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MOLECULAR MECHANISM FOR THE BIOSYNTHESIS AND REGULATION OF SECONDARY METABOLITES IN *LYSOBACTER*

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MOLECULAR MECHANISM FOR THE BIOSYNTHESIS AND REGULATION OF SECONDARY METABOLITES IN *LYSOBACTER*

By

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MOLECULAR MECHANISM FOR THE BIOSYNTHESIS AND REGULATION OF SECONDARY METABOLITES IN *LYSOBACTER*

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This thesis presents regulatory and biosynthetic mechanisms by which microorganisms produce secondary metabolites that can potentially be developed into drugs beneficial to humans. The first section shows the role of small signaling molecules in regulating the production of one of the novel antifungal metabolites, heat stable antifungal factor (HSAF), from *Lysobacter enzymogenes*.

In the second part of the thesis I report our attempts to isolate and characterize the biosynthesis of WBP, a new secondary metabolite from *Lysobacter antibioticus* OH13. I have included the *in-silico* analysis of the gene cluster for WBP and the predicted biosynthetic pathway based on analysis of the genes. I have also included the work to delete part of the gene responsible for the biosynthesis of WBP, which is still in progress.

Acknowledgement

I would first like to express my sincerest gratitude towards my advisor Liangcheng Du for giving me this tremendous opportunity to work in his lab. He was my mentor not only in the research I have done but also in all aspects of leadership. I would like to thank his postdoctoral fellow, Shanren Li, for all his advice, motivation, and friendship in almost all the steps of my work. Thanks to all current and former members of Du group for their guidance.

Table of Contents

Molecular Mechanism for the Biosynthesis and Regulation of Secondary Metabolites in *Lysobacter*

Introduction

Natural products from microorganisms and plants have been the best source of structurally profound drugs, particularly as anti-infective and anti-tumor agents (*1, 2*). However, the interest in natural products, especially by the pharmaceutical companies was compromised in the last two decades (*3, 4*). Some of the challenges that lead to this de-emphasis in natural product drug discovery includes: extremely low yields, limited supply, and complex structures posing enormous difficulty for structural modifications (*1, 3, 5*). However, the rapid advance in gene sequencing, gene synthesis, bioinformatics, and metabolomics has driven the natural product drug discovery process to a new era, by transforming the process from the tedious isolation, screening process to *in silico*-based bio-mining approaches that seek to eventually transform genomic information directly into biosynthetic output (*1, 5-7*). Therefore, so many new microorganisms are under investigation through these new approaches for the discovery of new bioactive compounds (*8-10*). *Lysobacter* is a new example, which is a genus of Gram-negative bacteria emerging as a new source of novel secondary metabolites (*11-15*).

The genus *Lysobacter* belongs to the family *Xanthomonadaceae* and consists of around 25 reported species, where *L. antibioticus, L. brunescens, L. enzymogenes* and *L. gummosus* are the first to be isolated from soil (*16, 17*). These gliding bacterial predators are known for their lytic activity against diverse organisms: bacteria, fungi, and worms. They produce several lytic

enzymes like proteases, glucanases, lipases, chitinases, as well as secondary metabolites (*16, 18-21, 27*). We have been studying the metabolites from *Lysobacter*, especially from *L. enzymogenes* for their potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (*22, 23*). WAP-8294A, lysobactin, and tripropeptins are some of the main antibacterial metabolites so far isolated and under extensive research in our group (*11, 13, 22, 24*). Another group of metabolites isolated from *L. enzymogenes* is dihydromaltophilin (HSAF) and analogs, which have potent activities against a wide range of fungi with a novel mode of action (*13, 15, 25, 26*). The compounds belong to the polycyclic tetramate macrolactams (PTM), which are emerging as a new class of secondary metabolites with distinct structures and a new mode of action (*15, 28*). The majority of these secondary metabolites are biosynthesized by nonribosomal peptide synthetase (NRPS) or/and polyketide synthetase (PKS) (*13, 15, 28*). However, only a few metabolites and their corresponding genes are known from *Lysobacter* species other than from the *L. enzymogenes* (*29*). To explore new secondary metabolites from *Lysobacter* species we obtained and analyzed the genome of *L. antibioticus* OH13 using antiSMASH for clustered natural product biosynthetic genes (*30, 31*). Thirteen hits of gene clusters for the secondary metabolite are identified including the phenazine cluster (*32*). Six gene clusters among the thirteen hits contain NRPS genes. Here in the Section II of this thesis we report the identification and characterization of one (WBP) of the NRPS gene clusters from *L. antibioticus* OH13. The WBP cluster appears partly similar to the WAP gene cluster but with one less NRPS module than the WAP cluster (*11*).

One of the main problems in developing natural products into potential drugs, as mentioned above, is their low production yield (*5*). For instance, the yield of HSAF is still very low in *L. enzymogenes* strains OH11 and C3, although this compound has been extensively studied (*15, 33*). One of the solutions to this problem is to thoroughly investigate the regulatory mechanism of HSAF production in this bacterium, which could lead to new approaches to facilitating its production through rational genetic and molecular engineering strategies. Several recent studies have shown that the antibiotic production in *L. enzymogenes* is a nutrient-dependent trait and is regulated by endogenous and/or xenogenous small molecules (*34-36*). However, the details of the signaling pathways, in particular how the small molecule signals are perceived by the *Lysobacter* cells, remain largely unknown. We recently identified a group of DSFs (diffusible signaling factors), *Le*DSF1-5, from *L. enzymogenes* strain OH11 and found that *Le*DSF3 regulates the biosynthesis of HSAF. The *Le*DSF3 signaling is mediated by a two-component regulatory system (TCS), RpfC/RpfG, which is responsible for sensing the DSF and for conveying the signal to the subsequent gene expression and HSAF production (*36*). RpfC is a membrane-bound sensor protein with histidine kinase activity (*37-39*). When the cell density increases and the DSF concentration reaches a threshold, the DSF-bound RpfC undergoes an autophosphorylation at its active site histidine residue. The autophosphorylation activates RpfC, which subsequently phosphorylates its partner protein RpfG, the intracellular response regulator of the TCS, resulting in the activation of the cyclic di-GMP phosphodiesterase activity of RpfG (*40-42*). The downstream signal transduction pathway is still unclear in *L. enzymogenes*, but

evidence suggested that the activated RpfG could then hydrolyze cyclic di-GMP, which is a second messenger involved in numerous cellular processes (*35, 36*). The decrease in c-di-GMP concentration in the cells could be sensed by downstream regulators, such as the global regulator Clp, a cAMP receptor-like protein (*43, 44*). Previous studies and our recent results showed that Clp controls antibiotic biosynthesis and lytic enzyme production in *L. enzymogenes* (*35, 44*). Thus, the DSF-regulated HSAF biosynthesis in *L. enzymogenes* is likely mediated by the RpfC/RpfG/Clp pathway. Furthermore, we also discovered another key regulator, DF (diffusible factor) that can help activate the transcription of the *hsaf pks/nrps* operon, resulting in increasing HSAF level (*34-36*). The DSF and DF systems work independently to carry out a positive regulation on the HSAF biosynthesis in *L. enzymogenes* (*34*). Unlike the DSF-family signal, where their regulatory pathway is through the two-component signaling transduction pathway (RpfC/RpfG) and regulator Clp (*34-36*), the DF structure and its mode of action for regulating HSAF biosynthesis remains poorly understood in *L. enzymogenes* to date. In Section I of this thesis, we showed that *L. enzymogenes* produced 3-HBA and 4-HBA via LenB2 under both *in vitro* and *in vivo* conditions. Importantly, we presented several lines of evidence to show that 4-HBA, and 3-HBA, served as a diffusible factor or a potential diffusible signaling molecule to modulate the antibiotic HSAF biosynthesis in *L. enzymogenes*.

SECTION I

3-Hydroxybenzoic Acid and 4-Hydroxybenzoic Acid are the Diffusible Factors Regulating the Heat-Stable-Antifungal-Factor Biosynthesis in *Lysobacter enzymogenes*

Abstract

Lysobacter enzymogenes, a Gram-negative microorganism, is a source of potentially novel bioactive secondary metabolites such as HSAF, WAP-8294A, and aryl polyene pigments. Extensive research has been carried out to study the mechanism of biosynthesis of these metabolites. However, the molecular regulations of these natural products are not well understood so far. In the present study, we provided the first report that 3-hydroxybenzoic acid (3-HBA) and 4-hydroxybenzoic acid (4-HBA) serve as diffusible factors capable of regulating HSAF biosynthesis in *L. enzymogenes*. We found *L. enzymogenes* utilized LenB2 as a bi-functional chorismatase to convert chorismate to 4-HBA and 3-HBA. Mutation of *lenB2* almost completely abolished the production of 4-HBA and 3-HBA, leading to a complete stop of HSAF production, whereas overexpression of *lenB*2 increased the production of HSAF. The results show that 3-HBA and 4-HBA are diffusible signaling molecules modulating the HSAF biosynthesis in *L. enzymogenes*. This finding expands our understanding of the biological roles played by the widely distributed benzoic acid derivatives in bacteria.

1.1. Background and Significance

Lysobacter species are gram-negative bacteria having lytic activity against a wide range of microorganisms including fungi, Gram-positive and Gram-negative bacteria, and nematodes (*29*). This activity is attributed to the production of several extracellular enzymes, secondary metabolites, and other unknown bioactive compounds (*29, 45*). Some of the many secondary metabolites identified so far from *L. enzymogenes* are WAP-8294A and HSAF, which have strong activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) and many fungal species, respectively (Figure 2) (*11, 15*). Several studies have been carried out so far to elucidate the mechanism of biosynthesis and activity of these two potential lead anti-infective compounds. WAP-8294A is a group of cyclic lipodepsidpeptides that are biosynthesized by two large NRPS in *L. enzymogenes* OH11 (*Le*OH11) (*11*); it has potent activity against MRSA strains (*11*). HSAF is a polycyclic tetramate macrolactam (PTM) with unusual chemical structure and novel mode of action against many pathogenic fungal species (*28, 33*). It is biosynthesized by a PKS/NRPS hybrid megaenzyme (*28*). Although many studies have been carried out to study the mechanism of biosynthesis of these two groups of compounds, few attempts are made to investigate the regulation of the production of HSAF and WAP-8294A in *Le*OH11 (*36*). We recently discovered several key systems/regulators, such as DSF (diffusible signal factor) and DF (diffusible factor) that are involved in the transcriptional activation of the *hsaf pks/nrps* operon, resulting in an increase of HSAF production (*34-36*). These two systems worked independently to carry out a

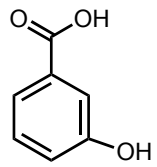
positive regulation on the HSAF biosynthesis in *L. enzymogenes* (*34*). Specifically, a DSF-family signal, referred to as *Le*DSF3 (13-methyltetradecanoic acid), was identified to control HSAF biosynthesis by a two-component signaling transduction pathway (RpfC/RpfG) and the global regulator Clp (*34-36*). In contrast, the structure of DF in *L. enzymogenes* has not been determined and its regulatory mechanism for HSAF biosynthesis remains poorly understood to date.

History of the DF signaling in bacteria can be traced back to 1997. It was initially discovered in the economically important phytopathogenic bacterium, *Xanthomonas campestris* pv. *campestris* (*Xcc*), which causes serious disease of black rot in crucifers (*46*). A novel, but uncharacterized secreted compound controlling the biosynthesis of the polyene yellow pigments (also called xanthomonadins) was found in this bacterium (*46*). The structure of DF was further identified as 3-HBA by He and colleagues (*47*). In addition, they showed that *Xcc* also produced 4-HBA in their later studies (*48*). Interestingly, the biosynthesis of both 3-HBA and 4-HBA in *Xcc* was dependent on an enzyme XanB2, a pteridine-dependent dioxygenase-like protein (Figure 1) (*47, 48*). Further evidence showed that XanB2 is a bi-functional chorismatase that converted chorismate, the end product of the shikimate pathway, to 3-HBA and 4-HBA in *Xcc* (*48*). Malfunction of XanB2 almost completely abolished production of 3-HBA and 4-HBA (*47, 48*). Moreover, XanB2 homologs are found to be widespread in different bacterial species under

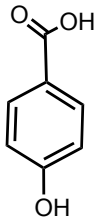
various ecological niches (*47*), suggesting the potential importance of this group of small molecules in the bacterial kingdom.

Although both 3-HBA and 4-HBA were produced in *Xcc* by the same shikimate pathway, the functions played by each molecule was, however, remarkably different. Abundant evidences have shown that 3-HBA played a vital role in modulating xanthomonadins biosynthesis, while 4-HBA was mainly involved in ubiquinone (CoQ8) coenzyme biosynthesis in *Xcc* (*47, 48*). But it is important to note that 4-HBA was found to be a precursor for CoQ8 biosynthesis, not as a signaling molecule (*48*). Furthermore, *X. oryzae pv. oryzae* (*Xoo*, the bacterial pathogen causing rice blight) was also found to produce 3-HBA and 4-HBA via the XanB2 homologue (PXO_3739) for the biosynthesis of xanthomonadin, CoQ8, and exopolysaccharide (*49*). Thus, despite the many studies in *Xanthomonas*, all available reports to date did not clearly address whether 4-HBA can function as a signal molecule (DF).

Figure 1. Structure of DF (3-Hydroxybenzoic acid and 4-Hydroxybenzoic acid)



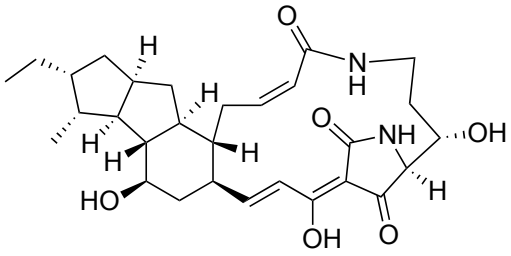
Chemical Formula: $C_7H_6O_3$
Molecular Weight: 138.12
3-Hydroxybenzoic acid



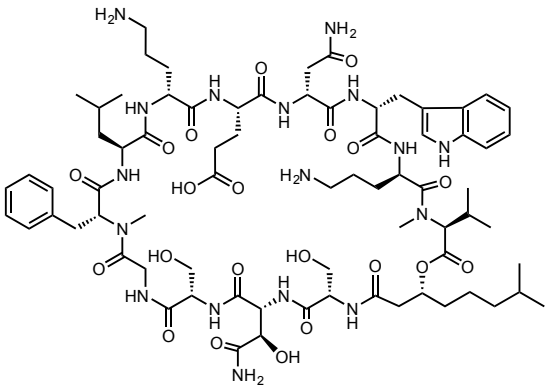
Chemical Formula: $C_7H_6O_3$
Molecular Weight: 138.12
4-Hydroxybenzoic acid

In the present study, we showed that *L. enzymogenes* also produced 3-HBA and 4-HBA via LenB2 under both *in vitro* and *in vivo* conditions. Importantly, we presented several lines of evidence to show that 4-HBA and 3-HBA served as signaling molecules to modulate the antibiotic HSAF biosynthesis in *L. enzymogenes*. To our knowledge, this is the first example to clearly demonstrate that 3-HBA and 4-HBA can serve as signaling molecules for antibiotic biosynthesis in bacteria. These findings deepen our current view on biological functions of 3-HBA and 4-HBA in bacterial physiology.

Figure 2. Structure of anti-infective agents produced by *Lysobacter*: HSAF and WAP-829A2



HSAF (antifungal)



WAP-8294A2 (antibacterial)

1.2. Materials and Methods

1.2.1. Bacterial strains, plasmids and growth conditions

Escherichia coli strains used for plasmid construction were routinely grown in LB (Luria Broth) at 37 °C, supplemented with gentamicin (Gm, 25μg/ml) as needed for solid and liquid media. *Lysobacter enzymogenes* stains were grown in LB medium or 1/10 TSB at 28 °C. When required, antibiotics were added into the medium according to the following final concentrations: kanamycin (Km), 100 μg/ml; and Gm, 150 μg/ml.

1.2.2. Isolation, purification, and structural identification of 3-HBA and 4-HBA in *L. enzymogenes*

The wild-type OH11 of *L. enzymogenes* was grown in 1/10 TSB (100 ml, 3000 shake flask, total 300 liters) at 28 °C with shaking at 200 rpm for 36-48 hours. The culture was acidified by HCl to a pH value of 2.0, followed by extraction with the same volume of ethyl acetate (EtOAc) until the filtrate was colorless. The combined filtrate, upon evaporation, yielded a crude extract, which was further partitioned between methanol and petroleum ether. The methanol layer was concentrated under vacuum to obtain a yellow syrupy material (7.4 g). The extract was then subjected to column chromatography (60 g silica gel 60 Merck, Darmstadt, Germany, column; chloroform-methanol, gradient elute; 250 ml for each gradient) and Sephadex LH-20 column chromatography (GE healthcare, Uppsala, Sweden) column were used. TLC analyses were performed with pre-coated silica gel 60 F254 plates (Merck, Darmstadt, Germany). NMR spectra were recorded on a Bruker Advance 400 spectrometer at 400/100 MHz (Bruker, Fällanden, Switzerland). Mass spectra were obtained on a LCQ mass spectrometer (Thermo, West Palm Beach, FL, USA). An Agilent 1120 HPLC system (Agilent, Santa Clara, CA, USA), with RF C18 columns (10.0 × 250 mm, 5 μm, for preparative HPLC; 4.6 × 150 mm, 3.5 μm, for analytic HPLC), was used in the studies. The HPLC program is described in (Table S2). All general chemical reagents were purchased from Sigma-Aldrich or Fisher Scientific.

1.2.3. Gene in-frame deletion in *L. enzymogenes*

A double cross-over homologue recombination strategy was used to generate an in-frame deletion of GOI (Genes of Interest) in *L. enzymogenes*, as described previously (*50*). In brief, two flanking regions of GOI were generated by PCR (polymerase chain reaction) amplification using various corresponding primer pairs (Table S1), and cloned into respective sites of the suicide-vector pEX18Gm. The final constructs were transformed into conjugal strain *E. coli* to conjugate with the wild type *L. enzymogenes* OH11 (*34, 50*). 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 the LB plates containing 10% (w/v) sucrose and Km (100 μg/mL) to select for correct construct by a second cross-over event. The resultant mutants were confirmed by PCR and sequencing (Table S1).

1.2.4. Construction of *lenb2* overexpression mutant

To construct *lenb2* overexpression strain, In-fusion cloning system (In-Fusion® HD Cloning Plus catalogue # 638909, Clontech Laboratories, Inc. A Takara Bio Company, www.clontech.com) was implemented. This method of cloning is ligase independent cloning of PCR product, where the reaction depends upon the 3' to 5' proofreading exonulease function of the polymerase developed from Vaccina virus DNA polymerase (*51*). It works by fusing DNA fragment and linearized vectors by recognizing a 15 bp overlap at their ends (*52*). The vector

pHmgA was linearized with *Pst*I/*Bam*HI restriction enzymes and the coding region of *lenb2* was amplified by designing forward and reverse primers having a 15 bp overlap, homologous to the site of the vector (Figure 3). The reaction mixture, which contained the enzyme, the linearized vector, and the purified PCR product of the *lenb2* gene, was incubated for 15 minutes in 50^{o}C. The vector, pHmgA, included one homologous region of *hmgA* gene, HSAF promoter (P_{HSAF}), and the plasmid containing the *lenb*2 gene is therefore expected to integrate into the *hmgA* gene in OH11 genome by homologous recombination (*17*). The *hmgA* gene encodes a homogentisate 1,2 dioxygenase, which catalyzes the oxidative cleavage of the aromatic ring of tyrosine/phenylalanine, a key step in aromatic amino acid degradation pathway. The disruption of *hmgA* gene blocks the oxidative cleavage reaction and leads to the accumulation of homgentisate, exhibiting black color in the mutant organisms and allowing for easy selection of the single crossover mutant into whose genomes the construct has been integrated. In the construct the coding region of *lenb2* was placed downstream from the strong promoter P_{HSAF}. The constructs were validated and transferred into *Le*OH11. The black colored colonies were selected and verified by diagnostic PCR (Figure 4)

Figure 3. Construction of pHmgA-P_{HSAF}-based vectors for overexpressing *lenb2* in *Lysobacter enzymogenes*

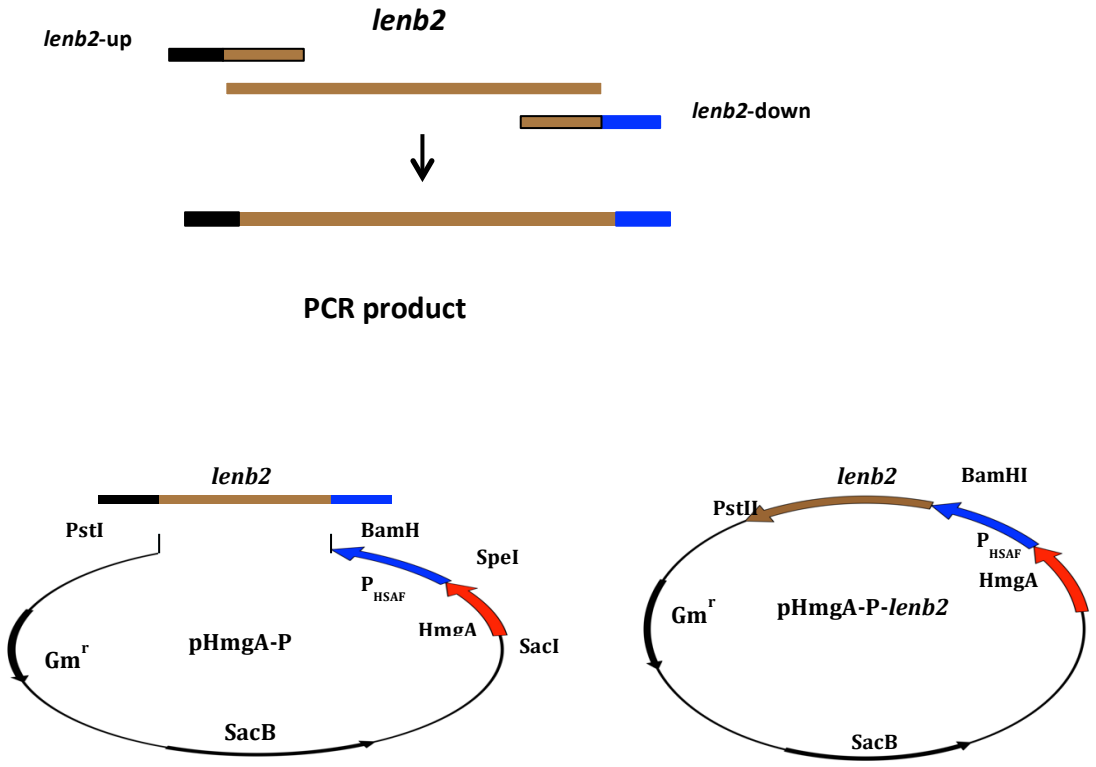
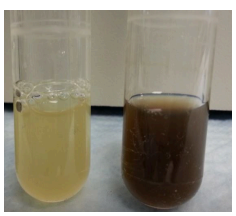
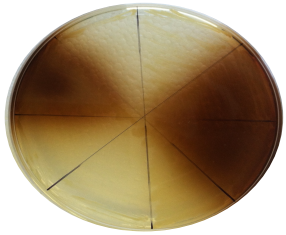


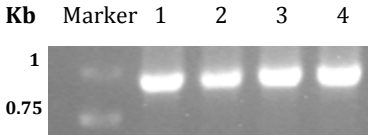
Figure 4. Genotypic and phenotypic confirmation of the mutant

Yellow: wild type *Le*OH11
Black: *hmgA* gene disruption mutant





Diagnostic PCR to amplify the expected 902 bp in the *lenb2* and HmgA region of the vector



1.2.5. Effect of 3-HBA and 4-HBA on HSAF production

3-HBA and 4-HBA (final concentration 0.2-10 μM) were added into 50 ml 1/10 TSB culture of various strains of *L. enzymogenes*. The cultures grew at 28 °C, 200 rpm for 2 days, and were extracted with the same volume of ethyl acetate. The organic phase was concentrated under vacuum, and the crude extract was dissolved with 0.5 ml methanol. A fraction (20 μl) of the methanol extract was injected in HPLC to analyze HSAF and analogs. For semi-quantification, the peak area of HSAF and analogs was measured to obtain the relative yield of the compounds.

1.2.6. RNA extraction, reverse-transcription PCR and real time-PCR

*Le*OH11 and its mutants were grown in 100 ml 1/10 strength TSB medium for 24 h. An aliquot of 3 ml cells was transferred to a sterile centrifuge tube and centrifuged for 3 min at 12,000 rpm. RNA was extracted from the strains using TRIZOL solution following the manufacturer's

instruction. For DNA removing and reverse transcription PCR, PrimerScript RT reagent Kit with gDNA Eraser Kit (TaKaRa biocompany) was used in this study. For real time-PCR assay, iQ SYBR green supermix kit (BIO-RAD company) was used. The primers for real-time PCR are listed in (Table S1), and 16S RNA was used as the reference (*36*).

1.3. Results and Discussion

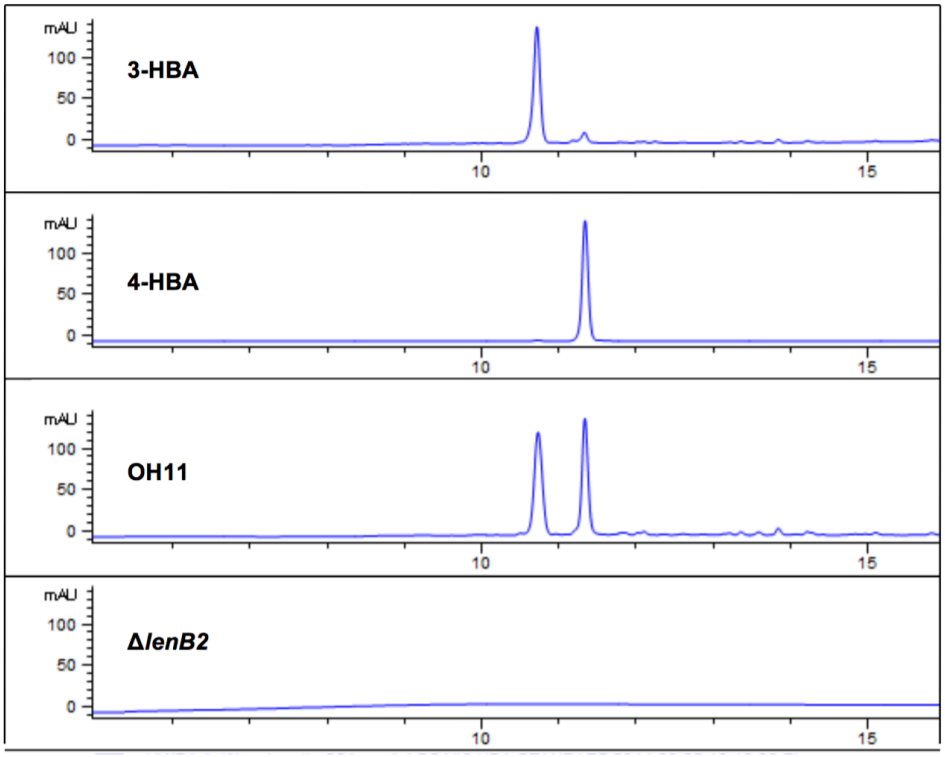
1.3.1. *L. enzymogenes* produced both 3-HBA and 4-HBA via LenB2

To explore whether *L. enzymogenes* can produce 3-HBA and 4-HBA, we cultivated *L. enzymogenes* OH11 in the HSAF-inducing medium (1/10 TSB), and collected cell-free culture after growth for 2 days. These cell-free cultures were extracted by EtOAc and concentrated, followed by HPLC separation to reveal two peaks that exhibited similar retention time of 4-HBA and 3-HBA as described in the previous *Xcc* study (*48*). These two peaks were further collected and their structures identified by NMR analyses. As shown in (Figure S1), both compounds gave distinct ^{1}H and ^{13}C chemical shifts similar to the 4-HBA and 3-HBA standards. The data demonstrate that *L. enzymogenes* produces 3-HBA and 4-HBA in the HSAF-producing medium.

Next, we investigated how 3-HBA and 4-HBA are synthesized in *L. enzymogenes*. Given that XanB2 from *Xcc* was shown to be a bi-functional enzyme requiring for both 3-HBA and 4-HBA production (*48*), we speculated that the XanB2 homologue LenB2 might play a similar function in *L. enzymogenes*. As shown in (Figure 5), we found that mutation of *lenB2* almost completely

impaired 3-HBA and 4-HBA production as identified by HPLC. These data suggest that LenB2 was responsible for the *in vivo* production of 3-HBA and 4-HBA in *L. enzymogenes*.

Figure 5. HPLC analysis of 3-HBA and 4-HBA in *L*eOH11 and *LenB2* mutant



1.3.2. Exogenous addition of 3-HBA and 4-HBA increases HSAF production

We tested the effect of 3-HBA and 4-HBA on the production of HASF and analogs. When 0.2 μM 3-HBA or 4-HBA was added into the cultures, the production of HSAF analogs increased in all treatments (Figure 6-7). 3-HBA exhibited a stronger effect (6.1 fold) than 4-HBA (0.9 fold). When the concentration of 4-HBA and 3-HBA increased to 1.0 μM, their effect on HSAF production became similar, with 6.3 fold increase by 3-HBA and 6.0 fold increase by 4-HBA.

Next, we evaluated the expression level of HSAF *pks-nrps*, the key gene for HSAF biosynthesis using Q-RT-PCR (Figure 8). In the wild type, the exogenous addition of 4-HBA (0.2 μM) increased *pks-nrps* expression by 2.2 fold, whereas 3-HBA (0.2 μM) increased *pks-nrps* expression by 4.6 fold (Figure 8). The results are in general agreement with the observed HSAF increase when HBA were exogenously added to the wild type culture (Figure 6-7). In addition, we evaluated the expression level of HSAF *ox4*, a tailoring gene for HSAF biosynthesis using Q-RT-PCR (Figure 9) (*28*). The exogenous addition of 4-HBA (0.2 μM) increased *ox4* expression by 2.1 fold, and 3-HBA (0.2 μM) increased the *ox4* expression by 4.7 fold (Figure 9) (*28*).

Figure 6. The effect of 3-HBA on HSAF yield. A *Le*OH11 wild type; B through E. *Le*OH11 wild type treated with 0.2, 1.0, 5.0, and 10 μM 3-HBA, respectively (from 48hrs culture extracts For the identity of the compounds, HSAF (1), alteramide A (2), 3-deOH-HSAF (3), and 3-deOH-altermide A (4)

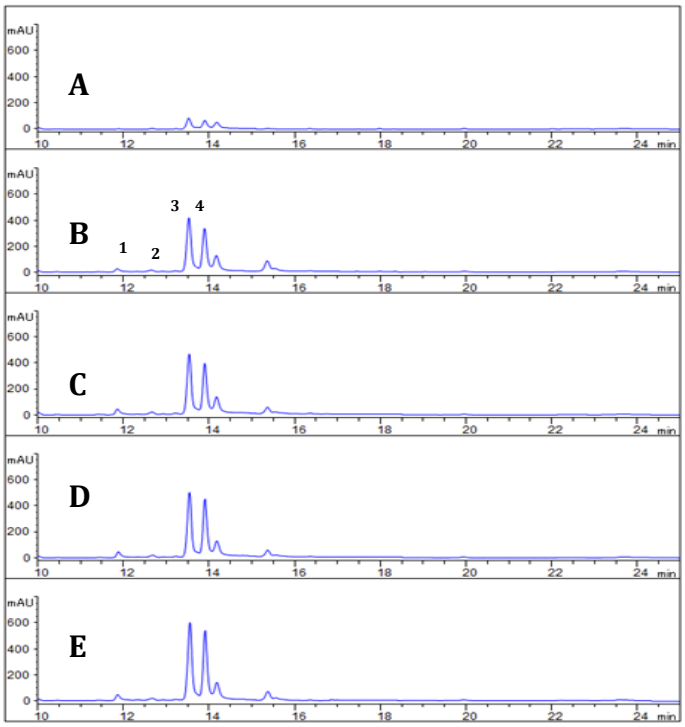


Figure 7. The effect of 4-HBA on HSAF yield. A. *Le*OH11 wild type; B through E. *Le*OH11 wild type treated with 0.2, 1.0, 5.0, and 10 µM 4-HBA, respectively. (The sample was taken from 48 hrs culture extracts). For the identity of the compounds, HSAF (1), alteramide A (2), 3-deOH-HSAF (3), and 3-deOH-altermide A (4)

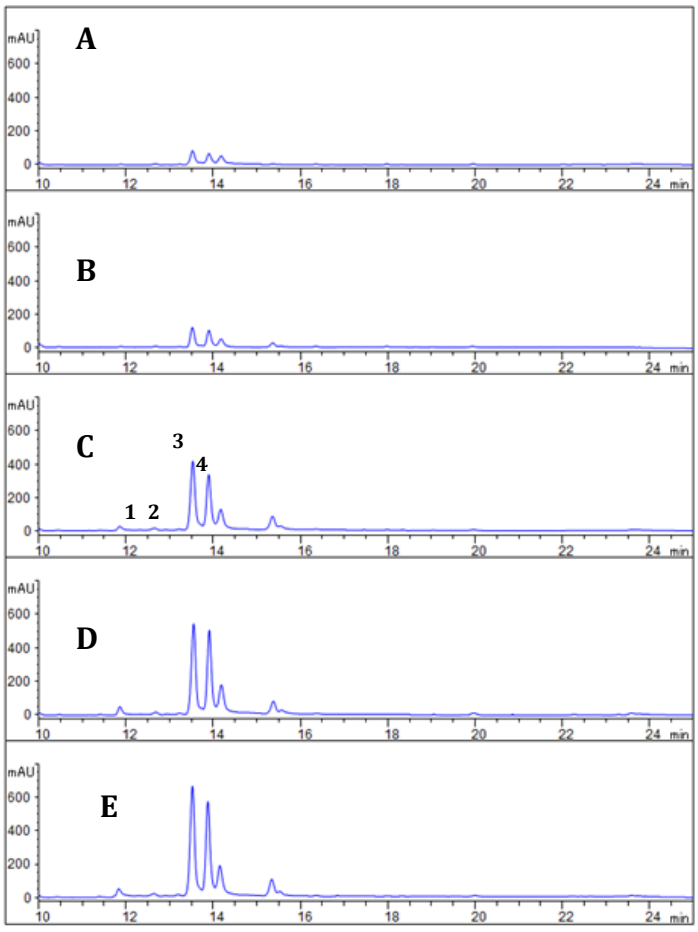


Figure 8. The effect of 3-HBA and 4-HBA on HSAF *pks-nrps* expression. A. *Le*OH11 wild type; B. *Le*OH11 wild type treated with 0.2 μM 4-HBA; C. *Le*OH11 wild type treated with 0.2 μM 3-HBA. (The data is generated from 3 replicates).

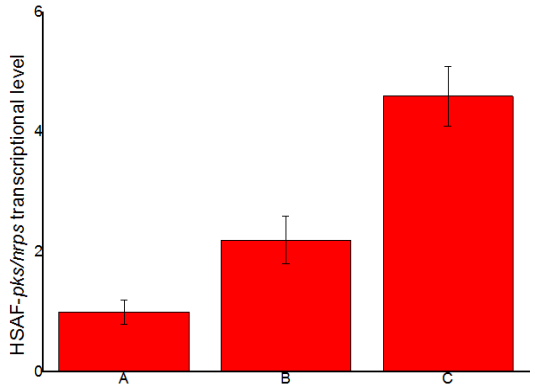
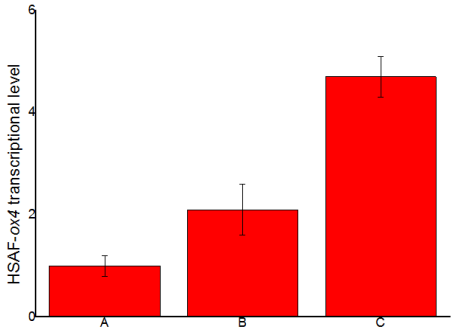


Figure 9. The effect of 3-HBA and 4-HBA on HSAF *ox4* expression. A. *Le*OH11 wild type; B. *Le*OH11 wild type treated with 0.2 μM 4-HBA; C. *Le*OH11 wild type treated with 0.2 μM 3-HBA. (The data is generated from 3 replicates).



1.3.3. LenB2 impacts the expression of HSAF biosynthetic genes

We compared the production and the expression level of HSAF in the strains: wild type *Le*OH1, overexpressed *lenb2* gene (*Le*OH11-pHmgA-P_{HSAF}-lenb2) and *lenb2* mutant strains (*55*). HPLC analysis of the metabolite extracts showed that the production of HSAF analogs increased by 5.6 fold in the overexpressed *lenb2* strain (Figure 10). This result coincides with the increased yield of HSAF upon exogenous addition of DF. Next, we evaluated the expression level of biosynthetic genes (*hsaf-nrps/pks* and *ox-4*) through real time PCR. As shown in (Figure 11-12), the expression level of both *hsaf-nrps/pks* and *ox-4* increased by about 1.75 fold in the overexpressed *lenb2* strains relative to the wild type *Le*OH11. The expression level of both HSAF genes decreased by about 0.5 fold in the Δ*lenb2* strains, compared to the wild type *Le*OH11. This result coincides with the exogenous addition of DF in *Le*OH culture medium.

Figure 10. HSAF yield in *Le*OH11 wild type (A), *lenb2* overexpressed strain (B), and Δ*lenb2* (C). (The sample was taken from 36hrs culture extracts). For the identity of the compounds, HSAF (1), alteramide A (2), 3-deOH-HSAF (3), and 3-deOH-altermide A (4)

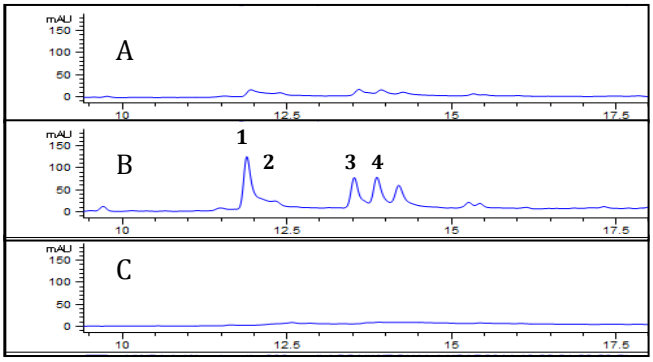


Figure 11. HSAF-*pks/nrps* expression in *Le*OH11 wild type (A), overexpressed *lenb2* (B), Δ*lenb2* (C). (The data is generated from 3 replicates).

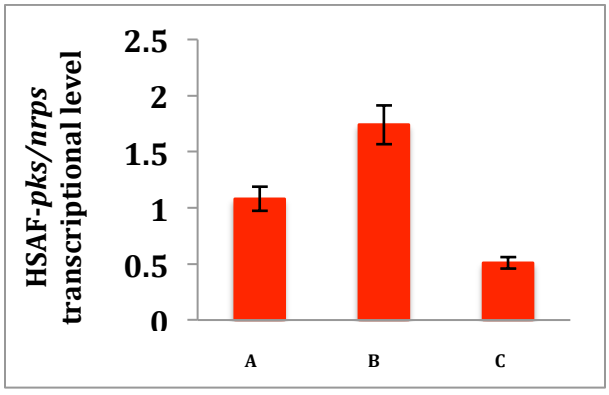
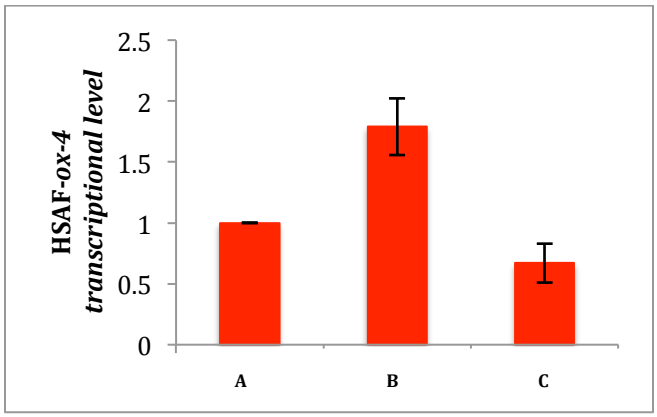


Figure 12. HSAF-*ox-4* expression in A. *Le*OH11 wild type; B. overexpressed *lenb2*; C. Δ*lenb2*. (The data is generated from 3 replicates).



1.3.4. Summary

3-HBA and 4-HBA have been predicted to be present in a variety of bacterial species (*48, 56*). However, their relations to antibiotic biosynthesis in bacterial biocontrol agents have never been demonstrated. In the present study, we provided the first example to show that 3-HBA and 4-HBA function as diffusible factors capable of regulating antibiotic HSAF production in *L. enzymogenes*.

In summary, this work demonstrates that 3-HBA and 4-HBA are signaling molecules that regulate the antifungal HSAF biosynthesis in *L. enzymogenes*. The role of these diffusible factors in antibiotic regulation has never been described in any DF producing bacterium. This finding widens our current view on the regulatory mode of DF in bacteria. Furthermore, it also facilitates the generation of high yield HSAF producing strains via modification of the 4-HBA signaling pathway in *L. enzymogenes*. Finally, given that DFs are widely produced by a variety of bacterial species, this study may trigger more studies on the function of DF in many other antibiotics.

1.4. Supporting Information

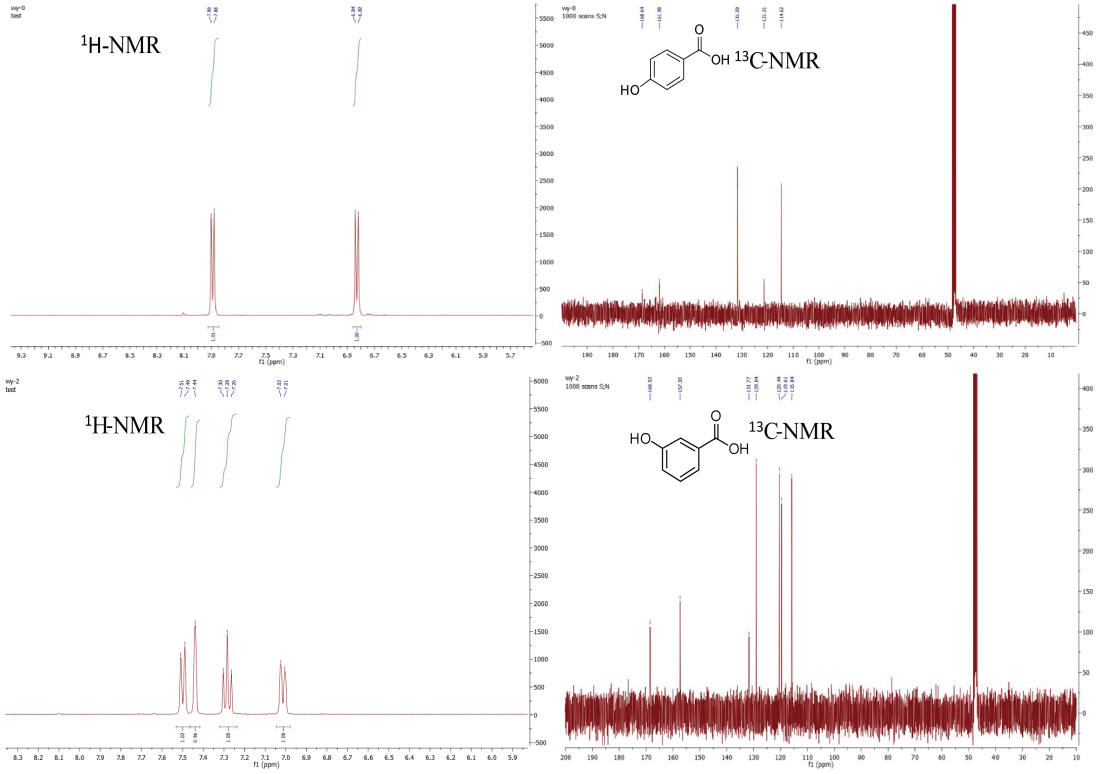
Table S1. Primers used in this study

Primer	Sequence
LenB2-F	CGCCATATGAGCGCGGCCGCCGCGACCGT (*NdeI*)
LenB2-R	CCCAAGCTTGCCGTGCACTCCGTCGATCT(*Hin*dIII)
RT-*lenB2*-F	CAGTTGGAAGAAACCCTGGC
RT-*lenB2*-R	CATGCACCAGGATCCGCG
16s-forw-realtime PCR	ACTTCGTGCCAGCAGCCG
16s-revs-realtime PCR	CCATTCCCAGGTTGAGCCC
HSAF-nrps-forw-realtime PCR	GCAGATTCCGCCGCACAT
HSAF-nrps-revs-realtime PCR	CGAAGCCGAACGAGTTGACC
HSAF-ox4-forw-realtime PCR	CGACGACGCCGACAAGATG
HSAF-ox4-revs-realtime PCR	TCGCCCATTGCCAGCACA
In-fusion-forw-lenB2	GAAAAAGAAGGATCATGAGCGCGGCCGCCGCG
In-fusion-revs lenB2	TTGATATCGAATTCCTGCAGTCAGCCGTGCACTCGGTC

Table S2. The gradient elution program for HPLC analysis (Mobile Phase A: acetonitrile contain 0.1% formic acid; Mobile Phase B: water containing 0.1% formic acid; flow rate: 1 mL/min; detect wavelength: 280 nm)

Time (min)	MP A (%)	MP B (%)
0	40	60
4	40	60
17	70	30
20	70	30
25	100	0
28	100	0
29	40	60

Figure S1. NMR spectra of 3-HBA and 4-HBA produced in *L. enzymogenes*.



Section II

Identification and Characterization of WBP Biosynthetic Gene Cluster from *Lysobacter antibioticus* OH13

Abstract

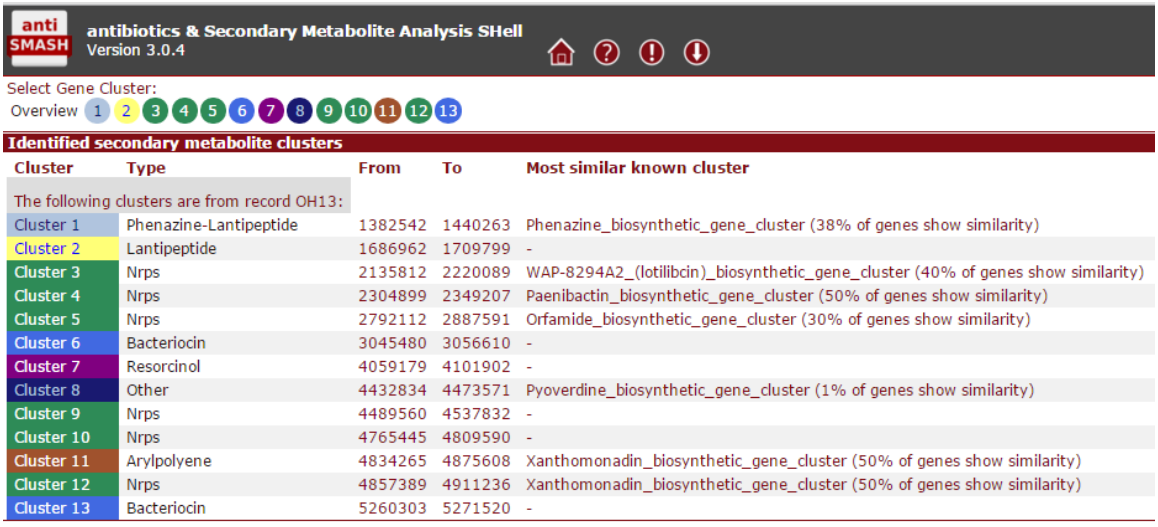
Lysobacter antibioticus OH13 (*La*OH13) is a Gram-negative bacterium known to produce several bioactive compounds. We analyzed the genome sequence of *La*OH13 using antiSMASH and found at least 13 gene clusters putatively responsible for the biosynthesis of natural products. Among the 13 clusters, six gene clusters contain nonribosomal peptide synthetases (NRPS) genes. One (designated WBP) of the gene clusters is similar to the WAP cluster that is responsible for the biosynthesis of WAP-8294A, a group of potent anti-MRSA antibiotics in *Lysobacter enzymogenes* OH11. The WBP cluster contains two huge open reading frames, together encoding 11 modules of NRPS, which is one module less than the WAP cluster. In this research, we set out to isolate the putative WBP metabolites from *La*OH13 through constructing a WBP mutant by deleting one of the NRPSs, WBPS1. The mutation abolished the production of several metabolites that were produced by the wild type. The isolation and structural determination of these compounds are currently undergoing.

2.1. Background and Significance

Lysobacter antibioticus OH13 is a ubiquitous environmental bacterium that belongs to the *Lysobacter* genus within the *Xanthomonadaceae* family (*16, 56*). The genus is emerging as a novel biocontrol agent against pathogens of crop plants including *Bipolaris sorokiniana, Uromyces appendiculatus,* and *Rhizoctonia solani* (*57-59*). We have been studying *Lysobacter* species as a new source of bioactive natural products (*11, 15, 17, 25, 26, 28, 54, 60*). We recently identified the biosynthetic genes for WAP-8294A, a group of cyclic lipodepsipeptides isolated from *Lysobacter enzymogenes*, with very potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (*11*). Cyclic depsipeptides are a large and diverse family of naturally occurring secondary metabolites with potent antibacterial activity (60, *61*). Most of the compounds are isolated from soil-borne or plant-associated bacteria (*62*). Cyclic lipodepsipeptides are composed of a lipid tail linked to a short oligopeptide which is cyclized to form a lactone or lactam ring either between two amino acids in the peptide chain or between an amino acid and amino- or hydroxyl-group bearing fatty acid moiety (*63*). The peptides are biosynthesized by the multi-functional enzymes, non-ribosomal peptide synthetase (NRPS) (*64,65*). Daptomycin is the leading antibiotic of this group already in the market for the treatment of systemic and life-threatening infections caused by Gram-positive bacteria such as MRSA and vancomycin resistant Staphylococcus aureus (VRSA) and enterococci (VSE) (*65-68*).

In exploring for new antibiotic compounds in *La*OH13 we obtained and analyzed the genome of *La*OH13 and found thirteen gene clusters (Figure 13). Among the 13 gene clusters, we found one cluster is likely to encode for NRPS that are similar to the WAP-829A NRPS. We predicted that the NRPS are to synthesize new cyclic lipodepsipeptides, WBP. The goal of this project is to identify the natural products and to characterize the putative WBP biosynthetic gene cluster from *La*OH13.

Figure 13. Putative natural product biosynthetic gene clusters identified from the genome of *Lysobacter antibioticus* OH13 using antiSMASH



Identified secondary metabolite clusters

Cluster	Type	From	To	Most similar known cluster
The following clusters are from record OH13:				
Cluster 1	Phenazine-Lantipeptide	1382542	1440263	Phenazine_biosynthetic_gene_cluster (38% of genes show similarity)
Cluster 2	Lantipeptide	1686962	1709799	-
Cluster 3	Nrps	2135812	2220089	WAP-8294A2_(lotilibcin)_biosynthetic_gene_cluster (40% of genes show similarity)
Cluster 4	Nrps	2304899	2349207	Paenibactin_biosynthetic_gene_cluster (50% of genes show similarity)
Cluster 5	Nrps	2792112	2887591	Orfamide_biosynthetic_gene_cluster (30% of genes show similarity)
Cluster 6	Bacteriocin	3045480	3056610	-
Cluster 7	Resorcinol	4059179	4101902	-
Cluster 8	Other	4432834	4473571	Pyoverdine_biosynthetic_gene_cluster (1% of genes show similarity)
Cluster 9	Nrps	4489560	4537832	-
Cluster 10	Nrps	4765445	4809590	-
Cluster 11	Arylpolyene	4834265	4875608	Xanthomonadin_biosynthetic_gene_cluster (50% of genes show similarity)
Cluster 12	Nrps	4857389	4911236	Xanthomonadin_biosynthetic_gene_cluster (50% of genes show similarity)
Cluster 13	Bacteriocin	5260303	5271520	-

2.2. Materials and Methods

2.2.1. Bacterial strains, plasmids, and general DNA manipulations.

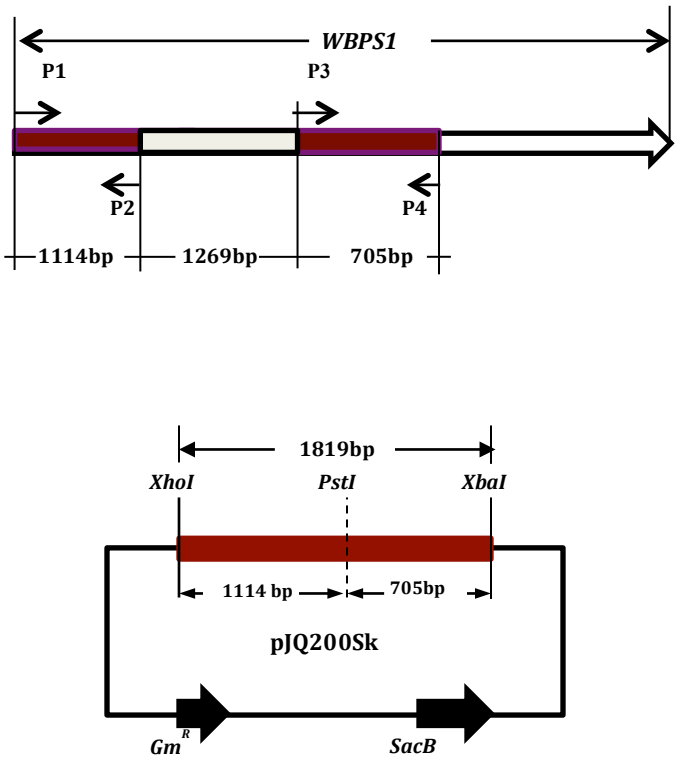
Escherichia coli XL1-blue strain was used as the host for general DNA propagation. *L. antibioticus* OH13 and other bacterial strains were grown in NA broth medium (0.5% peptone, 0.4% yeast extract, 1% glucose). Genomic DNA of *L. antibioticus* was prepared as previously described (*44*). Plasmid preparation and DNA gel extraction were carried out using kits from Qiagen. PCR primers were synthesized by Eurofins MWG Operon (distributed through Fisher Scientific). All other manipulations were performed according to standard methods (*69*).

2.2.2. Generation of gene deletion mutants

To construct vectors for in-frame deletion of the first A (adenylation) domain (A1) from *wbps1* (Figure 14), two DNA fragments were amplified from the upstream and downstream of each of these two genes using the primer pairs described in (Table S4). Genomic DNA from the wild type *L. antibioticus* OH13 was used as the PCR template. Each of the upstream fragments was digested with *Xho*I/*Pst*I, and each of the downstream fragments was digested with *Pst*I/*Xba*I. The upstream and downstream fragments of *wbps1* were cloned into the conjugation vector pJQ200SK to produce pJQ200SK- *wbps1leftright*. The resulting vectors were confirmed by sequencing and PCR. Several attempts were carried out to transfer the constructed vectors into *L.*

antibioticus wild type using electroporation but it is not yet successful.

Figure 14. Construction of vector for the deletion of the A1-domain in WBP_*nrps* gene



2.2.3. Production and analysis of metabolites in wild type *La*OH13

OH13 was grown in R_2A for 36-48 hrs, and an aliquot of 2 ml was transferred to a 250-ml flask containing 50 ml of fermentation medium (5% yeast extract, 5% protease peptone, 5% casein

hydrolysate, 5% glucose, 0.5% soluble starch, 3% sodium pyruvate, 3% dipotassium hydrogen phosphate, $MgSO_4 7H_2O$; pH 7.2). The culture was incubated at 28°C for 3 days with shaking at 200 rpm. To extract the metabolites, the 50-ml broth culture was collected. The supernatant was extracted with ethyl acetate (1/1, vol/vol). The organic phase was dried with a rotavapor (R-200; Buchi) to obtain the crude extract. The extract was dissolved in 2 ml methanol. A 20 µl aliquot of each extract was analyzed by high-pressure liquid chromatography (HPLC; 1220 Infinity LC, Agilent Technologies) using a column (Cosmosil 5C18-AR-II; 4.6 mm by 250 mm). Water-0.1% TFA (solvent A) and acetonitrile-0.1% TFA (solvent B) were used as the mobile phases with a flow rate of 1.0 ml/min. Hence the mass of WBP is predicted to be 1508 the sample was analyzed using LC-MS (HPLC: Cosmosil 5C18-AR-II, LC, MS: Finnigan mat, LCQ).

2.3. Results and Discussion

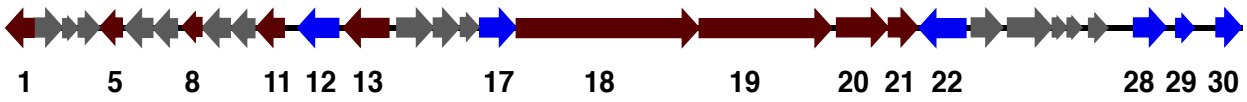
2.3.1. Sequence analysis of WBP gene cluster

In looking for novel antibiotics in *Lysobacter* species, we obtained and analyzed the genome of *La*OH13. We found at least 13 gene clusters probably responsible for the biosynthesis of several secondary metabolites; the majority of these gene clusters code for nonribosomal peptide synthetases (NRPS). One of the clusters grabbed our attention, as it contains two huge NRPSs that are embedded next to each other. The first NRPS (WBPS1) hosts 7 modules having 26

domains: 7 adenylation domains (A), 7 peptidyl carrier proteins (PCP), 7 condensation domains (C), 4 epimerase domains (E), and 1 methylation domain (M). The second (WBPS2) contains four modules having 15 domains 4 A, 4 PCP, 4 C, 2 E and 1 thioesterase (TE). WBP is named because of its similarity to the NRPS organization of the WAPS1 and WAPS2, previously characterized from *Lysobacter enzymogenes* OH11 (*11*).

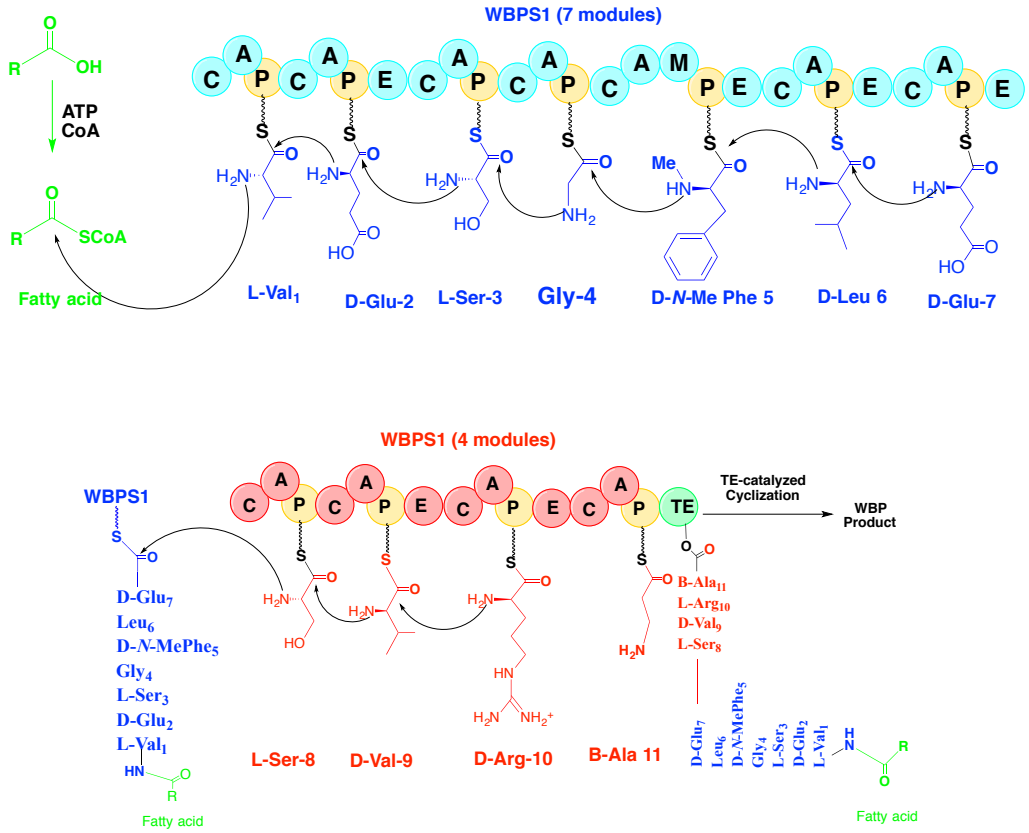
The substrate specificity of the adenylation (A) domains of the 11 NRPS modules was predicted based on sequence alignments of the 10-amino-acid "nonribosomal peptide codes" defined by Stachelhaus *et al*. (*70, 71*). Table S3 below shows the predicted "nonribosomal peptide codes" from the A domains of the 7-module of WBPS1, which would putatively activate and incorporate L-Val_1 , D-Glu_2 , L-Ser_3 , Gly_4 , D-N-Me-Phe_5 , D-Leu_6 , and D-Glu_7, and of the 4-module WBPS2, which would putatively activate and incorporate L-Ser_8, D-Val_9, D-Arg_{10}, and beta Ala_{11}. These putative substrates are used to predict the assembly line for the WBP biosynthesis, as shown in (Figure 16).

Figure 15 WBP gene cluster from *L. antibioticus* OH13



ORF	Bp	aa	Location	Blastx Homolog
1	1499		4539156 - 4540655	NAD-dependent epimerase/dehydratase
5	1115		4531279 – 4532394	glycosyl transferase group 1
8	1142		4535484 – 4536626	glycosyl transferase group 1
11	1499		4539156 - 4540655	exopolysaccharide biosynthesis domain protein
12	692		4540656 – 4541348	polysaccharide export protein
13	1445		541777 – 4543222	sugar transferase
17	1907		4547277 – 4549184	ABC transporter related protein
18	27915	9304	4549308 – 4577222	condensation domain-containing (7modules) **C A_1 PP C A_2 PP E C A_3 PP C A_4 PP C A_5 M PP E C A_6 PP C A_7 PP E**
19	16296	5431	4577203 – 4593498	condensation domain-containing (4modules) **C A_1 PP C A_2 PP E C A_3 PP E C A_4 PP TE**
20	1262		4593555 – 4594817	Decarboxylase, pyridoxal-dependent
21	218		4594951 – 4595169	mbtH-like protein
22	1289		4595329 – 4596618	major facilitator family transporter
28	1334		4602202 – 4603536	sensor histidine kinase
29	515		4603586 – 4604101	response regulator
30	1388		4604434 – 4605822	sigma-54 dependent transcriptional regulator

Figure 16. Proposed biosynthetic pathway for WBP products from *L. antibioticus* OH13.

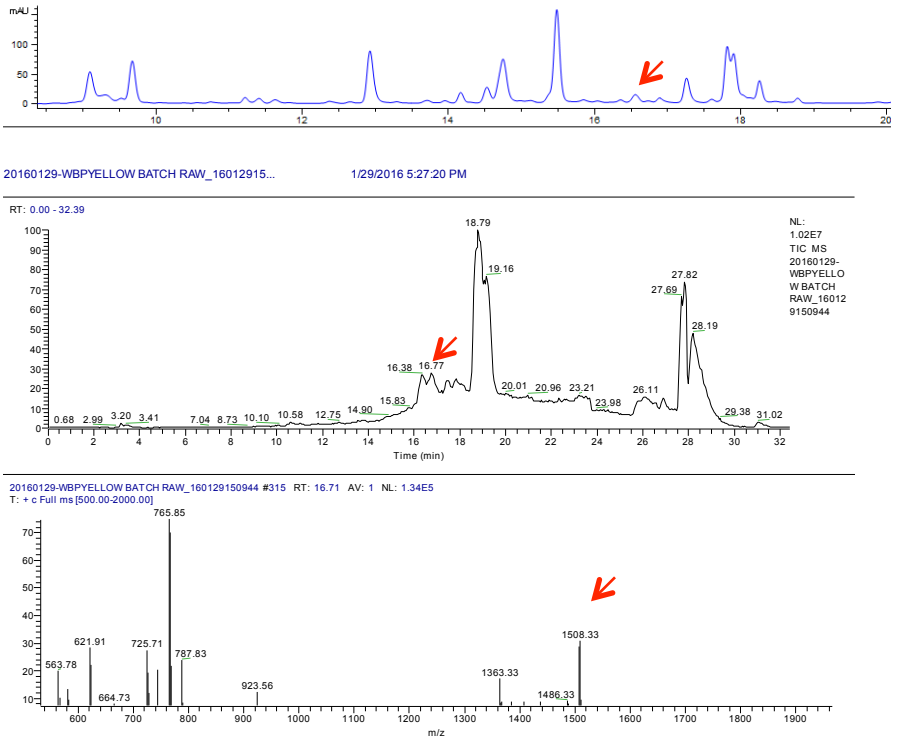


2.3.2. LC-MS analysis of crude extract from *Lysobacter antibioticus* OH13

Numerous attempts to generate WBP mutant strains were not successful. To identify the putative WBP from the crude extract of *L. antibioticus* OH13, we have used tandem Liquid-Chromatography-Mass Spectrometry (LC-MS) to trace the putative WBP compounds from the

crude extracts of *L. antibioticus* OH13 grown in the R_2A medium for three days. A peak with a mass of 1508 was identified at the retention time of 16.77 minutes (Figure 17). Bruijn *et al*. recently reported the same mass of 1508 for an unknown compound, which could also be the same products of the WBP gene cluster (*29*). Therefore, we are collecting the crude extracts of OH13 metabolites to isolate the compounds with the retention time around 16 minutes and ultimately characterize their structure using NMR.

Figure 17 HPLC (A) and LC-MS (B) of the crude extract of wild type *Lysobacter antibioticus* OH13



2.4. Supporting Information

Table-S3: Specificity of Adenylation domains of WBP NRPSs

SN		A-Domain	Signature	AA	Score (%)
1		A1	D A Y W W G G T F K	Val	100
2		A2	D T E D I G A V D K	Glu	70
3	**WBPS1**	A3	D V W H L S L V D K	Ser	90
4		A4	D I L Q L G L V W K	Gly	100
5		A5	D A W T I A A V C K	Phe	90
6		A6	D A M L I G A I C K	Leu	80
7		A7	D T E D V G C V D K	Arg/Glu	70
8		A8	D V W H V G S I G K	Ser	70
9	**WBPS2**	A9	D A Y W L G G T F K	Val	90
10		A10	D A A I V G E I W K	Arg	60
11		A11	I D W V S S I W D K	Ala-b	60

Table S4. Primers used for amplifying the left and right arms of *wbp_s1*

	Type	Primers
P1	S1 Left armForward (XhoI)	5’-CCGCTCGAGCACGCTCACCGCCTTCATCC-3’
P2	S1 left arm Reverse	5’-GTCGTTCCAGTCCAGCAGCAGCGCGAACCACATTTCCGACT-3’
P3	S1 Right arm Forarwrd	5’-AGTCGGAAATGTGGTTCGCGCTGCTGCTGGACTGGAACGAC-3’
P4	S1 Right arm Reverse (XbaI)	5’-TGCTCTAGATCAACAACGGCACCCACAAC-3’

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