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Biosynthesis, regulation, and engineering of natural products from *Lysobacter*

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Lysobacter is a genus of Gram-negative bacteria that was classified in 1987. Several *Lysobacter* species are emerging as new biocontrol agents for crop protection in agriculture. *Lysobacter* are prolific producers of new bioactive natural products that are largely underexplored. So far, several classes of structurally interesting and biologically active natural products have been isolated from *Lysobacter*. This article reviews the progress in *Lysobacter* natural product research over the past ten years, including molecular mechanisms for biosynthesis, regulation and mode of action, genome mining of cryptic biosynthetic gene clusters, and metabolic engineering using synthetic biology tools.

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1. Introduction

Lysobacter is a genus of Gram-negative bacteria in the Xanthomonadaceae family ubiquitously inhabiting soil and water.[1] The genus has attracted research interest in the past decade because it has emerged as a new group of biocontrol agents against pathogens of crop plants and as a new source for lytic enzymes and antimicrobial natural products. There are approximately 48 species in the genus *Lysobacter*. Since its classification in 1978, a number of species that were previously misidentified have been reclassified to *Lysobacter*, thus the number continues to increase.[2] *Lysobacter* exhibit the gliding motility but without flagella; they have a high genomic GC content (65.4% to 70.1%) and are prolific producers of extracellular lytic enzymes. These traits distinguish them from other taxonomically and ecologically related microbes. There are over 22 *Lysobacter* strains with genomes being publicly available. Among these, strains of the three species *L. enzymogenes*, *L. antibioticus*, and *L. capsici* have been given the most attention by researchers, owing to their potent biocontrol activities and the ability to produce multiple bioactive natural products.[3,4] The main groups of natural products isolated from the *Lysobacter* strains belong to polyketides and nonribosomal peptides. The biosynthesis of these natural products typically involves modular enzyme assembly lines.[5–8] A module of polyketide synthase (PKS) is typically consisted of the following domains: β-ketosynthase (KS), acyltransferase (AT), enoylreductase (ER), dehydratase (DH), ketoreductase (KR), and acyl carrier protein (ACP). A typical module of a nonribosomal peptide synthetase (NRPS) contains domains for condensation (C), adenylation (A), and a peptidyl carrier protein (PCP). At the end of the assembly line usually is a thioesterase (TE) that releases the polyketide chain or peptide chain. In 2012, we wrote a Highlight on this topic.[3] Since then, significant progress has been reported in the literature. This review covers the advances in research of *Lysobacter* natural products during the past ten years. We focus on molecular mechanisms for biosynthesis and regulation; we also review the progress on genome mining of cryptic biosynthetic gene clusters and metabolic engineering using synthetic biology tools.

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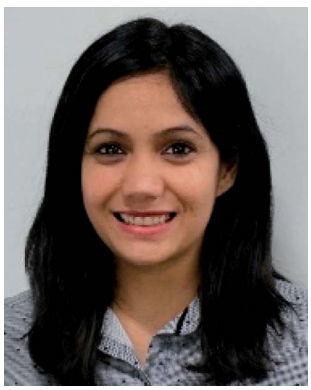
Amanda Lynn Miller received her B.S. degree in Chemistry from University of Nebraska-Lincoln in 2018. She is currently pursuing her PhD at Department of Chemistry, University of Nebraska-Lincoln under the guidance of Professor Liangcheng Du. Her research focus is on the chemical biology of new bioactive natural products from Lysobacter species.



Vimmy Khetrapal earned her B.S. degree (2010) in Chemistry, Industrial Microbiology, and Zoology from Panjab University, India, and M.S. degree (2013) in Biochemistry from Punjab Agricultural University, India. She is currently pursuing her PhD at Department of Chemistry, University of Nebraska-Lincoln, under the guidance of Professor Liangcheng Du. She is working on biosynthetic engineering and molecular regulation of bioactive natural products in Lysobacter enzymogenes.

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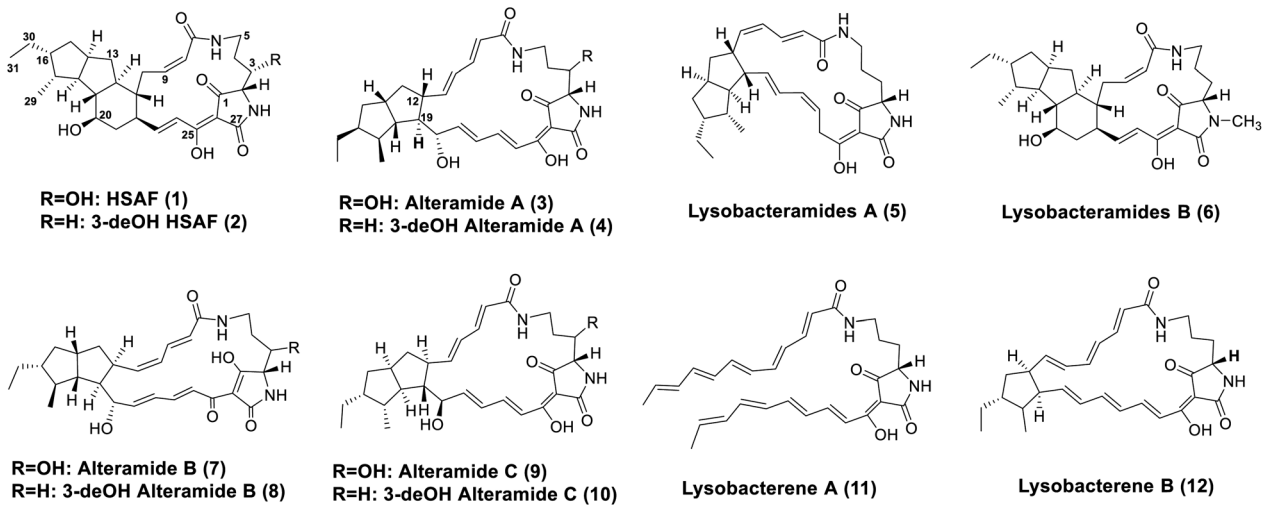


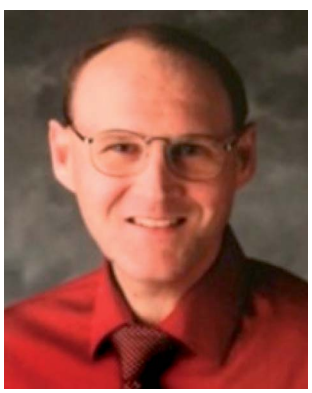
Fig. 1 Chemical structures of HSAF, alteramides and related compounds isolated from *Lysobacter enzymogenes*. Note that H12 of alteramide A (3) and 3-deOH alteramide A (4) was revised to be beta and on the opposite plane as H19 in PoTeM compounds isolated from a marine *Pseudoalteromonas* sp. OT59 associated with Pacific octocoral *Leptogorgia alba*.[18]

2. Natural products from *Lysobacter*

2.1 Polycyclic tetramate macrolactams (PoTeM)

2.1.1 Chemical structure, antibiotic activity, and mode of action. Several *Lysobacter* strains produce a group of distinct natural products belonging to the polycyclic tetramate macrolactams (PoTeM), including HSAF (heat stable antifungal factor), alteramides, and their 3-dehydroxyl analogues (3-deOH) (Fig. 1).[3,4,9–13] The structure of these compounds is characterized by a tetramate (2,4-pyrrolidinedione) within the macrolactam, which is fused to a cyclic system of 2–4 rings. HSAF was first isolated from *Lysobacter enzymogenes* strain C3, a biocontrol agent originally classified as *Stenotrophomonas maltophilia* C3 and isolated from grass foliage.[9,14,15] Yu *et al.* reported the initial chemical structure of HSAF (Fig. 1).[9] It is a tetramate-containing 17-membered macrolactam, which is fused with a 5/5/6-tricyclic system. HSAF shares the same plain structure as dihydromaltophilin, an antibiotic first isolated from a *Streptomyces* sp. with a 5/5/6-tricyclic skeleton as well.[16] The absolute configurations of HSAF were later studied by Xu *et al.*[11] The authors also reported alteramide A, 3-deOH HSAF, and two new HSAF analogues (lysobacteramides A and B) from *L. enzymogenes* C3. Ding *et al.* reported alteramide B, which is another potent HSAF analogue produced by *L. enzymogenes* C3.[17]

It should be noted that multiple stereoisomers exist for PoTeM with the same plain structure. For example, maltophilin isolated from *Stenotrophomonas maltophilia* R3089 and *Streptomyces* sp. and xanthobaccin A isolated from *Lysobacter* sp. strain SB-K88 have the same plain structure but differ by the stereochemistry in the cyclic systems.[16,19–21] The absolute configurations of the complex cyclic systems can, so far, only be confidently assigned by X-ray crystallography.[22] It is not entirely clear if HSAF isolated from *L. enzymogenes* is identical to dihydromaltophilin isolated from *Streptomyces* sp., and it is possible that some of the reported PoTeM might be the same compounds but with different names. Further studies are needed to clarify the issues.



Stephen Wright earned his B.S. degree from Wayne State College (Nebraska) in 2009 and his M.S. degree from University of Nebraska-Lincoln in 2013. He is currently completing his PhD degree at Department of Chemistry, University of Nebraska-Lincoln, under the guidance of Professor Liangcheng Du. He is studying the regulatory and biosynthetic mechanisms of natural products in Lysobacter species.

Dr Liangcheng Du is a Professor at Department of Chemistry, University of Nebraska-Lincoln. He is interested in discovery of new antibiotics and other bioactive natural products from Lysobacter species and other underexplored microorganisms. The current research includes the study of molecular mechanisms for biosynthesis and regulation, and metabolic pathway engineering of new natural products.

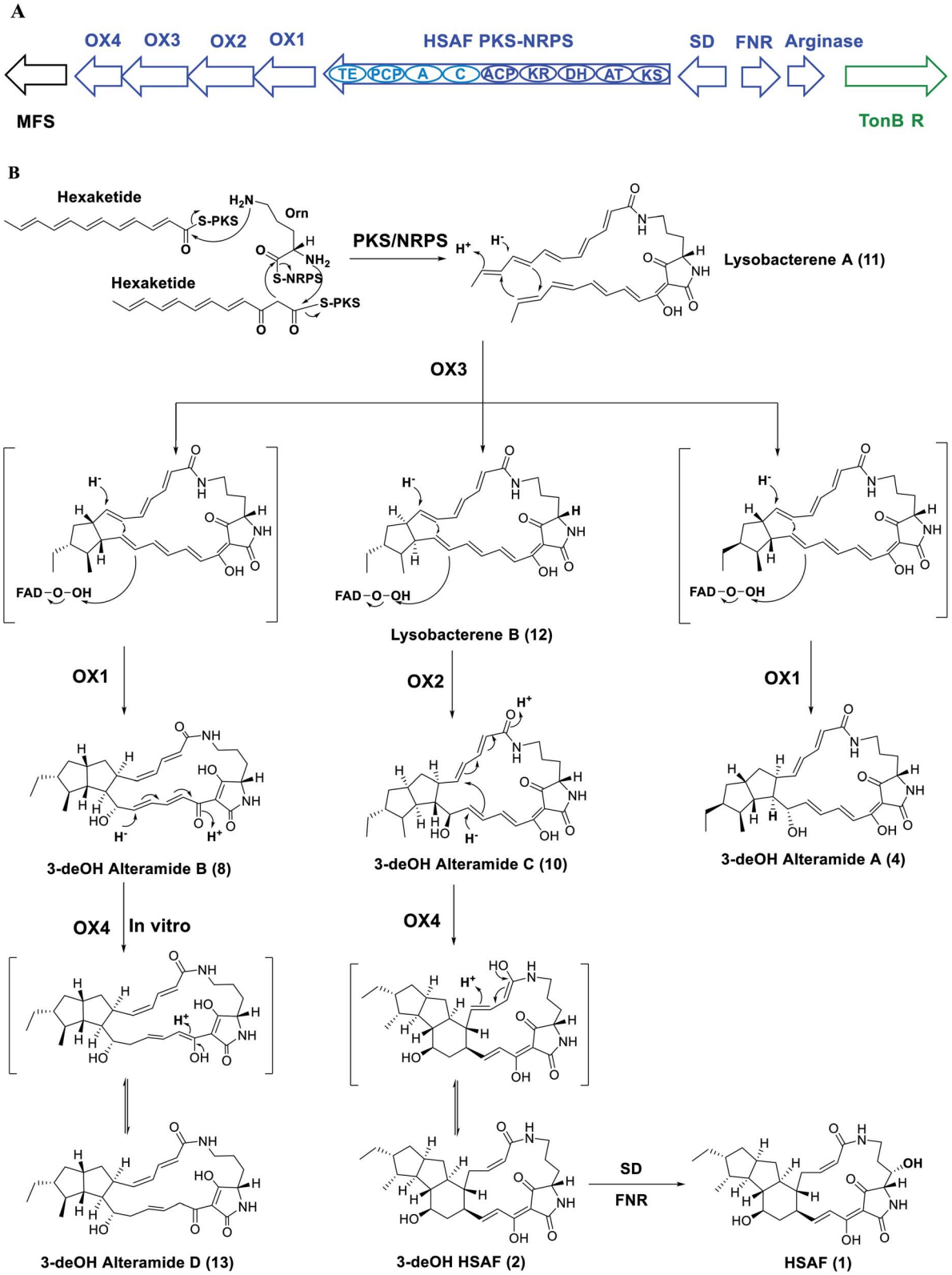


Fig. 2 Biosynthetic gene cluster (A) and proposed biosynthetic pathway (B) for HSAF and analogues from *Lysobacter enzymogenes*. MFS, major facilitator superfamily transporter; OX1–OX3, FAD-dependent oxidoreductase; OX4, alcohol dehydrogenase/zinc-binding protein; HSAF PKS-NRPS, polyketide synthase-nonribosomal peptide synthetase for HSAF; SD, fatty acid hydroxylase/sterol desaturase; FNR, ferredoxin NADP reductase/FAD/NADP-binding protein; TonB R, tonB-dependent outer membrane receptor. For the domains in the PKS module, KS, ketosynthase; AT, acyltransferase; DH, dehydratase; KR, ketoreductase; ACP, acyl carrier protein. For the domains in the NRPS module, C, condensation domain; A, adenylation domain; PCP, peptidyl carrier protein; TE, thioesterase domain. The figure is modified from Li *et al.* with permission.[13]

HSAF shows inhibitory activity against a broad spectrum of filamentous fungi and oomycetes, such as *Aspergillus nidulans*,[23] *Fusarium graminearum*,[24] *Uromyces appendiculatus*,[25] *Fusarium verticillioides*,[11] and *Bipolaris sorokiniana*,[26] as well as yeast cells, such as *Candida albicans*.[27] The mode of action towards filamentous fungi was tested using *A. nidulans*.[23] The results suggested that HSAF could interrupt the biosynthesis of sphingolipids by targeting a ceramide synthase, BarA, which is special to filamentous fungi. Further studies indicated that HSAF caused thickening of fungal cell walls due to an increased chitin level and accumulated sphingolipid intermediates.[28] These resulted in permanent blockage of the polarized growth of fungal hyphal tips. Thus, HSAF has a chemical structure and mode of action distinct from existing antifungal drugs and fungicides. Sphingolipids in fungal cells are structurally different from that in mammalian cells, making them an interesting new target for development of new antifungal antibiotics.[29] He *et al.* took the transcriptomics approach to examine the mechanism for HSAF during the inhibition of the Chinese pear fungal pathotype, *Alternaria alternata*.[30] They observed that multiple signaling pathways and essential cellular metabolisms were disrupted by HSAF. It was suggested that HSAF could breach metabolism networks in *A. alternata* and ultimately induce cell wall thickness and apoptosis in the pathogen. As for the structure–activity relationship, very little has been done. A preliminary study showed that the 3-hydroxyl on the ornithine residue could play a crucial role in the antifungal activity of HSAF.[31] When compared with HSAF in the inhibition of *Penicillium avellaneum*, 3-deOH HSAF displayed very little antifungal activity. When tested against *C. albicans*, HSAF could induce apoptosis through increasing production of reactive oxygen species (ROS).[27]

Alteramide A was first isolated from the bacterium *Alteromonas* sp. associated with the marine sponge *Halichondria okadai*.[32] Alteramide A exhibits antifungal properties towards *Penicillium citrinum*.[18] When an extract containing both alteramide A and HSAF isolated from *Streptomyces puniceus* L75 was used to treat tomato leaves, the extract exhibited significant inhibition against the tomato early blight fungal pathogen *Alternaria solani*.[33]

Alteramide B displays broad-spectrum inhibitory activity against phytopathogenic fungi, oomycetes and several yeasts.[17,34] Its mode of action was examined using *C. albicans*, which indicated that alteramide B could cause apoptosis through inducing ROS. Alteramide B appeared to bind tubulin and inhibited tubulin polymerization, leading to cell cycle arrest at the G2/M phase. This resulted in ROS induction and apoptosis.[17]

2.1.2 Biosynthetic mechanism

2.1.2.1 The HSAF biosynthetic gene cluster. The HSAF biosynthetic gene cluster (BGC) was initially identified from *L. enzymogenes* C3.[9] In the core of the BGC is a gene (*hsaf pks-nrps*) encoding a hybrid PKS-NRPS (polyketide synthase-nonribosomal peptide synthetase). The hybrid PKS-NRPS has a modular organization, including one PKS module and one NRPS module. The PKS module consists of five domains, ketosynthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), and acyl carrier protein (ACP); the NRPS module consists of four domains, condensation (C), adenylation (A), peptidyl carrier protein (PCP), and thioesterase (TE) (Fig. 2). The deletion of this gene eliminated HSAF production and the antifungal activity in strain C3, confirming that the hybrid PKS-NRPS is required for the antifungal HSAF biosynthesis. Based on the structural scaffold of HSAF, Yu *et al.* proposed that HSAF could be built from two separate hexaketide chains that would be linked *via* an amino acid (ornithine) residue, which forms amide bonds with the two polyketides through two Claisen condensation reactions (Fig. 2).[9] The five-membered tetramate ring in the structure is then formed by a carbon–carbon bond through the Dieckmann condensation between ornithine's carbonyl carbon and one hexaketide's α-carbon.

Later, the genome sequence of strain OH11 of *L. enzymogenes* became available, and when the BGC from strain C3 was compared with the genome sequence of strain OH11, it was surprising to realize that no other PKS genes were present in either the upstream or downstream regions of the hybrid PKS-NRPS.[10] This suggests that a single PKS module would have to act not only iteratively to assemble two hexaketide chains, but also separately so as to connect the two hexaketide chains with the NRPS-activated ornithine to form this distinct PoTeM structure. This was previously unrecognized among the bacterial modular PKSs.[35] In canonical bacterial modular PKS, such as erythromycin PKS, one PKS module is responsible for one cycle of carbon chain elongation that would add 2 carbons to the polyketide chain.[36] For the hexaketide-ornithine-hexaketide scaffold in HSAF, it would have required 11 modules (5 PKS modules, 1 NRPS module and 5 PKS modules). However, a single-module PKS-NRPS is sufficient for assembling the scaffold, which is now recognized as a general mechanism for all known PoTeM so far.

2.1.2.2 Formation of the polyene-tetramate-polyene scaffold. To verify the unusual biosynthetic mechanism, Lou *et al.* heterologously expressed the four NRPS domains (C-A-PCP-TE) in *E. coli* and purified the 148.6 kDa protein.[10] *In vitro* assays showed that the A domain specifically activated L-ornithine, and the four-domain NRPS was capable of catalyzing the proposed Claisen condensation reactions between acyl-*S*-ACP and ornithinyl-*S*-NRPS, forming the characteristic tetramate product. The structure of the biosynthetic product was confirmed by comparing with the chemically synthesized tetramate compound.[10] The results demonstrated that the single-module NRPS is in charge of forming the two amide bonds between the two amine groups of ornithine and the two polyketide chains, as well as the carbon–carbon bond between the carbonyl group of ornithine and the α-carbon of one polyketide chain (Fig. 2). This work provided experimental evidence for the previously unrecognized biosynthetic mechanism for hybrid polyketide-peptides in bacteria. Another uncommon phenomenon was revealed when studying the TE domain. During *in vitro* investigation of the heterologously expressed TE domain, it was found that the purified TE possessed both protease activity and peptide ligase activity, cleaving amide bonds and also forming amide bonds.[37] The results imply that this TE may play

a role in the formation of the amide bonds in the tetramate-containing macrolactam of HSAF.

In HSAF BGC, the *hsaf pks-nrps* gene is flanked by nine genes that were predicted to be involved in tailoring, transportation and regulation (Fig. 2A).[10] Notably, four *OX* genes that encode redox enzymes are tightly clustered together at the downstream of *hsaf pks-nrps*, and a gene encoding fatty acid hydroxylase/sterol desaturase (SD) is also tightly clustered with the *hsaf pks-nrps* at the upstream. A characteristic of nearly all known PoTeMs is that they carry a 3-hydroxyl group on the ornithine residue.[38] Li *et al.* studied the fatty acid hydroxylase/SD gene in strain C3.[31] After deleting this gene, they found HSAF disappeared from the mutant but a new compound emerged. The structure of the new compound was determined to be 3-deOH HSAF (Fig. 1). Next, they expressed the *SD* gene in *E. coli*, and when 3-deOH HSAF was fed to the *E. coli* cells carrying the *SD* gene, HSAF was produced *in vivo*. Furthermore, the SD enzyme extract from *E. coli* was capable of converting 3-deOH HSAF to HSAF *in vitro*, in the presence of NADPH (nicotinamide adenine dinucleotide phosphate). Co-expressing the ferredoxin reductase (FNR) gene, which is also located in the HSAF BGC (Fig. 2A), with the *SD* gene in *E. coli* considerably enhanced the 3-hydroxylation activity. The results demonstrated that the *SD* gene encodes a 3-hydroxylase to catalyze the 3-hydroxylation of ornithine residue during HSAF biosynthesis, and the 3-hydroxylation step is likely a post PKS-NRPS reaction in HSAF biosynthetic pathway.[31]

One particularly intriguing aspect in PoTeM biosynthesis is the mechanism by which the two polyketide chains are cross-cyclized to form the polycyclic system with distinct regioselectivity and stereoselectivity for each PoTeM. In an attempt to study the mechanism *in vitro*, Olson *et al.* chemically synthesized a 2,4,6,8,10-dodecapentanoic acid thioester (*N*-acetylcystamine, SNAC) as an analogue of the substrate for biosynthesis of HSAF.[39] The authors designed an elegant synthetic approach that overcame two major challenges. One was the ability to introduce a thioester in the presence of a highly reactive polyenoate, and the other was the ability to readily adapt the synthesis to analogues with varied chain lengths. However, the *in vitro* assays did not exhibit conversion of the dodecapentaenoate-SNAC into HSAF, suggesting that HSAF biosynthesis may require the polyunsaturated substrates to be tethered to the acyl carrier protein of PKS (Fig. 2B).

Subsequently, Li *et al.* took an alternative approach to study the biosynthetic mechanism.[12] They first isolated a cosmid clone from the genomic library of *L. enzymogenes* C3, which contains the entire HSAF BGC. This BGC was directly transformed into a *Streptomyces* host, but no HSAF-like compound was detected. Subsequently, the *Streptomyces* promoter *ermE** was used to replace the putative promoter located at the 5′-nontranslated region of the *hsaf pks-nrps* gene. The resulting construct, pSETHSAF3, was transformed into two different strains of *Streptomyces* with a "clean" metabolic background, to avoid potential effects from the native PKS and NRPS genes in the hosts. Strain SR107 was obtained from *Streptomyces* sp. LZ35 after deleting the host's four PKS gene clusters, and strain ZM12 was derived from *S. coelicolor* by deleting all ten PKS and NRPS gene clusters of the host. When pSETHSAF3 was introduced into strain SR107, the transformants produced multiple compounds that were absent in the host strain.[12] Two of the compounds were analyzed by HR-ESI-MS and 1D- and 2D-NMR, which identified the compounds as 3-deOH HSAF and a new stereoisomer of 3-deOH alteramide A. The pSETHSAF3 transformants of strain ZM12 also produced these compounds. The results demonstrated that the single-module hybrid PKS-NRPS in pSETHSAF3 is sufficient for the assembly of the PoTeM scaffold. Since 3-deOH compounds, rather than the final compounds (HSAF and alteramides) were produced, the *SD* gene in the construct might not be functional in the *Streptomyces* hosts. To find out, Li *et al.* also inserted the promoter *ermE** in front of the *SD* gene, which generated pSETHSAF4. However, no HSAF-like compounds were found in strain SR107 carrying pSETHSAF4. The result indicated *SD* might not be functional in this new construct. Alternatively, the insertion of the *ermE** cassette in front of the *SD* gene might have impaired the function of other genes in the BGC.[12]

Another strain was generated by introducing a third *Streptomyces* expression construct pSETHSAF5, which contained only the *hsaf pks-nrps* gene under the promoter *ermE**. The polyene tetramate intermediate (**11**) was detected by LC-MS/MS, supporting the idea that the single-module hybrid PKS-NRPS is capable of assembling two separate polyketide chains and connecting them with the ornithine residue to generate the PoTeM scaffold. This polyene tetramate intermediate was later named lysobacterene A (**11**) (Fig. 1 and 2B).[12]

In seeking for direct evidence, Li *et al.* also expressed the 5-domain PKS module (KS-AT-DH-KR-ACP) in *E. coli* and purified the giant protein (199.8 kDa) (Fig. 2A).[12] This PKS was converted to its holo form by incubating with coenzyme A (CoA) and Svp, a 4′-phosphopantetheinyl transferase.[40] Both the apo and holo forms were confirmed by Q-TOF-MS/MS analysis of the tryptic fragments and phosphopantetheine ejection assay. Next, the PKS was incubated with malonyl-CoA, acetyl-CoA, and NADPH, and the results showed that a hexaketide polyene was covalently attached to the ACP domain of the PKS *via* the 4′-phosphopantetheinyl thioester. Interestingly, the MS/MS data suggested that malonyl-CoA was used by the PKS as the starter as well as the extender. To verify, Li *et al.* further expressed the 5-domain PKS with a point-mutation at the active site cysteine to alanine (C176A) in the KS domain.[12] Then, the mutant PKS protein was purified, and the enzyme assays showed that the synthase transferred malonyl group from malonyl-CoA, but not the acetyl group from acetyl-CoA, to the ACP domain of the PKS.

Finally, Li *et al.* reconstituted the whole PKS-NRPS (~350 kDa) for HSAF biosynthesis *in vitro*.[12] The polyene-PKS was first generated and then incubated with the purified NRPS protein (containing 4 domains, C-A-PCP-TE) in the presence of ornithine and adenosine triphosphate (ATP). LC-HRMS data showed that the same polyene tetramate intermediate, lysobacterene A (**11**), as *in vivo* produced in the *Streptomyces* strain SR107 carrying the *hsaf pks-nrps* gene, was produced by the *in vitro* reconstituted PKS-NRPS. Altogether, the results proved the unusual iterative biosynthetic mechanism for bacterial polyketide–peptide natural products (Fig. 2). The polyene-tetramate-

polyene, lysobacterene A (**11**), is the precursor for the polycycles in HSAF and its analogues.

2.1.2.3 Formation of polycyclic system of PoTeM. Another intriguing aspect in PoTeM biosynthesis is how the polyene-tetramate-polyene scaffold is transformed into the polycyclic system. Studies on gene deletion mutants indicated that four *OX* genes are involved in formation of the 5/5/6-tricyclic system in HSAF and analogues produced by *L. enzymogenes*.[10] *OX1*, *OX2*, and *OX3* are homologous to each other and encode a cascade of NADPH-dependent flavin oxidoreductases; *OX4* encodes an NADPH-dependent alcohol dehydrogenase/Zn-binding protein (Fig. 2A). In order to understand the role of each of the *OX1–OX4* genes in forming various HSAF analogues, a "clean" host strain, SR111, was generated by deleting the seven native PKS gene clusters and one native NRPS gene cluster in *Streptomyces* sp. LZ35.[13] The expression construct, pIB-HSAF1, that contains the HSAF BGC except the genes for 3-hydroxylation was put under the control of *ermE** promoter and introduced into strain SR111. The resulting strain (SR111-HSAF1) generated ten compounds that were absent in the original host. Three of the compounds were identified as known HSAF analogues, including 3-deOH HSAF (**2**), 3-deOH alteramide B (**8**), lysobacterene A (**11**) (Fig. 1). The most abundant compound produced in strain SR111-HSAF1 was a new HSAF analogue (named lysobacterene B) (**12**). Its structure was established by extensive spectroscopic analyses. Lysobacterene B contains only the first ring (the outermost ring) of the cyclic system, and the stereochemistry of this ring is identical to that of HSAF. In addition, lysobacterene B retains the characteristic tetramate ring and contains a triene in both of the hexaketide chains.[13] These structural features suggest that the outermost ring was formed first in the biosynthesis of the cyclic system in HSAF and that lysobacterene B (**12**) is an intermediate for HSAF biosynthesis. The structures of the minor compounds produced in strain SR111-HSAF1 have not been fully established due to the very low yield and low stability, but spectroscopic data suggested that they are isomers/analogues of lysobacterene A (**11**) or lysobacterene B (**12**). The observation of multiple isomeric forms of compounds with "zero ring" or "one ring" implied that isomerization reactions had taken place after the nascent polyene-tetramate-polyene precursor was assembled by the hybrid PKS-NRPS. The isomerizations could proceed non-enzymatically, due to the reactive nature of the extended conjugation systems in the polyene chains.[13]

Next, Li *et al.* removed all four *OX* genes from strain SR111-HSAF1, and the resulting strain, SR111-HSAF2, only produced the zero-ring polyene-tetramate-polyenes including lysobacterene A (**11**), which is consistent with previously reported results.[12] In subsequent studies, they used a combinatorial approach, in which each of the *OX* genes was removed in turn from strain SR111-HSAF1.[13] They also tested various combinations of double or triple in-frame deletion of the four *OX* genes. Together, the observed PoTeM compounds indicated that *OX3* is the "gatekeeper" in charge of the formation of the first five-membered ring (Fig. 2B). The enzyme encoded by *OX3* has a relaxed selectivity, allowing it to convert the zero-ring precursor, lysobacterene A (**11**), to several one-ring stereoisomers including lysobacterence B (**12**). *OX2* and *OX1* are responsible for formation of the second five-membered ring in HSAF and in the alteramides, respectively, but with different selectivity. Depending on the stereochemistry, the one-ring precursors can be converted to two-ring products, 3-deOH alteramides A (**4**) and B (**8**) by *OX1* or 3-deOH alteramide C (**10**) by *OX2*. 3-deOH alteramide C (**10**) was a new compound, and to establish its structure, several different mutant strains were constructed in order to isolate a sufficient amount for spectroscopic studies.[13] Among the two-ring products, only 3-deOH alteramide C (**10**) has the same stereochemistry as that in 3-deOH HSAF (**2**), and when *OX4* was heterologously expressed in *E. coli*, the purified enzyme converted 3-deOH alteramide C (**10**) to 3-deOH HSAF (**2**), but converted 3-deOH alteramide B (**8**) to 3-deOH alteramide D (**13**) (Fig. 2B). The results not only

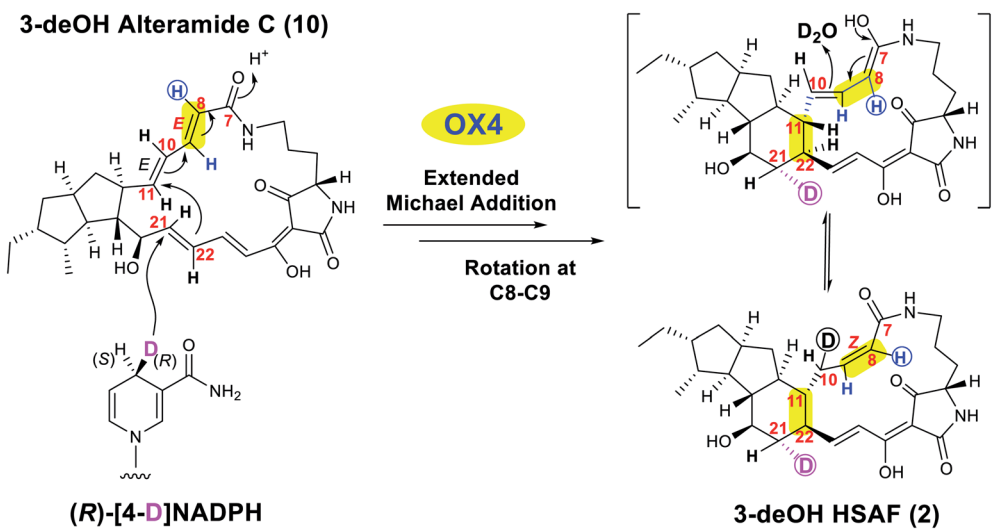


Fig. 3 Proposed mechanism for the OX4-catalyzed reductive cyclization of 3-deOH alteramide C (**10**) to form the six-membered ring in 3-deOH HSAF (**2**), *via* an extended Michael addition-like reaction (modified from Li *et al.*[41] with permission).

demonstrated that *OX4* is responsible for formation of the six-membered ring of 3-deOH HSAF, but also showed that the enzyme encoded by *OX4* only takes the precursor with the "correct" absolute configuration (3-deOH alteramide C, **10**) to form the three-ring product, 3-deOH HSAF (**2**).[13]

The presence of multiple *OX* genes is a hallmark for the structural diversity of the HSAF family. A further study of the regioselectivity and stereoselectivity of OX4 *via* deuterium isotope labeling revealed that OX4 selectively incorporates the *pro-R* hydride from NADPH to C21 and one proton from water to C10 of 3-deOH alteramide C (**10**), forming 3-deOH HSAF (**2**) (Fig. 3).[41] This regioselective incorporation of the NADPH hydride to C21 of 3-deOH alteramide C is also stereoselective, resulting in the 21*S* configuration of 3-deOH HSAF when a deuterium is added. Most intriguingly, the OX4-catalyzed reaction converts the *trans* double bond at C8-C9 in 3-deOH alteramide C (**10**) to a *cis* double bond in 3-deOH HSAF (**2**). This unusual transformation is consistent with a reductive cyclization of the polyene-containing precursor using an extended 1,6-Michael-like addition mechanism, in which C8–C9 can transiently exist as a single bond in the intermediate that can undergo a rotation. Thus, despite the sequence similarity to alcohol dehydrogenases/Zn-binding proteins, OX4 catalyzes a reductive cyclization of two polyene chains, reducing two carbon–carbon π bonds, forming a new carbon–carbon *sigma* bond, and isomerizing a carbon–carbon π bond during the formation of the six-membered ring of HSAF and analogues.[41] Future studies of the 3D structure of OX4, with bound substrate and cofactor NADPH, could reveal molecular insights into the unusual catalytic activity of this alcohol dehydrogenase homolog.

In addition to OX4, Li *et al.* investigated the function of OX2.[42] HSAF and alteramides carry a C20–hydroxyl group that is absent in most PoTeM natural products. Coincidently, the gene cluster for HSAF and alteramides in *L. enzymogenes* has four *OX* genes, while other PoTeM gene clusters contain two or three *OX* genes. As shown in Fig. 2, the products of the gene cluster containing *OX1*/*OX2* always carry this C20–OH. However, the HSAF gene cluster does not contain an obvious candidate for the hydroxylation, such as cytochrome P450 monooxygenases found in some PoTeM clusters.[43] To search for clues, Li *et al.* designed an *in vivo* approach, which combined genes from two distinct sources, the Gram-negative *L. enzymogenes* and the Gram-positive *Streptomyces* sp. S10.[42] They constructed pSET5035-HSAF$_{hybrid}$ that contains the *hsaf pks-nrps* gene, *OX*3 and *O*X4 from the HSAF biosynthetic gene cluster in *L. enzymogenes* and the *cbmB* gene from the combamide gene cluster in *Streptomyces* sp. S10 (Fig. 4).[43] Like OX2, the enzyme encoded by *cbmB* also catalyzes formation of the second five-membered ring in combamides, which lack the C20–OH. When the hybrid PoTeM gene cluster was introduced into heterologous *Streptomyces* hosts (strain SR111 and strain S001), the strains produced lysobacterene B (**12**, the one-ring precursor of HSAF), combamide D (**14**, a two-ring product lacking the C20–OH), pactamide A (**15**, a three-ring product lacking the C20–OH) (Fig. 4). Phylogenetic analysis showed that the OX enzymes that catalyze a coupled ring formation-hydroxylation fall into the same clade, such as OX1/OX2 for HSAF and alteramides,[13] SGR-OX1 for

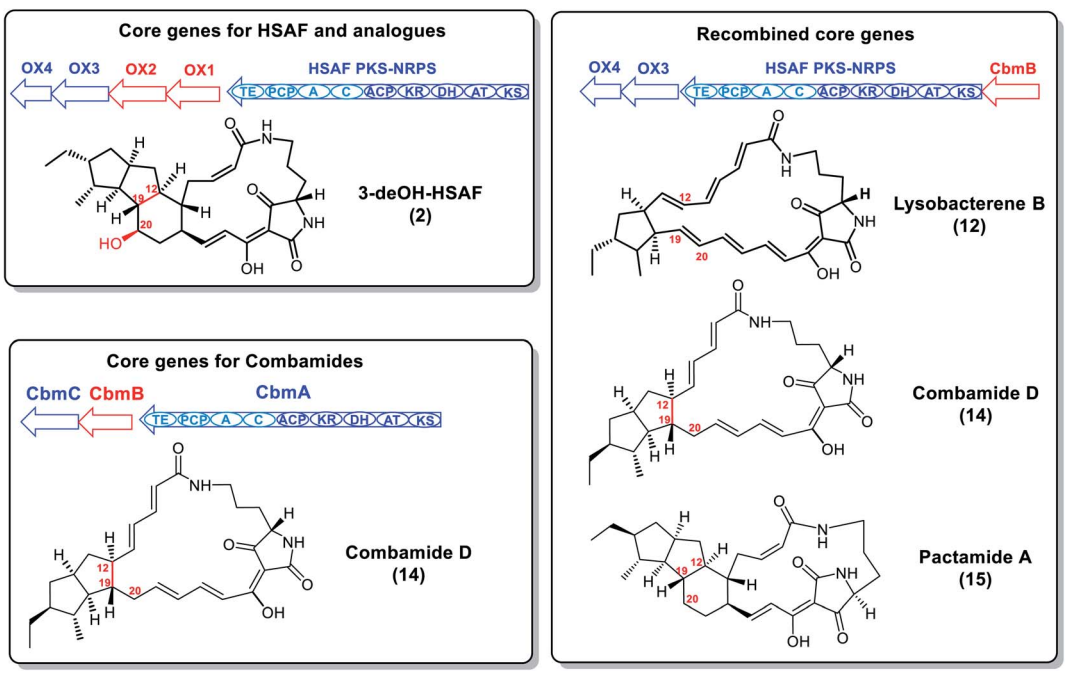


Fig. 4 Scheme for engineering a hybrid gene cluster containing the core genes for HSAF and analogues from *Lysobacter enzymogenes* with a combamide biosynthetic gene (*cbmB*) from *Streptomyces* sp. S10.[42] CbmB is a homologous enzyme of OX1/OX2 and also responsible for the second five-membered ring in combamides. The C20-hydroxy group is highlighted to indicate the structural feature shared by HSAF and alteramides, but not present in combamides. When the hybrid gene cluster was introduced into two strains (SR111 and strain S001) of *Streptomyces* three compounds were produced, and none of the compounds contain the C20-hydroxy group.

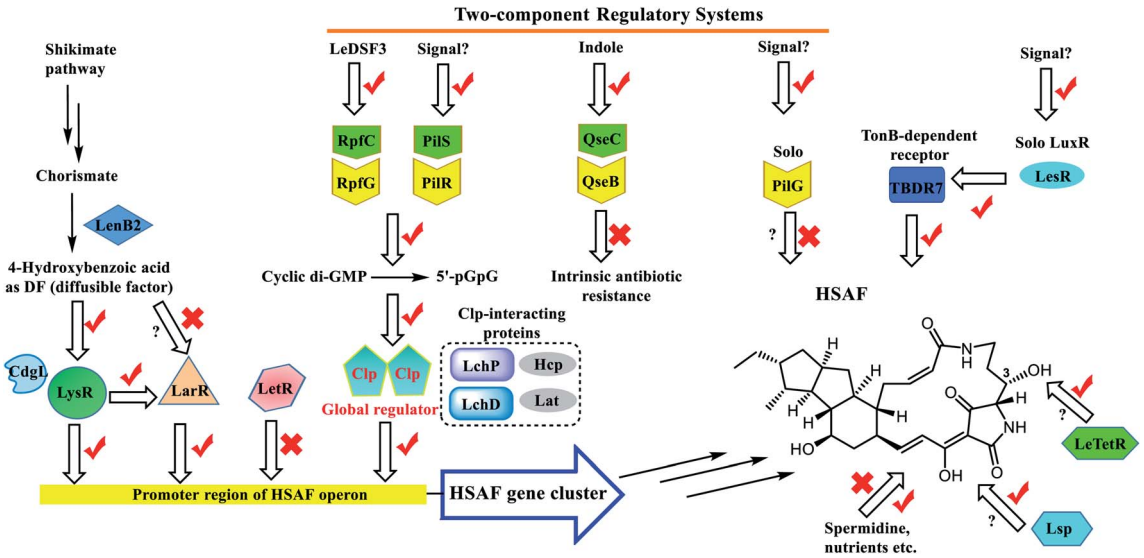


Fig. 5 Schematic representation of regulatory mechanisms for HSAF biosynthesis. Regulatory interactions are indicated by bold arrows with a check sign indicating a positive regulation and a cross sign indicating a negative regulation. Chemical reactions are indicated by line arrows. A question mark indicates that the signal or the regulatory mechanism is still not clear. For the details of the regulatory mechanisms, please refer to the text.

"compounds a–c",[44] and OX1 for frontamides,[38] while the enzymes that catalyze only the ring formation fall into another distinct clade, such as CbmB for combamides[43] and PtmB1 for pactamides.[45] Although direct evidence is yet to be obtained, these data support that the second-ring formation is coupled with the C20-hydroxylation in the biosynthesis of HSAF and analogues.[42] Like the reaction catalyzed by OX4, the reaction catalyzed by OX2 (also likely OX1) is very unusual and the molecular mechanism is worth further investigation.

2.1.3 Regulatory mechanisms. HSAF and analogues are the predominant natural products in *L. enzymogenes*. Although attractive compounds due to their distinct chemical structures and broad-spectrum antifungal activity, the large-scale production of these natural products has been challenging because their biosynthesis is very sensitive to environmental stimuli and nutrients. Several laboratories have conducted studies to understand the regulatory mechanisms affecting the biosynthesis of HSAF and analogues. These studies revealed a complex hierarchical network for HSAF regulation. The regulatory mechanisms include the global regulator Clp, two-component regulatory systems (TCS), specific transcription regulators, and small molecule signaling factors (Fig. 5). Given the diverse habitats of *Lysobacter*, these complex regulatory networks are likely necessary to allow *Lysobacter* to respond effectively to specific environmental conditions, such as microbial competition, available nutrients, and abiotic stresses. For example, recent studies have shown that a diverse array of *Lysobacter* genes are involved in the suppression of fungal hypha growth and spore germination, *via* regulation of production and secretion of HSAF and its analogues, as well as extracellular enzymes.[46]

2.1.3.1 Global regulator Clp. The role of the global regulator Clp has been the focus in several studies. Xu *et al.* recently reviewed this transcription factor in *L. enzymogenes*.[47] Clp is a cyclic-AMP-receptor (CRP)-like protein, which contains a conserved cyclic nucleotide (cNMP)-binding domain at the N-terminus and a helix-turn-helix (HTH) DNA binding domain at C-terminus.[48] Despite its name, Clp has been shown to specifically interact with the second messenger c-di-GMP (3′,5′-cyclic diguanosine monophosphate), rather than cAMP.[49] Clp can execute its regulatory functions by directly interacting with target DNA, and its functions can also be modulated by interacting with specific protein factors or small molecule signals (Fig. 5).

Kobayashi *et al.* first observed that disruption of the *clp* gene in *L. enzymogenes* C3 resulted in the loss of antifungal activity and lytic enzyme production.[50,51] Clp has since been reported to participate in production of lytic enzymes and antimicrobial compounds, as well as the process of fungal hyphae attachment. In the regulation of HSAF biosynthesis, Clp has been shown to directly bind to at least two sites (PA and PB) upstream of the *hsaf-pks/nrps* gene of HSAF BGC.[52] Both the PA and PB sites are required for Clp-mediated activation of transcription of the HSAF genes. The K_d for Clp binding to PA and PB was estimated to be 0.29 μM and 0.08 μM, respectively. Surprisingly, c-di-GMP primarily inhibited Clp binding to PA site, the lower affinity site, and the inhibition of PA site seemed sufficient to block the HSAF gene expression.

Clp can also interact with protein factors to modulate its regulatory activity (Fig. 5). One protein that can physically interact with Clp is LchP, a multi-domain protein located in the inner membrane.[52] LchP exhibits dual enzymatic activity, being able to both synthesize and degrade c-di-GMP. Through controlling the level of c-di-GMP, LchP regulates the equilibrium between free Clp (active) and c-di-GMP-bound Clp (inactive), and thus exerts control over downstream cellular

processes, including HSAF biosynthesis. Another Clp-interacting protein is LchD, which is also an inner-membrane protein that possesses enzymatic activity for c-di-GMP synthesis.[53] The binding to Clp stimulates LchD's activity to synthesize more c-di-GMP, resulting in increase of inactive Clp and a decrease of HSAF production. Interestingly, a structural protein (Hcp) of the inner tube of the type VI secretion system (T6SS) was recently found to bind to Clp.[54] The binding of Hcp to Clp seemed to prevent c-di-GMP from binding to Clp and thus block Clp inactivation by c-di-GMP, leading to an increase of HSAF production.

The activity of Clp can also be modulated by small molecules (Fig. 5). Two groups independently found that the biosynthesis of HSAF and analogues in *L. enzymogenes* OH11 was strictly controlled by spermidine, the most prevalent polyamine in bacteria.[55,56] The deletion of two key genes required for spermidine biosynthesis, *LeSDC* encoding *S*-adenosylmethionine decarboxylase and *LeADC* encoding arginine decarboxylase, led to a barely detectable level of HSAF and its analogues in the mutants, and loss of antifungal activity. Exogenous addition of spermidine into the media restored the production of HSAF and analogues, as well as the antifungal activity of the mutants. This showed that maintaining a proper level of spermidine in the cells is critical for antifungal metabolite production. Notably, *LeSDC* is closely clustered with the *clp* gene in the genome, and evidence suggested that spermidine may play an important role in maintaining Clp levels.[56]

In addition to being modulated by polyamines, Clp can be regulated by nutrient conditions of the growth media (Fig. 5). Puopolo *et al.* found that a lower quantity of nutrients was associated to a higher expression of *clp* in *L. capsici* AZ78.[57] This is consistent with the observation that a higher yield of HSAF and analogues was produced by *L. enzymogenes* grown in nutrient-poor media, since Clp is a positive regulator for HSAF biosynthesis.

It should be pointed out that Clp is also involved in regulation of other processes than natural product biosynthesis, such as type IV pilus (T4P)-driven twitching motility of *L. enzymogenes*.[58] Wang *et al.* investigated the Clp regulon, which is consisted of loci previously known to be regulated by Clp and of several new loci encoding antibiotic metabolite biosynthesis.[59] This study revealed that Clp could control the transcription of 775 genes belonging to 19 functional groups. A new regulatory factor encoded by *Lat* was also identified in this study (Fig. 5).[59] Lat (*Lysobacter* acetyltransferase) is a homologue of histone acetyltransferase Hpa2. The protein was found to control production of HSAF and function downstream of the Clp signaling pathway.

2.1.3.2 Solo LuxR of quorum sensing (QS) regulatory system. Bacteria coordinate gene expression in response to changes in population of their neighboring microorganisms and/or eukaryotic hosts.[60,61] One common approach to achieve this coordination is *via* a quorum sensing (QS) regulatory system, a cell-density-based regulatory mechanism mediated by chemical signal molecules in bacteria.[62–64] QS helps bacteria control a wide variety of bacterial phenotypes, including natural product biosynthesis, motility, biofilm formation, virulence, and symbiosis. The most common QS signals produced by Gram-negative bacteria are *N*-acyl homoserine lactones (AHLs). A typical AHL-responsive QS system consists of two protein components, LuxI and LuxR, where LuxR is a response regulator and LuxI is responsible for synthesizing the AHL signals, which interacts directly with the cognate LuxR protein at a high cell density or quorum concentration. As a consequence, a stable protein–AHL complex binds to certain gene promoter sequences known as *lux* boxes, to activate or repress target gene transcription. For a typical AHL-responsive QS, the LuxR protein is genetically paired with LuxI protein.[60,61] However, recent studies have shown many bacterial genomes containing another subgroup of LuxR-family proteins not genetically linked with a cognate LuxI. These LuxR-type homologues have been called orphan or solo LuxR proteins.[60,65]

In *L. enzymogenes*, Qian *et al.* identified a solo LuxR designated as LesR, which consists of an AHL-binding motif and an HTH DNA-binding motif (Fig. 5).[66] Over-expression of *lesR* resulted in a significant decrease of HSAF production and antimicrobial activities. LesR appears to act as an upstream regulator that controls expression of the downstream Clp.[67] Further studies using proteomics, bioinformatics and genetic approaches identified potential LesR targets, including HSAF biosynthetic genes. LesR could affect the expression of 33 genes, among which TBDR7, a member of the TonB-dependent receptor family, plays a crucial role in modulating HSAF production.[68] The transcription of *hsaf pks-nrps* for HSAF biosynthesis was almost completely abolished in the TBDR7-mutant. This suggests that LesR regulates TBDR7 and ultimately HSAF production (Fig. 5). However, it is unclear if *Lysobacter* produces AHL-type signals. Since LesR has a typical AHL-binding motif and an HTH DNA-binding motif, it is possible that LesR could bind to AHL signals produced by neighboring bacteria, and thus execute its subsequent regulatory effects.

2.1.3.3 Two-component regulatory system. The two-component regulatory system (TCS) is a prevailing mechanism for Gram-negative bacteria to sense, respond, and adapt to changes in the environmental stimuli.[69,70] The system consists of a membrane-bound histidine kinase and a cognate response regulator. The environmental stimuli are sensed by the kinase, which relays the signal to its partner, the response regulator protein, through phosphorylation. The response regulator usually contains multiple domains, including a receiver domain and DNA binding domain. Phosphorylation activates the response regulator to stimulate or suppress the expression of target genes in its regulon. There are 54 putative TCSs in the genome of *L. enzymogenes* and several TCS have been studied for their potential roles in natural product biosynthesis.[71]

2.1.3.3.1 RpfC/RpfG system. The diffusible signal factors (DSF) are a group of small molecule signals first identified from *Xanthomonas campestris* pv. *campestris* (Xcc).[72,73] The *Rpf* gene cluster encodes the enzymes responsible for DSF biosynthesis as well as proteins for regulation. At least two enzymes are involved in the biosynthesis of DSF. RpfF is a bifunctional enzyme, with enoyl-CoA hydratase and thioesterase activities;

RpfB is an acyl-CoA synthetase. RpfC and RpfG make up the TCS that is responsible for sensing and transducing the DSF signals, respectively. RpfC is a histidine kinase sensor protein, which also suppresses the biosynthesis of DSF if its intracellular domain binds to RpfF. However, when the extracellular DSF concentration reaches the threshold value, RpfC undergoes auto-phosphorylation and releases RpfF, which in turn becomes active and synthesizes more DSF. At the same time, RpfC phosphorylates its cognate response regulator RpfG, which possesses c-di-GMP phosphodiesterase activity and degrades the second messenger c-di-GMP.

The *rpf* gene cluster has been found in *L. enzymogenes*, with most genes showing a high similarity to their counterparts in *X. campestris* pv. *campestris*.[74,75] Prior to the studies of DSF signaling system, phenotypes associated with the DSF signaling were known in *L. enzymogenes* including motility, virulence, biofilm formation, as well as the production of extracellular polysaccharide (EPS) and extracellular enzymes.[66] Mutation of *rpfF* dramatically reduced HSAF production, indicating the crucial role of the DSF signaling pathway in the production of HSAF. Subsequently, several DSF-like molecules were identified from the culture of *L. enzymogenes* OH11, using a green fluorescence reporter strain of *X. campestris* that contained a *gfp* gene under the control of a DSF-inducible promoter.[76,77] The signal molecules were produced in an extremely low yield and tended to produce only in small-volume cultures. It took 3000 flasks of 100 mL cultures each to accumulate a sufficient amount of the molecules for analyses by ESI-MS, FT-IR, and NMR. One of the compounds that showed the most potent activity to induce the green fluorescence in the reporter strain was determined to be 13-methyltetradecanoic acid (*Le*DSF3).[77] Exogenous addition of *Le*DSF3 in *L. enzymogenes* cultures significantly increased the HSAF yield and the transcription of HSAF biosynthetic genes and expression of *clp*. Furthermore, no HSAF production was observed in the *clp* deletion mutant. These results clearly showed that *Le*DSF3 is a positive signal for production of HSAF, and that this regulation is mediated by the RpfC/RpfG-Clp signaling pathway (Fig. 5). As discussed above, a high level of c-di-GMP prevents the binding of the global regulator Clp to one of the two promoter sites on HSAF operon and results in reduction of gene expression and ultimately HSAF production.[52] The DSF signals activate the c-di-GMP phosphodiesterase activity of RpfG that degrades c-di-GMP, resulting in the release/activation of Clp for HSAF biosynthesis. Recently, an *rpfB* homolog (*rpfB2*) was found outside the canonical *rpf* gene cluster.[78] The *rpfB* gene encodes a fatty acyl-CoA ligase, and its enzyme activity can influence the free fatty acid metabolism and thus the production of the fatty acid-based DSF signals.

2.1.3.3.2 QseC/QseB system. A characteristic of *Lysobacter* is its intrinsic resistance to multiple antibiotics. Han *et al.* 2017 found the inactivation of the TCS QseC/QseB by an inhibitor (LED209) or by gene deletion of the TCS in *L. enzymogenes* resulted in the dramatically increased production of a metabolite that was barely detectable in the untreated culture (Fig. 5).[71] This metabolite was identified to be indole. Indole is known as an interspecies signal in gut microbiota and mammalian hosts.[79,80] To find out the role of indole in *Lysobacter*, Han *et al.* chemically synthesized a fluorescent indole probe that could label the cells.[71] Their results showed that the probe could label the wild type and the deletion mutant of QseB (cytoplasmic response regulator), but could not label the deletion mutant of QseC (membrane bound sensor). Most intriguingly, the authors found that indole caused the intrinsically resistant *L. enzymogenes* to become susceptible to multiple antibiotics. QseC containing point mutations at conserved amino acids also exhibited susceptibility to multiple antibiotics. The finding that indole could reverse the resistance of *L. enzymogenes* to common antibiotics may have implications for efforts to address drug-resistant pathogens. Additionally, the ability to use indole to reduce *L. enzymogenes*'s multiple-antibiotic resistance could broaden the array of selection markers available for use in genetic manipulations of *Lysobacter*.

To understand the mechanism underlying the indole-mediated reversal of intrinsic antibiotic resistance in *L. enzymogenes*, Wang *et al.* 2019 identified a dual-function importer (BtuD) that is able to transfer both vitamin B12 and antibiotics.[81] The authors showed that indole stimulated *btuD* expression and promoted import of extracellular vitamin B12. The overexpressed importer also caused *Lysobacter* to increase antibiotics uptake to a lethal level. Notably, this mechanism appears to be a common approach for multiple bacterial species. The findings are interesting as they suggest that some of the well-developed "old antibiotics" could be reused to fight against resistant pathogens by coupling with effective small signal molecules that can facilitate the reversal of the antibiotic resistance. The author also noted that the antibiotic resistance of *Lysobacter* could be restored by the quorum sensing signal *Le*DSF.[81] Furthermore, indole was found to cause *Lysobacter* to have irregular, random twitching motility with significantly increased speed through activating expression of type IV pilus genes (*pil*).[82] Notably, indole and *Le*DSF work synergistically in the regulation of twitching motility.

2.1.3.3.3 PilS/PilR system. The third TCS in *Lysobacter* that has been studied is the PilS/PilR system (Fig. 5). This system is usually involved in twitching motility but was recently also found to have an impact on the biosynthesis of HSAF. T4P (Type IV pilus) is involved in the twitching motility, which is observed as the bacterial surface movement *via* pili.[83,84] Regulation of T4P involves many signal transduction systems including the TCS PilS/PilR. Surprisingly, the response regulator PilR was found to control the antifungal antibiotic production *via* a c-di-GMP pathway.[85] In the *pilR* deletion mutant of *L. enzymogenes*, the HSAF production was significantly decreased and c-di-GMP level significantly increased. PilR of *L. enzymogenes* was able to bind to the *pilA* promoter and upregulate *pilA* expression, which in turn regulated pilus synthesis and twitching motility *via* a traditional pathway. However, the regulation of HSAF production by PilR was found to be independent of pilus formation, and exactly which c-di-GMP signaling pathway is controlled by PilS/PilR is not clear.

2.1.3.3.4 Solo PilG. Another TCS involved in T4P-dependent twitching motility in *L. enzymogene* is an orphan response

regulator PilG (Fig. 5).[86] Mutation of *pilG* abolished the twitching motility of *L. enzymogenes*. Unexpectedly, mutation of *pilG* also increased HSAF production and the transcription of its key biosynthetic gene *hsaf pks-nrps*, suggesting that PilG is a negative regulator of HSAF biosynthesis. However, the mechanism for PilG regulation of HSAF production is currently unknown, and the environmental stimuli of PilG in the HSAF regulation is unclear.

2.1.3.4 Other regulatory systems

2.1.3.4.1 TetR transcription factor. TetR is a family of transcription factors that control a wide range of gene expression in bacteria, including genes involved in multidrug resistance, osmotic stress, enzymes in different catabolic pathways, general metabolism, and bacterial pathogenicity.[87] They typically act as transcriptional repressors. Two TetR genes have been associated with HSAF production in *L. enzymogenes* OH11 (Fig. 5). *Le*1552 (*Let*R) was found to repress the transcription of HSAF biosynthetic genes.[88] In-frame deletion of *LetR* in OH11 significantly increased the HSAF level and key biosynthetic gene transcription, whereas overexpression of *LetR* in OH11 significantly reduced the HSAF level and the gene expression. Although it seems evident that *Let*R regulates the HSAF biosynthetic gene expression *via* a direct interaction manner, the specific binding sites of *Let*R still remains unclear.

Another TetR regulator (*Le*TetR) encoded by orf3232 was recently found to positively regulate the production of HSAF and alteramides (Fig. 5).[89] Deletion of *Le*TetR resulted in significant reduction of HSAF and alteramides, while the production of their biosynthetic precursors, 3-deOH HSAF and 3-deOH alteramides, was barely affected. This is an interesting finding as it implies that *Le*TetR selectively regulates the final step of the HSAF biosynthetic pathway, namely, the 3-hydroxylation of 3-deOH-HSAF. The 3-hydroxylation step converts the barely active precursors, the 3-deOH compounds, to the antifungals HSAF and alteramides.[31] The exact mechanism underlying this unusual TetR regulator also remains to be investigated.

2.1.3.4.2 LysR transcription factor. In addition to DSF, another type of small signal molecules, diffusible factors (DF), was identified in *X. campestris pv. campestris*.[90,91] These factors were identified as 3-HBA (3-hydroxybenzoic acid) and 4-HBA (4-hydroxybenzoic acid).[92] The biosynthesis of DF requires a pteridine-dependent oxygenase XanB2, which functions as a chorismatase (chorismate lyase) that transforms chorismate, the end-product of the shikimate pathway, to 4-hydroxybenzoate and pyruvate. This enzyme was found to participate in the biosynthesis of both 3-HBA and 4-HBA.[92] 3-HBA regulates the biosynthesis of xanthomonadin, a defensive pigment, while 4-HBA plays a significant role in Coenzyme Q8 biosynthesis. *L. enzymogenes* OH11 contains LenB2, a homologous of XanB2 (Fig. 5). LenB2 was found to work in the same way as XanB2 in converting chorismate to 4-HBA and 3-HBA.[93] A recent study

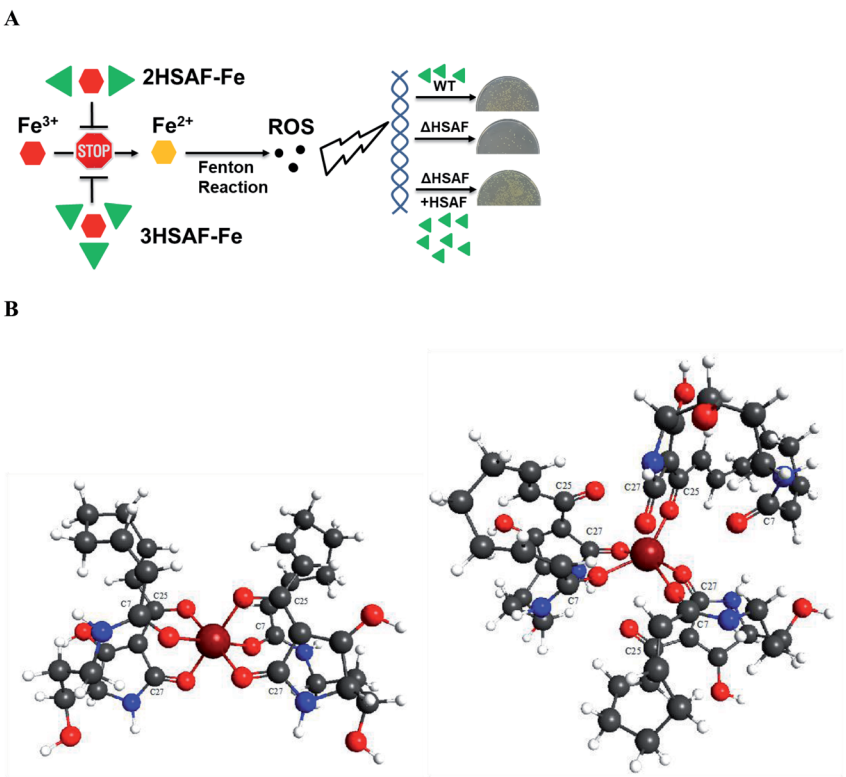


Fig. 6 (A) Schematic illustration of HSAF's biological function as a novel oxidative stress modulator for *Lysobacter enzymogenes* to survive under reactive oxygen species (ROS). (B) Molecular structure of 2HSAF–Fe^{3+} and 3HSAF–Fe^{3+} complexes obtained from the molecular mechanical force field method and quantum mechanical method. All PoTeM share the 2,4-pyrrolidinedione-embedded macrolactam, which contains the absolutely conserved carbonyl groups at C7, C25 and C27 that participate in the iron ion coordination. The figure is modified from Yu *et al.*[109] with permission.

suggested that 4-HBA is important for HSAF biosynthesis. 4-HBA could interact with $LysR_{Le}$, a LysR-family transcription factor that binds to the promoter region of HSAF operon and thus positively regulates HSAF production.[94] 4-HBA appears to stabilize the $LysR_{Le}$–DNA complex, resulting in strong binding of the transcription factor to the promotor region of HSAF operon.

2.1.3.4.3 LarR transcription factor. Three putative transcription factor genes, *Le*4806, *Le*4969, *Le*3904, were also identified within the 4-HBA regulatory pathway. Among these, *Le*4806 encodes a LarR transcription factor that can bind to the promoter of the HSAF biosynthetic gene operon, leading to positive regulation of HSAF production (Fig. 5). LarR is a downstream component of the 4-HBA regulatory pathway.[94] $LysR_{Le}$ can function as a receptor of 4-HBA and also has the ability to bind to the promotor of *LarR*. Surprisingly, 4-HBA and $LysR_{Le}$ affect the *LarR* transcription in opposite ways. 4-HBA suppresses the transcription of *LarR* while $LysR_{Le}$ promotes its transcription, which suggests that 4-HBA could utilize two independent pathways to control HSAF production. In one pathway, 4-HBA interacts with $LysR_{Le}$ and enhances its binding to the promotor region of HSAF, leading to increase of HSAF production. In the other pathway, 4-HBA interacts with an unidentified factor and leads to the suppression of *LarR*.

2.1.3.4.4 CdgL. Recently, CdgL, a co-activator of the transcription activator LysR, was investigated.[95] CdgL increased the apparent affinity of LysR to the HSAF operon promoter in the absence of c-di-GMP (Fig. 5). When c-di-GMP concentration increased, CdgL binds to c-di-GMP, which results in the disassembly of the CdgL–LysR–DNA complex and the release of the LysR–DNA complex. The disassembly of the complex lowers the HSAF operon expression, and consequently HSAF production.

2.1.3.4.5 Ax21. Proteins of the Ax21 family are known to be involved in cell–cell signaling and virulence related traits. In 2017, Wang *et al.* identified three genes encoding *Lysobacter* small proteins, Lsp1, Lsp2 and Lsp3, which are homologous to Ax21 proteins in *L. enzymogenes* OH11 (Fig. 5).[96] The authors found that the small proteins could positively regulate HSAF and WAP-8294A2 biosynthesis.

2.1.4 Transport and physiological functions. HSAF and its analogues are present both inside and outside of *L. enzymogenes* cells. However, very little is known about their transport. In 2018, Meers *et al.* reported that outer membrane vesicles (OMV) could play an important role in the export of HSAF and analogues in *L. enzymogenes* C3.[97] Gram-negative bacteria are prone to release OMV, usually in response to stress-related conditions.[98–100] When OMV sprout from the outer membrane, they simultaneously encapsulate numerous contents, such as genetic materials, enzymes, periplasmic and membrane-bound proteins, toxins and virulence factors. The molecules are delivered to outside of the cells to play various roles in interactions with other microbes and the surrounding environment.

Meers *et al.* found that strain C3 produced OMV in 1/10 tryptic soy broth (TSB) or minimal medium in either late log or stationary phase cultures.[97] The OMV had a size around 130–150 nm in diameter and contained lipopolysaccharides, nucleic acids, proteins and antifungal molecules. The antifungal activity of the OMV was tested against the yeast *Saccharomyces cerevisiae* and the filamentous fungus *Fusarium subglutinans*. The C3 OMV directly inhibited their growth, and the inhibition was dependent on physical contact with the yeast or fungal cells. Moreover, when the OMV samples were treated with heat at 70 °C for 30 min, the growth inhibitory activity remained entirely unchanged. Further analyses indicated that the OMV contained most if not all of the heat-stable antifungal activity. OMV were also extracted from a C3 mutant strain, in which the *hsaf pks-nrps* gene for HSAF biosynthesis was disrupted, and the OMV from the mutant exhibited no antifungal activity. This was the first report of the OMV-mediated export of HSAF and its analogues. Interestingly, the antifungal compounds appeared to be membrane-bound and were not diffusive in aqueous conditions.

L. enzymogenes is a facultative predator of surrounding microorganisms.[101] The membrane-bound compounds in OMV could be delivered and transferred into fungal cells through membrane–membrane interactions, which could serve as a way for the predatory *L. enzymogenes* to prey on the fungi.[97] Growing evidence has shown that OMV play a significant role in host–pathogen interactions.[102,103] Recently, Yue *et al.* reported an OMV-mediated co-delivery of HSAF and analogues with a lytic polysaccharide monooxygenase (LPMO) in strain OH11 of *L. enzymogenes*.[104] Several families of LPMO have been reported from various microorganisms, but this type of enzyme has not previously been reported in *Lysobacter*. LPMO is a group of copper-containing enzymes that catalyze oxidative cleavage of the glycosidic bonds of polysaccharides, such as cellulose and chitin, to produce oligomeric products of 1,5 δ-lactones and aldonic acids.[105–108] Unlike hydrolases of polysaccharides, such as chitinase and β-1,3-glucanases, LPMOs use the copper-activated molecular oxygen to oxidize the substrate and are capable of cleaving recalcitrant polysaccharides with crystalline structures in fungal cell walls. Yue *et al.* revealed a synergistic strategy used by the predatory *L. enzymogenes* OH11 during interactions with fungal prey.[104] OH11 uses LPMO as a "fungal cell wall opener" and the antifungal HSAF and analogues as "warheads". Both the LPMO and the antifungal small molecules are packaged together in OMV as a lethal cargo that is delivered to fungal prey. Once the tough cell walls are broken in by the wall-opener enzyme LPMO, the other enzymes such as the hydrolases could join the degradation process. Moreover, the enzyme-cleaved fungal cell wall products are strong inducers for production of HSAF and analogues, which in turn inhibit fungal growth and kill the prey. The killed fungal cells then become substrates for LPMO and other enzymes. This study revealed a "smart" strategy utilized by the predatory *Lysobacter* when preying on fungi. Furthermore, the study also elucidated one biological function of HSAF and analogues: their important role in *Lysobacter* predation on fungi.

Apart from the role in predation, HSAF and analogues have recently been found to function as oxidative stress modulators.[109] Yu *et al.* observed brown-orange substances that were formed in *L. enzymogenes* OH11 culture or in HSAF extracts,

when the cultures or the extracts were combined with various iron salts. The substances were absent in the culture or extract from the HSAF mutants. HPLC and mass spectrometry detected the formation of 2HSAF–Fe^{3+} and 3HSAF–Fe^{3+} complexes, the existence of which was supported by molecular mechanical and quantum mechanical calculations.[109] In 2HSAF–Fe^{3+} complex, each of the two HSAF molecules provides three oxygen atoms (at C7, C25, and C27 carbonyl groups) to form 3 coordinate bonds to the iron ion (Fig. 6). In 3HSAF–Fe^{3+} complex, two HSAF molecules used the oxygen atoms at C7 and C27 carbonyl groups, and one HSAF molecule used the oxygen atoms at C25 and C27 carbonyl groups. Since the carbonyl oxygen at C7, C25 and C27 are absolutely conserved in all PoTeM, the data suggest that similar iron complexes could also be formed in other PoTeM producers. The structure of 2,4-pyrrolidinedione-embedded macrolactam that is common to all PoTeM may represent a new iron-chelating scaffold of microbial metabolites. Future studies should look into other PoTeM–metal complexes and their potential physiological functions.

The HSAF–Fe^{3+} complexes exhibited absorptions at ~410–600 nm that gave the observed orange-brown appearance. The association constant (K_a) of HSAF–Fe^{3+} was determined to be 2.71×10^6 M^{-1}, which is significantly smaller than that of typical siderophores. This suggests that HSAF and analogues in strain OH11 may not function as siderophores for iron acquisition from the environment. To investigate the potential physiological functions of the compounds in *Lysobacter*, Yu *et al.* conducted a series of *in vivo* and *in vitro* experiments. Their data showed that HSAF protected DNA from degradation in high Fe^{3+} concentration, H_2O_2, or under UV radiation (Fig. 6). In contrast, the HSAF mutants barely survived under oxidative stresses and exhibited markedly increased production of reactive oxygen species (ROS). The mutants could be rescued by addition of HSAF, which significantly lowered the ROS production. Thus, HSAF and analogues in OH11 can modulate oxidative damages to the cells, in addition to their antifungal activity. This was a previously unrecognized biological function for this group of natural products.[109]

2.2 Cyclodepsipeptides

Cyclodepsipeptides are a family of cyclic molecules with a vast array of biological activities such as antifungal, antibiotic, antitumor, anti-inflammatory, and insecticidal activities.[110] *Lysobacter* species are considered "peptide production specialists", because they are particularly rich in nonribosomal peptide natural products.[4] Several nonribosomal peptides have been isolated and identified from *Lysobacter*. These include the cyclodepsipeptides WAP-8294As, WBP-29479A1, tripropeptins, lysocins, and lysobactin. The peptides typically contain 8–12 amino acid residues, many of which are nonproteinogenic amino acids carrying various modifications. Most of the peptides also contain a β-hydroxyl fatty acyl chain of varied chain length and shape (linear or branched). All cyclodepsipeptides possess complex chemical structures and exhibit potent antibiotic activity.

2.2.1 WAP-8294A

2.2.1.1 Chemical structures, antibiotic activity, and mode of action of WAP-8294A. WAP-8294As are a family of at least 19 closely related cyclic lipodepsipeptides isolated from *Lysobacter* sp. WAP-8294 and *L. enzymogenes* OH11 (Fig. 6).[111,112] The peptides have potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA).[113] Within the family, WAP-8294A2 (**16**, Lotilibcin) reached phase I/II clinical studies by aRigen Pharmaceuticals in 2009 and later was reacquired by Dakota Life Science in 2017, which intended to develop A2 as a new chemical entity (NCE) for treatment of skin infections, pneumonia, bacterial endocarditis, and osteomyelitis.

A2 is the most abundant component of the family produced by *Lysobacter* strains and also the most extensively studied WAP-8294A. Its structure was established by MS, 1D and 2D NMR, as well as total chemical synthesis.[111,114] Several members of the family, such as A1 (**18**), A4 (**20**), Ax8 (**22**), Ax9 (**23**), and Ax13 (**24**), were isolated as minor components, and their structures were proposed based on ESI-MS/MS analysis. The rest of the family were detected only by HPLC, and their structures have not been fully established. Recently, six new WAP-8294A compounds (AZ1, AZ2, AZ3, AZ4, AZ6 and AZ7; **25–30**) were identified in *L. enzymogenes* OH11 using UHPLC-HR-MS/MS and molecular networking analysis.[115] The structure of the compounds differs from A2 in one of the amino acid residues (AZ1, AZ2, AZ3, and AZ6) or in (*R*)-3-hydroxy-fatty acid (AZ4) (Fig. 6). Zhu *et al.* also identified AZ5 (**22**), which lacks the *N*-methyl group on L-Val, indicating that AZ5 is the same as Ax8, a previously reported minor compound.[112] In addition, an unusual compound, AZ7 (**30**), was also identified in this work. The molecular mass of AZ7 is significantly smaller than all known WAP-8294As (Fig. 7). The study showed that AZ7 is a cyclic peptide with only 9 residues, which are in the same sequence and composition as the number 3 residue (L-Ser) through number 11 residue (D-Orn) of A2. This peptide does not contain the (*R*)-3-hydroxy-fatty acid moiety, the first two amino acid residues, L-Ser and D-*threo*-β-OH-Asn, and the last amino acid residue, L-*N*-MeVal.[115] This structure raised an interesting question regarding the origin of AZ7: whether the 9-residue peptide is a degradation product of parent compounds, or an aberrant biosynthetic product due to an unusual start (from L-Ser) and premature release of the peptide from the NRPS assembly line, through a cyclization between L-Ser and D-Orn.

Itoh *et al.* conducted a total synthesis of A2 and its deoxy analogue (deoxy-WAP8294A2, **17**), which showed an equal potency against various strains of Gram-positive bacteria including MRSA.[114] They also found that the mode of action of the antibiotics was due to membrane disruption, which depended on the presence of menaquinone, an essential factor for the bacterial respiratory chain. Yu *et al.* showed that A2 exhibited potent (MIC 0.2–0.8 μg mL^{-1}) and selective activity against *S. aureus* among a panel of 54 clinically relevant isolates of Gram-positive bacteria.[116] In 2020, Chen *et al.* performed another total synthesis of A2 and multiple analogues.[117] They also conducted a systematic structure–activity relationship (SAR) study of A2, through evaluating the antibiotic activity of

eleven alanine-scanning analogues and eight lysine-scanning analogues of A2.

2.2.1.2 Biosynthesis of WAP-8294A. During the genome mining of natural products in *L. enzymogenes* OH11, a potent antibiotic was isolated and identified to be WAP-8294A2.[118] This led to the identification of the BGC for WAP-8294A family in *L. enzymogenes* OH11. The BGC contains three structural genes, with ORF1 (*wapC*) encoding the NRPS-associated protein MbtH, ORFs 2 and 3 (*wapB* and *wapA*) encoding two multi-module NRPS, WAPS1 and WAPS2 (Fig. 8). WAPS1 and WAPS2 together make up a total of 45 domains for the 12 modules responsible for the recognition, activation, thiolation, condensation, and modification of the 12 amino acids of WAP-8294A.[118]

Members of the WAP-8294A family differ mainly in the 3-hydroxyl fatty acyl chain. The main component A2 contains (*R*)-3-hydroxy-7-methyloctanoic acid. The mechanism by which the fatty acyl group is incorporated into WAP-8294A is still not very clear. The BGC in *L. enzymogenes* OH11 does not contain an acyl-CoA ligase (ACL) gene. Chen *et al.* identified seven putative ACL genes in the OH11 genome, and gene deletion experiments

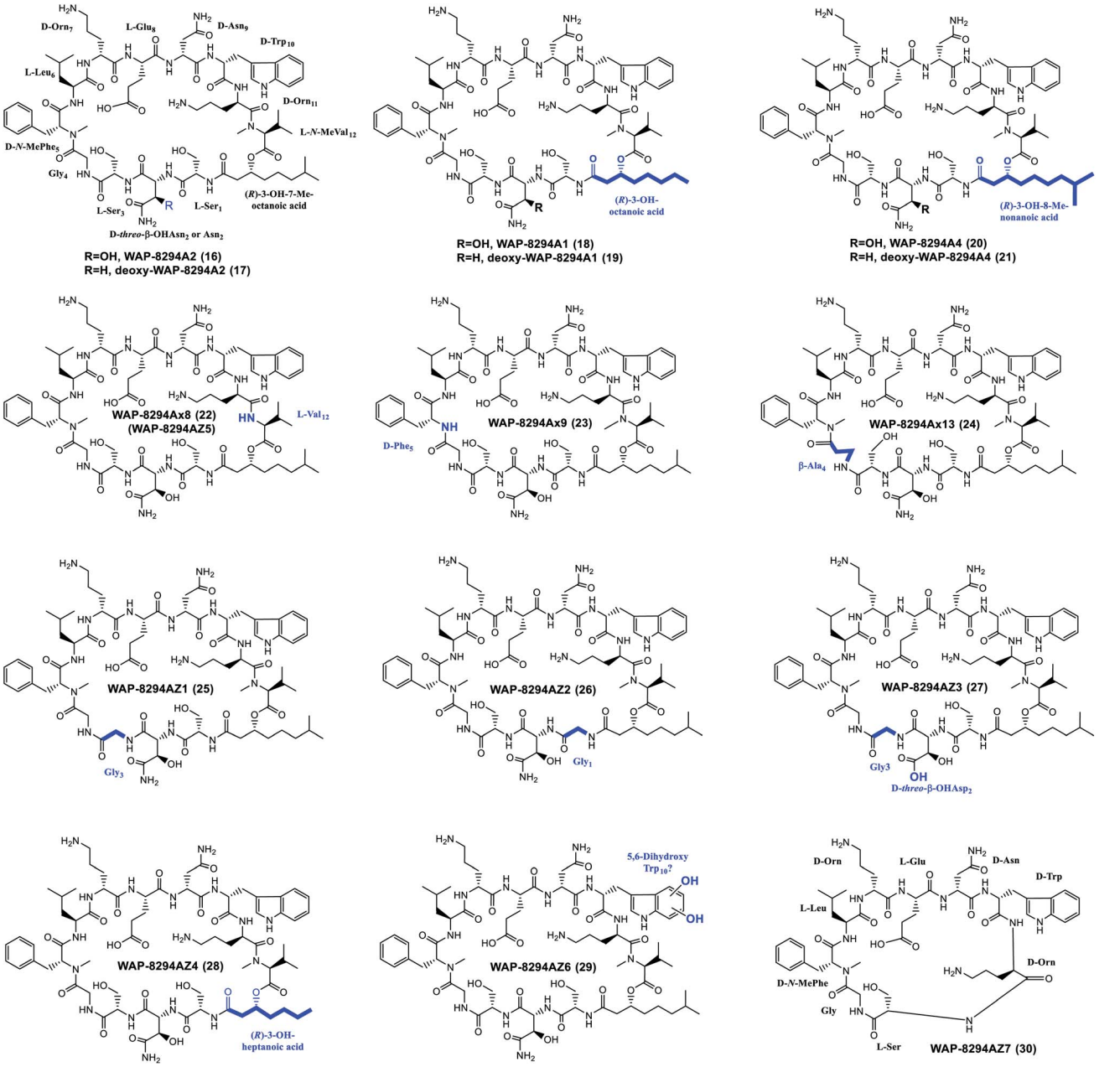
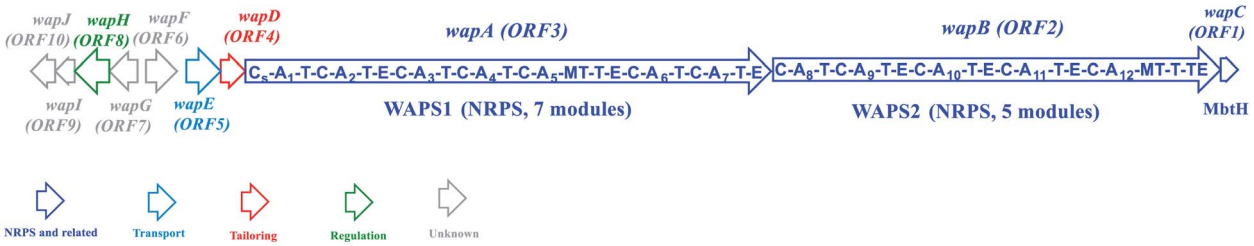


Fig. 7 Chemical structures of WAP-8294A congeners isolated from *Lysobacter*. The structural moieties highlighted in blue are to show the differences from WAP-8294A2, the predominant congener produced by *Lysobacter*. WAP-8294AZ7 is a cyclic peptide with only 9 residues and does not contain the (*R*)-3-hydroxy-fatty acid moiety, the first two amino acid residues, L-Ser and D-*threo*-β-OH-Asn, and the last amino acid residue, L-*N*-MeVal, of WAP-8294A2.



ORF	Proposed function	ORF	Proposed function
wapC	MbtH-like protein	*wapF*	Pectin lyase fold/virulence factor
wapB	Nonribosomal peptide synthetase	*wapG*	Lytic endopeptidase preproenzyme
wapA	Non-ribosomal peptide synthetase	*wapH*	TonB-dependent receptor
wapD	TauD-like dioxygenase	*wapI*	SapC-related protein
wapE	Efflux transporter	*wapJ*	Pass1-related protein

Fig. 8 The *wap* biosynthetic gene cluster and predicted functions of ORFs. The domains are as follows: C_s, $C_{starter}$ which is the condensation domain that acylates the first amino acid residue with the β-hydroxy fatty acid; C, condensation; A, adenylation; T, thiolation; MT, methyltransferase; E, epimerase; and TE, thioesterase.

showed that A2 production was affected by multiple ACL genes.[119] Among the genes, the deletion of ACL6 exhibited the most significant impact on A2 production. To find more clues, the authors chemically synthesized several (*R*)-3-hydroxy fatty acids and their acyl SNAC (*N*-acetylcysteamine thioesters) as putative substrates for the ACLs. Feeding experiments and *in vitro* enzyme assays using six purified ACL enzymes showed that ACL6 is the main player for fatty acyl activation and incorporation in A2 biosynthesis. The involvement of multiple functionally overlapping ACLs revealed the molecular basis for the acyl chain diversity in WAP-8294As.[119]

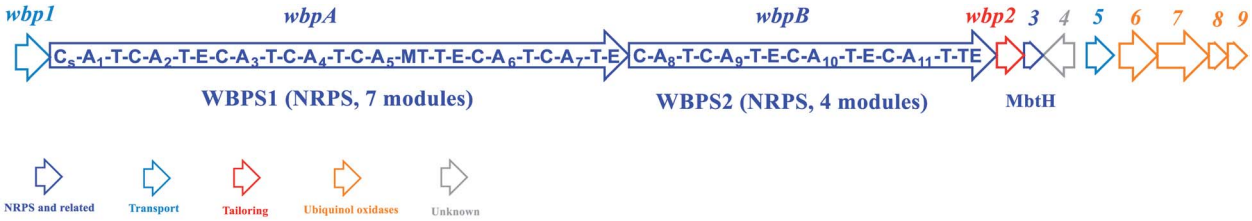
Seven accessory genes, *wapD-J* (ORFs 4–10), are clustered with the three structural genes, *wapA-C* (Fig. 8). The functions of the accessory genes have not been fully determined, but probably are related to modification, regulation and resistance of WAP-8294As.[118] Recently, Zhu *et al.* conducted a series of genetic manipulations on the accessory genes including ORF4 (*wapD*), which is homologous to genes encoding 2-ketoglutarate dependent dioxygenases.[115] The *wapD* mutants with a single-point mutation at the active sites produced deoxy-WAP-8294A (**17**, **19**, **21**) (Fig. 7). The result indicates that the ORF4-encoding enzyme catalyzes the β-hydroxylation of the D-asparagine residue in WAP-8294As.

2.2.2 WBP-29479A1

2.2.2.1 Chemical structures, antibiotic activity, and mode of action of WBP-29479A1. WBP-29479A1 is a new cyclic lipodepsipeptide isolated from *L. antibioticus* ATCC 29479 using the genome mining approach (Fig. 9).[120] Among the putative BGCs in the genome of strain 29 479, a cryptic gene cluster (*wbp*) was found to contain two large NRPS genes (*wbpA* and *wbpB*) that were different from any known BGC. Notably, the cluster contains four genes (*wbp6*–*wbp9*) encoding four subunits of the cytochrome *o* ubiquinol oxidase complex, which are the terminal enzymes of an electron transport chain for bacterial respiration.[121,122] Through testing a variety of culture conditions, the authors were able to detect a compound (WBP-29479A1, **31**) that was produced only under certain conditions. Deletion of *wbpB* gene abolished the production of **31**. The structure of **31**, including six nonproteinogenic amino acid residues and the 3-hydroxy fatty acid, was established by ESI-HRMS, 1D and 2D-NMR.[120] The absolute configuration of the amino acids was determined by the advanced Marfey's method.[123] The structure of γ-aminobutyric acid residue was confirmed by comparing with standard α-, β-, and γ-aminobutyric acid, and the absolute configuration of (*R*)-3-hydroxy-7-methyloctanoic acid was established by comparing with the chemically synthesized D,L-3-OH-7-methyloctanoic acid and the biologically produced (*R*)-3-hydroxy-7-methyloctanoic acid.

WBP-29479A1 is a potent anti-MRSA compound, with MIC values of 0.25–2 μg mL^{-1}. It also has activity against *S. epidermidis* with MIC ≤0.25 μg mL^{-1}. Its antibiotic activity is dependent on the presence of menaquinone, an essential component for bacterial electron transfer chain. In contrast, ubiquinone, which is involved in mammalian electron transfer chain, had no effect on the antibiotic activity.[120] This mode of action of WBP-29479A1 is similar to that of WAP-8294A2.[114]

2.2.2.2 Biosynthesis of WBP-29479A1. The two genes, *wbpA* and *wbpB*, in the BGC are predicted to encode a total of 40 domains, making up the 11 modules of the NRPS assembly line that is responsible for recognition, activation, thiolation, condensation, and modification of the 11 amino acids of WBP-29479A1.[120] The module organization of the assembly line is co-linear with the composition of the peptide (Fig. 9). Like the BGC for WAP-8294A, the BGC for WBP-29479A1 does not contain an acyl CoA-ligase gene, suggesting that *in trans* acyl-CoA ligases may be responsible for the activation and incorporation of the (*R*)-3-hydroxy fatty acid. Although only one compound has been identified so far, it seems likely that the *wbp* cluster does produce multiple congeners with varied (*R*)-3-hydroxy-fatty acyl chains. The biosynthetic genes in the BGC remain to be characterized through biochemical approaches or expression in heterologous hosts. Most intriguingly, the functions of the four



ORF	Proposed function
wbp1	ABC transporter ATP-binding protein
wbpA	Nonribosomal peptide synthetase
wbpB	Non-ribosomal peptide synthetase
wbp2	PLP-dependent decarboxylase
wbp3	MbtH-like protein
wbp4	Membrane protein
wbp5	MFS permease
wbp6	Ubiquinol oxidase subunit II
wbp7	Cytochrome o ubiquinol oxidase subunit I
wbp8	Cytochrome o ubiquinol oxidase subunit III
wbp9	Cytochrome o ubiquinol oxidase subunit IV

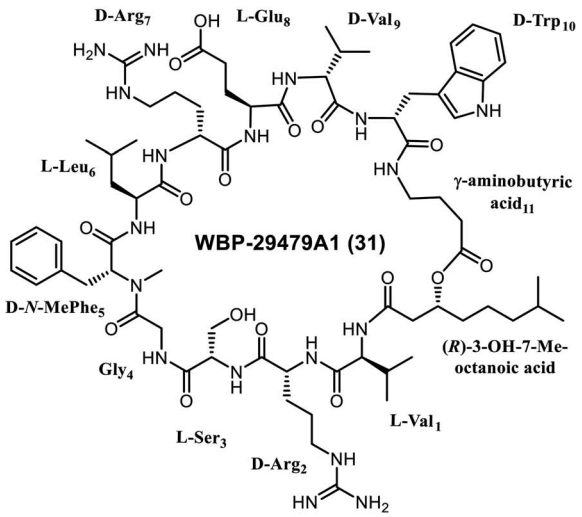


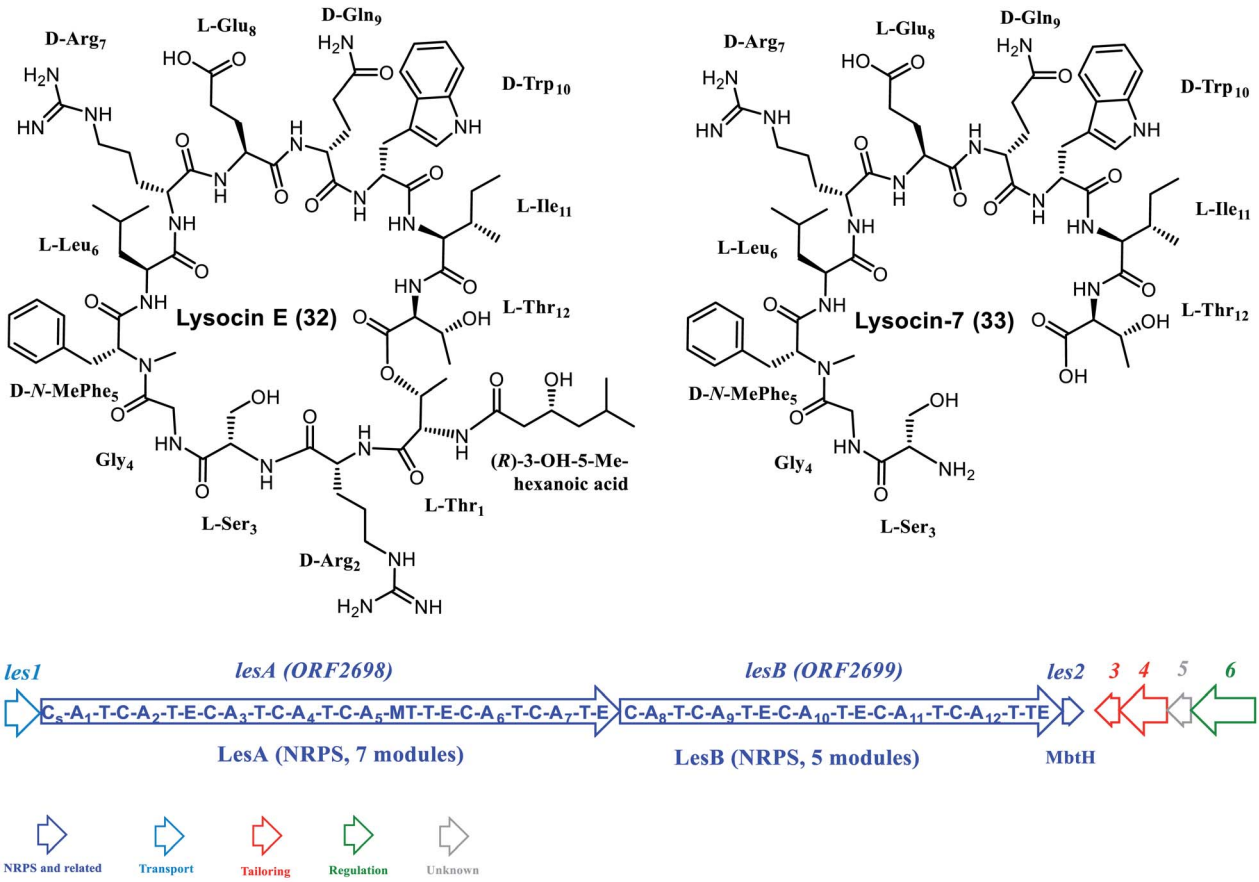
Fig. 9 The *wbp* biosynthetic gene cluster, predicted functions of ORFs in the gene cluster, and chemical structure of the main product, WBP-29479A1.

tightly clustered genes (*wbp6*–*wbp9*) that code for the cytochrome *o* ubiquinol oxidase complex are worth further investigation. It remains to be seen if these four genes are related to self-protection of the producer, as WBP-29479A1 is a menaquinone-dependent membrane-disrupting compound and the cytochrome complex encoded by *wbp6*–*wbp9* may provide a compensation for the disrupted electron transport chain in membranes.

2.2.3 Lysocins

2.2.3.1 Chemical structures, antibiotic activity, and mode of action of lysocins. Hamamoto *et al.* first reported lysocins isolated from *Lysobacter* sp. RH2180-5.[124] All the nine lysocin congeners are cyclic lipodepsipeptides comprising twelve amino acid residues with an ester linkage to a (*R*)-3-hydroxy fatty acid (Fig. 10). Interestingly, several amino acid residues and their sequences appear conserved in all three families of cyclic lipopeptides, WAP-8294A, WBP-29479A, and lysocins. For example, the sequence, L-Ser, Gly, D-*N*-MePhe, L-Leu, D-Arg/D-Orn, L-Glu, D-Asn/D-Gln/D-Val, and D-Trp, is conserved in all three families. However, the fatty acyl chain of lysocins is not part of the cyclic system. This is the main difference from the other two families. Among the congeners, lysocin E (**32**) showed the best anti-MRSA activity with a MIC of 4 μg mL^{-1}. The mode of action of lysocin E is through interacting with menaquinone in the bacterial membrane to achieve the bactericidal activity, which is similar to the mode of action of WAP-8294A and WBP-29479A families.

Elegant solid-phase strategies have been developed for chemical synthesis of lysocin E and its enantiomeric, epimeric, and *N*-demethylated analogs.[125] Interestingly, the antibacterial activity of the synthetized enantiomer of lysocin E is comparable to that of the natural isomer. SAR studies showed that the cationic functionalities of two arginine residues, the fatty acyl group and the indole ring of the tryptophan are essential for the mode of action of lysocin E.[126] A high-throughput strategy has been developed for the preparation of thousands of lysocin E analogues and large-scale SAR analyses.[127] Over two thousand cyclic peptides were synthesized from an integrated 26-step chemical synthesis, and about a dozen analogues exhibited antimicrobial activity superior or comparable to that of lysocin E.



ORF	Proposed function	ORF	Proposed function
les1	ABC transporter ATP-binding protein	*les3*	Copper amine oxidase
lesA	Nonribosomal peptide synthetase	*les4*	NADH:ubiquinone oxidoreductase
lesB	Non-ribosomal peptide synthetase	*les5*	Hypothetical protein
les2	MbtH-like protein	*les6*	TonB-dependent receptor

Fig. 10 Chemical structure of lysocin E and a congener (lysocin-7), the *les* biosynthetic gene cluster (Cluster-8) in *Lysobacter* sp. 3655, and the predicted functions of ORFs in the gene cluster.

Recently, Yu *et al.* found *Lysobacter* sp. 3655 that could produce lysocins.[128] Through testing various culture media and growth conditions, the authors detected a minute amount of lysocin E and three other congeners (probably isomers of lysocin A/B, C/D/F/G, and H/I, respectively) in *Lysobacter* sp. 3655. Further synthetic biology work produced several engineered strains of *Lysobacter* sp. 3655 that produced at least 19 lysocin-like compounds. Among these, 13 appeared to be new lysocin-related products, including lysocin-7 (**33**), which is likely derived from a degradation of the parent lysocins (Fig. 10).[128]

2.2.3.2 Biosynthesis of lysocins. Although several lysocins have been isolated from *Lysobacter* sp. RH2180-5 and a large number of analogues have been synthesized using chemical methods, little is known about the biosynthesis of lysocins. The main reason is that the BGC for lysocins was not identified until recently. An *in silico* study reported a putative lysocin BGC (*les*) in *Lysobacter* sp. RH2180-5;[129] however, the putative BGC in strain RH2180-5 has not been experimentally confirmed. Recently, Yu *et al.* found that *Lysobacter* sp. 3655 contains a cryptic BGC (Cluster-8) that includes several core genes homologous to the *les* genes in *Lysobacter* sp. RH2180-5 (Fig. 10). In particular, two multi-module NRPS genes (*orf2698* and *orf2699*) are homologous to *lesA* and *lesB*, respectively. The core genes are apparently located in the same operon and presumably share the same promoter. However, the cryptic lysocin genes in *Lysobacter* sp. 3655 were barely transcribed, and the wild type produced a negligible amount of lysocins. To activate the cryptic lysocin BGC, Yu *et al.* screened for strongly transcribed housekeeping genes in strain 3655 and engineered strains with a significantly increased transcription of the cryptic genes and a markedly increased lysocin yield.[128] With readily detectable production of lysocins in the engineered strains, the authors showed that deletion of the putative *les* genes abolished the lysocin production in strain 3655, and complementation of the deleted genes in the mutants restored the lysocin production. The results demonstrated that Cluster-8 in *Lysobacter* sp. 3655 is the lysocin BGC (Fig. 10).

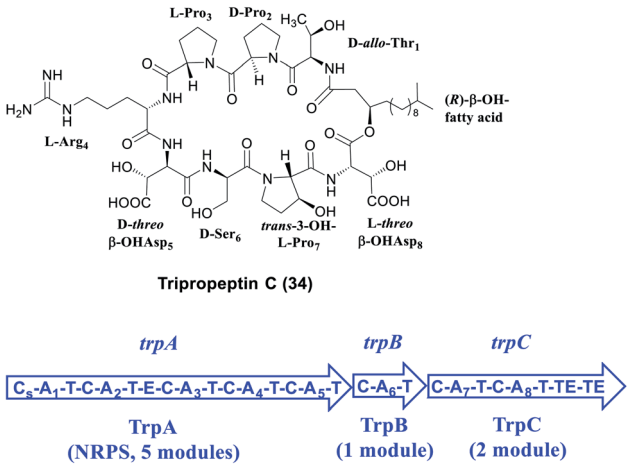


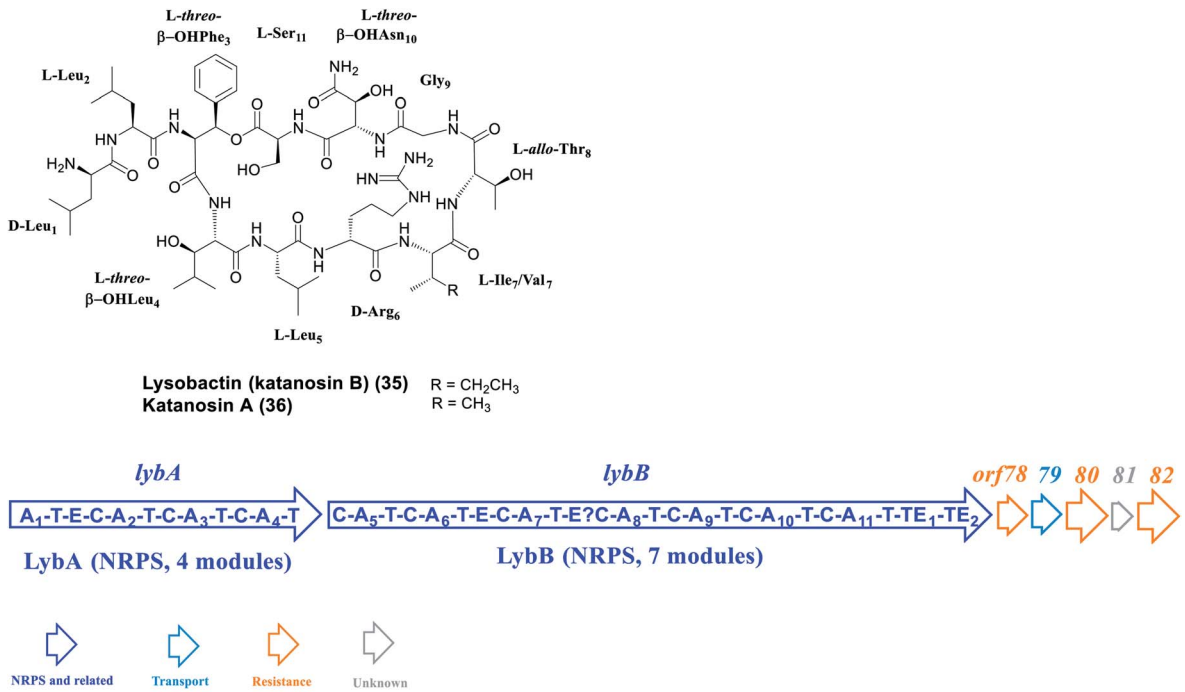
Fig. 11 Chemical structure of tripropeptin C and the *trp* biosynthetic gene cluster in *Collimonas fungivorans* strains Ter331 and Ter6.[136]

The two genes, *lesA* and *lesB*, in the lysocin BGC are predicted to encode a total of 43 domains, making up the 12 modules of the NRPS assembly line that is responsible for recognition, activation, thiolation, condensation, and modification of the 12 amino acids of lysocins. Similar to the BGC of WAP-8294A and WBP-29479A1, the module organization of the assembly line is co-linear with the composition of the peptide (Fig. 10). Again, the lysocin BGC does not contain an acyl CoA-ligase gene, suggesting that *in trans* acyl-CoA ligases may be responsible for the activation and incorporation of different (*R*)-3-hydroxy-fatty acids in different lysocin congeners.

2.2.4 Tripropeptins

2.2.4.1 Chemical structures, antibiotic activity, and mode of action of tripropeptins. Tripropeptin C (**34**) is a calcium-ion dependent cyclic lipodepsipeptide produced by *Lysobacter* sp. (Fig. 11).[130,131] It shows potent *in vitro* activity against Gram-positive pathogens, including MRSA, penicillin-resistant *Streptococcus pneumoniae* and vancomycin-resistant enterococci. It is also active *in vivo* in a MRSA mouse model, with a ED_{50} value similar to that of vancomycin.[132] Tripropeptin C inhibits cell wall biosynthesis, but the mechanism appears different from that of other peptidoglycan-targeting antibiotics, such as β-lactams, vancomycin and bacitracin. Tripropeptin C blocks the lipid cycle of cell wall biosynthesis by complex formation with undecaprenyl pyrophosphate and does not appear to have a cross-resistance with peptidoglycan-targeting antibiotics.[132]

Several derivatives of tripropeptin C with modifications to the carboxyl groups have been chemically synthesized. These compounds showed reduced antibacterial activity, highlighting the importance of the two free carboxyl groups in tripropeptin.[133] Notably, tripropeptin C not only has potent antibiotic



ORF	Proposed function
lybA	Non-ribosomal peptide synthetase
lybB	Nonribosomal peptide synthetase
orf78	β-lactamase
orf79	ABC transporter
orf80	Acriflavin resistance protein
orf81	Membrane fusion protein precursor
orf82	Acriflavin resistance protein

Fig. 12 Chemical structure of lysobactin (katanosin B) and katanosin A, the *lyb* biosynthetic gene cluster in *Lysobacter* sp. ATCC 53042, and the predicted functions of ORFs in the gene cluster.

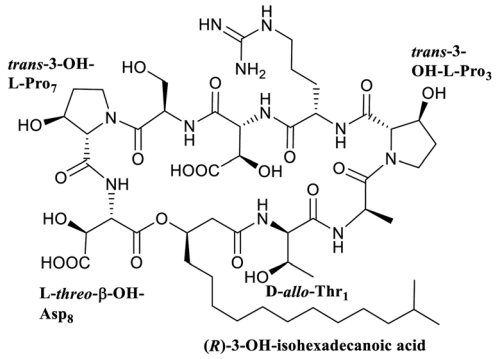
activity but also is able to reverse β-lactam antibiotic resistance in MRSA.[134] The cyclic lipodepsipeptide is able to revitalize and synergistically potentiate the activity of beta-lactams against MRSA. The mechanism of the reversal is tripropeptin C's inhibition of expression of the β-lactam-inducible *blaZ* and *mecA* genes, which encode β-lactamase and foreign penicillin-binding protein, respectively.[135] The combined injection of tripropeptin C and ceftizoxime into a mouse/MRSA septicemia model showed a synergistic therapeutic efficacy compared with each drug alone. This finding is interesting as it could lead to an effective new therapeutic strategy for MRSA treatment.

2.2.4.2 Biosynthesis of tripropeptins. Tripropeptins are relatively simple among the cyclodepsipeptides and have been known for a long while; however, their biosynthetic genes have not been reported in *Lysobacter*. Instead, a putative tripropeptin BGC was reported in *Collimonas*, a Betaproteobacterial genus consisting mostly of soil bacteria that are able to feed on fungi as food.[136] The BGC was identified by a genome mining approach through sequencing six strains belonging to three *Collimonas* species. Within the six genomes, the tripropeptin BGC was found only in *C. fungivorans* strains Ter331 and Ter6, which also exhibited antibacterial activity against *Staphylococcus aureus*. Coincidently, the genome of the other four strains, belonging to *C. pratensis* and *C. arenae*, do not contain the tripropeptin BGC and do not show antibacterial activity. The gene cluster contains three NRPS genes, *trpA*, *trpB* and *trpC*, which encode five, one, and two modules, respectively, for the incorporation of the eight amino acids in tripropeptins (Fig. 11). Interestingly, the final NRPS module contains two TE domains, a feature that is also found in other *Lysobacter* NRPS gene clusters such as lysobactin.[137] The mutation of *trpA* in *C. fungivorans* Ter331 led to loss of the antibacterial activity, suggesting that tripropeptin is involved in the antibacterial activity. However, isolation and analysis of tripropeptins from the strains and mutants have not been reported.[136]

2.2.5 Lysobactin (katanosin B)

2.2.5.1 Chemical structures, antibiotic activity, and mode of action of lysobactin. Lysobactin (katanosin B, **35**) is a branched cyclodepsipeptide isolated from *Lysobacter* sp. SC13,067 (ATCC-53042) (Fig. 12).[138,139] Unlike the peptides described in the previous sections, lysobactin does not contain a fatty acyl chain. Strain ATCC-53042 was first isolated in 1988 from a leaf litter sample. The structure of lysobactin was determined by Tymiak *et al.* in 1989.[140] At the same time, scientists at the Shionogi Research Laboratories in Osaka, Japan, were able to isolate two peptide-related antibiotics named katanosin A (**36**) and B from a fermented culture of a bacterial strain related to the genus *Cytophaga*.[141] These two compounds differed only by one amino acid residue, L-Val in katanosin A and L-Ile in katanosin B. Through structural determination it was found that katanosin B had the same structure as lysobactin.[142]

The peptide core of lysobactin consists of 11 amino acid residues, of which six are non-proteinogenic, including two D-configured amino acids (D-Leu_1 and D-Arg_6), three β-hydroxylated amino acids (L-*threo*-β-$OHPhe_3$, L-*threo*-β-$OHLeu_4$, and L-*threo*-β-$OHAsn_{10}$), and one *allo*-threonine (L-*allo*-Thr_8) (Fig. 11).[143–145] The β-hydroxyl group of L-*threo*-β-$OHPhe_3$ and the carboxyl group L-Ser_{11} form an ester bond, which completes the depsipeptide ring.



Plusbacin A_3 (37)

Fig. 13 Chemical structure of plusbacin A3 from *Lysobacter firmicutimachus* sp. nov (formerly classified as *Pseudomonas* sp. PB-6250).

Lysobactin is a very potent antibiotic against human pathogens. It showed strong activity against Gram-positive and Gram-negative bacteria, especially toward methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE).[138,139] The minimum inhibitory concentrations (MIC) were determined to be 0.39 μg mL^{-1} and 0.78 μg mL^{-1} respectively, which were 2 to 50-fold lower than that of vancomycin. Due to this remarkable antibiotic activity, lysobactin is a promising drug candidate in the fight against bacterial infections by superbugs. The potent activity has inspired several research groups to chemically synthesize the partial or total structure of this cyclodepsipeptide.[143–145] A very efficient solid phase synthesis of lysobactin and other structural analogs was performed by Hall *et al.* in 2012, which required only a single purification step for the final product.[146] Synthetic analogues such as desleucyllysobactin (removal of N-terminal D-Leu_1) and the hydrolyzed lactone (a linear lysobactin) did not possess antibacterial activity. But when the desleucyllysobactin was reacylated with D- or L-Ala, the D-analogue, D-alanyl-desleucyllysobactin, was 10-fold more active in antibacterial activity. The results indicated that a D-amino acid at the N-terminus and a rigid conformation due to the macrolide are important for its antibacterial activity.[140]

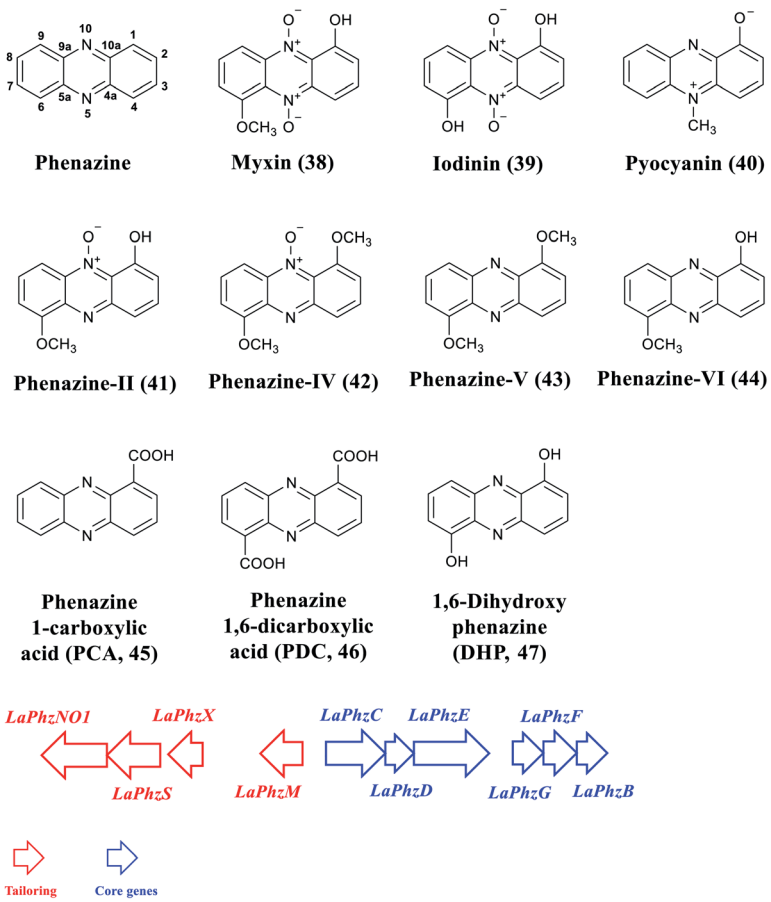
The mode of action of lysobactin had been studied.[147,148] Lysobactin was known to inhibit peptidoglycan (PG) biosynthesis leading to antibacterial activity similar to vancomycin. Unlike vancomycin, lysobactin also inhibited the formation of lipid intermediates and transglycosylation, suggesting that the mode of action is different from that of vancomycin. This feature would make lysobactin a potential candidate for treatment of VRE infections.[148] The cellular molecular mechanism was established by Lee *et al.* in 2016.[147] It was shown, using enzyme inhibition assays, that lysobactin binds to PG precursor Lipid II to form a distinctive 1 : 1 stoichiometric complex that inhibits the enzymes involved in biosynthesis of lipid-linked PG precursors. In contrast, other NRPS-derived antibiotics, such as ramoplanin and teixobactin, bind to Lipid II with a ratio of 2 : 1

(inhibitor:substrate). Lysobactin is a substrate binder that recognizes and binds to the reduced end of lipid-linked cell wall precursors.

2.2.5.2 Biosynthesis of lysobactin. Bernhard *et al.* first identified part of the lysobactin BGC in 1996,[149] and Hou *et al.* identified the complete lysobactin BGC in 2011.[137] Two NRPS, LybA and LybB, contain a total of 35 domains, making up the 11 modules that are corresponding to the 11 amino acid residues of lysobactin (Fig. 12). An interesting observation was the unusual tandem thioesterase (TE) domains found in the end of the final module of LybB. The two individual domains, TE1 and TE2, were assigned different functions *via in vitro* biochemical characterization. TE1 is entirely responsible for the macrocyclization and release of product, whereas TE2 is involved in the hydrolytic cleavage of substrates from misprimed PCPs. There has not been much study of the molecular mechanism for biosynthesis and regulation since the report of lysobactin BGC in 2011. Owing to its potent activity, lysobactin is worth more efforts in synthetic biology and pathway engineering to increase the yield, and to generate new analogues.

2.2.6 Plusbacins. Plusbacins were originally isolated by Shoji *et al.* in 1991 from a fermentation broth of the strain *Pseudomonas* sp. PB-6250,[150] which was in 2016 reclassified as a species of *Lysobacter*.[151] The 16S rRNA gene sequence of strain PB-6250 showed 99.2% similarity to that of *Lysobacter enzymogenes* DSM 2043. After comparing with several closely related *Lysobacter* strains, it was concluded that strain PB-6250 should be affiliated to the *Lysobacter* genus and was renamed *Lysobacter firmicutimachus* sp. nov.[151]

The structures of plusbacins A_1–A_4 and B_1–B_4 were elucidated in 1992 by Shoji *et al.*[152] Among the plusbacins, A3 (**37**) has received the most attention (Fig. 13). Plusbacin A3 has been subject to several chemical total syntheses and studies of its mode of action.[148,153–157] Plusbacins are cyclic lipodepsipeptides containing eight amino acids cyclized through a lactone linkage between L-*threo*-β-hydroxyaspartic acid and a (*R*)-3-hydroxyl fatty acid chain.



ORF	Proposed function	ORF	Proposed function
LaphzNO1	Cyclohexanone monooxygenase	*LaphzD*	Isochorismatase
LaphzS	Flavin-containing monooxygenase	*LaphzE*	Anthranilate synthase
LaphzX	Antibiotic biosynthesis monooxyenase	*LaphzG*	FMN-dependent oxidase
LaphzM	SAM-dependent methyltransferase	*LaphzF*	DHHA isomerase
LaphzC	DAHP synthase	*LaphzB*	Phenazine biosynthesis protein

Fig. 14 Chemical structure of phenazine natural products, the *Laphz* gene cluster and proposed function of each ORF in *L. antibioticus* OH13.[168] DHHA, 2,3-dihydro-3-hydroxyanthranilate; DAHP, 3-deoxy-D-arabinoheptulosonate-7-phosphate.

The amino acids of plusbacins A_1–A_4 were composed of two L-*trans*-3-hydroxyprolines and one of D-*threo*-β-hydroxyaspartic acid, L-*threo*-β-hydroxyaspartic acid, D-*allo*-threonine, D-serine, D-alanine and L-arginine, respectively (Fig. 13). In plusbacins B_1–B_4, one of the L-*trans*-3-hydroxyprolines is replaced by L-proline. The main differences among plusbacins A_1–A_4 and B_1–B_4 are in their fatty acid chains. The fatty acid was 3-hydroxy-tetradecanoic acid for A_1 and B_1, 3-hydroxy-isopentadecanoic acid for A_2 and B_2, 3-hydroxy-isohexadecanoic acid for A_3 and B_3, and 3-hydroxy-hexadecanoic acid for A_4 and B_4.[152]

Plusbacins exhibited potent inhibitory activity against Gram-positive bacteria, but showing little activity against Gram-negative bacteria.[150] Plusbacin A_3 gave a MIC of 0.78 to 3.13 μg mL^{-1} against MRSA and VanA-type vancomycin-resistant enterococci (VRE).[148] At a concentration close to the MIC, plusbacin A_3 inhibited the incorporation of *N*-acetylglucosamine into *Staphylococcal* cell wall peptidoglycan. *In vitro* studies with a wall-membrane particulate fraction of *S. aureus* revealed that plusbacin A_3 inhibited the formation of lipid intermediates and nascent peptidoglycan, with IC_{50} of 2.3 and 0.4 μg mL^{-1}, respectively. Further evidence suggested that plusbacin A_3 has a dual mode of action.[156] Surprisingly, the activity of plusbacin A_3 was not suppressed by the presence of *N*-acetyl-L-Lys-D-Ala-D-Ala, a tripeptide mimic binding domain of the lipid intermediates terminus to vancomycin. Thus, plusbacin A_3 holds strong possibility for use in the treatment of vancomycin-resistant organisms.[148]

So far, the research on plusbacins has focused on chemical synthesis and mode of action. The BGC for plusbacin biosynthesis has not been reported, and the molecular mechanism for biosynthesis and regulation are yet to be investigated.

2.3 Phenazines

2.3.1 Chemical structures, antibiotic activity, and mode of action. Phenazines are nitrogen-containing tricyclic aromatic compounds that share the dibenzopyrazine core structure (Fig. 14). The compounds have been isolated from many microorganisms and exhibit diverse biological activities.[158,159] The heterocyclic system of phenazines is decorated with various functional groups depending on the producer microorganisms, and the decorating groups often dictate diverse activity of the compounds.[160] For example, myxin (1-hydroxy-6-methoxyphenazine-*N*5,*N*10-dioxide, **38**) is a topical broad spectrum antibiotic (Cuprimyxin, a myxin-copper(II) complex) that had been used as a veterinary drug for decades.[161] The mode of action of myxin is probably inhibition of DNA synthesis.[160,162] Through a free radical mechanism, myxin is able to cleave both DNA strands under aerobic and anaerobic conditions. Additionally, myxin was shown to inhibit DNA template-controlled RNA synthesis. Iodinin (**39**) is another well-known phenazine natural product with antimicrobial and cytotoxic properties.[163,164] Iodinin and myxin were shown to promote apoptotic cell death in multiple cancer cell lines with genetic features that indicated poor patient prognosis.[165–167] It has been documented that, similar to myxin, iodinin can interact with DNA through intercalation of DNA's C-G base pairs.[162]

Several synthetic phenazines with halogen groups exhibit potent antagonistic activity against biofilms and persister cells of methicillin-resistant *Staphylococcus aureus* (MRSA).[169–171] Clofazimine (Lamprene) is a synthetic phenazine that has been used for leprosy treatment and is on the World Health Organization's List of Essential Medicines.[172] Recently, clofazimine was shown to be a broadly active inhibitor for SARS-CoV-2.[173]

Phenazines also are redox-active metabolites with important biological functions.[159,174–176] Pyocyanin (1-hydroxy-*N*5-methylphenazine, **40**) produced by *Pseudomonas aeruginosa* functions as a virulence factor for infection.[177–179] It was shown that pyocyanin can function as an electron shuttle to help the aerobic pathogen to maintain redox homeostasis in anoxic environments such as biofilms or human tissues.[175,180] Pyocyanin can also serve as a signal to control gene expression and community behavior in divergent bacteria.[174,181] Recently, phenazines were shown to solubilize phosphorus through reductive dissolution of iron oxides, which enhances phosphorus bioavailability and thus increase microbial growth in phosphorus-limited environments.[182]

In *L. antibioticus* OH13, Zhao *et al.* isolated six phenazines including four *N*-oxides (**38–39**, **41–44**, Fig. 12).[168] The OH13 culture produced phenazine-II (**41**) as the most abundant product, which was isolated as an orange pigment. Its structure, 6-methoxy-1-phenazinol-*N*10-oxide, was established by ESI-MS and NMR. For the two known *N*-oxides, myxin (**38**) was isolated as a dark red pigment, and iodinin was isolated as a purple pigment in strain OH13. Phenazine-IV (**42**) was isolated as yellow powder and identified as 1,6-dimethoxy-phenazinol-*N*5-oxide. Phenazine-V (**43**) was isolated as yellow powder and identified as a non-oxide phenazine, 1,6-dimethoxyphenazine. Phenazine-IV and phenazine-V were new phenazine natural products. Phenazine-VI (**44**) was isolated as yellow powder and identified as 1-hydroxy-6-methoxyphenazine.

Naturally occurring heterocyclic aromatic *N*-oxides are rare, but this type of natural products often possesses potent activities. For example, orellanine isolated from the toadstool

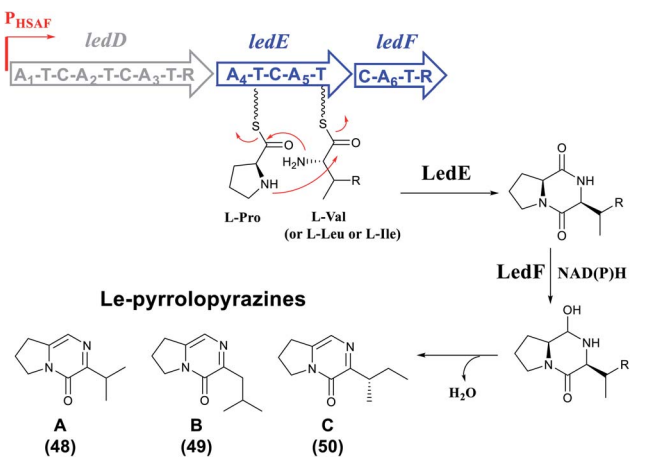


Fig. 15 Identification of Le-pyrrolopyrazines A-C from *L. enzymogenes* OH11 *via* genome mining of a cryptic BGC (*led*), modified from Li *et al.* with permission.[196]

mushroom (*Cortinarius orellanus*) is a deadly poison.[183] The six phenazine compounds from *L. antibioticus* OH13 were tested against a panel of 14 bacterial species, most of which were pathogens of crop plants.[168] Myxin showed potent activity against most of the tested strains, with an impressive MIC of 0.05 μg mL^{-1} against MRSA. The other compounds showed a varied degree of activity.

2.3.2 Biosynthetic mechanism of phenazines. The biosynthetic mechanism for the core structure of phenazines has been elucidated in multiple microorganisms.[158,184,185] The "core biosynthetic genes" for phenazines are conserved throughout diverse microorganisms. The core *phz* BGC consists of six or seven genes, *phzA(B)CDEFG*.[186] Depending on the microorganisms, the core genes can yield phenazine-1-carboxylic acid (PCA, **45**) or phenazine-1,6-dicarboxylic acid (PDC, **46**) (Fig. 14).

Despite the well-understood biosynthetic mechanism for phenazine core structure, it was unclear how the *N*-oxide was installed on the aromatic heterocycles. Zhao *et al.* sequenced the genome of *L. antibioticus* OH13 and identified a 10-gene BGC (*Laphz*) (Fig. 14).[168] Six of the genes, *LaphzB*, *LaphzC*, *LaphzD*, *LaphzE*, *LaphzF*, and *LaphzG* are homologous to the core phenazine genes. The mutation of three selected core genes abolished production of all phenazines, confirming that the *Laphz* cluster is indeed responsible for phenazine biosynthesis in stain OH13.

In addition, the BGC contains four putative modification genes, *LaphzNO1*, *LaphzS*, *LaphzX*, and *LaphzM* (Fig. 14). *LaphzM* shares a sequence similarity to *phzM*, which encodes a methyltransferase in *P. aeruginosa*.[187] PhzM catalyzes the *N*-methlyation reaction in pyocyanin biosynthesis. *LaphzS* is similar to *phzS* in *P. aeruginosa*, which encodes a flavin-containing monooxygenase that catalyzes a decarboxylative hydroxylation to convert PCA (or probably 5-methyl-PCA) to 1-hydroxyphenazine (or pyocyanin).[188,189] *LaphzNO1* shows a sequence similarity to genes coding for ChnB cyclohexanone monooxygenases, flavin-dependent enzymes catalyzing the Baeyer–Villiger reaction of cyclic ketones to produce lactones.[190,191] However, a Baeyer–Villiger reaction is not known to be involved in phenazine biosynthesis. Finally, the protein encoded by *LaphzX* exhibits a sequence similarity to ActVA-Orf6 and TcmH, which are small, cofactor-free enzymes catalyzing oxidation of phenolic compounds to corresponding quinones.[192,193] Since neither *LaphzNO1* nor *LaphzX* had an orthologue in other characterized *phz* clusters, Zhao *et al.* hypothesized that either *LaphzX* or *LaphzNO1* could be responsible for the observed *N*-oxidation of phenazines in OH13.[168]

When *LaphzNO1* gene was deleted, the mutant was no longer able to produce the four *N*-oxide compounds (**38–39**, **41–42**).[168] Meantime, the non-oxides (**43–44**) were dramatically increased in the mutant. *LaphzNO1* was then expressed in *E. coli*, and the purified enzyme was able to convert the non-oxide **44** to the oxide **41** in the presence of NADPH and FAD. When the hydroxyl on C1 or C6 is methylated, LaphzNO1 was unable to make the *N*-oxidation, suggesting the requirement of a free hydroxyl at the beta-carbon to the nitrogen atom. The enzyme is a light yellow protein with absorptions at 442 and 382 nm, confirming that LaphzNO1 is a flavoprotein.

To understand the selectivity of LaphzNO1, LaphzS was also investigated.[168] The *LaphzS* deletion mutant produced PDC (**46**) as the main product, while phenazines **38–39**, **41–44** were abolished. *LaphzS* was then expressed in *E. coli*, and purified LaphzS was able to convert PDC to a new product, which was identified as 1,6-dihydroxyphenazine (DHP, **47**). The activity of LaphzS requires NADH and FAD. Thus, LaphzS from strain OH11 is bifunctional, catalyzing both decarboxylation and hydroxylation of the heteroaromatic rings. Unlike phzS from *P. aeruginosa*,[188,189] LaphzS is able to catalyze the decarboxylative hydroxylation on both C1 and C6 of the PDC ring.

DHP (**47**) generated by LaphzS has a free hydroxyl on the beta-position of both nitrogen atoms, and LaphzNO1 converted DHP to the *N5*,*N10*-dioxide, iodinin (**39**), confirming that LaphNO1 is able to catalyze *N*-oxidation at both N5 and N10.[168] This result further support the idea that the *N*-oxidation by LaphzNO1 requires a free hydroxyl group at the beta-position as the methoxy at C1 or C6 blocks the *N*-oxidation. Together, the *in vivo* and *in vitro* studies showed that LaphzNO1 is an NADPH-dependent, flavin-containing *N*-monooxygenase in phenazine biosynthesis. It is interesting to reveal that a Baeyer–Villiger monooxygenase homolog actually catalyzes the aromatic *N*-oxidation of phenazines. Additionally, the data provided experimental evidence for PDC being the precursor of myxin. A probable mechanism for the LaphzNO1-catalzyed *N*-oxidation could involve the flavin hydroperoxide FADH–OOH, generated from $FADH_2$ and O_2, undergoing an attack by the aromatic nitrogen of phenazines. This would result in the release of FADH–OH and production of the *N*-oxide products. FAD would be regenerated from FADH–OH by a dehydration.

In 2018, Jiang *et al.* reported the function and structure of LaphzM from *L. antibioticus* OH13.[194] *LaphzM* encodes a SAM (*S*-adenosyl-L-methionine)-dependent *O*-methyltransferase that catalyzes the conversion of iodinin (**39**) to myxin (**38**). The substrate selectivity study revealed that LaphzM is responsible for formation of both monomethoxy and dimethoxy in all phenazines isolated from strain OH13. LaphzM was also able to use phenazines with non-, mono-, or di-*N*-oxide, as substrates. Although LaphzM exhibits a relaxed substrate selectivity, it appeared to catalyze only *O*-methylation, not *N*-methylation. This is in contrast to phzM from *P. aeruginosa*, which catalyzes *N*-methylation in pyocyanin biosynthesis.[187] Actually, when PCA was used as the substrate for LaphzS and LaphzM, the reactions produced a small amount of 1-hydroxyphenazine and then 1-methoxyphenazine, rather than *N*5-methylphenazine and then pyocyanin (**40**). Thus, while LaphzS is similar to phzS to catalyze the decarboxylative hydroxylation of PCA to produce 1-hydroxyphenazine, LaphzM is unlike phzM in catalyzing the *N*-methylation. LaphzM was the first characterized phenazine *O*-methyltransferase.

Jiang *et al.* also determined the X-ray crystal structure of LaphzM at 1.4 Å resolution, with bound cofactor *S*-adenosyl-L-homocysteine (SAH).[194] LaphzM has a dimeric structure, with the N-terminus portion of each monomer forming the dimerization domain and the C-terminus of each monomer forming the catalytic domain with the α/β Rossmann fold. The overall

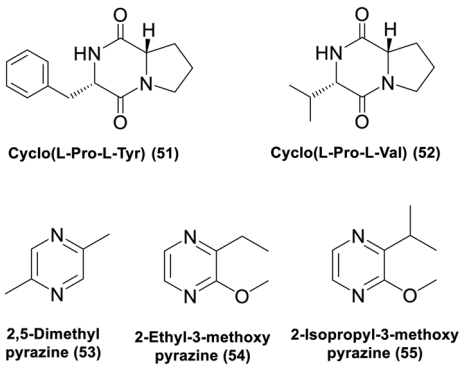


Fig. 16 Volatile organic compounds from *L. capsici* AZ78.

fold of LaphzM is similar to that of many other *O*-methyltransferases.

LaphzX in the *Laphz* BGC may be shown to be related to the self-protection of the producer from myxin, if the *LaphzX*-encoded enzyme could be proven to catalyze deoxidation of the myxin *N*-oxides in *L. antibioticus*.[195] The mutation of *LaphzX* did not lead to obvious phenazine-related compounds, and more biochemical characterization of this enzyme is needed to elucidate the exact function of this gene. Recently, Zhao *et al.* found that the *lexABC* genes that encode a RND (Resistance-Nodulation-Division) efflux pump contributed to self-protection from myxin produced in *L. antibioticus*.[195] The RND genes are clustered with *lexR*, a putative LysR-type transcriptional regulator gene, but not with the phenazine BGC (*Laphz*) in the genome. The deletion of *lexABC* or *lexR* led to no myxin production, and the mutants exhibited significantly increased susceptibility to exogenous myxin. This study showed that the RND-type efflux pump confers myxin resistance in *L. antibioticus*. Interestingly, the study also noted that deoxidation at the nitrogen atoms and methylation at the hydroxyl of myxin contributed to self-protection when myxin was produced at a low level.

2.4 Others

2.4.1 Pyrrolopyrazines. Although *Lysobacter* genomes contain a large number of BGCs for natural products, most of the genes are silent under laboratory conditions. Genome mining is an active area in natural product research; however, natural product discovery in *Lysobacter* through genome mining is still in its infancy. In 2017, Li *et al.* employed the promoter replacement strategy to activate a cryptic gene cluster (*led*) that contains three NRPS genes (*ledD*, *ledE*, and *ledF*) coding for six NRPS modules in *L. enzymogenes* OH11 (Fig. 15).[196] The transcription of the *led* genes in the wild type was barely detectable as indicated by quantitative reverse transcription PCR (qRT-PCR). Upon replacement with P_{HSAF}, the transcription of the *led* genes increased by 7–34 fold. Interestingly, although *ledD* was the first gene under P_{HSAF} control, the second gene *ledE* was expressed the highest. Product analysis showed that the strain produced three new compounds, which were identified to be pyrrolopyrazines, Le-pyrrolopyrazines A–C (**48–50**) (Fig. 15).

The pyrrolopyrazines apparently result from the condensation of two amino acids, which are not "co-linear" to the 6-module NRPS assembly line. Gene deletion experiments confirmed that two NRPS genes (*ledE* and *ledF*) are required for biosynthesis of the pyrrolopyrazines, but one NRPS gene (*ledD*) is not required.[196] Further experiments using targeted single-point mutations and product analyses revealed a "module/domain portable mechanism" for the biosynthesis of the pyrrolopyrazines, which selectively recruits LedE and LedF. This work shows the remarkable flexibility and versatility of the modular assembly line of NRPS. This study was the first example in *Lysobacter* species of a cryptic BGC being activated to produce new natural products.

2.4.2 Volatile compounds. Several antimicrobial 2,5-diketopiperazines, monoalkyl-methoxypyrazines and dialkyl-methoxypyrazines have been isolated from *Lysobacter capsici* AZ78, which is a strain isolated from the rhizosphere of tobacco plants (Fig. 16).[197–199] These compounds appear to be produced in nutrient-rich conditions, which is in contrast to HSAF and analogues that are produced only in conditions where nutrients are depleted. Among the 2,5-diketopiperazines, cyclo(L-Pro-L-Tyr) (**51**) exhibited strong *in vitro* and *in vivo* activity against *Phytophthora infestans* and *Plasmopara viticola*, and cyclo(L-Pro-L-Val) (**52**) showed a cytotoxic activity similar to chloramphenicol (25 μg mL^{-1}) against *Rhodococcus fascians*, a phytopathogenic Gram-positive bacterium. Several other volatile compounds were also detected. Their identity was not entirely clear, but probably were mono- and dialkylated methoxypyrazines (**53–55**).[200] Recently, Puopolo and coworkers used a combined MALDI-qTOFMSI and UHPLC-HRMS/MS to analyze the metabolome of AZ78.[201] This work identified 2,5-diketopiperazines, as well as WAP-8294A and HSAF in *L. capsici* AZ78. The authors also predicted several putative cyclic lipodepsipeptides, macrolactones and macrolides based on MALDI-qTOFMSI data. However, most of the predicted compounds remain to be isolated and structurally determined. Additionally, other volatile compounds have been detected

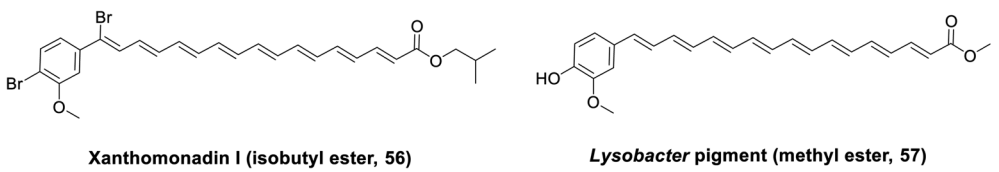


Fig. 17 Chemical structure of xanthomonadin I from *Xanthomonas campestris* pv. *campestris*, and the proposed structure of one of the yellow pigments isolated from *L. enzymogenes* OH11.[203]

from AZ78, such as pyrazines and NH_3. These compounds contribute to the antagonistic effects of AZ78 against plant pathogenic microorganisms, including *Pythium ultimum*, *Rhizoctonia solani*, and *Sclerotinia minor*.[199,202]

2.4.3 Aryl polyenes. *Lysobacter* species exhibit a characteristic yellow-orange appearance. The nature and function of the pigments conferring the characteristic appearance is not very clear. In 2013, Wang and coworkers used the transposon mutagenesis approach to identify a "white" colony of *L. enzymogenes* OH11, which carried mutations in a stand-alone (type II) PKS gene cluster.[203] Specific deletions of the PKS genes resulted in abolition of the yellow pigments, confirming that the BGC is required for the pigment production in *L. enzymogenes* OH11. The BGC was predicted to consist of 17 ORFs, including genes encoding stand-alone KS (ketosynthase), AT (acyltransferase), ACP (acyl carrier protein), KR (ketoreductase), DH (dehydratase), tailoring enzymes, transporters, and regulators.

The pigments were unstable and quickly became insoluble once purified. The exact chemical structure of the pigments has not been determined. Based on data from mass spectra and a comparison with the yellow pigments known as xanthomonadins (**56**) from *Xanthomonas campestris* pv. *campestris*,[204–208] the pigments from *Lysobacter* belong to xanthomonadin-like aryl polyenes (**57**) (Fig. 17). Combined with the organization of the pigment BGC and feeding experiment, the authors proposed that the *Lysobacter* pigments are analogues of xanthomonadins, which use 3-hydroxybenzoic acid (3-HBA) as the precursor for the pigment biosynthesis.[203] It should be pointed out that, although a number of xanthomonadins had been reported from various *Xanthomonas*, xanthomonadin I (isolated as isobutyl ester, **56**) is the only compound with an established structure (by X-ray crystallography).[206] The fully conjugated polyenes are highly unstable, probably due to their highly photosensitive nature.

The stand-alone (type II) PKS for *Lysobacter* polyene pigments is unusual, because polyene-type polyketides are typically biosynthesized by modular PKS (Type I). Type II PKS always contains the heterodimer KS-CLF (chain-length factor) as the key catalytic component for chain initiation and elongation.[209] However, *Lysobacter* aryl polyene PKS complex only contains the KS (ORF17), but not the CLF. Instead, a hypothetical protein encoded by ORF16 is located immediately next to ORF17. ORF16–17 homologs are widespread in numerous uncharacterized microbial genomes.[203] The deletion of ORF16 also eliminated pigment production, and homology modeling suggested that ORF16 protein shares a structural similarity to the N-terminal half of CLF. All ORF16 homologs share a conserved glutamine (Q166 of *Lysobacter* ORF16) that is the conserved active site of known CLF. Subsequently, the authors conducted a point-mutation of glutamine (Q166A) and found the mutant gave white colonies, in which the pigment production was abolished. The results suggest that the hypothetical protein (ORF16) may be a novel type of CLF.[203] It will be very intriguing to further investigate the large number of homologs of the unusual KS/CLF pairs in the database.

The white mutants were significantly more sensitive to visible light, UV radiation, or H_2O_2 treatment than the wild type or the mutants with restored yellow pigments. *Lysobacter* yellow pigments are non-carotenoid polyenes that can serve as "sunscreens" for photo-survival, which is important for these ubiquitous microorganisms.[203] As biocontrol agents, the epiphytic survival of *Lysobacter* species is essential for the bacteria to effectively perform the crop protection against invading pathogens.

3. Metabolic engineering in *Lysobacter*

As prolific producers of lytic enzymes and bioactive natural products, *Lysobacter* species exhibit a great potential as new biocontrol agents and sources of bioactive compounds. However, many of the products are produced only under very special conditions and often in very low yield. In the past several years, attempts have been made to increase production of the bioactive products and to enhance the biocontrol abilities against bacterial and fungal diseases, as the antagonistic activity of *Lysobacter* is mainly attributable to potent antimicrobial natural products.[210,211]

3.1 PoTeM

As discussed above, HSAF and analogues produced by *L. enzymogene* C3 and OH11 have been the most extensively studied natural products in *Lysobacter*. Other PoTeMs such as xanthobaccins A, B, and C were reported from *L. capsici* SB-K88, which can protect sugar beets by inhibiting the damping-off disease caused by *Pythium* spp.[20,21] Several metabolic engineering approaches have been used to increase the production of the PoTeMs or to produce new products.

Wang *et al.* in 2013 reported the first genetic manipulation of natural product biosynthesis in *Lysobacter*.[212] *Lysobacter* species are naturally resistant to many antibiotics commonly used in selection of genetic transformants. This intrinsic antibiotic resistance has hampered genetic manipulation in *Lysobacter*. The researchers identified a new locus *hmgA*, which is required for tyrosine/phenylalanine metabolism, through random mutagenesis using a transposon.[212] The disruption of *hmgA* led to accumulation of dark brown pigments on the pale-yellow background of *Lysobacter* colonies. This provided a facile method for site-specific gene integration, which is based on a yellow-to-black color change as a visual selection marker for putative transformants. Furthermore, a series of plasmid vectors were constructed and tested in *L. enzymogenes* OH11. Immediately behind the *hmgA* gene, promoter P_{HSAF} (538-bp) for constitutive expression of *hsaf-pks/nrps* gene and putative ribosomal binding sites (Shine-Dalgarno sequence) were placed in front of the gene of interest. As proof of principle, *wapH* gene (ORF8) encoding a putative TonB-dependent regulator was tested as the target (Fig. 8). The manipulation of *wapH* led to 7-fold increase in HSAF yield and 2-fold increase in WAP-8294A yield in the black color strains.

A number of regulator genes have been explored for yield increase of natural products in *L. enzymogenes*. One approach is the deletion of negative regulators to generate high-yield

strains. Wang *et al.* in 2017 identified a TetR family gene, *letR* (*Le*1552), which acts as a transcriptional repressor through direct binding to the promoter region of HSAF biosynthesis operon and consequently reduces the HSAF production.[88] By inactivating the *letR* gene, the authors improved the titer of HSAF by 2-fold over the wild type strain. In another study, deletion of the gene encoding a TCS response regulator, PilG, resulted in about 2.2-fold increase in HSAF production.[86] Furthermore, deletion of genes encoding enzymes for c-di-GMP synthesis has also been found helpful to increase production of the antibiotics. As described above, HSAF biosynthesis is inhibited by increased intracellular levels of c-di-GMP, which regulates many bacterial cellular processes through binding to a wide variety of intracellular receptors.[85] C-di-GMP is synthesized by diguanylate cyclases (DGCs) through condensing two GTP molecules. To explore this second messenger, Ren *et al.* in 2020 constructed a strain lacking seven active DGC genes, which lowered the amount of intracellular c-di-GMP and resulted in 2.3-fold increase of HSAF production.[213] Further fermentation optimization of this strain led to around 200-fold increase of HSAF titer as compared to the wild type.

Another approach is through redirecting the metabolic flux and supplementing precursors in *L. enzymogenes*.[34] The PoTeM pathway was redirected to alteramide B from HSAF, through knocking out *OX2*/*OX4* genes in strain OH11 (Fig. 2). The resulting strain produced alteramide B as the main product, with a minimal amount of HSAF. The yield of alteramide B in this strain was further improved by adding arginine into media. Together, these manipulations led to a 24-fold increase of yield, reaching 893.32 $\pm$ 15.57 mg L^{-1}, one of the highest yields of PoTeM reported so far.

In addition to producing high-yielding *Lysobacter* strains, metabolic engineering has also been applied to produce new analogues. In 2019, Li and coworkers first demonstrated the feasibility of producing new PoTeM compounds through combinatorial biosynthesis using the genes from the HSAF gene cluster and the combamide gene cluster (Fig. 4).[42] Upon introducing the hybrid construct into two different heterologous hosts, three PoTeMs were produced. Two of the products were known compounds, lysobacterene B (**12**) and combamide D (**14**), and the third was a new compound that was identified as pactamide A (**15**).[42] The results demonstrate that combinatorial biosynthesis is feasible in *Lysobacter* species to produce new PoTeM compounds.

3.2 WAP-8294A

Several approaches have been explored to improve WAP-8294A production in *L. enzymogenes* OH11.[116,212] As described above, Wang *et al.* attempted to increase WAP-8294A yield through increasing expression of the TonB-dependent receptor gene (ORF8) in the WAP gene cluster, which resulted 2-fold increase in an engineered *L. enzymogenes* strain.[212]

In 2018, Yu *et al.* applied the CRISPR/dCas9 system to *Lysobacter* for the first time to enhance the expression of the WAP-8294A genes.[116] The authors also refactored four putative self-protection genes in the BGC into a single operon and put them under the control of P_{HSAF}. Upon introducing the CRISPR/dCas9-ω3 system, the transcription of the WAP genes increased 5 to 48-fold. Further manipulations by refactoring the self-protection genes led to 20 to 65-fold increase in gene transcription. The yield of WAP-8294A compounds in the engineered strains increased 4 to 9-fold, comparing to the starting strain. This work demonstrated that the combined use of CRISPR/dCas9-mediated gene expression and refactoring of self-protection genes can be an effective approach to yield improvement in *Lysobacter*.

In 2019, Chen *et al.* conducted a systematic investigation on the nutritional and environmental conditions that could affect WAP-8294A production in an engineered strain of *L. enzymogenes*, in which the key biosynthetic gene for the main metabolite HSAF had been deleted.[214] An activity-based method was developed for quick screening of optimal nutritional and environmental conditions, which led to 10-fold increase in small-scale cultures and approximately 15-fold increase in scale-up fermentation (14 liters). Interestingly, the ratio of WAP-8294A2 to WAP-8294A1 in the strains could be manipulated through changing the culture media.

In addition to the yield improvement of the known WAP-8294A compounds, recent efforts have sought to generate new analogues through manipulating the biosynthetic genes. Zhu *et al.* attempted to manipulate the accessory genes in the BGC to produce new WAP-8294A analogues (**25–30**) in *L. enzymogenes* (Fig. 7).[115] WAP-8294A2 and several deoxy analogues have been chemically synthesized.,[114,117] which showed that deoxy-WAP-8294A2 is also a potent antibacterial compound. However, the chemical synthesis approach is unlikely to be practical because it required dozens steps of highly elaborate chemical reactions. The study by Zhu *et al.* indicated that *Lysobacter* strains can be engineered to produce deoxy WAP-8294A (**17**, **19**, **21**) through minimal manipulations of the biosynthetic genes, without introducing foreign DNA.[115] However, the yield of the new compounds was extremely low, and further effort is needed to optimize the metabolic flows in *Lysobacter* strains so that the expression of WAP biosynthetic pathway could be enhanced.

3.3 Lysocins

In wild-type *Lysobacter* sp. 3655, lysocins were produced only under certain conditions, and the compounds was barely detectable by HPLC.[128] Using strong promoters for constitutively expressed housekeeping genes from *Lysobacter* sp. 3655, Yu *et al.* generated engineered *Lysobacter* strains.[128] Most notably, the engineered strains produced both the known lysocins and a large number of new lysocins (Fig. 10). The studies showed the feasibility of using synthetic biology approaches to produce new lysocins and to study the mechanism for lysocin biosynthesis.

Among the new lysocin-like compounds produced by the engineered strains, lysocin-7 (**33**) is particularly interesting (Fig. 10).[128] This is a linear decapeptide derivative of lysocins, only containing the portion of the 3rd through 12th amino acid residues of lysocin E (**32**). Three structural units, L-Thr_1 and D-Arg_2, and 3-hydroxy fatty acid, which are common to all previously reported lysocins, are missing in this compound. In

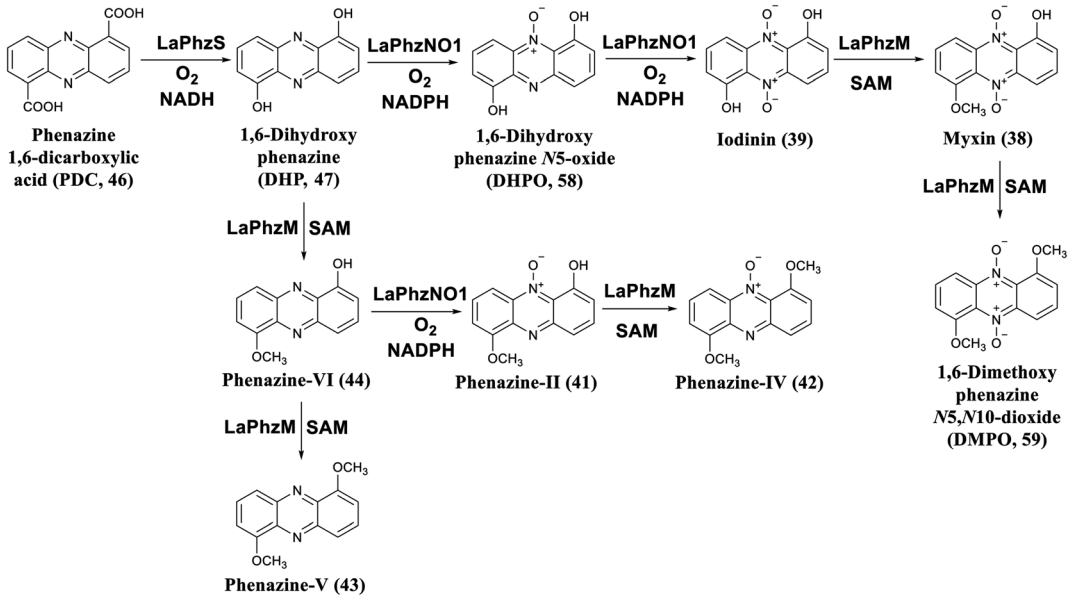


Fig. 18 Chemoenzymatic synthesis of phenazines using the ring-decorating enzymes from the heterologously expressed *LaPhz* genes in *L. antibioticus* OH13.[194]

contrast to other lysocins, this compound had no bioactivity. Further experiments indicated that lysocin-7 is most likely a degraded product of lysocins, rather than an aberrant biosynthetic product through module-skipping of the first two NRPS modules. This compound might be the result of a self-protection mechanism of the producer *Lysobacter*, given the potent antibacterial activity of lysocins.

3.4 Phenazines

The characterization of several phenazine modification enzymes provided an opportunity to exploit the thousands of existing phenazine scaffolds as new antibiotics through metabolic engineering and chemoenzymatic syntheses. Jiang *et al.* demonstrated a one-pot biosynthesis of myxin by *in vitro* reconstitution of the three phenazine-ring decorating enzymes.[194] Using purified LaPhzM, LaPhzS, and LaPhzNO1, a series of decorated phenazines including myxin (**38**), iodinin (**39**) and 1,6-dihydroxy phenazine *N*5-oxide (DHPO, **58**) were produced *in vitro* from PDC (**46**) (Fig. 18). Although it had been proposed that PDC is one of the two common precursors for all phenanzine natural products, this was the first time that PDC was experimentally demonstrated to be the precursor of iodinin and myxin. The study also established the steps from PDC to myxin in the biosynthetic pathway. Additionally, the *in vitro* synthesis produced a new compound, 1,6-dimethoxyphenazine *N*5,*N*10-dioxide (DMPO, **59**), which had not been observed in strain OH13 (Fig. 18).

4. Discussion and conclusion

In the past ten years, significant progress has been made in the research of natural products from *Lysobacter*, particularly in molecular mechanisms for the biosynthesis and regulation of HSAF and its analogues in two strains of *L. enzymogenes*. New cyclodepsipeptides with potent antibiotic activity have been identified, and their biosynthetic gene clusters and molecular modes of action have been revealed. The biosynthesis of phenazine core structures has been known from studies of other microorganisms, but *Lysobacter* has provided a great opportunity to elucidate the enzymes and their catalysis in the formation of phenazine *N*-oxides.

However, there are still many aspects remaining to be studied. First, essentially nothing is known about the regulatory and resistant mechanisms for the cyclodepsipeptides found in *Lysobacter* species. So far, the main effort in this area has been devoted to isolation, structural determination, and chemical synthesis. Second, the mining of *Lysobacter* genomes for new natural products is still in its infant stage. Over 22 genomes of *Lysobacter* strains are publicly available, including strains belonging to the most interesting species, *L. antibioticus*, *L. capsici*, and *L. enzymogenes*, species that encompass biocontrol agents of phytopathogenic microorganisms.[2] de Bruijn and coworkers have conducted genomics and metabolomics analyses of five *Lysobacter* strains representing the four species, including *L. enzymogenes*, *L. capsici*, *L. gummosus* and *L. antibioticus*.[210] Multiple putative BGCs including NRPS and PKS clusters are found in each of the strains. So far, only a few of the BGCs have been investigated, and the majority of the BGCs remain to be explored. For example, although *L. enzymogenes* is the most extensively studied species in this genus, only four BGCs have the products identified (HSAF, WAP-8294A, xanthomonadin-like pigments, and pyrrolopyrazines) among the 18 putative BGCs in its genome. This large number of putative BGCs in other *Lysobacter* species remains to be explored. Third, tools for genetic manipulations in *Lysobacter* is still lacking. *Lysobacter* species are intrinsically resistant to

multiple antibiotics that are commonly used in genetic transformant selection. Many strains of *Lysobacter* are not genetically transformable. Despite the efforts to develop simple and efficient methods, there are only a few tools available so far. Future research should set out to identify new selection markers, powerful promoters, and innovative approaches to tackle the low gene expression level and often extremely poor product yield.

Finally, more efforts are needed in the studies of application of *Lysobacter* as new biocontrol agents. A key factor for the success of this biological approach is the discovery of new microbial strains that can produce potent natural products with novel chemistry and modes of action. Lin *et al.* recently reviewed the antifungal strategies of *L. enzymogenes* as a mighty biocontrol agent, which possesses multiple types of weapons for long-range, medium-range and short-range killing of microbial pathogens.[215] With the constant emergence of resistant pathogens and the recognized risks of toxic pesticides to human health and the environment, the search for safe and effective alternative strategies for pest control has become increasingly important. Biocontrol measures can provide green and effective management tools for crop diseases. A number of application products based on *L. enzymogenes* have been tested and provided promising new means for biocontrol of diseases of agricultural crops. One interesting application was found in protection of books. The decolored cell-free supernatant of strain C3 of *L. enzymogenes* was able to effectively protect books from mold damage.[216]

5. Conflicts of interest

There are no conflicts to declare.

6. Acknowledgements

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