Phytopathology Research

RESEARCH Open Access

Coordination of cyclic di-GMP and 4-hydroxybenzoic acid in regulating antifungal antibiotic biosynthesis in *Lysobacter enzymogenes*

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Abstract

Small molecules are able to regulate numerous cellular processes through binding to various bacterial receptor proteins, but the mechanisms and functions by which these chemicals coordinate and execute remain poorly understood. 4-hydroxybenzoic acid (4-HBA) and cyclic di-GMP (c-di-GMP) are two such molecules with distinct structures that are produced in *Lysobacter enzymogenes* to synergistically affect the secretion of an antifungal antibiotic, known as heat-stable antifungal factor (HSAF). In our earlier studies, we showed that CdgL, a YajQ-like protein without DNA-binding domain, was able to physically interact with LysR, a transcription factor, to enhance its binding affinity toward the upstream region of the HSAF biosynthesis operon promoter, hence increasing the HSAF biosynthesis. Interestingly, 4-HBA or c-di-GMP can bind to its cognate receptor of LysR or CdgL, respectively, to regulate the HSAF biosynthesis. Further, c-di-GMP acts by binding to CdgL to induce the dissociation of the CdgL-LysR complex, leading to decreased downstream expression. We now showed that CdgL controlled the transcription of *lenB2*, which encodes an oxygenase to convert chorismate to 4-HBA. Notably, overexpression of *cdgL* was found to stimulate *lenB2* transcription, which likely increased the intracellular 4-HBA content. Also, 4-HBA could bind to LysR to interrupt the LysR-CdgL complex formation and release of CdgL, which caused a lower affinity of LysR toward DNA and hence decreased HSAF operon expression. These findings, along with our earlier report, allow us to propose a coordination mechanism demonstrating how the HSAF biosynthesis is co-regulated by 4-HBA and c-di-GMP through interactions with their cognate receptors. This new mechanism shall shed light on improving the HSAF yield for practical usage.

Keywords: C-di-GMP, 4-HBA, Antifungal antibiotic, *Lysobacter*, CdgL, LysR, HSAF

Background

Bacteria of the genus *Lysobacter* is gradually recognized as a rich source of crop protecting agents, due to their great ability to produce abundant extracellular lytic enzymes and antimicrobial secondary metabolites (Folman et al. 2003; Kobayashi et al. 2005; Qian et al. 2009; Xie et al. 2012). *L. enzymogene* OH11 is one of such plant-associated soil proteobacteria. It can secrete a diffusible antifungal antibiotic, known as heat-stable antifungal factor (HSAF), into the surrounding environment to kill neighbor fungal pathogens through targeting their sphingolipids biosynthesis pathway (Yu et al. 2007; Li et al. 2008; Zhao et al. 2017, 2019).

The biosynthesis of HSAF in *L. enzymogenes* is controlled by a gene cluster known as HSAF biosynthesis gene operon (Lou et al. 2011; Wang et al. 2017), whose transcription is influenced by multiple factors, including small molecules and transcription factors (Qian et al.

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2013; Wang et al. 2014; Chen et al. 2017; Su et al. 2017, 2018). 4-hydroxybenzoic acid (4-HBA) is one of such small molecules that is generated from a conserved shikimate pathway in *Xanthomonas campestris* and *L. enzymogenes* (Zhou et al. 2013; Su et al. 2017). Previously, we showed that the *L. enzymogenes* LenB2, a petridine-dependent dioxygenase-like protein, can convert chorismate, the end-product of the shikimate pathway, to 4-HBA (Su et al. 2017). Mutation of *lenB2* lowers the production of 4-HBA, leading to blockage of HSAF production (Su et al. 2017). Exogenous supplement of the physiological amount of 4-HBA into the *lenB2* mutant culture can rescue the HSAF production back to the wild type level (Su et al. 2017). The regulatory pathway of 4-HBA involves LysR, a transcription factor that directly binds to the upstream region of the HSAF biosynthesis operon promoter (pHSAF), to activate the operon transcription (Su et al. 2017). Indeed, the direct 4-HBA-LysR binding (K_d, 5 μM) was confirmed by an in vitro assay (Su et al. 2017). To date, however, it remains unclear whether the binding of 4-HBA with LysR could strengthen the transcription of the HSAF biosynthesis operon under the in vivo condition or not.

Besides 4-HBA, we found cyclic di-GMP (c-di-GMP), another small molecule, is also engaged in regulating the HSAF biosynthesis (Chen et al. 2017; Xu et al. 2018; Qian et al. 2020). Indeed, c-di-GMP is a ubiquitous second messenger in bacteria that functions by binding with protein receptors or RNA riboswitches (Römling et al. 2005, 2013). We have shown that elevated intracellular c-di-GMP levels inhibit HSAF biosynthesis, and further identified CdgL as a c-di-GMP-binding protein that is required for HSAF production (Han et al. 2020). CdgL is a protein homologous to YajQ from plant pathogenic *X. campestris*, in which it directly interacts with c-di-GMP to contribute to bacterial virulence (An et al. 2014). In our earlier study, we found that at low intracellular c-di-GMP level, CdgL forms a stable protein complex with LysR to strengthen the binding affinity of LysR with pHSAF, the promoter region of the HSAF biosynthesis operon, thereby enhancing HSAF operon expression (Han et al. 2020). At high intracellular c-di-GMP level, c-di-GMP binding to CdgL can induce the disassembly of the CdgL-LysR complex to release CdgL from the LysR-DNA complex, leading to decreased operon expression (Han et al. 2020). These earlier findings collectively reveal that HSAF biosynthesis in *L. enzymogenes* can be fine-tuned by designing an interconnected cellular network constituted by LenB2, 4-HBA, LysR, c-di-GMP, and CdgL. However, the mechanism of how these components are linked remains unclear.

In the present study, we showed that inactivation of the c-di-GMP receptor CdgL remarkably impaired the transcription of *lenB2*, leading to lower amounts of both 4-HBA and HSAF. Overexpression of *cdgL*, in contrast, could promote the *lenB2* transcription and inhibit the HSAF yield. We further found that higher amount of *lenB2* transcription is correlated with increasing 4-HBA levels, which can disassociate the LysR-CdgL complex, leading to blocked HSAF generation. These observations, along with our earlier finding of c-di-GMP-induced disassembly of the CdgL-LysR complex (Han et al. 2020), led us to propose a model addressing how the regulatory effects of two small molecules (4-HBA and c-di-GMP) in HSAF biosynthesis are linked through targeting the same protein complex (CdgL-LysR) formed by their respective receptors. The potential significance for this newly-discovered mechanism in facilitating ecological adaption of *L. enzymogenes* in natural niches is also discussed.

Results

CdgL stimulated the transcription of *lenB2*

We have carried out a CdgL-mediated transcriptomics study and discovered that the transcription of the HSAF biosynthesis operon was positively controlled by CdgL (Han et al. 2020). A closer analysis of the transcriptomics data led us to observe that *lenB2* formed another CdgL regulon, with the inactivation of CdgL blocking the *lenB2* transcription (Han et al. 2020). To confirm this result, we measured the mRNA levels of *lenB2* in the *cdgL* mutant and found that the *lenB2* level was reduced by approximately 10 folds to that of wild-type (Fig. 1a). As expected, the plasmid-borne *cdgL* gene restored the *lenB2* transcript abundance back to normal (Fig. 1a). These results suggest that intracellular CdgL stimulated, directly or indirectly, the expression of *lenB2*. To validate this finding, we further investigated the effect of a plasmid-boren *cdgL* on the growth of wild-type OH11 for checking the *lenB2* transcript level. Introduction of the plasmid-borne *cdgL* (Additional file 1: Figure S1) into the wild-type OH11 decreased its growth at the stationary phase, but not at the logarithmic phase (Fig. 1b). Based on this finding, we subsequently collected bacterial samples at OD_{600} of 1.0 (Fig. 1b) for qRT-PCR, and the result of Fig. 1c showed that introducing the plasmid-borne *cdgL* significantly increased the *lenB2* transcript abundance by approximately 15 folds compared to that of wild type carrying an empty vector.

CdgL regulated production of 4-HBA and 3-HBA

Findings of CdgL in stimulating *lenB2* transcription revealed a potential role of CdgL in 4-HBA production, as we already knew that LenB2 is required for the generation of 4-HBA (Su et al. 2017). By HPLC analysis, we found that the 4-HBA level was indeed significantly reduced in the *cdgL* mutant, while the plasmid-borne *cdgL* complementation restored the amount of 4-HBA to the wild-type level (Fig. 2a). Since we have previously shown

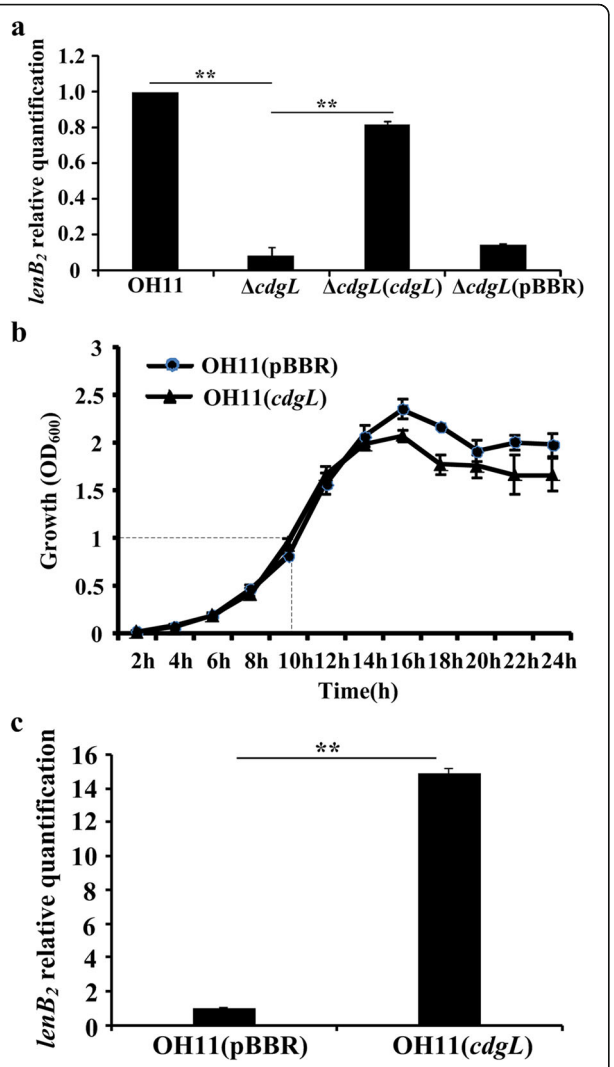

Fig. 1 CdgL controlled the transcription of *lenB2* in *L. enzymogenes*. **a** qRT-PCR analyses of *lenB2* mRNA abundance in the wild-type OH11 and its derivatives. **b** The growth of the wild-type OH11 carrying a plasmid-borne *cdgL* in the HSAF-producing medium, 1/10 TSB. Cells at OD_{600} of 1.0 (indicated by dashed lines) were collected for qRT-PCR assay. **c** Effect of *cdgL* expression in the wild-type OH11 on the mRNA abundance of *lenB2*. Δ*cdgL*, the *cdgL* deletion mutant; Δ*cdgL* (pBBR) and Δ*cdgL* (*cdgL*), the mutant strain carrying an empty vector or a plasmid-borne *cdgL*; OH11(*cdgL*), the wild-type strain carrying a plasmid-borne *cdgL*. In all assays, average data from three experiments are shown, ± SD. $^{**}P < 0.01$

that LenB2 is essential for the production of both 4-HBA and 3-HBA in vivo, with 4-HBA regulating the HSAF biosynthesis and 3-HBA involved in a yellow-pigment development (Su et al. 2017), we thus further quantified the amounts of secreted 3-hydroxybenzoic acid (3-HBA) in the test strains (Fig. 2a). As expected, mutation of *cdgL* also remarkably impaired the 3-HBA yield and yellow-pigment amounts, which were in agreement with those of the control strain, the *lenB2* mutant (Fig. 2a-d). Exogenous supplement of 3-HBA at physiological range or introducing the plasmid-borne *cdgL* into the *cdgL* mutant both rescued the observed deficiencies (Fig. 2a-d). These results revealed that CdgL could control 4-HBA and 3-HBA synthesis through altering the transcription of *lenB2*.

CdgL accumulation impaired HSAF biosynthesis

Previously, we found that CdgL is required for the c-di-GMP-mediated HSAF generation (Han et al. 2020). This, along with the involvement of CdgL in 4-HBA synthesis, indicated that CdgL might also participate in the 4-HBA-mediated pathway to control HSAF production. Considering that overexpression of *cdgL* could stimulate the transcription of *lenB2* (Fig. 1c), we thus assumed that wild-type OH11 containing a plasmid-borne *cdgL* may produce a higher amount of HSAF. Unexpectedly, the data shown in Fig. 3a did not seem to support this hypothesis, since we found that wild-type OH11 with a plasmid-borne *cdgL*, like the *cdgL* mutant (Han et al. 2020), also displayed a significant reduction in the amount of HSAF. This phenotype was further confirmed by the reduced transcription abundance of *lafB* in the *cdgL* overexpression strain compared to that of the vector control (Fig. 3b). Considering that overexpression of *cdgL* promoted the expression of *lenB2*, we thus tested to see whether the lower amount of HSAF in the *cdgL* overexpression strain is associated with the higher *lenB2* expression. For this purpose, we overexpressed *lenB2* in wild-type OH11, and indeed found that it reduced the HSAF yield compared to that of the vector control (Fig. 3c). However, we still could not conclude that reduction in the amount of HSAF in the *lenB2* overexpression strain is either due to the increased levels of 4-HBA, or to the accumulated levels of 3-HBA since we previously showed that LenB2 could convert chorismate to both 4-HBA and 3-HBA (Su et al. 2017). To address this issue, we overexpressed *ubiC* in the wild-type OH11, which is proven to explicitly convert chorismate to 4-HBA, as described previously (Siebert et al. 1994). We subsequently found that the wild-type OH11 carrying an overexpressed *ubiC* indeed produced lower amounts of HSAF compared to the vector control (Fig. 3d). Taken together, these results reveal that the *cdgL* overexpression could stimulate *lenB2* expression, the translation of which seems to generate higher amounts of 4-HBA to block HSAF biosynthesis.

4-HBA disrupted the LysR-CdgL association

To test the above hypothesis, we further investigated the potential effect of 4-HBA on the LysR-CdgL association, as disruption of this binary complex has been shown to block HSAF production, according to our earlier report (Han et al. 2020). For this purpose, we carried out a series of microscale thermophoresis (MST) assays to

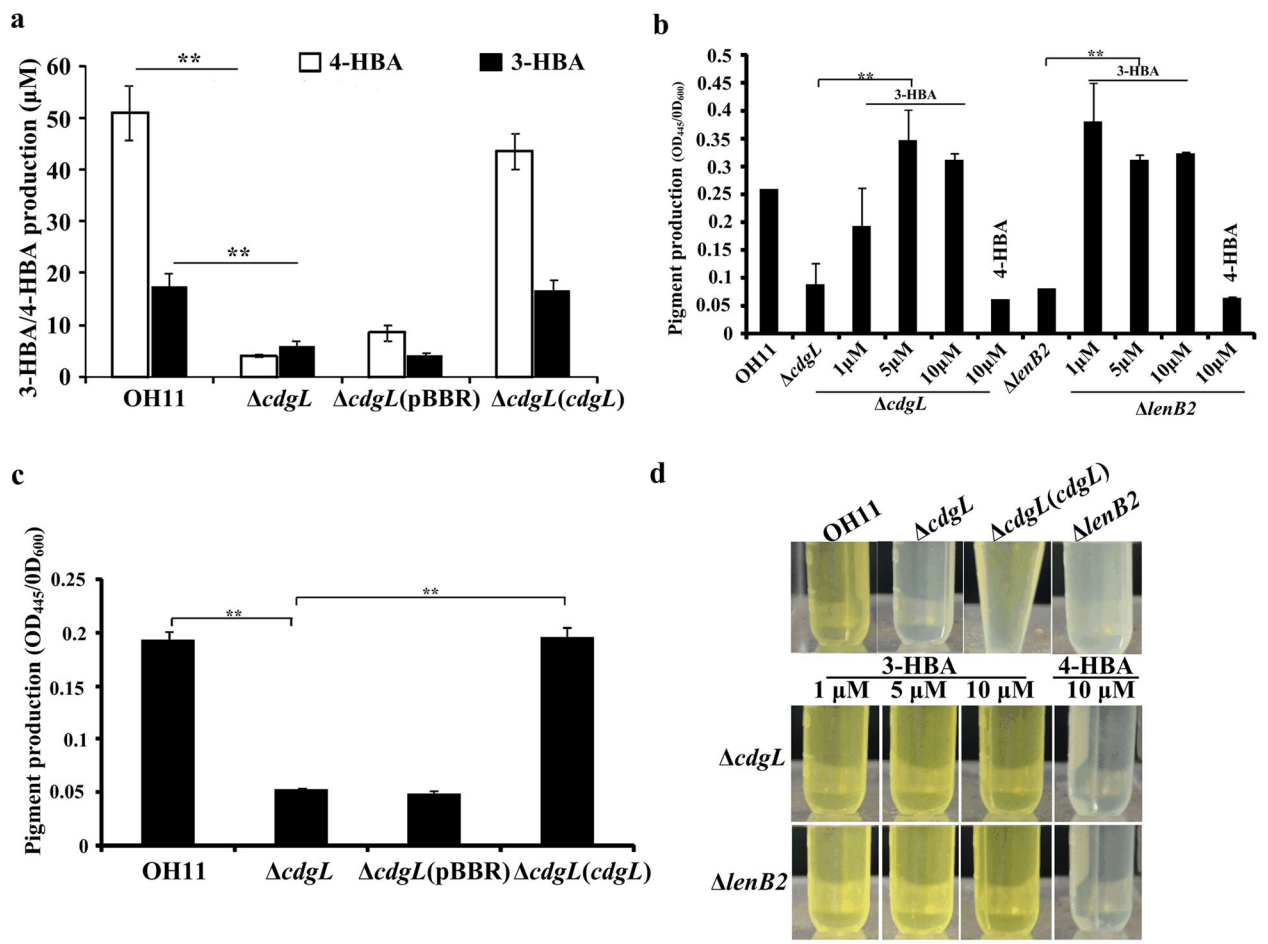

Fig. 2 Effect of CdgL on the production of 4-HBA, 3-HBA, and yellow-pigment in *L. enzymogenes*. **a** Quantification of 3-HBA and 4-HBA levels produced in the wild-type OH11 and its derivatives as measured by HPLC. **b** Effect of exogenous addition of 3-HBA or 4-HBA on the production of yellow pigment of the Δ*cdgL* or Δ*lenB2* strain. **c** Effect of CdgL on the production of yellow pigment in *L. enzymogenes*. **d** Representative results showing the yellow or white phenotype from the test strains. OH11, the wild-type strain; Δ*cdgL*, the *cdgL* deletion mutant; Δ*cdgL* (pBBR) and Δ*cdgL* (*cdgL*), the mutant strain carrying an empty vector or a plasmid-borne *cdgL*; Δ*lenB2*, the *lenB2* deletion mutant. Average data from three experiments are shown, ± SD. $^{**}P < 0.01$

quantify the CdgL-LysR binding affinity in the presence or absence of 4-HBA. We found that 4-HBA indeed strongly inhibited the CdgL-LysR binding. Without 4-HBA, LysR-His_6 could bind to GST-CdgL with a high affinity (K_d, 0.56 μM, Fig. 4a), which is consistent with our recent report (Han et al. 2020). Yet, in the presence of 4-HBA at concentrations ranging from 0.5 to 5 μM, the binding affinity of LysR-His_6 with GST-CdgL was gradually decreased (Fig. 4b-d), with the binding completely abolished when the level of 4-HBA reached 5 μM (Fig. 4d). As a control, 3-HBA, the structural analog of 4-HBA, exhibited only a small inhibition effect on the LysR-CdgL binding at a concentration of 5 μM (Fig. 4e). These results suggested that 4-HBA binds specifically to LysR (Su et al. 2017) to impair the LysR-CdgL formation, resulting in a lower HSAF production. To further support this hypothesis, we carried out a series of EMSA assay. Results of Fig. 4f showed that the CdgL-LysR-DNA ternary complex formation was still very stable in the presence of moderately lower 4-HBA concentrations of 5 and 10 μM. However, 5 μM of 4-HBA could efficiently disassociate the CdgL-LysR complex as determined by the MST method (Fig. 4d). The precise reason for this inconsistent phenomenon is unclear, but the MST technique may be more sensitive in detecting the 4-HBA-induced disassembly of the CdgL-LysR complex than that of EMSA. In agreement, when a higher concentration (20 μM) of 4-HBA (Su et al. 2017) was applied, the amount of the CdgL-LysR-DNA complex decreased significantly while the LysR-DNA binary complex reappeared (Fig. 4f). Notably, 4-HBA did not interact with the test DNA probe, according to our earlier work (Su et al. 2017) or CdgL (Additional file 1: Figure S2). Therefore, these data suggest that 4-HBA very likely

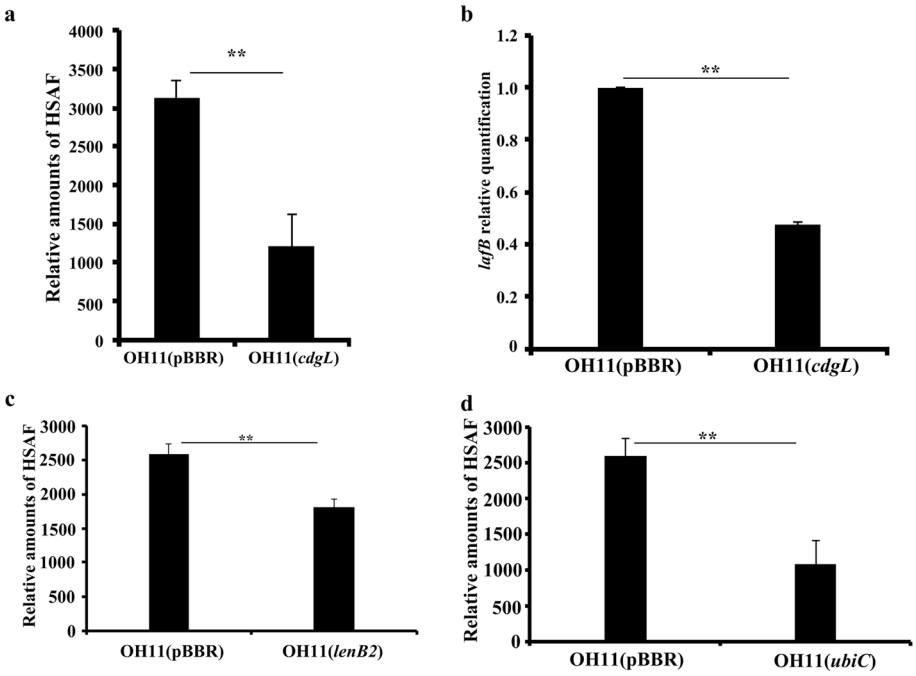

Fig. 3 CdgL and LenB2 accumulations blocked HSAF biosynthesis. **a** Quantification of HSAF levels measured by HPLC in the wild-type OH11 carrying a plasmid-borne *cdgL*. Relative amounts of HSAF (y-axis) are expressed as peak intensities from the HPLC chromatogram per unit of bacterial optical density, OD_{600} (Qian et al. 2013). **b** qRT-PCR analyses of *lafB* mRNA abundance in the wild-type OH11 carrying a plasmid-borne *cdgL*. **c**, **d** Quantification of HSAF levels measured by HPLC in the wild-type OH11 carrying a plasmid-borne *lenB2* (**c**) or *ubiC* (**d**). OH11(pBBR), the wild-type strain carrying an empty vector; OH11(*cdgL*), the wild-type OH11 possessing a plasmid-borne *cdgL*; OH11(*lenB2*), the wild-type strain carrying a plasmid-borne *lenB2*; OH11(*ubiC*), the wild-type strain carrying a plasmid-borne *ubiC*. Average data from three experiments are shown, ± SD. $^{**}P < 0.01$

binds with LysR (Su et al. 2017) to change the CdgL-LysR complex conformation, leading to CdgL release from the LysR-CdgL-DNA ternary complex. Without CdgL, the LysR-DNA binding was impaired, which decreased the extent of HSAF generation.

Discussion

It seems that *L. enzymogenes* has evolved a strategy to secrete HSAF as an antifungal weapon to kill surrounding fungi as foods in nutrient-poor condition and in the presence of fungi (Qian et al. 2009), while HSAF production would be needless to prevent energy loss in a nutrient-rich environment or in the absence of fungi (Li et al. 2006; Yu et al. 2007). However, the underlying mechanism remains poorly understood. Our current work, along with those previously published (Su et al. 2017; Han et al. 2020), allow us to propose a rational mechanism (Fig. 5) regarding how *L. enzymogens* fine-tunes the HSAF biosynthesis. We argued that overexpression of *cdgL*, stimulated by an unknown environmental signal (likely the presence of fungi), could induce the accumulation of CdgL, which boosted the transcription of *lenB2* (via an unidentified mechanism) to increase intracellular 4-HBA levels. Next, 4-HBA binding to LysR (Su et al. 2017) could impair the LysR-CdgL complex formation to release CdgL from the LysR-CdgL-DNA ternary complex. This would weaken the binding affinity of LysR to the upstream region of the HSAF operon promoter, resulting in lower HSAF operon expression and production. This strategy could enable *L. enzymogenes* to gain a better ecological fitness to turn on HSAF production to kill nearby fungi for food or to turn off this event to avoid energy waste in a relatively nutrient-rich environment or in the absence of fungi.

Notably, our findings presented in this work have also provided some clues to connect the pathways of 4-HBA and c-di-GMP, two structurally unrelated small molecules yet acting synergistically to regulate HSAF biosynthesis. Previously we showed that c-di-GMP binding to its receptor CdgL can weaken the CdgL-LysR binding and lead to impaired HSAF biosynthesis (Han et al. 2020). Our present study further showed that 4-HBA also used a similar strategy by binding to a different receptor protein of LysR (Su et al. 2017) to impair the CdgL-LysR complex formation. Thus, either 4-HBA or c-di-GMP could regulate the HSAF biosynthesis through binding and targeting to its cognate receptor in the same protein complex in a synergistic way. To the best of our knowledge, such a mechanism linking the interplay of

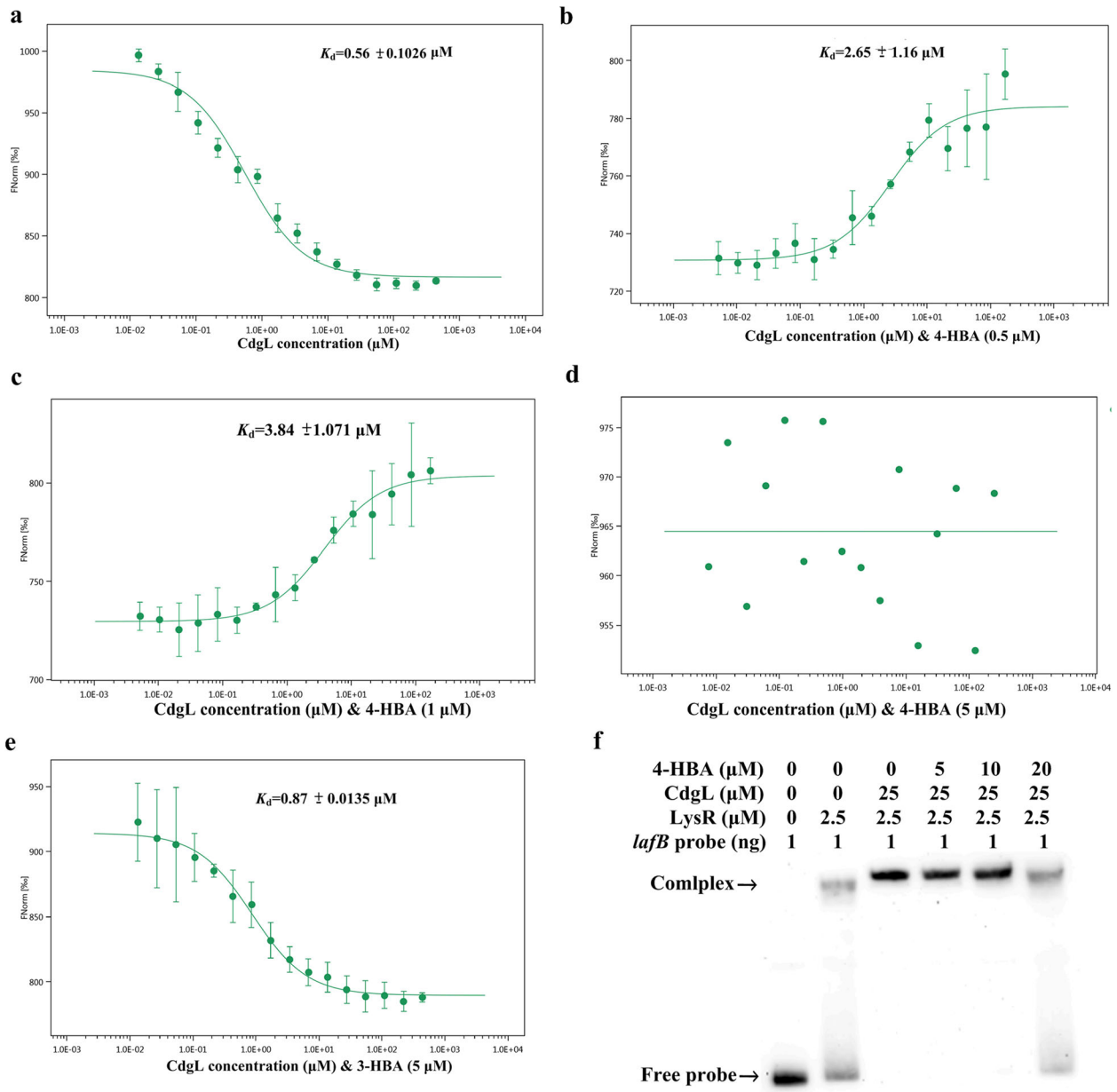

Fig. 4 Effect of 4-HBA on the CdgL-LysR binding and the CdgL-LysR-DNA ternary complex formation. **a** Microscale thermophoresis (MST) showing that GST-CdgL interacted with LysR-His6 with a K_d of 0.56 μM. FNorm was plotted on a linear y-axis in per mil (‰) against the total concentration of thetitrated partner on a $\log_{10}$ x-axis as described previously (Seidel et al. 2013). **b**, **c**, **d** Characterization of the LysR-CdgL binding affinity via MST in the presence of various concentrations of 4-HBA: 0.5 μM (**b**),1 μM (**c**), or 5 μM (**d**). **e** Effect of 3-HBA on the interaction between CdgL and LysR. **f** Effect of 4-HBA on the LysR-CdgL-DNA ternary complex formation determind by electrophoretic mobility shift assay

two small molecules has never been documented for bacteria to date. According to an earlier study (Wang et al. 2018), 4-HBA can act as a versatile intermediate for numerous value-added bio-products, such as resveratrol and ubiquinone. Unlike these cases, we demonstrated that 4-HBA could play a regulatory/signaling role in *L. enzymogenes*, since it could bind to and disrupt the LysR-CdgL complex formation to inhibit HSAF biosynthesis. Such a function of 4-HBA is also specific since its structural analog 3-HBA failed to do the job. Thus, our findings also shed light on the functional and mechanistic diversity of 4-HBA in bacterial cells.

In our earlier work (Su et al. 2017), we showed that exogenous addition of 0.5-μM 4-HBA that is 10-fold lower than the affinity of 4-HBA binding with LysR (K_d, 5 μM) is already effective in restoring HSAF production in the *lenB2* mutant to the wild-type level. Thus, 4-HBA in *L. enzymogenes* might have other unidentified,

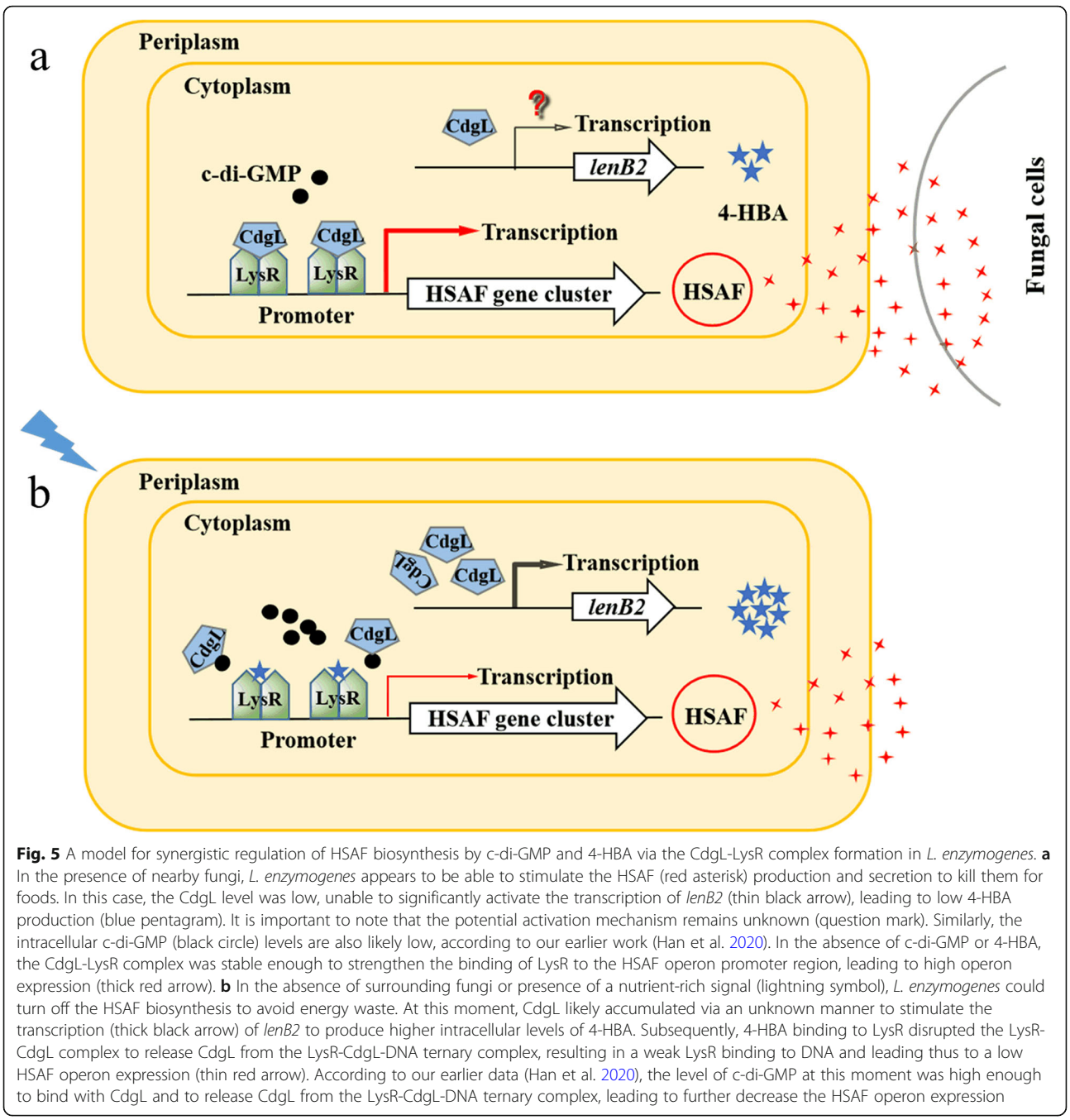

Fig. 5 A model for synergistic regulation of HSAF biosynthesis by c-di-GMP and 4-HBA via the CdgL-LysR complex formation in *L. enzymogenes*. **a** In the presence of nearby fungi, *L. enzymogenes* appears to be able to stimulate the HSAF (red asterisk) production and secretion to kill them for foods. In this case, the CdgL level was low, unable to significantly activate the transcription of *lenB2* (thin black arrow), leading to low 4-HBA production (blue pentagram). It is important to note that the potential activation mechanism remains unknown (question mark). Similarly, the intracellular c-di-GMP (black circle) levels are also likely low, according to our earlier work (Han et al. 2020). In the absence of c-di-GMP or 4-HBA, the CdgL-LysR complex was stable enough to strengthen the binding of LysR to the HSAF operon promoter region, leading to high operon expression (thick red arrow). **b** In the absence of surrounding fungi or presence of a nutrient-rich signal (lightning symbol), *L. enzymogenes* could turn off the HSAF biosynthesis to avoid energy waste. At this moment, CdgL likely accumulated via an unknown manner to stimulate the transcription (thick black arrow) of *lenB2* to produce higher intracellular levels of 4-HBA. Subsequently, 4-HBA binding to LysR disrupted the LysR-CdgL complex to release CdgL from the LysR-CdgL-DNA ternary complex, resulting in a weak LysR binding to DNA and leading thus to a low HSAF operon expression (thin red arrow). According to our earlier data (Han et al. 2020), the level of c-di-GMP at this moment was high enough to bind with CdgL and to release CdgL from the LysR-CdgL-DNA ternary complex, leading to further decrease the HSAF operon expression

membrane-bound, or cytoplasmic sensors or receptors other than LysR. In addition, several other interesting questions remain: For example, how CdgL, a protein without any known DNA binding domain, affects the transcription of *lenB2*? Does it work with a transcription factor? Also, does 4-HBA affect the synthesis of c-di-GMP or vice versa? In-depth investigations are required for these issues.

Conclusions

At present, it is clear that a single bacterial cell could produce a variety of small molecules with diversified functions. How these chemicals establish cellular communication is not fully understood. The biocontrol bacterium, *L. enzymogenes*, produces two small molecules, 4-HBA and c-di-GMP, both of which could control the antifungal HSAF biosynthesis. In this work, we show that the c-di-GMP-binding receptor CdgL promoted the transcription of *lenB2* to increase the intracellular levels of 4-HBA. As a potential feedback, 4-HBA binds with the transcription factor LysR to disrupt the CdgL-LysR complex, resulting in low HSAF production. Therefore, *L. enzymogenes* seems to be able to adopt a LysR-CdgL

complex to interconnect the 4-HBA and c-di-GMP pathways to control the HSAF biosynthesis synergistically in a rather elegant way. Thus, our findings reveal a previously uncharacterized mechanism by which two small molecules are functionally linked. Engineering this novel mechanism may be helpful for enhancing the HSAF yield to serve as an applicable bio-fungicide.

Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in the Additional file 1: Table S1. *Escherichia coli* strains were grown in LB medium with appropriate antibiotics at 37 °C. *L. enzymogenes* strains were cultivated in LB medium or 1/10 Tryptic Soy Broth (TSB) at 28 °C. When required, appropriate antibiotics (kanamycin, Km, and gentamicin, Gm) were added into media.

Complementation and overexpression

The coding region of the CdgL-Flag fusion was PCR-amplified by primers (Additional file 1: Table S2). The purified product was cloned into the broad-host vector pBBR1-MCS5 to generate a final construct (Additional file 1: Table S1). This construct was transformed into the wild-type OH11 or mutants by electroporation as described previously (Qian et al. 2012), with the transformants selected on the LB agar containing Km and Gm.

Quantitative RT-PCR, qRT-PCR

The wild-type OH11 and its derivatives were cultivated in 1/10 TSB medium and collected at OD_{600} of 1.0. Bacterial RNA Kit (No. R6950–01, OMEGA, China) was used to extract total RNA from collected cells. To remove genomic DNA, 400 ng RNA samples were treated with RNase inhibitors and DNase I (No.E1091, OMEGA, China). Next, 2 μg RNA sample was used to synthesize cDNA using PrimerScript™ RT reagent Kit with gDNA Eraser (No. RR047A, TaKaRa, Japan). qRT-PCR was carried out by an Applied Biosystems 7500 system using the primers listed in the Additional file 1: Table S2 with the following program: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s, before fluorescence detection followed by a melting curve determined with 1 cycle of 95 °C for 15 s, 60 °C for 60 s and 95 °C for 30 s. The 16S rDNA was used as an internal control as described previously (Qian et al. 2013, 2014). Data were analyzed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). The hypothesis test of percentages (*t*-test, $P = 0.05$ or 0.01) was used to determine the significant differences in gene expression.

Quantification of yellow pigments

Extraction of yellow pigment was carried out as described previously (Zhou et al. 2013). In brief, cells from 8 mL of the culture of the wild-type OH11 and its derivatives were harvested at the stationary phase (OD_{600}, 3.0). The collected cells were re-suspended in 750 μL methanol, followed by addion of the same volume of chloroform via thoroughly mixing, which were further subjected to centrifugation at 13,000 rpm for 10 min for collecting the supernatant. The amount of pigment was expressed as the absorbance of the crude pigment extract (absorbance at 445 nm) (Poplawsky and Chun 1997; Zhou et al. 2013)

HSAF extraction and quantification

HSAF was extracted from 50 mL of *L. enzymogenes* cultures grown in 1/10 TSB for 24 h at 28 °C with shaking at 200 rpm. A total of 4 mL of culture was mixed with an equal volume of ethyl acetate with 12 μL hydrochloric acid. After shaking, the ethyl acetate phase was collected and evaporated to dryness. The final HSAF-containing residue was dissolved in 200 μL of methanol. HSAF was then determined by HPLC and quantified as per unit of OD_{600} as described earlier (Yu et al. 2007; Qian et al. 2013). All tested strains were carried out in triplicate, with each analysed in three technical replicates. Data were analyzed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). The hypothesis test of percentages (*t*-test, $P = 0.05$ or 0.01) was used to determine significant differences in HSAF level.

Extraction and quantification of 3-HBA and 4-HBA

3-HBA and 4-HBA were extracted from 15 mL of *L. enzymogenes* cultures grown in 1/10 TSB for 2 days at 28 °C with shaking at 200 rpm. Cells were removed by centrifugation (13,000 rpm for 3 min). The harvested supernatant was mixed with an equal volume of ethyl acetate, followed by shaking at 28 °C for 30 min. The ethyl acetate phase was collected by centrifugation (13, 000 rpm for 10 min) and further evaporated to dryness. Both 3-HBA and 4-HBA were detected and quantified using HPLC, as described previously (Su et al. 2017).

Microscale thermophoresis, MST

The affinity of protein-protein interaction was determined by MST using Monolith NT.115 (NanoTemper Technologies, Germany) as described previously (Su et al. 2017; Xu et al. 2018; Han et al. 2020). For the LysR-CdgL binding assay, the purified LysR-His was labeled with the fluorescent dye NT-647-NHS (Nano Temper Technologies) via amine conjugation. A constant concentration (40 μM) of the labeled protein in the MST buffer (50 mM Tris, pH 7.5, 150 mM NaCl,10 mM $MgCl_2$, 0.05% Tween 20) was titrated against GST-CdgL

dissolved in MST buffer at concentrations ranging from 440 μM to 13.4 nM. The MST premium-coated capillaries (Monolith NT.115 MO-K005, Germany) were used to load the samples into the MST instrument at 25 °C using 80% MST and 20% LED power. FNorm was plotted on a linear y-axis in per mil (‰) against the total concentration of the titrated partner on a $\log_{10}$ x-axis, as reported previously (Seidel et al. 2013). The remaining tests were run similarly to the LysR-CdgL binding assay. All experiments were performed in triplicate. Data were analyzed using Nanotemper Analysis software 2.2.4.4577 (NanoTemper Technologies).

Electrophoretic mobility shift assay, EMSA

EMSA assay was carried out as follows: Biotin-labeled fragment containing the promoter region of *lafB* (pHSAF) was amplified using the 5′-end biotinylated primers listed in the Additional file 1: Table S2. Purified proteins and DNA probe was mixed and incubated in the test system for 30 min at 28 °C according to the specifications of LightShift® Chemiluminescent EMSA Kit (ThermoFisher, Waltham, USA). The reaction system was loaded onto the polyacrylamide gel, electrophoresed, transferred to a nylon membrane and crosslinked as described in the manufacturer protocol. The biotinylated DNA fragments were detected by chemiluminescence using Versa Doc imaging system (Bio-Rad, Philadelphia, USA).

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s42483-020-00053-y.

Additional file 1: Table S1. Strains and plasmids used in this study. **Table S2.** Primers used in this study. **Figutre S1.** Western blot analyses of the CdgL-Flag fusion expression in *Lysobacter enzymogenes*. **Figure S2.** MST showing no detectable GST-CdgL binding to 4-HBA.

Abbreviations

4-HBA: 4-hydroxybenzoic acid; C-di-GMP: Bis-(3′-5′)-cyclic diguanosine monophosphate; EMSA: Electrophoretic mobility shift assay; HSAF: Heat-stable antifungal factor; HPLC: High-performance liquid chromatography; MST: Microscale thermophoresis

Acknowledgments

Not applicable.

Authors' contributions

GQ conceived the project. GQ and SH designed experiments. SH carried out experiments. SH, MY and LL analyzed data and prepared figures and tables. GQ wrote the manuscript draft. GQ, AMF, and SHC revised the manuscript. All authors read and approved the final manuscript.

Funding

This study was supported by the Natural Science Foundation of Jiangsu Province (BK20190026; BK20181325), the National Natural Science Foundation of China (31872016), the Fundamental Research Funds for the Central Universities (KJJQ202001; KYYJ201904; KYT201805 and KYTZ201403), Jiangsu Agricultural Sciences and Technology Innovation Fund [CX (18)1003] and Innovation Team Program for Jiangsu Universities (2017).

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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Received: 18 March 2020 Accepted: 5 May 2020
Published online: 19 May 2020

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