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Identification of an Anti-MRSA Cyclic Lipodepsipeptide, WBP-29479A1, by Genome Mining of *Lysobacter antibioticus*

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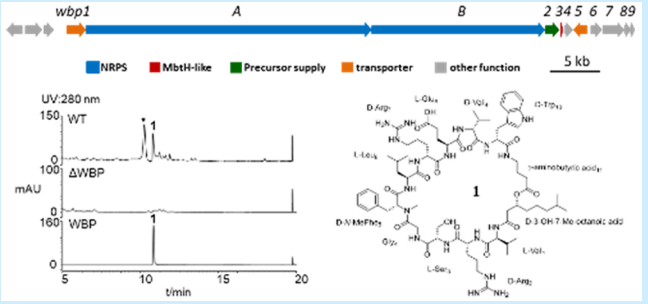
Supporting Information

ABSTRACT: *Lysobacter* are ubiquitous in the environment but remain largely underexplored, although the bacteria are considered "peptide specialists". Here, we identified a new cyclic lipodepsipeptide, WBP-29479A1 (**1**), through genome mining of *L. antibioticus* ATCC 29479. **1** is biosynthesized by a large NRPS gene cluster, and its structure, including the six nonproteinogenic residues and 3-hydroxy fatty acid, was determined by extensive spectroscopic analyses and chemical derivatization. **1** exhibits potent anti-MRSA activity in a menaquinone-dependent manner.



Microbial natural products and their derivatives have been a primary source of most successful antibiotics.[1] Since the discovery of the first antibiotics in the early 20th century, soil bacteria (particularly *Actinomycetes*) and fungi have provided the vast chemical scaffolds of the antibiotics in current use. A recent report on natural product discovery over the past 70 years revealed that continuing to search for natural products in the same producing organisms using the same approaches is associated with a high probability of rediscovering the same chemical scaffolds.[2] Looking into new microbial arenas that have not been well-explored is a profitable approach to discover natural products with novel structures. *Lysobacter* is a genus of Gram-negative bacteria that was not classified until 1978 and has remained largely underexplored.[3] These ubiquitous environmental gliding bacteria have attracted the attention of researchers in agricultural crop protection and new antibiotic discovery, because several *Lysobacter* species are prolific producers of lytic enzymes and bioactive natural products.[4−6] Notably, *Lysobacter* is particularly rich in biosynthetic gene clusters (BGC) for cyclic lipopeptides and considered "peptide specialists".[6] Currently, only a few cyclic lipopeptides are marketed, although these antibiotics have enjoyed enormous success, such as daptomycin (Cubicin, Merck). However, daptomycin-resistant pathogens have emerged.[7] *Lysobacter* provides a new opportunity to discover new scaffolds of cyclic lipopeptides, such as WAP-8294A2 (Lotilibcin), which is structurally distinct from daptomycin and, importantly, shows a high efficacy against daptomycin-resistant pathogens.[8,9] The goal of this study is to address the need to expand the current repertoire of bioactive cyclic lipopeptides from *Lysobacter*. Herein, we report the identification of a cryptic gene cluster and structural elucidation of a new cyclic lipodepsipeptide, WBP-29479A1 (**1**), from *Lysobacter antibioticus* ATCC 29479. The activity tests showed that WBP-29479A1 has potent activity against Gram-positive bacteria in a menaquinone-dependent manner.

In the genome of *L. antibioticus* ATCC 29479, we found a BGC (*wbp*, GenBank Accession No. MN205987) that is different from all known BGCs from *Lysobacter*. It contains two large nonribosomal peptide synthetase (NRPS) genes (see Figure 1A, as well as Table S1 in the Supporting Information). *wbpA* encodes a 7-module NRPS, with a N-terminal condensation (C) domain, which is a type C^{III} domain typically found in NRPS for *N*-fatty acyl nonribosomal peptides.[10−12] *wbpB* encodes a 4-module NRPS, including a C-terminal thioesterase (TE) domain for product release.[13] In addition, the cluster contains genes coding for a MbtH-like protein, putative transporters, and auxiliary enzymes. Unusual among *Lysobacter* BGCs for cyclic lipodepsipeptides, *wbp* cluster contains four genes (*wbp6*−*wbp9*) predicted to encode four subunits of the cytochrome *o* ubiquinol oxidase complex, terminal enzymes of an electron transport chain for bacterial respiration.[14,15] These features suggest that the *wbp* cluster

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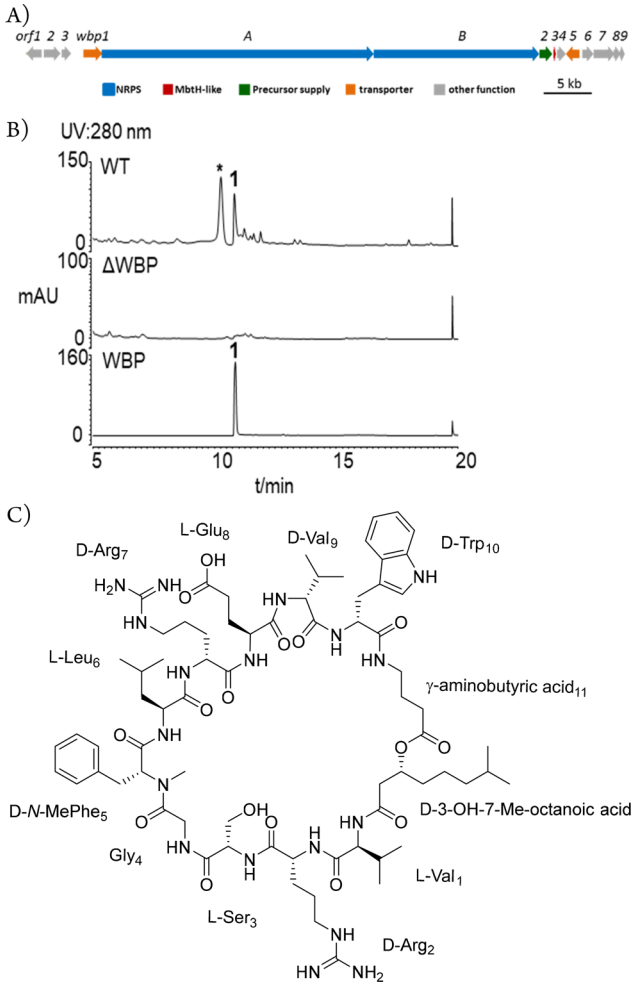

Figure 1. (A) Organization of the *wbp* biosynthetic gene cluster in *L. antibioticus* ATCC 29479. (B) HPLC analysis of WBP-29479A1 (**1**) production in the wild type (WT) and the mutant strain (ΔWBP) of ATCC 29479. The asterisk symbol (*) denotes a phenazine compound (see the Supporting Information for structure), which is not biosynthetically related to **1**. (C) Chemical structure of WBP-29479A1.

may be responsible for the biosynthesis of new peptide product(s) with intriguing biological properties.

To identify the product of the *wbp* cluster, we first must determine proper conditions for production of the product. Our previous experience has shown that most of the *Lysobacter* BGCs are silent or active only under certain culture conditions.[16] While there are sophisticated ways to activate BGCs, we decided to take a simple approach to identify the potential product of *wbp* cluster. Based on the organization of the *wbp* cluster and the predicted substrate specificity of the 11 adenylation (A) domains in WbpA and WbpB (see Tables S1 and S2 in the Supporting Information), we predicted the potential product of the *wbp* cluster would have a basic structure made from a 11-amino acid peptide with a N-terminal fatty acyl cap. Therefore, we decided to take advantage of this information to search for metabolites with a molecular mass in the range of 1300−1600 from *L. antibioticus* ATCC 29479 cultures growing under various conditions and media (NB, R_2A, WAP, 170, and 514a; see the Supporting Information for details). Liquid chromatography−high-resolution mass spectroscopy (LC-HRMS) analysis showed that the extract from *L. antibioticus* ATCC 29479 in the 514a medium contained a clear peak, **1** (WBP-29479A1), with m/z 743 for $[M+2H]^{2+}$ (see Figure S1 in the Supporting Information). This metabolite was not clearly produced in the other media, and we thus used 514a for the subsequent experiments.

To determine if the *wbp* cluster is responsible for **1** production, we generated a *wbp* mutant (ΔWBP) by deleting the key NRPS gene *wbpB* (see Figure S2 in the Supporting Information). High-performance liquid chromatography (HPLC) analysis showed that the mutant was no longer able to produce **1** (Figure 1B), indicating that *wbp* is required for **1** production. Interestingly, the deletion of the *wbp* gene led to disappearance of phenaznine compounds that also are produced by *L. antibioticus*. To further establish the relatedness between *wbp* and **1**, we performed a scale-up fermentation (8 L) of strain ATCC 29479 and obtained 32 mg of pure **1**, after a series of column chromatography purifications.

Compound **1** was isolated as a white powder. The molecular formula of **1** was established as $C_{72}H_{112}N_{18}O_{16}$ by electrospray ionization high-resolution mass spectroscopy (ESI-HRMS) (m/z 743.4326 for $[M+2H]^{2+}$) (see Figure S1C in the Supporting Information). Several α-amide proton signals in ^1H nuclear magnetic resonance (NMR) and carbonyl carbon signals at δ 174.4−170.4 in ^{13}C NMR were observed, suggesting that **1** was a peptide (see Table S3 and Figures S3−S6 in the Supporting Information). A detailed analysis of the 2D NMR data (HSQC, HMBC, and $^1H-^1H$ COSY) of **1** established the presence of two valine residues, two arginine residues, and one residue of serine, glycine, *N*-methylphenylalanine (*N*-MePhe), leucine, glutamic acid, tryptophan, and γ-aminobutyric acid, along with 3-OH-7-methyloctanoic acid (see Table S3 and Figures S7−S13 in the Supporting Information). The sequence of the amino acids was established through HMBC correlations from the NH protons to carbonyl carbons of adjacent residues, nuclear Overhauser effect (NOE) correlations from α-NH protons to α-amide protons of adjacent residues, and ESI/MS/MS analysis (see Figure S3). The analyses showed that the amino acids in **1** have a sequence as follows: 3-OH-7-methyloctanoic acid, Val_1, Arg_2, Ser_3, Gly_4, *N*-$MePhe_5$, Leu_6, Arg_7, Glu_8, Val_9, Trp_{10}, γ-aminobutyric $acid_{11}$ (see Figure S3). The closure of the macrocyclic ring was deduced from an HMBC correlation from δ 5.15 to the carbonyl at δ 172.6s. Thus, the planar structure of **1** was established (see Figure 1C).

The absolute configuration of the amino acids in **1** was established by the advanced Marfey's method.[17] After acid hydrolysis of **1**, the free amino acids in the hydrolysate were derivatized with L-FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and D-FDAA, followed by LC-MS analysis. The L-FDAA derivatives of the two Val residues in hydrolyzed **1** gave two peaks in LC-MS, and the D-FDAA derivatives of the two Val residues also gave the same two peaks (see Figure S5a in the Supporting Information). The result indicates that both D-Val and L-Val are present in **1**. Module-1 and module-9 of the NRPS assembly line contain an A domain for Val incorporation, but only module-9 contains an epimerase (E) domain (see Tables S1 and S2). Therefore, we assigned Val_1 as L and Val_9 as D. In contrast, the L-FDAA derivatives and the D-FDAA derivatives of the two Arg residues in hydrolyzed **1** each only gave one peak in LC-MS, indicating that the two Arg residues have the same configuration (see Figure S5a). However, the elution order of the L-FDAA derivative and the

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D-FDAA derivative seemed to suggest that the configuration of the Arg would be L, based on results reported in the literature,[17] instead of the D configuration, as predicted by the bioinformatics analysis (recall Tables S1 and S2). Besides, the retention time of the L-FDAA derivative and the D-FDAA derivative of Arg in hydrolyzed **1** were close to each another (Figure S5a). To clarify the question, we carried out another set of experiments to identify the absolute configuration of Arg in **1**. We compared the FDAA derivatives of Arg in hydrolyzed WBP-29479A1 with authentic D-Arg and L-Arg standards. The experiments determined the Arg in **1** to be the D configuration (Figure S5b). Similarly, we used standard α-, β-, and γ-aminobutyric acid to confirm that γ-aminobutyric acid is present in **1** (see Figure S5cc). For the rest of the amino acid residues in hydrolyzed **1**, each of the FDAA derivatives was also analyzed, and the results confirmed their respective absolute configurations, as predicted by bioinformatics analysis (Figure S5a). For 3-OH-7-methyloctanoic acid, **1** was hydrolyzed in acid media and derivatized with 3,5-dinitroaniline (DA) (Figure S6). Two standard compounds, the chemically synthesized D,L-3-OH-7-methyloctanoic acid and the biologically produced D-3-OH-7-methyloctanoic acid (by hydrolyzing WAP-8294A2, which is known to contain a D-configuration fatty acid[18]) were also derivatized with DA. The identity of the derivatives was confirmed by high-resolution mass spectroscopy (HRMS), and the absolute configuration of 3-OH-7-methyloctanoic acid in **1** was assigned to the D configuration, based on a chiral HPLC comparison of the derivatives (Figure S6).

L. antibioticus ATCC 29479 exhibited antibacterial activity against *Staphylococcus aureus*, and this activity was mostly lost in the *wbpB* mutant (Figure 2). This suggests that **1** is a main contributor to the antibacterial activity in *L. antibioticus* ATCC 29479. Indeed, the purified **1** showed potent activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) strains, including *S. aureus* ATCC 43300, *S. aureus* USA300 JE2, *S. aureus* USA 300 LAC, and *S. aureus* LAC 13C with minimum inhibitory concentration (MIC) values of 0.25, 8, 2, and 2 μg/mL, respectively. Compound **1** was also active against *S. epidermidis* 1457 WT with MIC $\leq$ 0.25 μg/mL (see Tables S4 and S5 in the Supporting Information). The antibiotic activity of **1** is selective toward Gram-positive bacteria and exhibits little activity against Gram-negative bacterial pathogens, such as *E. coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, as well as the fungal pathogens *Cryptococcus neoformans* and *Candida albicans* (see Table S5). Besides, compound **1** showed >32 μg/mL of CC_{50} (concentration that causes 50% cytotoxicity) against human embryonic kidney cells, and >32 μg/mL HC_{10} (concentration that causes 10% hemolytic activity) against human red blood cells.

To understand the mode of action, we tested the effect of menaquinone (MK) and ubiquinone (UQ) on the antibiotic activity of **1**. MK is an essential component for bacterial electron transfer in respiratory chain, while UQ is an important factor of mammalian electron transfer chain.[19] WAP-8294A2 was used as control, as it is also a cyclic lipodepsipeptide with potent activity that is selective against Gram-positive bacteria and is known to be MK-dependent.[8] The result showed that MK, but not UQ, significantly increased (>160 fold) the MIC of **1** and WAP-8294A2 against *S. aureus* (Figure 2). Together, our data support that the mode of action of **1** occurs by acting on a bacterial membrane component that is essential for the respiratory chain.

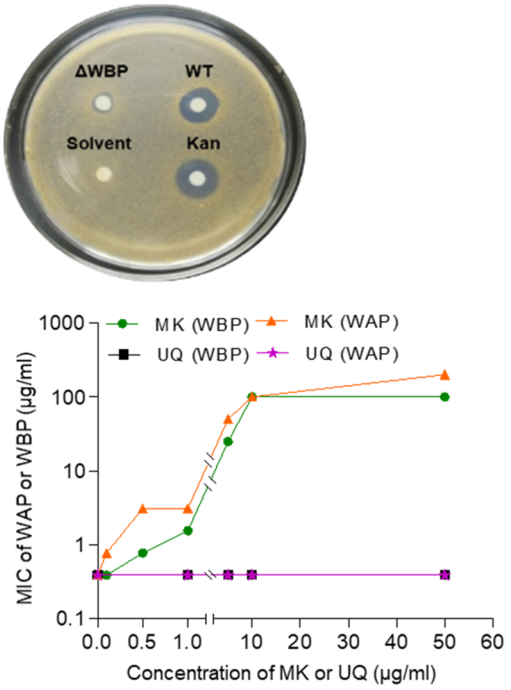

Figure 2. Antibacterial activity test of the wild type and the mutant. Top figure represents when *Staphylococcus aureus* ATCC 25923 was used as the testing organism. [Legend: WT = wild type of *L. antibioticus* ATCC 29479; ΔWBP = *wbpB* deletion mutant; Solvent = MeOH with 5 mM TFA, as a negative control; Kan = kanamycin (0.1 μg), as a positive control. Bottom figure depicts the effect of menaquinone (MK) and ubiquinone (UQ) on the antibiotic activity of WBP-29479A1 and WAP-8294A2 against *S. aureus* ATCC 25923. More activity test results are summarized in Tables S4 and S5.

In summary, we have successfully identified a new anti-MRSA cyclic lipodepsipeptide, WBP-29479A1, through genome mining of *Lysobacter*, a genus of Gram-negative bacteria emerging as a new source of bioactive natural products. The peptide portion of **1** is made from 11 amino acids, including 6 nonproteinogenic amino acids. The domain and module organization of the WBP NRPS assembly line is colinear with the composition of the peptide (see Figure 3).

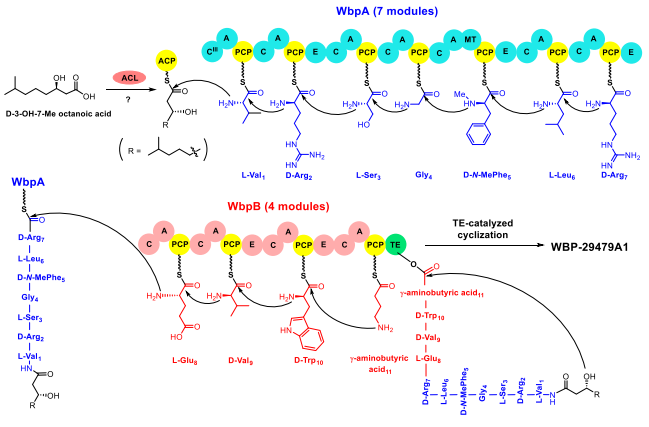

Figure 3. Proposed biosynthesis for WBP-29479A1 (**1**) in *L. antibioticus* ATCC 29479.

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The lipo-portion of **1** is a D-3-hydroxy fatty acid. Interestingly, no gene for acyl CoA-ligase-like enzyme is present in the *wbp* cluster, suggesting *in trans* acyl-CoA ligases may be responsible for the activation and incorporation of the fatty acids, as seen in WAP-8294A.[20] It conceivable that the *wbp* cluster could produce other congeners of **1** with varied D-3-hydroxyfatty acyl chains, probably because of the action of multiple acyl-CoA ligases similar in WAP-8294A. Compound **1** has potent anti-MRSA activity and exhibits a MK-dependent antibiotic activity, which is similar to that of WAP-8294A2 and lysocin E.[8,21−23] There are a large number of NRPS gene clusters in *Lysobacter* genomes remaining to be explored. Our study demonstrates that *Lysobacter* is a rich source of bioactive peptide natural products. Together with WAP-8294A2 and lysocin E, WBP-29479A1 provides a new cyclic lipodepsipeptide lead for the development of novel antibiotics with a unique menaquinone-dependent mechanism of action.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b02333.

Details of experimental procedures, primer list, gene cluster organization, construction of plasmid for gene deletion, generation and verification of mutant, metabolite production and isolation, chemical derivatization, and spectroscopic data (PDF)

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Author Contributions

L.D., Y.L., and Y.S. designed the experiments, analyzed and interpreted the data; M.S. performed the experiments with the support of Y.L., H.W., and L.S.; N.R.A. and M.C.-S. performed the activity tests; L.D., Y.L., and M.S. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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