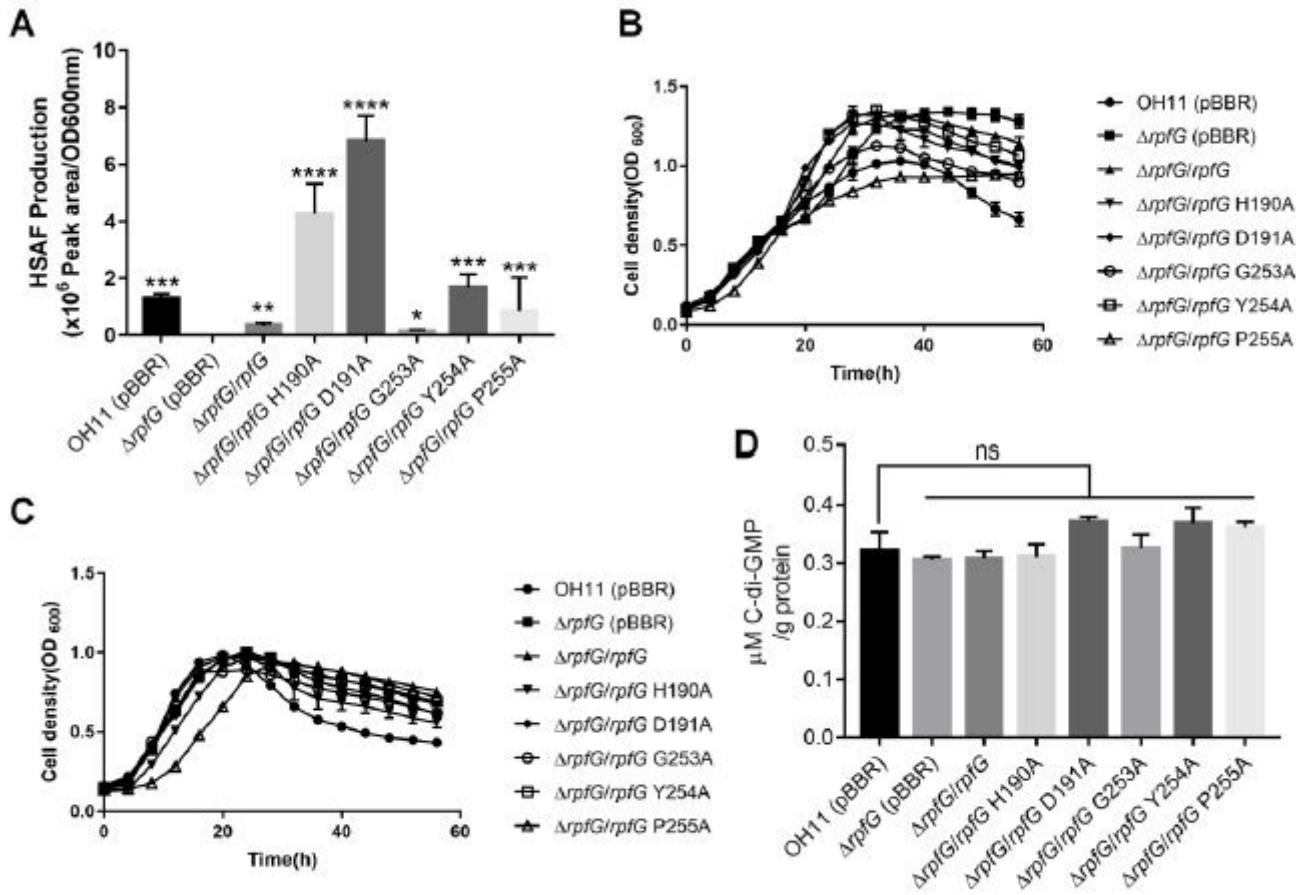


Figure 2

RpfG-mediated regulation of HSAF production does not depend on its PDE activity. (A) Quantification of HSAF produced by the rpfG mutant strain and strains complemented with rpfG or the rpfG site-directed mutant genes grown in 10% TSB medium. (B) Growth curves of the rpfG mutant strain and strains complemented with rpfG or the rpfG site-directed mutant genes in rich LB medium. (C) Growth curves of the rpfG mutant strain and strains complemented with the rpfG or rpfG site-directed mutant genes in 10% TSB medium. (D) Intracellular c-di-GMP concentrations in the wild type, rpfG mutant and strains complemented with rpfG or the rpfG site-directed mutant genes. Error bars, means \pm standard deviations (n = 3). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, assessed by one-way ANOVA. All experiments were repeated three times with similar results.

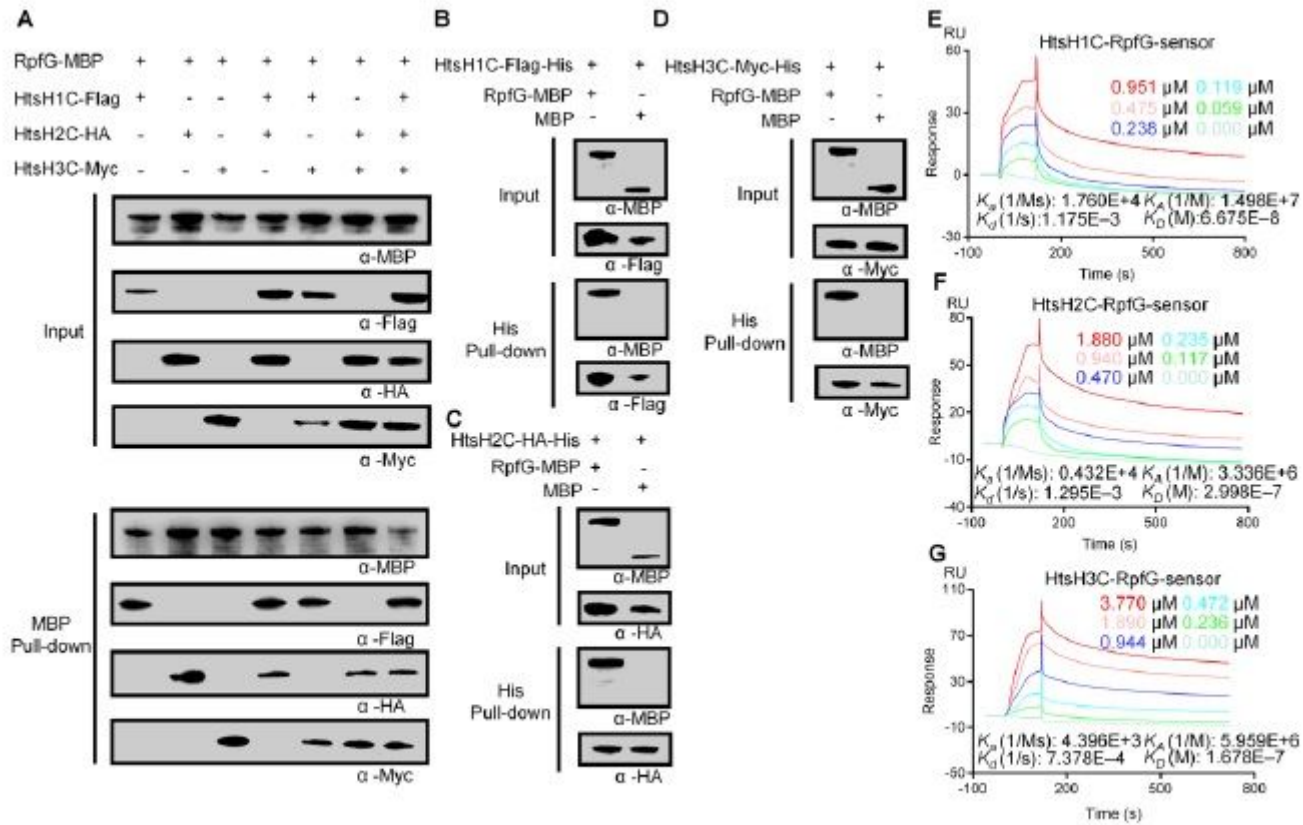


Figure 3

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-nitrilotriacetic acid (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 $K_D = 0.06675 \mu\text{M}$. (F) SPR showing that HtsH2C-HA-His forms a complex with RpfG-MBP with $K_D = 0.2998 \mu\text{M}$. (G) SPR showing that HtsH3C-Myc-His forms a complex with RpfG-MBP with $K_D = 0.1678 \mu\text{M}$.

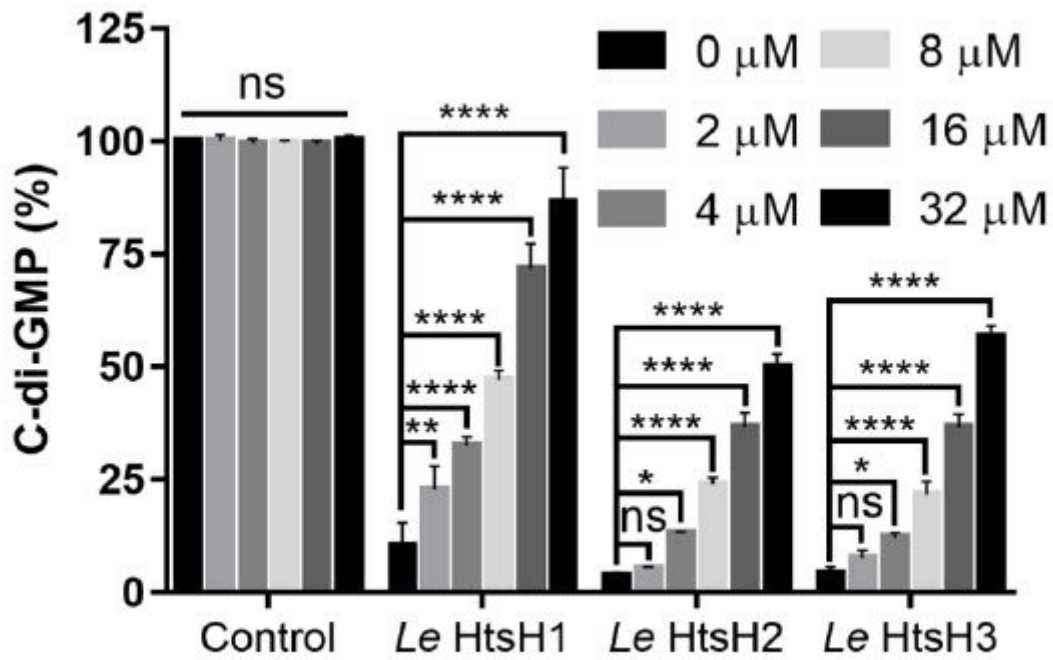


Figure 4

Impact of HtsH1, HtsH2, and HtsH3 on RpfG enzyme activity. The addition of HtsH1C-Flag-His, HtsH2C-HA-His, and HtsH3C-Myc-His proteins to an RpfG-MBP protein solution decreased its c-di-GMP phosphodiesterase activity. For convenience of comparison, the peak of c-di-GMP in the MBP solution was defined as 100% and used to normalize the c-di-GMP level ratios at 20 min.

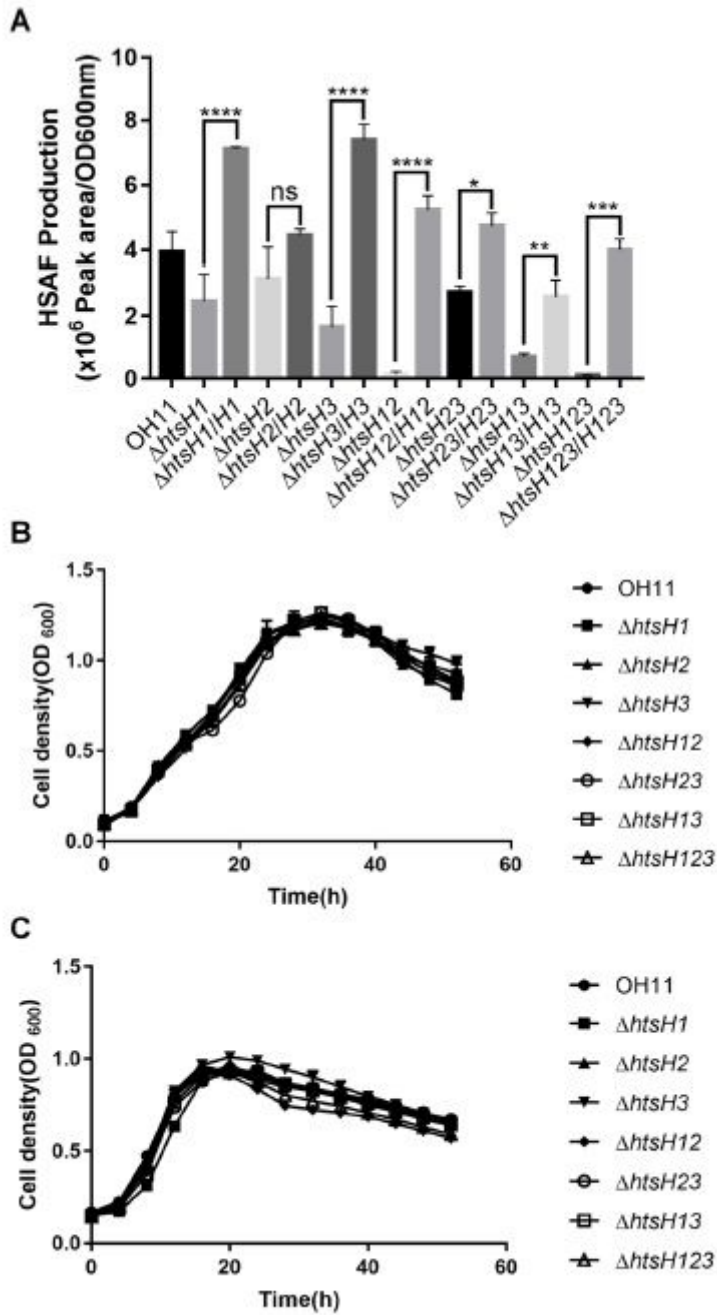


Figure 5

Deletion of *htsH1*, *htsH2*, and *htsH3* resulted in decreased HSAF production. (A) Quantification of the HSAF produced by the *htsH1*, *htsH2* and *htsH3* mutant strains and strains complemented with the *htsH1*, *htsH2*, and *htsH3* genes grown in 10% TSB medium. (B) Growth curves of the *htsH1*, *htsH2*, and *htsH3* mutant strains and strains complemented with the *htsH1*, *htsH2*, and *htsH3* genes in LB-rich medium. (C) Growth curves of the *htsH1*, *htsH2*, and *htsH3* mutant strains and strains complemented with the *htsH1*, *htsH2*, and *htsH3* genes in 10% TSB medium. Error bars, means \pm standard deviations ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, assessed by one-way ANOVA. All experiments were repeated three times with similar results.

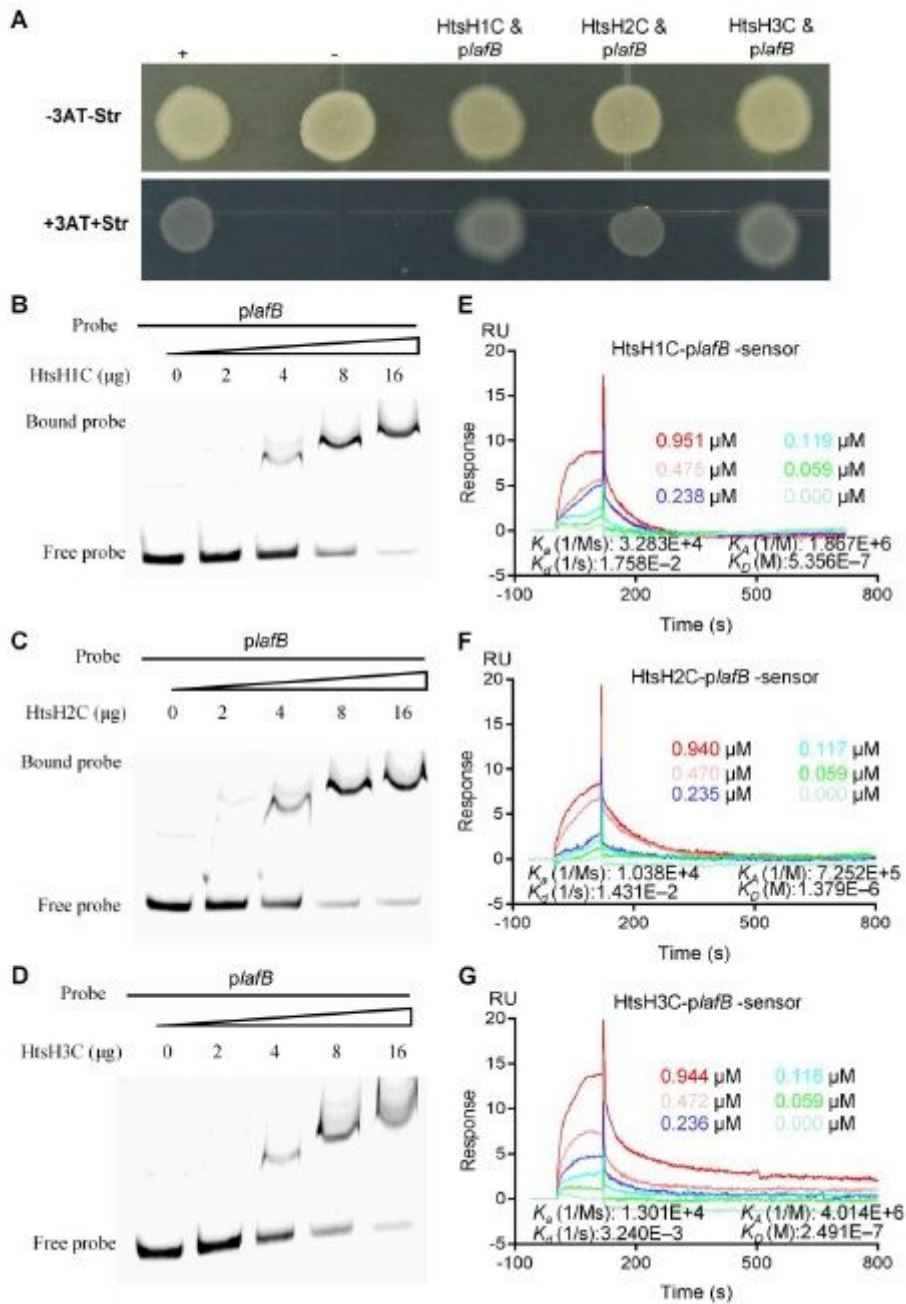


Figure 6

HtsH1, HtsH2, and HtsH3 directly bound the promoter of the HSAF biosynthesis gene (*plafB*). (A) Direct physical interaction between HtsH1, HtsH2, and HtsH3 and the *lafB* promoter region was detected in *E. coli*. Experiments were performed according to the procedures described in the Materials and 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 & *plafB*, cotransformant harbouring pTRG-HtsH1C and pBXcmT-*plafB*; HtsH2C & *plafB*, cotransformant harbouring pTRG-HtsH2C and pBXcmT-*plafB*; HtsH3C & *plafB*, cotransformant harbouring pTRG-HtsH3C and pBXcmT-*plafB*. -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, 1, 2, 4 or 8 μg) was added to reaction mixtures containing 50 ng of probe DNA, and the reaction mixtures were separated on polyacrylamide gels. (E) SPR showing that HtsH1C-Flag-His forms a complex with *plafB* with $K_D = 0.5356 \mu\text{M}$. (F) SPR showing that HtsH2C-HA-His forms a complex with *plafB* with $K_D = 1.379 \mu\text{M}$. (G) SPR showing that HtsH3C-Myc-His forms a complex with *plafB* with $K_D = 0.2491 \mu\text{M}$.

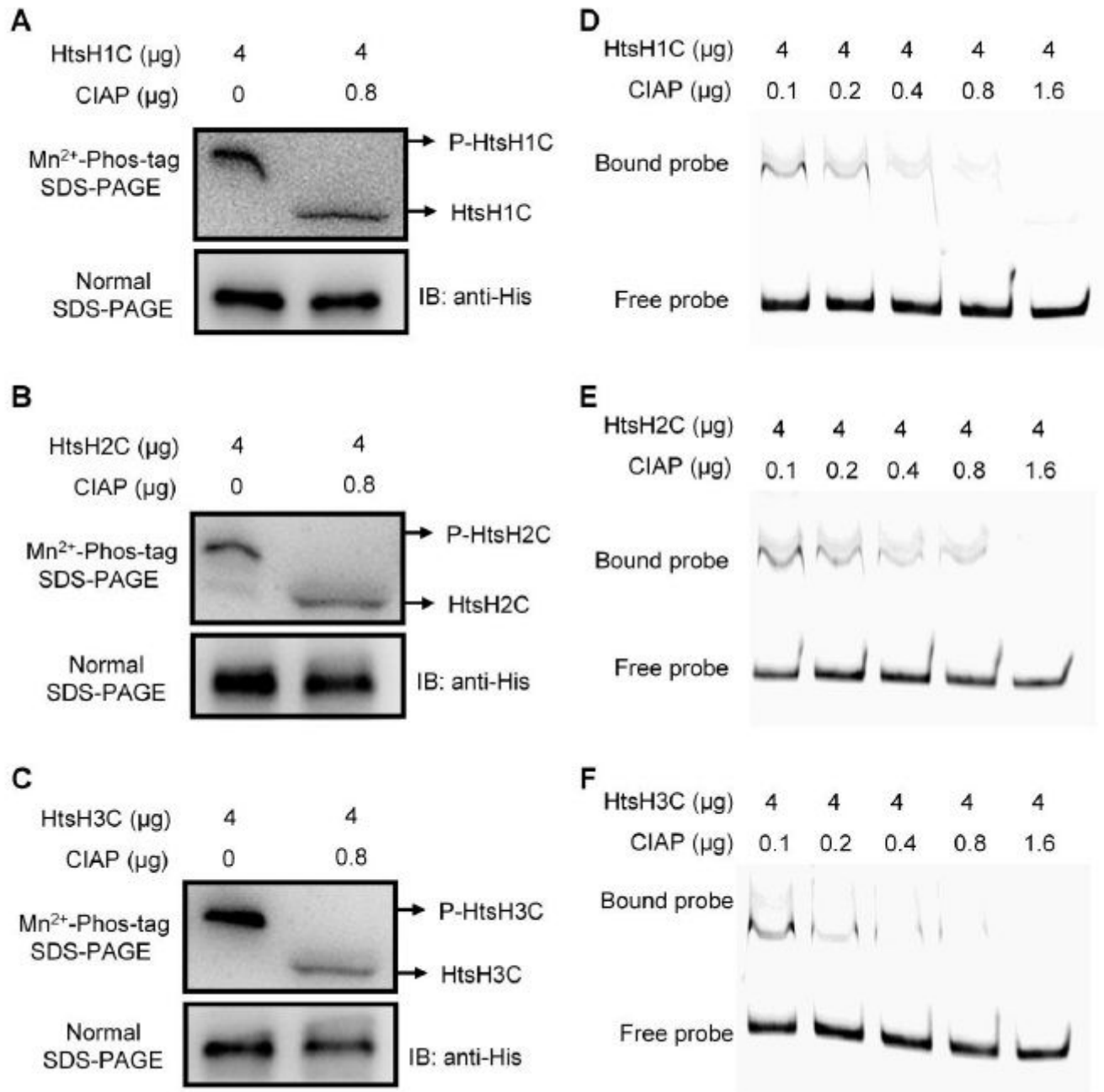


Figure 7

HtsH1, HtsH2, and HtsH3 directly target *plafB* depending on their PDE activity. (A-C) Phosphorylation analysis of HtsH1C-Flag-His, HtsH2C-HA-His, or HtsH3C-Myc-His with calf intestine alkaline phosphatase (CIAP) at concentrations ranging from 0.1 μg to 1.6 μg in the reaction mixtures (20 μL , 28°C, 60 min)

using Mn²⁺-Phos-tag SDS-PAGE. (D-F) Gel shift assay showing that HtsH1C-Flag-His, HtsH2C-HA-His, or HtsH3C-Myc-His does not directly target *plafB* with calf intestine alkaline phosphatase (CIAP) at concentrations ranging from 0.1 μg to 1.6 μg in the reaction mixtures (20 μL , 28°C, 60 min). HtsHs (0, 1, 2, 4 or 8 μg) were added to reaction mixtures containing 50 ng of probe DNA, and the reaction mixtures were separated on polyacrylamide gels.

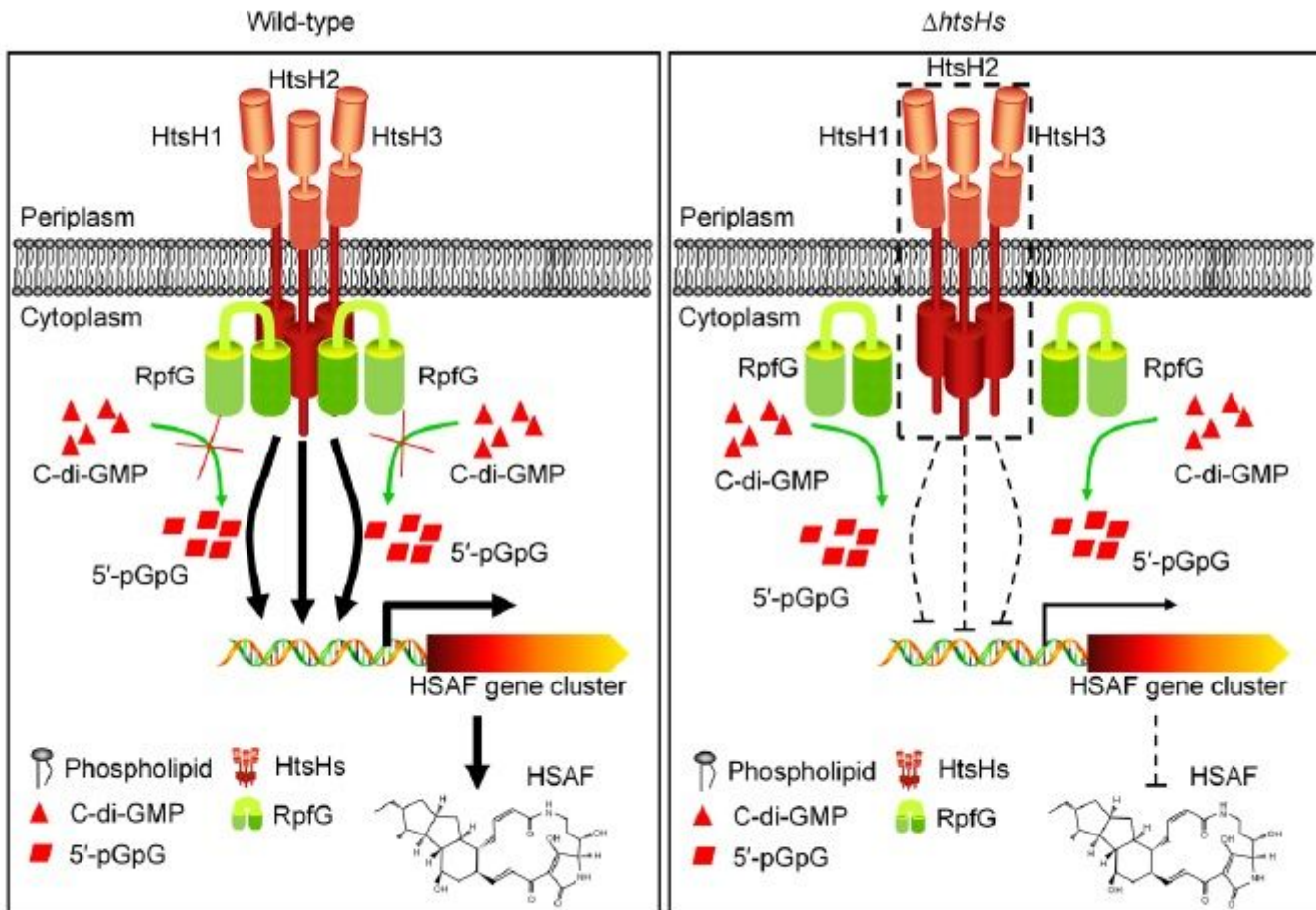


Figure 8

Schematic of the proposed RpfG directly interacting with three HyTCS proteins (HtsH1, HtsH2, and HtsH3) to regulate HSAF biosynthesis. The potential regulatory pathways and interactions of RpfG with HtsH1, HtsH2, and HtsH3 are proposed according to our observations and previous studies. RpfG and HtsH1, HtsH2, or HtsH3 interactions affect the PDE activity of RpfG. Phosphorylated HtsH1, HtsH2, and HtsH3 can directly target the promoter of HSAF biosynthesis genes to regulate HSAF production in *L.* enzymogenes.

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