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Heat-Stable Antifungal Factor (HSAF) Biosynthesis in *Lysobacter enzymogenes* Is Controlled by the Interplay of Two Transcription Factors and a Diffusible Molecule

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ABSTRACT *Lysobacter enzymogenes* is a Gram-negative, environmentally ubiquitous bacterium that produces a secondary metabolite, called heat-stable antifungal factor (HSAF), as an antifungal factor against plant and animal fungal pathogens. 4-Hydroxybenzoic acid (4-HBA) is a newly identified diffusible factor that regulates HSAF synthesis via *L. enzymogenes* LysR ($LysR_{Le}$), an LysR-type transcription factor (TF). Here, to identify additional TFs within the 4-HBA regulatory pathway that control HSAF production, we reanalyzed the LenB2-based transcriptomic data, in which LenB2 is the enzyme responsible for 4-HBA production. This survey led to identification of three TFs (Le4806, Le4969, and Le3904). Of them, LarR (Le4806), a member of the MarR family proteins, was identified as a new TF that participated in the 4-HBA-dependent regulation of HSAF production. Our data show the following: (i) that LarR is a downstream component of the 4-HBA regulatory pathway controlling the HSAF level, while $LysR_{Le}$ is the receptor of 4-HBA; (ii) that 4-HBA and $LysR_{Le}$ have opposite regulatory effects on *larR* transcription whereby *larR* transcript is negatively modulated by 4-HBA while $LysR_{Le}$, in contrast, exerts positive transcriptional regulation by directly binding to the *larR* promoter without being affected by 4-HBA *in vitro*; (iii) that LarR, similar to $LysR_{Le}$, can bind to the promoter of the HSAF biosynthetic gene operon, leading to positive regulation of HSAF production; and (iv) that LarR and $LysR_{Le}$ cannot interact and instead control HSAF biosynthesis independently. These results outline a previously uncharacterized mechanism by which biosynthesis of the antibiotic HSAF in *L. enzymogenes* is modulated by the interplay of 4-HBA, a diffusible molecule, and two different TFs.

IMPORTANCE Bacteria use diverse chemical signaling molecules to regulate a wide range of physiological and cellular processes. 4-HBA is an "old" chemical molecule that is produced by diverse bacterial species, but its regulatory function and working mechanism remain largely unknown. We previously found that 4-HBA in *L. enzymogenes* could serve as a diffusible factor regulating HSAF synthesis via $LysR_{Le}$. Here, we further identified LarR, an MarR family protein, as a second TF that participates in the 4-HBA-dependent regulation of HSAF biosynthesis. Our results dissected how LarR acts as a protein linker to connect 4-HBA and HSAF synthesis, whereby LarR also has cross talk with $LysR_{Le}$. Thus, our findings not only provide fundamental insight regarding how a diffusible molecule (4-HBA) adopts two different types of TFs for coordinating HSAF biosynthesis but also show the use of applied microbiology to increase the yield of the antibiotic HSAF by modification of the 4-HBA regulatory pathway in *L. enzymogenes*.

KEYWORDS 4-HBA, HSAF, *Lysobacter*, MarR

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The Gram-negative genus *Lysobacter* comprises a group of ubiquitous environmental bacteria, emerging as a rich resource for discovering new antibiotics (1). Of them, *Lysobacter enzymogenes* is the best-studied species and serves as an important biocontrol resource that has an efficient antagonistic effect on pathogenic filamentous fungi and oomycetes and plant parasitic nematodes (2–5). The antagonistic effects of this species are partly due to production of a polycyclic tetramate macrolactam (PTM)-type antifungal secondary metabolite, called heat-stable antifungal factor (HSAF), whose structure is remarkably different from structures of fungicides on the market (6, 7). The HSAF *pks* and *nrps* genes that code for a hybrid polyketide synthase (PKS) and a nonribosomal peptide synthetase (NRPS) are responsible for HSAF biosynthesis in *L. enzymogenes* (8, 9). Although HSAF has great potential to be developed as a biopesticide or antifungal drug, the original yield (1.8 μg/ml) of HSAF in *L. enzymogenes* is relatively low even in HSAF-inducing medium (4, 8). This fact restricts the extensive application of HSAF not only in the control of plant diseases but also in the inhibition of animal pathogens, especially in the case of antibiotic resistance (10, 11). Based on our knowledge, in addition to heterologous expression of the HSAF biosynthetic gene cluster (9), artificial synthesis, and optimized fermentation, understanding the regulation mechanism of HSAF biosynthesis is also greatly beneficial in constructing high-yield HSAF strains that improve the production of HSAF.

In order to reach this goal, we have identified three key transcription factors (TFs) that control HSAF production. These include the LuxR family protein LesR (a negative regulator), the global regulator Clp (a positive regulator), and the TetR family protein LetR (a negative regulator) (12–14). Apart from these regulators, we also found small-molecule metabolites, such as diffusible signal factor (DSF; a type of fatty acid compound) and diffusible factor (DF), that participate in the biosynthesis of HSAF (15). We along with our collaborators further showed that the RpfC/RpfG two-component system and Clp mediate the DSF signaling pathway and that *L. enzymogenes* LysR ($LysR_{Le}$) is involved in the DF regulatory cascade (16). The DF was recently identified as 4-hydroxybenzoic acid (4-HBA) in *L. enzymogenes*, and this molecule is predicted to be produced by a wide range of bacterial species (16). The *L. enzymogenes* 4-HBA is synthesized by LenB2 (a pteridine-dependent dioxygenase-like protein) using chorismate, the end product of shikimate pathway, as the substrate (16). $LysR_{Le}$ links the 4-HBA cascade to HSAF synthesis because, on one hand, according to our recent work (16), $LysR_{Le}$ could bind to the *lafB* gene (the originally described HSAF PKS/NRPS gene) promoter (also called the HSAF promoter, abbreviated as pHSAF) and, as a result, directs expression of HSAF biosynthetic genes and HSAF production; on the other hand, $LysR_{Le}$ interacts with 4-HBA directly. Binding with 4-HBA appears to partly promote the binding of $LysR_{Le}$ to pHSAF *in vitro*. However, at this moment we cannot conclude that binding of 4-HBA affects the binding of $LysR_{Le}$ to pHSAF, which would explain the change in HSAF output due to transcriptional activation (16). Our previous findings raise a great possibility that 4-HBA may be involved in stabilizing an $LysR_{Le}$-DNA (pHSAF) complex with an unidentified protein in *L. enzymogenes* (16). Nevertheless, these earlier findings provide a first TF ($LysR_{Le}$) linking 4-HBA regulation to HSAF biosynthesis in *L. enzymogenes*.

The objective of this study was to identify new potent TFs within the 4-HBA regulatory pathway that control HSAF levels and further dissect their genetic/biochemical relationship with $LysR_{Le}$. Here, we show that LarR (Le4806), an MarR family protein, is the second regulator connecting the 4-HBA cascade to HSAF synthesis. First, 4-HBA negatively regulates the transcription of *larR*; second, LarR positively controls HSAF levels by direct binding to pHSAF, similar to that of $LysR_{Le}$; third, *larR* transcription is positively controlled by $LysR_{Le}$ as $LysR_{Le}$ could bind to the *larR* promoter, but LarR failed to directly bind to the $lysR_{Le}$ promoter. Finally, we show that although $LysR_{Le}$ and LarR both serve as key components of the 4-HBA regulatory pathway, both regulators appear to employ independent mechanisms of modulating HSAF biosynthesis. Therefore, our results reveal that antifungal antibiotic HSAF biosynthesis in *L. enzymogenes* is modulated by the interplay of two transcription factors ($LysR_{Le}$ and LarR) and a

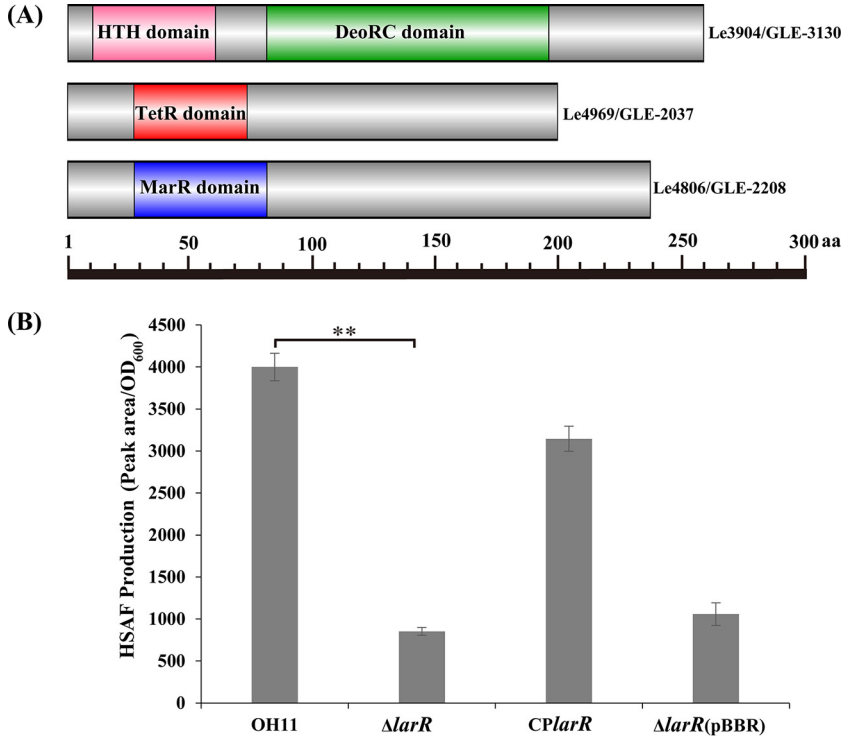

FIG 1 LarR (Le4806) is one of three transcription factors that belong to the LenB2 regulon and control HSAF production in *L. enzymogenes* OH11. (A) Bioinformatics analyses of the domain organization of three transcription factors (TFs) that belong to the LenB2 regulon. These three TFs belong to the MarR (Le4806), TetR (Le4969), and DeoRC (Le3904) protein families, and the protein numbers GLE-2208, GLE-2037, and GLE-3130, respectively, correspond to their homologues in *L. enzymogenes* C3, as indicated. (B) *In vivo* production of HSAF was modulated by *larR* in *L. enzymogenes* OH11. OH11, wild-type strain; Δ*larR*, the *larR* deletion mutant; CP*larR*, the complemented strain of Δ*larR* containing a plasmid-borne *larR*; Δ*larR*(pBBR), the *larR* mutant containing an empty vector (pBBR1-MCS5). Data of triplicate experiments are shown. **, $P < 0.01$. aa, amino acid.

diffusible molecule (4-HBA), presenting a new fundamental mechanism underlying a conserved bacterial chemical molecule (4-HBA) in functional performance. From an applied microbiology point of view, our findings also open a way to improve the yield of the antibiotic HSAF by engineering the components of the 4-HBA regulatory pathway (i.e., LarR) in *L. enzymogenes*.

RESULTS

LarR is an MarR family transcription factor that is transcriptionally repressed by 4-HBA and positively controls HSAF production. To discover any new TFs within the 4-HBA regulatory pathway controlling HSAF production, we reanalyzed the published LenB2-based transcriptomic data, according to which LenB2 is the enzyme responsible for 4-HBA production (15, 16). This investigation led to the identification of three TFs (Le4806, Le4969, and Le3904) from the LenB2 regulon. According to their functional domains, these three TFs belong to the MarR (Le4806), TetR (Le4969), and DeoRC (Le3904) family proteins, respectively (Fig. 1A). To understand their roles in HSAF production, each TF coding gene was accordingly deleted in frame (see Fig. S1A in the supplemental material), and HSAF levels were quantified from each generated mutant. The results showed that deletion of *le4969* or *le3904* from the wild-type OH11 did not sharply influence HSAF yield, whereas deletion of *le4806* (designated *larR*) almost abolished HSAF production (Fig. 1B and S1B). These results revealed that *larR* may regulate the biosynthesis of HSAF. To confirm this conclusion, an *larR* expression plasmid (Table 1) was introduced into the *larR* mutant, which almost restored HSAF production to the level of the wild type (Fig. 1B and S2A). Under similar test conditions, introduction of an empty vector to the *larR* mutant did not rescue HSAF production.

TABLE 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Description[a]	Source or reference
Lysobacter enzymogenes strains		
OH11	Wild type, Km^r	5
Δ*lenB2* strain	*lenB2* in-frame deletion mutant, Km^r	15
Δ*le4969* strain	*le4969* in-frame deletion mutant, Km^r	This study
Δ*le3904* strain	*le3904* in-frame deletion mutant, Km^r	This study
Δ*larR* strain	*larR* in-frame deletion mutant, Km^r	This study
Δ*larR* (*larR*) strain	Δ*larR* harboring plasmid pBBR-*larR*, Gm^r Km^r	This study
Δ*larR*(pBBR) strain	Δ*larR* harboring plasmid pBBR1-MCS5, Gm^r Km^r	This study
Δ*lenB2* Δ*larR* strain	*lenB2* and *larR* in-frame deletion mutant, Km^r	This study
Δ*lenB2* Δ*larR* (*lenB2*) strain	Δ*lenB2* Δ*larR* strain harboring plasmid pBBR1-*lenB2*, Gm^r Km^r	This study
Δ*lenB2* Δ*larR* (*larR*) strain	Δ*lenB2* Δ*larR* strain harboring plasmid pBBR1-*larR*, Gm^r Km^r	This study
Δ*lenB2* Δ*larR*(pBBR) strain	Δ*lenB2* Δ*larR* strain harboring plasmid pBBR1-MCS5, Gm^r Km^r	This study
Δ*larR* Δ$lysR_{Le}$ strain	*larR* and $lysR_{Le}$ in-frame deletion mutant, Km^r	This study
Δ*larR* Δ$lysR_{Le}$ (*larR*) strain	Δ*larR* Δ$lysR_{Le}$ strain harboring plasmid pBBR1-*larR*, Gm^r Km^r	This study
Δ*larR* Δ$lysR_{Le}$ ($lysR_{Le}$) strain	Δ*larR* Δ$lysR_{Le}$ strain harboring plasmid pBBR1-$lysR_{Le}$, Gm^r Km^r	This study
Δ*larR* Δ$lysR_{Le}$(pBBR) strain	Δ*larR* Δ$lysR_{Le}$ strain harboring plasmid pBBR1-MCS5, Gm^r Km^r	This study
Escherichia coli strains		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	15
BL21(DE3)	F^- *ompT* $hsdS_B$(r_B^- m_B^-) *gal dcm* (DE3)	16
XL1-Blue MRF′ Kan	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB* $lacI^qZ$ΔM15 Tn*5* (Km^r)]	28
Plasmids		
pEX18GM	Suicide vector with a sacB gene, Gm^r	31
pEX18-*lenB2*	pEX18GM with two flanking fragments of *lenB2*, Gm^r	15
pEX18-*larR*	pEX18GM with two flanking fragments of *larR*, Gm^r	This study
pEX18-*le4969*	pEX18GM with two flanking fragments of *le4969*, Gm^r	This study
pEX18-*le3904*	pEX18GM with two flanking fragments of *le3904*, Gm^r	This study
pBBR1-MCS5	Broad-host-range vector with a P_{lac} promoter	32
pBBR-*lenB2*	pBBR1-MCS5 cloned with a 1,553-bp fragment containing intact *lenB2* and its predicted promoter	15
pBBR-*larR*	pBBR1-MCS5 cloned with a 1,223-bp fragment containing intact *larR* and its predicted promoter	This study
pBBR-$lysR_{Le}$	pBBR1-MCS5 cloned with a 1,467-bp fragment containing intact $lysR_{Le}$ and its predicted promoter	This study
pSS122	Promoter-probe plasmid containing a promoterless *uidA*, Gm^r	27
p-*larR*	The promoter region (470 bp) of *larR* cloned into pSS122, Gm^r	This study
pTRG	The plasmid used for protein expression in bacterial one- or two-hybrid assay, Tet^r	28
pTRG-*larR*	pTRG with the coding region of *larR*, Tet^r	This study
pTRG-GacS	pTRG with the coding region of *gacS*, Tet^r	33
pBXcmT	The plasmid used for DNA cloning in bacterial one-hybrid assay, $Chlo^r$	28
pBXcmT-*lafB*	pBXcmT with pHSAF (the predicted *lafB* promoter region), $Chlo^r$	14
pBT	The plasmid used for protein expression in bacterial two-hybrid assay, $Chlo^r$	28
pBT-$lysR_{Le}$	pBT with the coding region of $lysR_{Le}$, $Chlo^r$	This study
pBT- GacS	pBT with the coding region of *gacS*, $Chlo^r$	33
pTRG-$lysR_{Le}$	pTRG with the coding region of $lysR_{Le}$, Tet^r	This study
pBXcmT-*larR*	pBXcmT with pLarR (the predicted *larR* promoter region), $Chlo^r$	This study
pET30a	Inducible expression vector, C-terminal His tag, Km^r, IPTG inducible	16
pET30a-$lysR_{Le}$	Plasmid used for protein expression in BL21(DE3), Km^r	16

[a]Km^r, Gm^r, Amp^r, Tet^r, and $Chlo^r$ are kanamycin, gentamicin, ampicillin, tetracycline, and chloramphenicol resistance markers, respectively.

Moreover, mutation of *larR* did not affect the growth ability of the wild type in the test HSAF-inducing medium (Fig. S2B). Finally, we performed detailed sequence analyses and found that LarR contains all conserved domains or motifs expressed by the well-studied MarR family proteins (Fig. S3), confirming that LarR is an MarR-like protein. Taken together, these results strongly suggest that LarR participated in regulating the biosynthesis of HSAF.

According to our earlier report (15), *larR* transcription is negatively controlled by LenB2. To validate this finding, we performed a promoter activity assay. The recombined construct consists of the *larR* promoter and a promoterless glucuronidase (GUS) gene, *uidA*. This construct (p-*larR*) was introduced into the wild-type OH11 and the *lenB2* mutant. We found that the *larR* promoter exhibited significantly higher promoter activity (GUS activity) in the background of the *lenB2* deletion than in the OH11

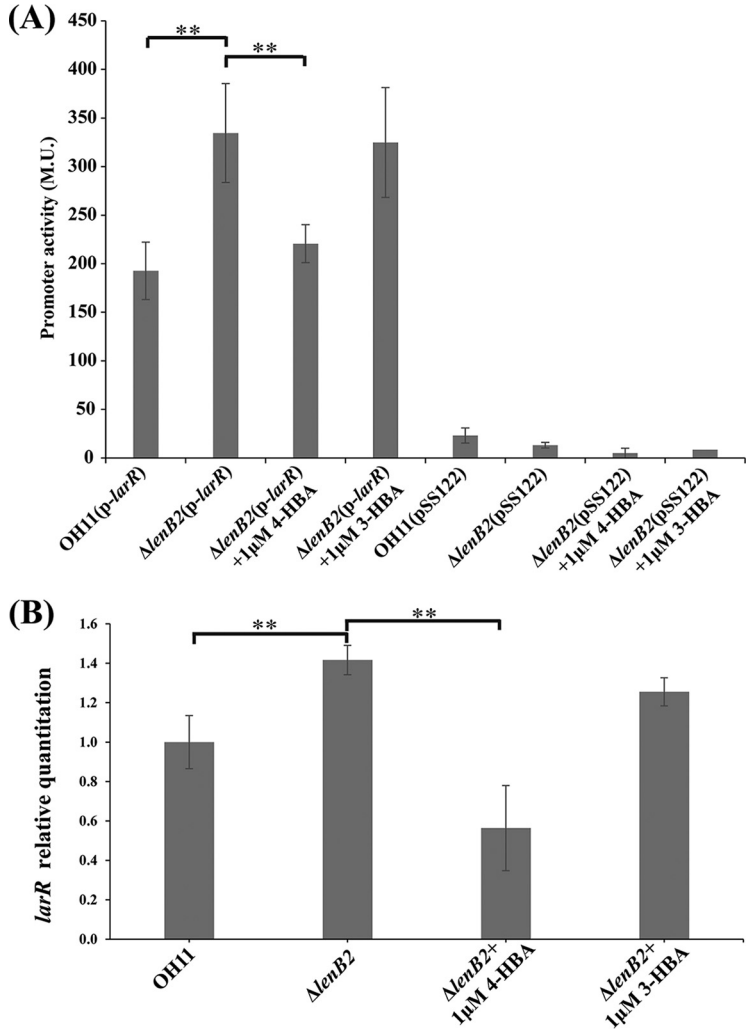

FIG 2 4-HBA negatively controls the transcription of *larR*. (A) Supplementation with 4-HBA, but not 3-HBA, at 1 μM had a significant effect on suppression of the activity (GUS activity) of *larR* in the *lenB2* mutant. P-*larR* represents the *larR* promoter. pSS122 is an empty vector that was both introduced into the wild-type OH11 and the *lenB2* mutant, generating OH11(pSS122) and Δ*lenB2*(pSS122), respectively. (B) Addition of 4-HBA, but not 3-HBA, at 1 μM remarkably inhibited the transcription of *larR* in the *lenB2* mutant as determined by qRT-PCR. Data of triplicate experiments are shown. **, $P < 0.01$.

wild-type strain (Fig. 2A), which is in agreement with our earlier finding mentioned above (15). LenB2 could catalyze chorismate to generate 3-HBA and 4-HBA, whereas only 4-HBA is related to the biosynthesis of HSAF (16). Therefore, only 4-HBA at a concentration of 1 μM was added to the culture medium of the Δ*lenB2*(p-*larR*) strain because such a low concentration of 4-HBA is sufficient to act as a diffusible factor in restoring the *lenB2* mutant to produce wild-type HSAF (16). In accordance with this, applying 4-HBA to the Δ*lenB2*(p-*larR*) mutant significantly reduced the *larR* promoter activity to a level similar to that of the wild-type OH11, while supplementation of 3-HBA in the culture medium of the Δ*lenB2*(p-*larR*) mutant had only a minor effect (Fig. 2A), suggesting that 4-HBA plays a key role in suppressing *larR* transcription. The empty plasmid pSS122 was also introduced into the culture medium of the wild-type OH11 or Δ*lenB2* strain to serve as a negative control in the testing of promoter activity. The strains that contained the empty vector displayed almost no GUS activity regardless of the presence or absence of 4-HBA or 3-HBA (Fig. 2A). Moreover, quantitative reverse transcription-PCR (qRT-PCR) assays showed that the molecule 4-HBA repressed *larR* expression in the background of an *lenB2* gene deficiency, but 3-HBA did not perform

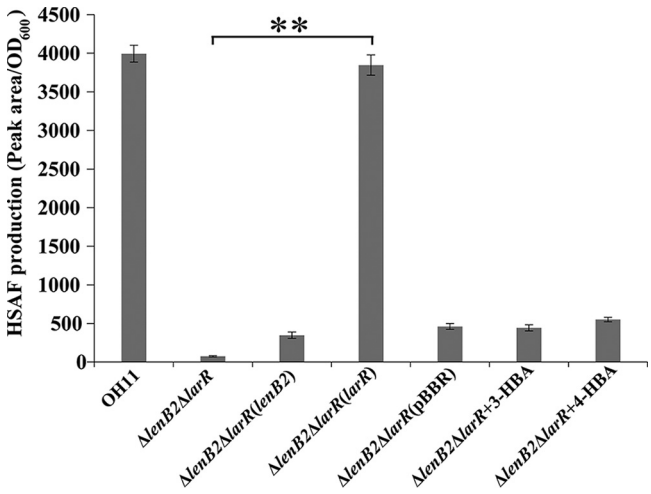

FIG 3 LarR is a downstream component of the LenB2 regulatory pathway controlling HSAF production in *L. enzymogenes* OH11. Single introduction of *larR*, but not *lenB2*, rescued the deficiency of the double mutant (Δ*lenB2* Δ*larR* strain) in producing HSAF. Addition of 3-HBA or 4-HBA to the double mutant had no effect on this function. Δ*lenB2* Δ*larR*, strain with deletion of both *lenB2* and *larR*; Δ*lenB2* Δ*larR* (*lenB2*), complementation of *lenB2* in the Δ*lenB2* Δ*larR* strain; Δ*lenB2* Δ*larR* (*larR*), complementation of *larR* in the Δ*lenB2*Δ*larR* strain; Δ*lenB2* Δ*larR*(pBBR), the Δ*lenB2* Δ*larR* mutant containing an empty vector, pBBR1-MCS5. Data of triplicate experiments are shown. **, $P < 0.01$.

such a function under similar test conditions (Fig. 2B). In conclusion, these results collectively suggest that LarR is involved in regulating HSAF biosynthesis and that *larR* transcription is negatively controlled by 4-HBA in *L. enzymogenes*.

LarR is within the 4-HBA regulatory pathway and directly binds to the HSAF promoter. The above results suggest that LarR is a downstream component of the 4-HBA regulatory cascade in modulation of HSAF biosynthesis. To provide more supporting evidence, we generated a mutant (Δ*lenB2* Δ*larR* strain) lacking both *larR* and *lenB2* (Table 1), and its identity was confirmed by RT-PCR (Fig. S4A). The ability of this double mutant to produce HSAF was tested. As expected, deletion of *lenB2* and *larR* almost completely impaired HSAF production (Fig. 3 and S4B). Then, single-gene complementation of the Δ*lenB2* Δ*larR* double mutant was accomplished by introducing plasmid-borne *lenB2* or *larR* (Fig. S4A). The results showed that individual introduction of *larR* into the Δ*lenB2* Δ*larR* double mutant significantly rescued HSAF production deficiency to almost the wild-type level, whereas the single introduction of *lenB2* did not yield a similar result (Fig. 3 and S4B). As a control, transformation of an empty vector did not restore the HSAF yield. These data suggest that *larR* is downstream of *lenB2 in vivo* in *L. enzymogenes*. Subsequently, we added 3-HBA and 4-HBA to the culture medium of the double mutant *in vitro* and tested HSAF production. The results, as shown in Fig. 3 and S4B, were consistent with those of the Δ*lenB2* Δ*larR* (*lenB2*) strain, suggesting that in the absence of LarR, addition of 4-HBA could not rescue HSAF production deficiency, providing another piece of evidence to highlight the importance of LarR in the 4-HBA regulatory cascade controlling HSAF production. Taken together, the results reveal that LarR was functionally located in the 4-HBA regulatory pathway and modulated HSAF production.

How does LarR control HSAF biosynthesis? To address this question, we tested whether LarR has an ability to bind pHSAF, resulting in directing HSAF gene expression and HSAF production. To test this hypothesis, we used a bacterial one-hybrid reporter system to test the direct binding of LarR to pHSAF. As shown in Fig. 4A, we clearly observed that the transformed *Escherichia coli* strain that contained both LarR and pHSAF grew very well on selective medium, as did the positive control, whereas the negative control did not successfully grow under similar conditions. This result reveals that direct binding of LarR to pHSAF occurred under the test conditions. To further verify the above finding, an electrophoretic mobility shift assay (EMSA) was carried out.

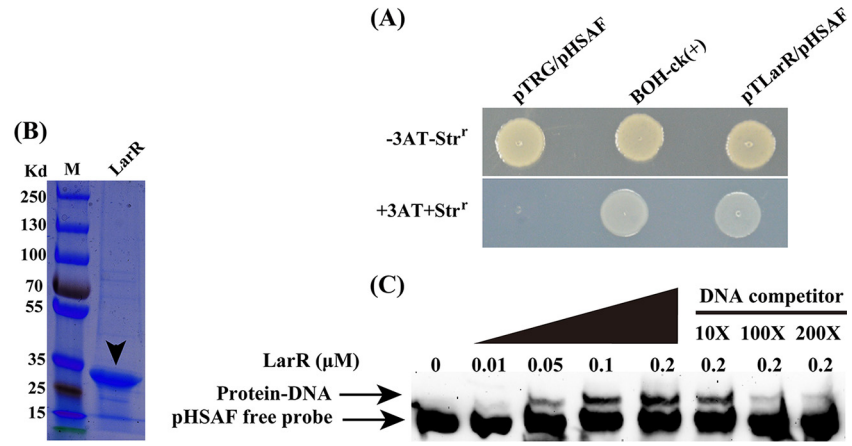

FIG 4 LarR directly bound to the HSAF promoter. (A) The direct physical interaction between LarR and the HSAF promoter, pHSAF (the promoter of *lafB*, the key biosynthetic gene of HSAF) was detected in *E. coli*. Experiments were performed according to the procedures described in the Materials and Methods section. BOH-CK(+), cotransformant containing pBX-R2031 and pTRG-R3133, used as a positive control; pTRG/pHSAF, cotransformant containing pBXcmT-*lafB* and the empty pTRG, serving as a negative control; pTLarR/pHSAF, cotransformant possessing both pTRG-*larR* and pBXcmT-*lafB* (Table 1). −3AT-Str^r, plate without selective medium; +3AT+Str^r, plate with selective medium. (B) SDS-PAGE of the His-tagged, purified LarR, as indicated. Lane M, molecular mass marker. (C) LarR bound to the HSAF promoter (pHSAF) *in vitro* as determined by an EMSA. The free DNA (the labeled pHSAF) and protein-DNA complex are indicated by arrows. The unlabeled probe (cold probe) at a 100- or 200-fold excess to the reaction mixtures can efficiently and competitively inhibit the binding of LarR to the labeled DNA probe (pHSAF).

His-tagged LarR protein was purified (Fig. 4B). As shown in Fig. 4C, the concentration-dependent protein-DNA complex formation that is triggered by LarR was evidently detected (from 0.1 to 2 μM) and could be specifically and competitively inhibited by an unlabeled HSAF promoter probe (cold probe) at a 100- or 200-fold excess concentration. To further validate the binding specificity of LarR to pHSAF, we selected the promoter region of *le1974* (p1974; 295 bp) as a new probe to test whether it could competitively inhibit LarR-pHSAF complex formation. *le1974* encodes a GGDEF domain-containing protein potentially responsible for synthesizing c-di-GMP, an intracellular nucleotide second messenger (17). To the best of our knowledge, p1974 should be unrelated to the binding capacity of LarR to pHSAF. Our results showed that addition of p1974 at different concentrations into the EMSA mixture containing LarR and pHSAF did not inhibit formation of the complex LarR-pHSAF (Fig. S5). In agreement, LarR could not bind to p1974 under the *in vitro* EMSA conditions (Fig. S5). These discoveries, together with the results of HSAF yield (Fig. 1B), powerfully support the hypothesis that LarR could specifically bind to the HSAF promoter and regulate HSAF biosynthesis.

LysR$_{Le}$ could directly bind to the *larR* promoter. The above results provide strong evidence to show that LarR is a second key TF, in addition to LysR$_{Le}$, within the 4-HBA cascade regulating HSAF synthesis. Thus, it is of great interest to question the relationship between LysR$_{Le}$ and LarR. As both TFs could bind to the HSAF promoter, we first investigated whether there is an interaction between the two factors in the binding of the HSAF promoter. For this purpose, a BacterioMatch II bacterial two-hybrid experiment was performed, as described in detail in Materials and Methods. Our results show that the transformed *E. coli* strain that contained both the LarR and LysR$_{Le}$ proteins did not grow any more on the selective medium, but the positive control grew well (Fig. S6). These results suggest that LarR and LysR$_{Le}$ may not interact with each other during their binding to the HSAF promoter.

Since 4-HBA affects *larR* transcription as described above (Fig. 2A), we investigated whether 4-HBA could control *larR* transcription via LysR$_{Le}$ because LysR$_{Le}$ is the 4-HBA receptor and has a DNA-binding domain (16). To test this hypothesis, we first tested the potential binding of LysR to the *larR* promoter (pLarR) by employing the bacterial one-hybrid reporter system described above. As shown in Fig. 5A, we clearly observed

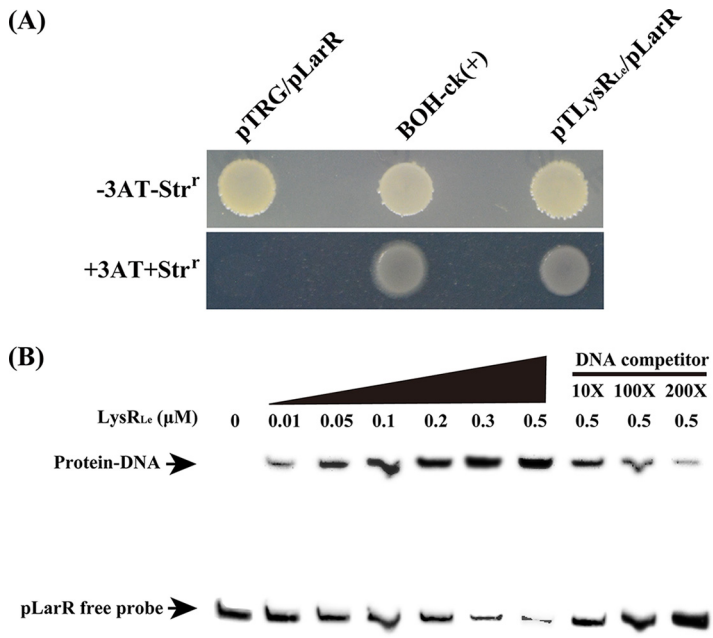

FIG 5 LysR$_{Le}$ directly bound the promoter of *larR*. (A) The direct physical interaction between LysR$_{Le}$ and the *larR* promoter region was detected in *E. coli*. BOH-CK(+), cotransformant containing pBX-R2031 and pTRG-R3133, used as a positive control; pTRG/pLarR, cotransformant containing pBXcmT-*larR* and the empty pTRG, used as a negative control; pTLysR$_{Le}$/pLarR, cotransformant containing both pTRG-*lysR$_{Le}$* and pBXcmT-*larR* (Table 1). pLarR, the *larR* promoter described in the text; −3AT-Str^r, nonselective medium plate; +3AT+Str^r, selective medium plate. (B) LysR$_{Le}$ bound to the *larR* promoter region *in vitro* as determined by an EMSA. The arrows indicate the free DNA (the labeled pLarR) and protein-DNA complex. The unlabeled probe (cold probe) at a 10- to 200-fold excess could efficiently and competitively inhibit the binding of LysR$_{Le}$ to the labeled DNA probe (pLarR).

that the transformed *E. coli* strain that contained both the LysR$_{Le}$ regulator and pLarR grew very well on the selective medium, as did the positive control; however, the negative control did not successfully grow under similar conditions. This result indicated that direct binding of LysR$_{Le}$ to pLarR occurred under the test conditions.

To better verify the above findings, an EMSA was carried out. As shown in Fig. 5B, concentration-dependent protein-DNA (pLarR) complex formation, triggered by LysR$_{Le}$, was obviously detected (from 0.01 to 0.5 μM) and could be competitively repressed by an unlabeled *larR* promoter probe (cold probe) at a 100- or 200-fold excess concentration, suggesting that LysR$_{Le}$ could specifically bind to pLarR *in vitro*. As further supporting evidence, we found that p1974 at different concentrations could not inhibit LysR$_{Le}$-pLarR complex formation (Fig. S7). Consistent with this, LysR$_{Le}$ failed to bind p1974 under the *in vitro* EMSA conditions (Fig. S7). Next, given that LysR$_{Le}$ is the receptor of 4-HBA (16), a series of different concentrations of 4-HBA was added to the EMSA system to test whether 4-HBA enhances or represses the binding of LysR$_{Le}$ to pLarR. The results (Fig. S8) showed that 4-HBA at all test concentrations neither enhanced nor repressed the interaction of LysR$_{Le}$ with pLarR; these results matched those of the negative control, 3-HBA. These data imply that LysR$_{Le}$ could bind to pLarR without the influence of 4-HBA or 3-HBA *in vitro*. It is also important that LarR could not bind to the *lysR$_{Le}$* promoter under the *in vitro* EMSA conditions (Fig. S9). Taken together, our results showed that LysR$_{Le}$ could specifically bind to pLarR, suggesting that LysR$_{Le}$ may control the transcription of *larR* (see below).

LysR$_{Le}$ and 4-HBA play opposite roles in *larR* transcription. To explore whether LysR$_{Le}$ has a regulatory effect on *larR* transcription, we quantified the relative expression of *larR* in the *lysR$_{Le}$* mutant by qRT-PCR. The results (Fig. 6) showed that, compared to wild-type OH11, the *larR* expression in the *lysR$_{Le}$* mutant was significantly low, suggesting that LysR$_{Le}$ positively regulates *larR* transcription. This finding is in contrast to the case of 4-HBA, where 4-HBA negatively controls *larR* transcription. This obser-

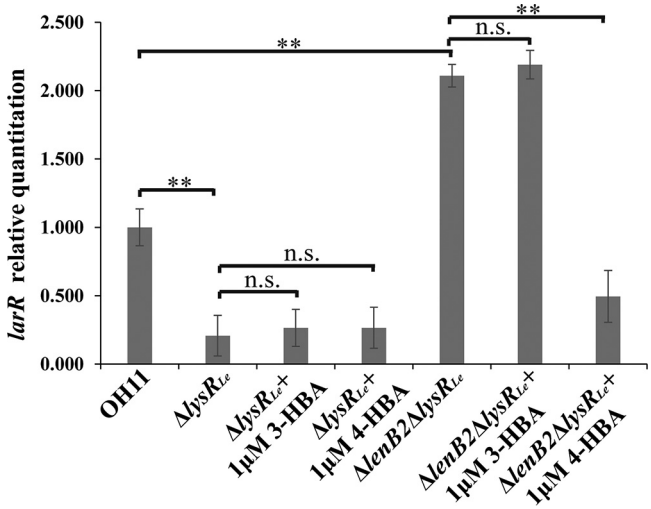

FIG 6 4-HBA and $LysR_{Le}$ play opposite roles in *larR* transcription, with 4-HBA having a bigger effect. Mutation of $lysR_{Le}$ significantly impaired *larR* transcription. Addition of 4-HBA or 3-HBA to the $lysR_{Le}$ mutant (Δ$lysR_{Le}$ strain) did not rescue its deficiency in transcribing *larR*. Double mutation of *lenB2* and $lysR_{Le}$ (Δ*lenB2* Δ$lysR_{Le}$ strain) sharply increased *larR* transcription, while supplementation of 4-HBA but not 3-HBA in this double mutant could significantly restore *larR* transcription compared to that of the double mutant. Data of triplicate experiments are shown. **, $P < 0.01$; n.s., not significant.

vation prompted us to determine the coregulatory effect of 4-HBA and $LysR_{Le}$. We thus generated a double mutant lacking both *lenB2* and $lysR_{Le}$ (Table 1). Surprisingly, we found that *larR* expression in this double mutant was significantly higher than that in the wild-type OH11 (Fig. 6). Adding 4-HBA but not 3-HBA could remarkably suppress *larR* expression in the background of double mutations (Fig. 6). These results collectively revealed that 4-HBA and $LysR_{Le}$ play opposite roles in *larR* transcription, with 4-HBA having a bigger effect.

$LysR_{Le}$ and LarR appear to independently regulate HSAF biosynthesis. All of the results described above suggest that both LarR and $LysR_{Le}$ are key regulators of the 4-HBA cascade in modulating HSAF biosynthesis, where 4-HBA and $LysR_{Le}$ control the transcription of *larR* in opposite ways, suggesting that $LysR_{Le}$ and LarR may regulate HSAF production independently. To test such a hypothesis, a double mutant (Δ*larR* Δ$lysR_{Le}$ strain) having deletions of both *larR* and $lysR_{Le}$ was generated (Table 1), followed by testing of its HSAF yield. As shown in Fig. 7, we observed that this double mutant almost lost the ability to produce HSAF; its HSAF yield was lower than that of the *larR* or $lysR_{Le}$ single mutant. Single introduction of the plasmid-borne *larR* or $lysR_{Le}$ into this double mutant had no visible effect on rescuing the HSAF production deficiency, suggesting that $LysR_{Le}$ and LarR may independently regulate HSAF production at the genetic level.

DISCUSSION

4-HBA is a newly identified diffusible factor that regulates antifungal antibiotic HSAF biosynthesis in *L. enzymogenes* (16). This chemical molecule is further predicted to be widely produced by a diverse range of bacterial species (16, 18), but the functionality and underlying mechanism remain poorly understood. In *L. enzymogenes*, we previously showed that $LysR_{Le}$, an LysR family TF, could serve as the 4-HBA receptor mediating the 4-HBA functional performance (16). Here, we have identified LarR, a member of the MarR protein family, as a second TF participating in 4-HBA-dependent HSAF biosynthesis, whereby 4-HBA and $LysR_{Le}$ have opposite regulatory effects on *larR* transcription, with 4-HBA having a bigger effect. These findings establish a bridge to connect one diffusible molecule (4-HBA) to two different types of TFs ($LysR_{Le}$ and LarR) in control of the same phenotype (HSAF production) in *L. enzymogenes*. Our results thus show that the biosynthesis of a unique secondary metabolite (HSAF) in an agriculturally

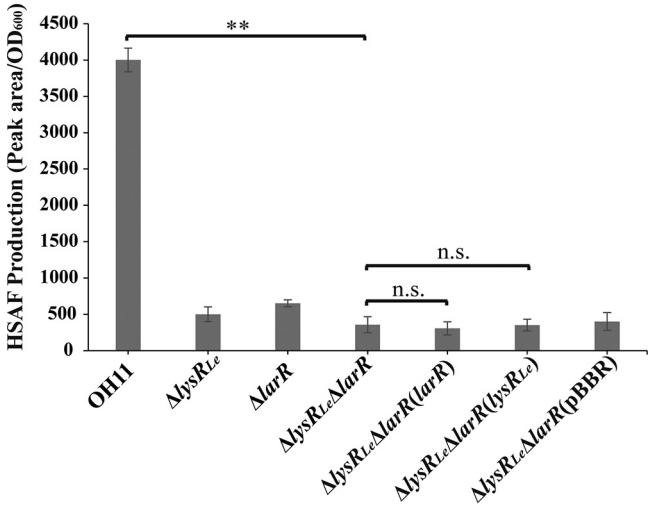

FIG 7 $LysR_{Le}$ and LarR are likely to independently control HSAF production. Double mutation of *larR* and $lysR_{Le}$ (Δ*larR* Δ$lysR_{Le}$) significantly impaired HSAF production, while single introduction of *larR* or $lysR_{Le}$ could not rescue the deficiency of the double mutant in producing HSAF. Δ*larR* Δ$lysR_{Le}$ (*larR*), the complementation of *larR* in the Δ*larR* Δ$lysR_{Le}$ strain; Δ*larR* Δ$lysR_{Le}$ ($lysR_{Le}$), the complementation of $lysR_{Le}$ in the Δ*larR* Δ$lysR_{Le}$ strain; Δ*larR* Δ$lysR_{Le}$(pBBR), the mutant Δ*larR* Δ$lysR_{Le}$ containing an empty vector, pBBR1-MCS5. Data of triplicate experiments are shown. **, $P < 0.01$; n.s., not significant.

important bacterium (*L. enzymogenes*) is controlled by the interplay of two TFs with 4-HBA, a conserved bacterial chemical molecule, which expands our current understanding of the working mechanism used by 4-HBA in bacteria. Our findings may trigger additional studies in 4-HBA-producing bacteria. The fundamental knowledge generated from the present study is greatly helpful in improving HSAF yield by supplying 4-HBA as a direct fermentation supplement and/or by generating higher-HSAF-producing strains via genetic and metabolic engineering of the regulators within the 4-HBA regulatory pathway.

The MarR family proteins are a large group of TFs widely distributed in bacterial and archaeal domains (19). This group of protein regulators could control bacterial detoxification in response to multiple antibiotics, toxic chemicals, or both (20, 21). Here, we identify LarR, an MarR-like protein that regulates the biosynthesis of HSAF, an antifungal secondary metabolite, via a direct binding mechanism to the HSAF promoter. This finding associates an MarR-like protein with the area of natural product (HSAF) biosynthesis, expanding the role of MarR family proteins in bacteria. As documented previously, MarR-like proteins prefer to form homodimers to bind gene promoter regions via their winged helix-turn-helix (wHTH)-type DNA binding domains, leading to control of expression of the respective genes (22–25). The protein-DNA interactions could be affected by specific phenolic compounds/ligands, such as salicylate, ethidium, and benzoate (22, 26). Earlier reports, along with our finding that LarR is within the 4-HBA regulatory pathway and could control HSAF production by directly binding to the HSAF promoter, raise a possibility that 4-HBA, a phenolic compound, may serve as the ligand of LarR. However, our results did not support this idea because the microscale thermophoresis (MST) data show no binding of LarR to 4-HBA (see Fig. S10 in the supplemental material). We further found that several 4-HBA structural analogs, including 3-HBA (3-hydroxybenzoic acid), 2-HBA (2-hydroxybenzoic acid), 3,4-HBA (3,4-hydroxybenzoic acid), 3,5-HBA (3,5-hydroxybenzoic acid), and 2,5-HBA (2,5-hydroxybenzoic acid) all failed to interact with LarR (Fig. S10). These findings collectively suggest that an unidentified phenolic ligand or other types of ligand may interact with LarR in *L. enzymogenes*. Thus, searching additional ligands of LarR will be the focus of our future study, which will facilitate our deep understanding of the underlying mechanism involved in the regulation of HSAF biosynthesis by LarR.

A notable finding of the present study was that $LysR_{Le}$, the 4-HBA receptor,

positively modulates *larR* transcription by directly binding to its promoter, establishing a genetic bridge to connect these two TFs that are both within the 4-HBA regulatory pathway. However, the binding of $LysR_{Le}$ to the *larR* promoter (pLarR) was not affected by 4-HBA *in vitro* (Fig. S8) although $LysR_{Le}$ binds 4-HBA directly (16). The mechanism underlying such a phenomenon is unclear at this time, but it is possible that under the *in vivo* conditions, the $LysR_{Le}$-pLarR complex may be affected by 4-HBA in combination with an unidentified protein in *L. enzymogenes*. Testing such a possibility is in progress in our laboratory. Although $LysR_{Le}$ established cross talk with *larR* by binding to its promoter (Fig. 5), LarR did not seem to perform similarly with $lysR_{Le}$ as LarR failed to bind the $lysR_{Le}$ promoter (Fig. S9). Furthermore, LarR is also not likely to interact with $LysR_{Le}$, as determined by a bacterial two-hybrid assay (Fig. S6). Based on our present understanding, it is thus likely that LarR did not establish cross talk with $LysR_{Le}$ by binding to the $lysR_{Le}$ promoter or interacting with $LysR_{Le}$. Another interesting observation made in the present study was that $LysR_{Le}$ and 4-HBA play opposite roles in *larR* transcription. $LysR_{Le}$ promoted the transcription of *larR* by directly binding to its promoter (Fig. 5 and 6), while 4-HBA suppressed the transcription of *larR* (Fig. 2). The repression of *larR* transcription by 4-HBA is likely to be independent of $LysR_{Le}$ as addition of 4-HBA could significantly decrease *larR* transcription in the background of the *lenB2* and $lysR_{Le}$ double mutation in the absence of $LysR_{Le}$ (Fig. 6). These findings suggest that an unknown factor, probably independent of $LysR_{Le}$, may mediate inhibition of *larR* transcription by 4-HBA in *L. enzymogenes*. Thus, it is possible that 4-HBA may utilize two independent pathways to control HSAF production in *L. enzymogenes*. One is mediated by $LysR_{Le}$, whereby 4-HBA directly interacts with $LysR_{Le}$ and appears to partly enhance $LysR_{Le}$ binding to pHSAF *in vitro*, leading to direct HSAF production (16). The other is LarR dependent. In this case, 4-HBA is likely to employ unidentified factor(s) (i.e., 4-HBA binding protein), probably independent of $LysR_{Le}$, to suppress *larR* transcription. To support this idea, our genetic data further show that regulation by $LysR_{Le}$ and LarR of HSAF production was independent at a genetic level (Fig. 7). However, at this time, it is unclear whether the two regulators ($LysR_{Le}$ and LarR) compete with each other in their binding to pHSAF. Addressing this and related issues, i.e., mapping the binding sites of $LysR_{Le}$ and LarR in pHSAF, is absolutely necessary for future study. It is also of great interest to understand why 4-HBA needs to adopt two different types of TFs ($LysR_{Le}$ and LarR) to coordinate HSAF biosynthesis. We do not know the exact answer, but it is likely that perhaps the two TFs play regulatory roles at different times and/or cell localizations as well as under different conditional stimulus responses. Such hypothesized molecular strategies may efficiently enable *L. enzymogenes* to acquire flexibilities or adaptabilities in determining when and how to generate HSAF via the 4-HBA regulatory network.

In summary, we expanded the proposed model of 4-HBA in regulating HSAF biosynthesis (Fig. 8). In this model, LenB2 uses chorismate, the end product of shikimate pathway, to produce 4-HBA (16). This molecule further employed two different types of TFs to mediate the regulation of 4-HBA in the control of HSAF production. One TF is $LysR_{Le}$, which could bind the HSAF promoter and thereby direct HSAF biosynthetic gene expression and HSAF production (16). In this process, 4-HBA interacts with $LysR_{Le}$ to partly enhance the binding of $LysR_{Le}$ to the HSAF promoter (16). The other TF is LarR, which can also bind to the HSAF promoter, but LarR did not bind 4-HBA. 4-HBA negatively controls *larR* transcription, probably via an uncharacterized factor, while LysR has a positive effect on *larR* transcription by directly binding to its promoter region. Our results thus suggest that the interplay of 4-HBA with two different TFs plays a key role in regulating HSAF biosynthesis in *L. enzymogenes*, which has not been reported in other 4-HBA-producing bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids that were used in this study are listed in Table 1. *Escherichia coli* strains DH5α, XL1-Blue MRF′ Kan, and BL21(DE3) were used for plasmid construction, bacterial one- and two-hybrid assays, and protein expression, respectively. All *E. coli* strains that were used for plasmid construction were usually grown in Luria broth

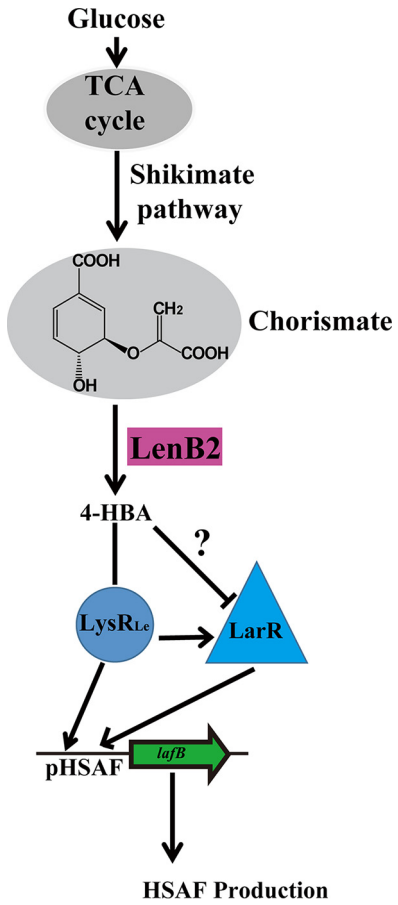

FIG 8 An expanding model for LarR-mediated regulatory pathway of 4-HBA in modulating HSAF biosynthesis in *L. enzymogenes*. LenB2 catalyzes the end product of the shikimate pathway, chorismate, to produce 4-HBA. 4-HBA further employs two different types of TFs to mediate the regulation of 4-HBA in controlling HSAF production. One TF is the reported $LysR_{Le}$, which could bind the HSAF promoter, thus directing HSAF biosynthetic gene (i.e., *lafB*) expression and HSAF production (16). In this process, 4-HBA may partly enhance the binding of $LysR_{Le}$ to the HSAF promoter (16). The other is LarR, presented in this study, which can also bind to the HSAF promoter; however, LarR did not bind 4-HBA. 4-HBA negatively controls *larR* transcription, probably via an uncharacterized factor (indicated by a question mark), while LysR has a positive effect on *larR* transcription by directly binding to its promoter region. LarR failed to bind the promoter of $lysR_{Le}$. Thus, the interplay of 4-HBA with two TFs within its regulatory cascade plays a key role in regulating HSAF biosynthesis in *L. enzymogenes*, which has not been discovered in other 4-HBA-producing bacteria. TCA, tricarboxylic acid.

(LB) at 37°C, supplemented with kanamycin (Km; 25 μg/ml) and gentamicin (Gm; 25 μg/ml) or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 100 μg/ml) as needed for solid and liquid media. *Lysobacter enzymogenes* strains were grown in LB medium or 1/10 tryptic soy broth (TSB) at 28°C. When required, antibiotics were added to the medium to the following final concentrations: kanamycin, 100 μg/ml; Gm, 150 μg/ml.

Promoter activity assay. The promoter region of *lafB*, also called the HSAF promoter (pHSAF), was amplified by PCR and cloned into the promoter-probe plasmid pSS122 (Table 1), which carries a promoterless *uidA* gene that encodes GUS activity (27). This combined construct was transformed into the wild-type OH11 and its derivatives by electroporation. Next, overnight cultures of strains containing constructed reporter plasmids in the HSAF-inducing medium (10% TSB) were centrifuged at 12,000 rpm at 4°C for 3 min, and the cells were collected. Then, cells were resuspended in 600 μl of GUS buffer (50 mM sodium phosphate, pH 7.0, 1 mM EDTA, and 14.3 mM β-mercaptoethanol), and 23 μl of 3% Triton X-100 and sodium lauroyl sarcosinate was added. The mixture was incubated at 30°C for 10 min. Last, 100 μl of 25 mM *p*-nitrophenyl-β-D-glucuronic acid (PNPG) (Sigma, USA) was added. Time for all test sample reactions in this assay is less than 10 min, but the precise time for each sample varied. During the assays, when a yellow pigment developed for each sample, 280 μl of Na_2CO_3 solution (1 M) was added to stop the reaction, and the respective reaction time for each sample was immediately recorded in seconds. The promoter activity was calculated as described previously (27). The biological experiments were performed in triplicate, and each biological replicate was assayed three times to reduce technical error.

Bacterial one-hybrid assay. The bacterial one-hybrid reporter system was shown to efficiently test physical interactions between the transcription factors and the promoter of target genes (28, 29), as

TABLE 2 Primers used in this study

Primer	Sequence (restriction enzyme)[a]	Purpose
Le3904-1F	CCCGGTACCGCGGGCGTGCGGGCGAGGGC (KpnI)	To amplify a 201-bp upstream homologuous arm of *le3904*
Le3904-1R	CCCTCTAGAAGGCGGTGGCGTTGCTGCGG (XbaI)	
Le3904-2F	CCCTCTAGAACTTCCTCGGCGTGTGCGGC (XbaI)	To amplify a 634-bp downstream homologuous arm of *le3904*
Le3904-2R	CCCAAGCTTGGCGATGAAGAAGGCGATGC (HindIII)	
Le4969-1F	CCCGGTACCCGGGCTTGCGTGGAGTGAGG (KpnI)	To amplify a 347-bp upstream homologuous arm of *le4969*
Le4969-1R	CCCTCTAGATGTCGCCCCCCTCGCCCGCT (XbaI)	
Le4969-2F	CCCTCTAGACGGGGGCGGCGGCGAGGATG (XbaI)	To amplify a 544-bp downstream homologuous arm of *le4969*
Le4969-2R	CCCAAGCTTGAGGACCGCCAGATTCACCG (HindIII)	
Le4806-1F	CCCGGTACCAAGGGCGGGCGTGGGGCGGG (KpnI)	To amplify a 220-bp upstream homologuousarm of *le4806* (*larR*)
Le4806-1R	CCCTCTAGAACGAAGCGGGCGAGGGCGAT (XbaI)	
Le4806-2F	CCCTCTAGACGGACAGGAACAGCAGGGCG (XbaI)	To amplify a 399-bp downstream homologue arm of *le4806* (*larR*)
Le4806-2R	CCCAAGCTTACGGACGGGAGGTGGAGGAT (HindIII)	
Le4806-cF	CGGGGTACCAGTTCGATCAGCCCGTCCC (KpnI)	To amplify a 1223-bp DNA fragment containing intact *larR* and its promoter
Le4806-cR	CCCAAGCTTTCAGGGCGAGCGCGCGCCGG (HindIII)	
Le4806-F	CGCCATATGGCCATGTCCCTCAGCCCGCT (NdeI)	To express and purify LarR in *E. coli* BL21
Le4806-R	CCCAAGCTTGGGCGAGCGCGCGCCGGGCG (HindIII)	
RT-larR-F	TCATCTCGTCGATCCAGCTG	To amplify a 232-bp DNA fragment to verify *larR* transcription
RT-larR-R	GACCACTTCGAGACCTACAAG	
RT-lenb2-F	CAGTTGGAAGAAACCCTGGC	To amplify a 193-bp DNA fragment to verify *lenB2* transcription
RT-lenb2-R	CATGCACCAGGATCCGCG	
pTLarR-F	CGGGATCCGCCATGTCCCTCAGCCCGCT (BamHI)	To amplify a 717-bp fragment containing the coding region of *larR*
pTLarR-R	CCGCTCGAGGGGCGAGCGCGCGCCGGGCG (XhoI)	
pTLysR_{Le}-F	CGGGATCCGCTCACGATCTCAACGACAC (BamHI)	To amplify a 1,167-bp fragment containing the coding region of *lysR*$_{Le}$
pTLysR_{Le}-R	CCGCTCGAGCTTATCGTCGTCATCCTTGT (XhoI)	
p-larR-F	CGGAATTCACCGTAGCCGGTCAATAGGTT (EcoRI)	To amplify a 470-bp fragment containing the *larR* promoter region
p-larR-R	GCTCTAGAACCGTAGCCGGTCAATAGGTT (XbaI)	
pBT-LysR_{Le}-F	TTGCGGCCGCAATGGCTCACGATCTCAACGA (NotI)	To amplify a 1,167-bp fragment containing the coding region of *lysR*$_{Le}$
pBT-LysR_{Le}-R	CCGCTCGAGTTACGCCAACGCCGCATC (XhoI)	
16S-F	ACGGTCGCAAGACTGAAACT	qRT-PCR (an internal control)
16S-R	AAGGCACCAATCCATCTCTG	
q-larR-F	CCTGCTGTTCCTGTCCGA	qRT-PCR
q-larR-R	CCTTGTAGGTCTCGAAGTGGT	
p1974-F 1	TGGTGCTGGGCATCGTCG	To amplify a 295-bp DNA fragment containing the *le1974* promoter region
p1974-R	GTCCCGGCCCGCTCCTGCCT	

[a]Restriction sites are underlined.

exemplified by the interaction between LarR and its target DNA (pHSAF) in the present study. As described previously, the bacterial one-hybrid reporter system consisted of three modules. The plasmids pBXcmT and pTRG were separately used for cloning the bait DNA and expressing a target protein. The *E. coli* XL1-Blue MRF′ Kan strain (Table 1) is the host strain used to propagate the recombined pBXcmT and pTRG vectors (28, 29). In the present study, the HSAF promoter region (491 bp) of *L. enzymogenes* OH11 was cloned into pBXcmT, generating the recombinant vector pBXcmT-*lafB* (Table 1); in addition, the coding region of *larR* (717 bp) was cloned into pTRG, creating the final construct pTRG-*larR* (Table 1). The vectors pBXcmT-*lafB* and pTRG-*larR* were cotransformed into the XL1-Blue MRF′ Kan strain. If direct physical binding occurred between *larR* and the HSAF promoter, the positively transformed *E. coli* strain that contained both pBXcmT-*lafB* and pTRG-*larR* was expected to grow well on the selective medium, which is a minimal medium (M9)-based medium that contains 5 mM 3-amino-1,2,4-triazole (3-AT), 8 μg/ml streptomycin (Str), 12.5 μg/ml tetracycline, 34 μg/ml chloramphenicol, and 30 μg/ml kanamycin, as described previously (28, 29). Moreover, a cotransformant containing the vectors pBX-R2031/pTRG-R3133 served as a positive control (28), while the cotransformant containing the empty pTRG and pBXcmT-*lafB* plasmids was used as a negative control in the present study. All of these cotransformants were spotted onto selective medium and grown at 28°C for 3 or 4 days, at which point they were photographed.

Genetic methods. A double-crossover homologous recombination strategy was used to generate an in-frame deletion of the gene of interest (GOI) in *L. enzymogenes*, as described previously (30). In brief, two flanking regions of the GOI were generated by PCR amplification using various corresponding primer pairs (Table 2) and cloned into the respective sites of the suicide vector pEX18Gm (Table 1) (31). The final constructs were transformed into wild-type OH11 or its derivatives by electroporation. Next, *Lysobacter* transformants on the LB plates were selected by adding Km (100 μg/ml) and Gm (150 μg/ml) in the absence of sucrose. Positive colonies were further cultivated on LB plates that contained 10% (wt/vol) sucrose and Km (100 μg/ml) to select for the correct construct that was generated by a second crossover event. The final mutants were confirmed by PCR and sequencing (see Fig. S1A in the supplemental material).

For complementation, a plasmid-borne method was utilized to generate the complemented strains, as described previously (12, 15). In brief, the DNA fragment that contained the full-length GOI and its predicted promoter region was amplified by PCR with different conjugated primer pairs (Table 2) and

cloned into the broad-host-range vector pBBR1-MCS5 (Table 1) (32). The final construct was transformed into competent cells of the GOI mutant by electroporation to generate the corresponding complemented strains; the identity of these strains was confirmed by PCR with the primer pairs that are shown in Table 2.

HSAF extraction and quantification. Extraction and quantification of the antifungal factor HSAF from various *Lysobacter* strains by high-performance liquid chromatography (HPLC) (Agilent 1260; USA) were performed as described previously (7, 8, 15). HSAF was extracted from 25-ml *L. enzymogenes* cultures that were grown in 1/10 TSB for 48 h at 28°C with shaking at 200 rpm. HSAF was detected using HPLC and quantified per unit of optical density at 600 nm (OD_{600}) as described previously (7, 13, 15). Three biological replicates were used, and each was analyzed in three technical replicates.

RT-PCR and q-RT PCR. Reverse transcription-PCR (RT-PCR) was performed as described previously (12, 15, 16). Briefly, the wild-type OH11 *L. enzymogenes* strain and its derivatives were cultivated in 1/10 TSB until the OD_{600} reached 1.0. The cells of each strain were collected by centrifugation (13,000 rpm) at 4°C for 1 min. Total RNA from these cells was extracted using a bacterial RNA kit (catalog no. R6950-01; Omega, China) according to the manufacturer's instructions. To remove genomic DNA, the eluted RNA samples were treated with RNase inhibitors and DNase I (catalog no. E1091; Omega, China). RNA integrity was examined by electrophoresis using 1.2% agarose gels. Then, 2 μg of each RNA sample was chosen for cDNA synthesis using a PrimeScript RT reagent kit with genomic DNA (gDNA) eraser (catalog no. RR047A; TaKaRa, Japan). The subsequent semiquantitative RT-PCR and quantitative RT-PCR (qRT-PCR) assays were performed to amplify the 16S rRNA gene and the GOI with the primer pairs listed in Table 2; the 16S rRNA gene was used in this study as an internal control as described previously (12, 15).

Protein expression and purification. Expression and purification of the target protein were performed as described previously (14, 16). In brief, *larR* was amplified by PCR with the primer pairs listed in Table 2. After enzymatic digestion (NdeI/HindIII), this gene was cloned into a pET-30a vector for protein expression in *E. coli* strain BL21(DE3) (Table 1). The resultant strain was cultivated in LB broth (containing Km at 30 μg/ml) overnight at 37°C. Then, a total of 2 ml of overnight culture was transferred into 300 ml of fresh LB medium that contained 30 μg/ml Km and was then grown at 37°C with shaking at 200 rpm until an OD_{600} of 0.6 was reached. Subsequently, isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) was added to the culture to a final concentration of 1 mM, and the culture was allowed to grow at 18°C for 12 h. Then, the cells were collected by centrifugation (13,000 rpm) at 4°C and resuspended in 25 ml of protein extract buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA) that was supplemented with the protease inhibitor phenylmethylsulfonyl fluoride ([PMSF] 10 mM). The cells were briefly lysed by sonication with a Sonifier 250 (Branson Digital Sonifier, Branson, USA), and the cell lysate was centrifuged at 13,000 rpm at 4°C for 30 min. Soluble protein fractions were collected and mixed with preequilibrated Ni^{2+} resin (GE Healthcare, USA) for 1 h at 4°C; the resin was then placed in a column and extensively washed with binding buffer (50 mM Na_3PO_4, 30 mM NaCl, and 10 mM imidazole). The desired protein was finally eluted in 50 mM Na_3PO_4, 30 mM NaCl, and 250 mM imidazole. Protein purity was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and another purification step in which an Amicon Ultra filter unit (Millipore, USA) was used to remove imidazole as well as to exchange the storage buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA and 5% glycerol). Finally, the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Sangon Biotech, China).

EMSA. An electrophoretic mobility shift assay (EMSA) was carried out as follows. Briefly, a biotin-labeled fragment that contained the HSAF promoter region was amplified by PCR using 5′ biotin-labeled primers (Table 2). The biotin-labeled target DNA and protein extract were incubated for 20 min at room temperature according to the protocols of a LightShift Chemiluminescent EMSA kit (Thermo Scientific, USA). Then, the reaction products were loaded onto a polyacrylamide gel, electrophoresed, transferred to a nylon membrane, and cross-linked. Finally, the biotinylated DNA fragments were treated with a chemiluminescent nucleic acid detection module and detected using a VersaDoc imaging system (Bio-Rad, USA).

Bacterial two-hybrid assay. A BacterioMatch II two-hybrid system was used to determine the potential interaction of two proteins. In detail, the encoding region for each target protein was cloned into pBT (containing a chloramphenicol resistance gene) and pTGR (containing a tetracycline resistance gene). Then, the two constructions were cotransformed into the *E. coli* reporter strain XL1-Blue MRF′ Kan, which is kanamycin resistant. If the bait protein interacts with the target protein, the transcription of the *HIS3* reporter gene will be activated, producing imidazoleglycerol-phosphate dehydratase. As a result, the cotransformed strain could grow in the presence of the compound 3-amino-1,2,4-triazole (3-AT), which is a competitive inhibitor of the product of the *HIS3* gene. A second reporter gene, *aadA*, encoding a protein that confers streptomycin (Str) resistance, provides an additional mechanism to validate the protein-protein interaction. In this experiment, the cotransformed cells were spotted on the selective medium, which is a minimal medium (M9)-based medium containing 5 mM 3-AT, 12.5 μg/ml Str, 12.5 μg/ml tetracycline, 34 μg/ml chloramphenicol, and 30 μg/ml kanamycin. Furthermore, the vectors, pBT-GacS and pTRG-GacS were constructed in this work (Table 1), and the cotransformant containing both vectors served as a positive control because the cytoplasmic domain of GacS from *Pseudomonas aeruginosa* is known to interact with itself (33). The cotransformant containing the empty pTRG and pBT vectors was used as a negative control in this study. All cotransformants were spotted onto the selective medium and grown at 28°C for 3 to 4 days and then photographed. LB agar is a nonselective medium containing 12.5 μg/ml tetracycline, 34 μg/ml chloramphenicol, and 30 μg/ml kanamycin. The purpose of this medium is to ensure that both vectors are successfully transformed into the host *E. coli* XL1-Blue MRF′ Kan.

Microscale thermophoresis assay. The binding affinity between LarR and 4-HBA as well as its analogue was determined by microscale thermophoresis (MST) using a Monolith NT.115 machine (NanoTemper Technologies, Germany) (34). Briefly, purified LarR was fluorescently labeled with NT-647-NHS dye (available from NanoTemper Technologies GmbH, Germany) via amine conjugation. A constant concentration (500 μM) of the labeled target protein (LarR) in standard MST buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, and 0.05% Tween 20) was titrated against increasing concentrations of 4-HBA and its analogue, which were dissolved in methanol and diluted to working concentrations with MST buffer. MST premium-coated capillaries (Monolith NT.115 MO-K005; Germany) were used to load the samples into the MST instrument at 25°C using 40% MST power and 20% LED power. Laser on and off times were set at 30 and 5 s, respectively. All experiments were performed in triplicate. Data analyses were performed using NanoTemper Analysis software, version 1.2.101 (NanoTemper Technologies, Germany).

Data analysis. All analyses were conducted using SPSS, version 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 production, promoter activity, and gene expression levels of the test *L. enzymogenes* strains.

Accession number(s). The sequence data of the present study have been submitted to the NCBI GenBank under accession numbers MG266897 (Le4806/LarR), MG266898 (Le4969), MG266895 (Le3904), MG266896 (Le1974), MG266894 (Le1703/$LysR_{Le}$), and MG266893 (Le1457/LenB2).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01754-17.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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F.L., G.Q., and Z.Q.F. conceived the project and designed experiments. Z.S and S.H carried out experiments. Z.S., S.H., F.L., G.Q., and Z.Q.F. analyzed data. Z.S. and G.Q. wrote the manuscript draft. F.L. and Z.Q.F. revised the manuscript.

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