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Identification of the Biosynthetic Gene Cluster for the anti-MRSA Lysocins through Gene Cluster Activation Using Strong Promoters of Housekeeping Genes and Production of New Analogs in *Lysobacter* sp. 3655

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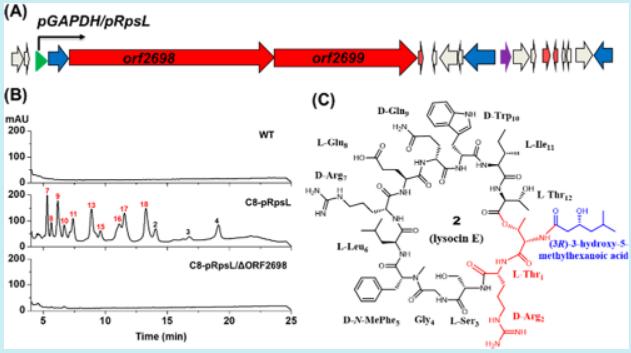
ABSTRACT: The Gram-negative gliding bacteria *Lysobacter* represent a new and rich source for bioactive natural products. In an effort to discover new antibiotics, we found a cryptic biosynthetic gene cluster (BGC) in *Lysobacter* sp. 3655 that shared a high similarity with the putative lysocin BGC identified *in silico* previously from *Lysobacter* sp. RH2180-5. Lysocins are cyclic lipodepsipeptides with potent activity against MRSA (methicillin-resistant *Staphylococcus aureus*) using a novel mode of action, but the lysocin BGC had not been experimentally verified so far. Using an activity-guided screening, we isolated the main antibiotic compound and confirmed it to be lysocin E. However, the putative lysocin BGC was barely transcribed in the wild type, in which lysocins were produced only in specific conditions and in a negligible amount. To activate the putative lysocin BGC, we screened for strongly transcribed housekeeping genes in strain 3655 and found several powerful promoters. Upon engineering the promoters into the BGC, the lysocin gene transcription was significantly enhanced and the lysocin yield was markedly increased. With readily detectable lysocins production in the engineered strains, we showed that lysocin production was abolished in the gene deletion mutant and then restored in the complementary strain, even when grown in conditions that did not support the wild type for lysocin production. Moreover, the engineered strain produced multiple new lysocin congeners. The determination of the lysocin BGC and the *Lysobacter* promoters will facilitate the ongoing efforts for yield improvement and new antibiotic biosynthesis using synthetic biology strategies.



KEYWORDS: *Lysobacter, natural products, antibiotics, lysocins, promoter engineering*

Lysobacter are gliding Gram-negative bacteria ubiquitously inhabiting in soil and water.[1] A number of *Lysobacter* species are prolific producers of extracellular lytic enzymes and recognized as a promising source of new bioactive natural products, which confer the species the potential both as biocontrol agents and as producers of new drug leads. Several types of bioactive natural products have been identified from *Lysobacter*, including cyclic peptides, cephem-type β-lactams, and polycyclic tetramate macrolactams (PoTeM).[2] Non-ribosomal peptides are the most common natural products found in *Lysobacter*, such as the cyclodepsipeptide lysobactin, the cyclic lipodepsipeptides tripropeptin family, WAP-8294A family, lysocin family, and WBP-29479A1.[3−11] These compounds typically contain 8−12 amino acid residues, and some also contain a β-hydroxyl fatty acyl chain of varied chain length and shape (linear or branched). Many of the amino acids are nonproteinogenic and carry modifications. All of the cyclopeptides have important biological activities. For example, WAP-8294A2, tripropeptins, and lysocin E show potent anti-MRSA activity. The MIC (minimum inhibitory concentration) of WAP-8294A2, which reached clinical studies, was reported to be 14-fold lower than that of vancomycin.[12,13] Lysobactin showed 4-fold greater activity than vancomycin.[14,15] Notably, cyclopeptides such as WAP-8294A2 and WBP-29479A1 exhibited strong selectivity against drug-resistant *Staphylococcus* strains among a panel of clinical isolates of pathogens.[11,16]

Due to their excellent antibiotic activity, the *Lysobacter* cyclopeptides have been a very active area of research for the

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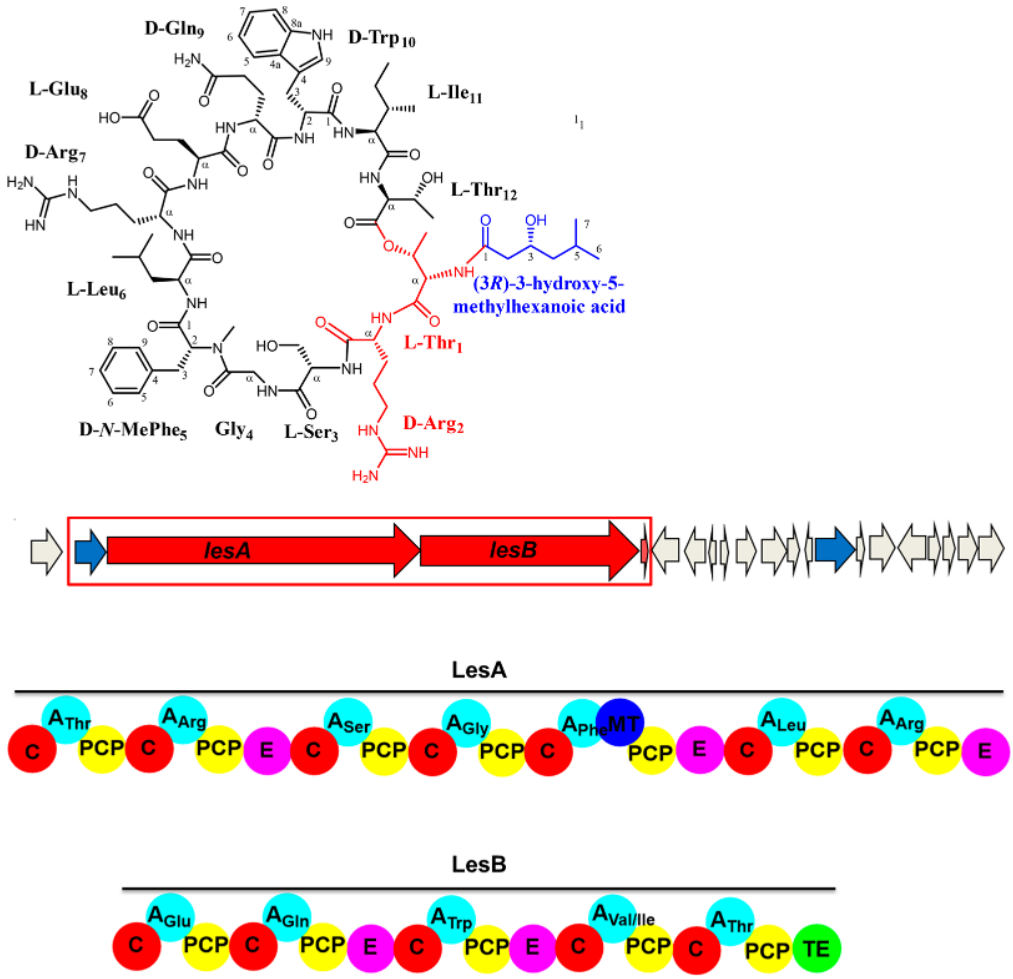

Figure 1. Chemical structure of lysocin E and the putative lysocin biosynthetic gene cluster (*les*) identified *in silico* from *Lysobacter* sp. RH2180-5.[19]

mode of action. Lysobactin inhibits peptidoglycan biosynthesis via formation of a 1:1 complex with Lipid I and Lipid II, which causes septal defects and catastrophic cell envelope damage.[3] WAP-8294A2, lysocin E, and WBP-29479A1 share a similar antibacterial mechanism, which is the menaquinone-dependent lysis of the bacterial membranes.[6,8,11] Tripropeptin C blocks the lipid cycle of cell wall biosynthesis by complex formation with undecaprenyl pyrophosphate.[17]

Among the *Lysobacter* cyclopeptides, lysocins represent the latest discovery of a promising lead for anti-MRSA antibiotic development. They were first isolated from *Lysobacter* sp. RH2180-5 using the silkworm infection model.[8] The structure is a macrocycle comprising 12 amino acid residues with an ester linkage (Figure 1). In the family, 9 lysocin congeners have been reported, among which lysocin E showed the best anti-MRSA activity with a MIC of 4 μg/mL. Total chemical synthesis of lysocin E was achieved via an elegant solid-phase strategy, and its enantiomeric, epimeric, and *N*-demethylated analogs also were synthesized simultaneously.[9] The antibacterial activity of the synthesized lysocin E and its enantiomer is comparable to that of the natural isomer, indicating the unimportance of chiral recognition in its antibacterial mechanism. Moreover, it was found 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.[18] Recently, a high-throughput strategy had been developed which enabled the preparation of thousands of lysocin E analogs and large-scale structure−activity relationship analyses.[10] A total of 2401 cyclic peptides were generated from an integrated 26-step chemical synthesis, and among the 23 that were identified, 11 analogs exhibited antimicrobial activity superior or comparable to that of lysocin E. So far, the research on lysocins has focused on the chemical synthesis, mode of action, and structure−activity relationship.[8−10,18] However, little is known about the biosynthesis of lysocins. The main reason is that the biosynthetic gene cluster for lysocin had not been experimentally determined, although an *in silico* study identified the putative lysocin BGC (*les*) in *Lysobacter* sp. RH2180-5.[19]

In this study, we found that *Lysobacter* sp. 3655 is a producer of lysocins and identified a cryptic BGC (Cluster-8, accession number MT410681) in its genome that is homologous to the *les* cluster in *Lysobacter* sp. RH2180-5. We also found that the putative *les* genes were barely transcribed in the wild type of *Lysobacter* sp. 3655, in which only a negligible amount of lysocins was produced. We then identified strong promoters of housekeeping genes in *Lysobacter* sp. 3655 and constructed engineered strains, in which both the transcription level and the lysocin yield were significantly enhanced. Using the engineered strains, we were able to demonstrate that Cluster-8 in *Lysobacter* sp. 3655 is indeed the lysocin BGC.

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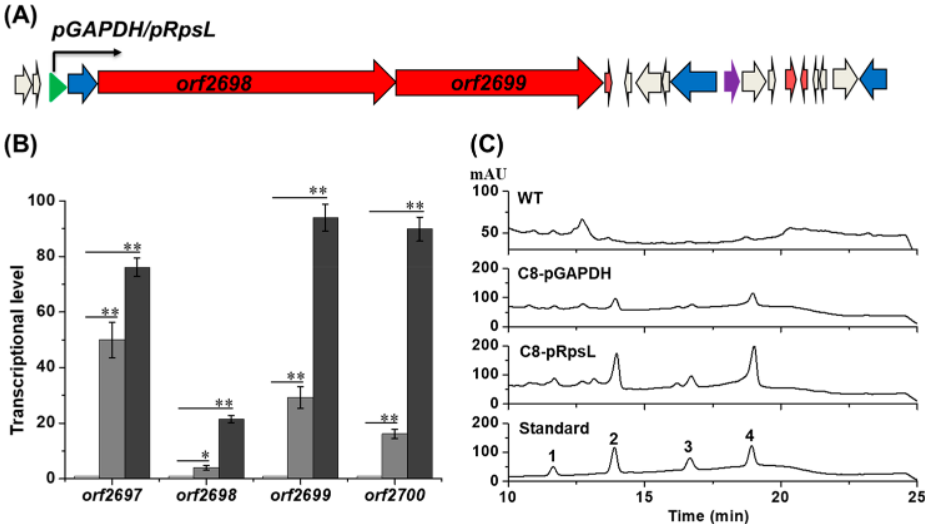

Figure 2. Activation of transcription of the putative lysocin biosynthetic gene cluster (Cluster-8, *les* homologous) and of production of lysocin congeners in *Lysobacter* sp. 3655 in GBS solid medium. (A) Genetic organization of Cluster-8, with the four core genes (*orf2697*-2700) under the control of the constitutive promoter *pGAPDH* and *pRpsL* in the engineered strains of *Lysobacter* sp. 3655. (B) qRT-PCR analysis of the four core genes in the wild type (WT, the first column) and the engineered strains (C8-pGAPDH, the second column; C8-pRpsL, the third column) at 48 h. Data are presented as averages of three independent experiments, with each conducted in triplicate. *, $P < 0.05$; **, $P < 0.01$. (C) HPLC analysis of lysocin congeners in WT, C8-pGAPDH, and C8-pRpsL at 72 h.

Furthermore, the results showed that new lysocin congeners can be synthesized through the synthetic biology approach.

RESULTS AND DISCUSSION

Isolation and Structural Analysis of Lysocins from *Lysobacter* sp. 3655. To isolate potential antibiotic compounds from strain 3655, we tested a variety of culture conditions. As shown in Figure S1A, extracts from strain 3655 generated clear inhibition zones against the testing organism, *Bacillus subtilis*, only in the GBS medium particularly in solid medium, while other culture conditions (liquid or solid PSE, 0.1TSB, and R2A) showed very little or no activity. We subsequently scaled up the fermentation in GBS solid medium and used the bioassay-guided fractionation on LC columns (RP-C18 and Sephadex LH-20) (Figure S1B) to collect the active fractions. This process yielded four compounds **1**−**4**, as shown on LC-MS, with molecular mass apparently consistent with several known lysocins (Figures S1B−D and S2).

Despite the very low overall yield, compound **2** was produced in a relatively higher amount than the other compounds. We subsequently conducted scaleup preprations (6 L solid culture) and obtained **2** (6 mg) for further spectroscopic analyses. The HR-ESI-MS of **2** gave m/z 809.4417 for $[M+2H]^{2+}$ and 1617.8748 for $[M + H]^{+}$ (Figure S3A), which is consistent with the molecular formula of lysocin E, $C_{75}H_{116}N_{20}O_{20}$ (calculated mass 1616.8675) (Figure S3B). Analyses of ^{13}C NMR (Table S1 and Figure S4A) and HSQC data (Figure S4B), as well as comparison with previously published data,[8] supported that **2** is lysocin E. The other compounds, **1**, **3**, and **4**, were isolated in a minute amount (1−2 mg), which were analyzed by mass spectrometry (Figure S2). The spectra indicated that **1** has the same mass as lysocin A/B, **3** is consistent with the mass of lysocins C/D/F/G, and **4** agrees with the mass of lysocin H/I.[8] Together, the results from the spectroscopic analysis, antibiotic assay, and the high sequence similarity between the biosynthetic genes from strain 3655 and that from the reported lysocin-producing *Lysobacter* sp. (Figure S5), support that compound **2** is lysocin E, and compounds **1**, **3**, and **4** are lysocin congeners (probably isomers of lysocin A/B, C/D/F/G, and H/I, respectively).[8]

Identification of Promoters for Highly Expressed Housekeeping Genes, Activation of Lysocin Gene Expression and Improvement of Lysocin Yield through Promoter Engineering. *Lysobacter* sp. 3655 could produce lysocin compounds in GBS solid medium, but the yield was still neglible. The compounds extracted from a 100 mL GBS culture was barely detectable on HPLC (Figure S6), which severely hindered the study of the biosynthetic mechanism for lysocins. One obvious problem was that there was no clear phenotypic change in the HPLC profiles between the wild type and mutants, due to the low yield in the wild type. Compared to the putative lysocin BGC from *Lysobacter* sp. RH2180-5,[19] Cluster-8 in the genome of *Lysobacter* sp. 3655 shares the same set of core genes (*orf2697*-*orf2700*) (Figure S5). Two huge NRPS genes (*orf2698* and *orf2699*) are homologous to *lesA* and *lesB*, respectively; one transporter gene (*orf2697*) and one MbtH gene (*orf2700*) are flanking the NRPS genes. The four core genes are apparently located in the same operon and presumably share the same promoter located upstream of *orf2697*. These features inspired us to test the promoter engineering approach to enhance the expression of these genes.

As a relatively new genus of bacteria, *Lysobacter* species have very few genetic tools available for biosynthetic pathway manipulations.[16,20] To search for strong constitutive promoters, we examined the expression of six housekeeping genes from *Lysobacter* sp. 3655. A previous report in *Streptomyces* showed that the promoter of some housekeeping genes could be used to activate silent BGC.[21] Among the six housekeeping genes we tested, *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) and *rpsL* (30s ribosomal protein S12) showed the highest expression, giving 12−15-fold higher than that of *pyK* (pyruvate kinase) (Figure S7). The expression of *gyrB* (DNA gyrase subunit B), *rpoA* (RNA polymerase subunit α), and *rpoB* (RNA polymerase subunit β) was about 3−9-fold higher

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than that of *pyK*. Consequently, we chose the promoters of *gapdh* and *rpsL* for the activation of the putative lysocin BGC.

The strategy was through homologues recombination to insert the strong promoter of highly expressed housekeeping genes in front of *orf2697*, presumably the location of the native promoter that controls the putative lysocin BGC. Two engineered strains, C8-pGAPDH and C8-pRpsL, were constructed using this approach, which inserted the promoter of *gapdh* and *rpsL*, respectively (Figure 2A and Figure S8). As expected, the transcription of *orf2697*, *orf2698*, *orf2699*, and *orf2700* was significantly enhanced in both engineered strains when compared to that of the wild type (Figure 2B). In strain C8-pGAPDH, the transcription of *orf2697-orf2700* increased 50-, 4-, 29-, and 16-fold, respectively; in strain C8-pRpsL, the transcription of *orf2697-orf2700* increased 76-, 21-, 94-, and 90-fold, respectively (Figure 2B). The results showed that the promoter of *rpsL* was more powerful than that of *gapdh* in enhancing the expression of the putative lysocin genes. Next, we analyzed the lysocin production in the engineered strains. In the wild-type extract, lysocin compounds were barely detectable by HPLC. However, lysocins were readily detectable in the crude extract of C8-pGAPDH and C8-pRpsL (Figure 2C). Particularly, the amount of lysocins in strain C8-pRpsL significantly increased and was higher than that in strain C8-pGAPDH, which was in accordance with the transcription assays. We were able to obtain 21 mg compound **2** and 18 mg compound **4** from 6 L of C8-pRpsL in YME liquid culture, compared to 6 mg **2** and 2 mg **4** from 6 L of the wild-type strain in the GBS solid medium. The result also supports that expression of the core genes, *orf2697-orf2700*, is necessary for the lysocin biosynthesis.

Experimental Verification of the Lysocin Biosynthetic Gene Cluster. With the readily detectable lysocins produced in the engineered strains, we now could test if the putative lysocin BGC (Cluster-8 in *Lysobacter* sp. 3655) is responsible for the production of the compounds. First, we deleted one NRPS gene (*orf2698*) in strain C8-pRpsL (Figure S9). No lysocin compounds could be detected in the deletion mutant (C8-pRpsL/ΔORF2698) (Figure 3). Next, we generated a complementary strain (C8-pRpsL/ORF2698C) by reintroducing *orf2698* into the mutant (Figure S10). As expected, the production of lysocins in the complementary strain was restored to that of C8-pRpsL (Figure 3). The result clearly demonstrated that Cluster-8 is the lysocin BGC in *Lysobacter* sp. 3655. Comparing Cluster-8 from *Lysobacter* sp. 3655 to the putative lysocin BGC from *Lysobacter* sp. RH2180-5,[19] only four genes (*orf2697-orf2700*) are common to both clusters, whereas the flanking genes of *orf2697-orf2700* did not appear to be related to lysocin biosynthesis (Table S2). Notably, in addition to compounds **1**–**4** found in the wild type (strain 3655), a large number of new peaks were detected in the engineered strain (C8-pRpsL) and the complemented strain (C8-pRpsL/ORF2698C). These new peaks also disappeared in the NRPS mutant (C8-pRpsL/ΔORF2698). This suggests that the production of the new peaks is also dependent on the lysocin genes and that the insertion of the constitutive promoter *pRpsL* into the lysocin BGC might have induced production of new lysocin congeners (Figure 3).

Production of New Lysocin Congeners in Engineered Strains. As described above, we found that GBS solid medium was the best for lysocin production in the wild type, as well as in the promoter engineered strains (C8-pGAPDH and C8-pRpsL) (Figure 2C). However, this medium contains a relatively high concentration of soybean oil (1.6%), which complicates the extraction and isolation of the lipopeptides. Since promoter *pRpsL* supports constitutive transcription of the lysocin biosynthetic genes, and the transcription level was significantly improved in engineered strains (Figure 2B), it is reasonable to speculate that the engineered strain could constitutively produce lysocins in culture media other than GBS solid medium. We subsequently tested six liquid media (YME, M813m, LBM, PSE, NB, and YPG), and the result clearly showed that the engineered strain C8-pRpsL could produce various lysocin compounds in all tested media (Figure 4 and Table S3).

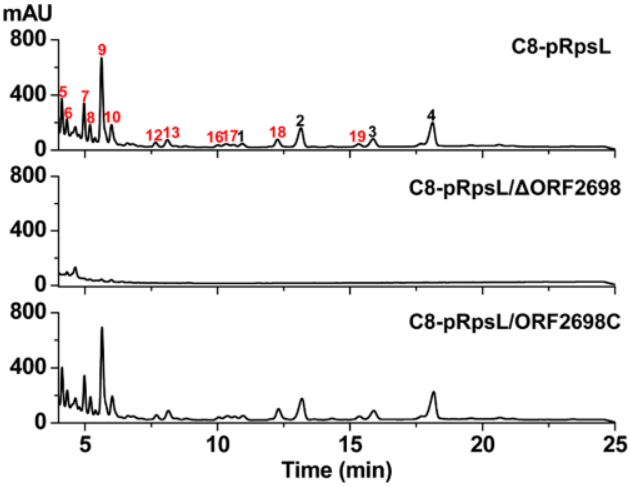

Figure 3. HPLC analysis of lysocin production in the engineered strain C8-pRpsL, the NRPS *orf2698* (*lesA*) deletion mutant (C8-pRpsL/ΔORF2698), and the complementary strain C8-pRpsL/ORF2698C. The strains were grown in GBS solid medium for 72 h. The MS data suggest that compounds **1**–**4** are known lysocin congeners (**1** lysocin A/B, **2** lysocin E, **3** lysocin C/D/F/G, **4** lysocin H/I), whereas **5**–**19** are probably lysocin-related products produced in the engineered strain.

The wild type could not produce any detectable lysocins in the six liquid media (except NB medium that produced a minute amount) (Figure 4). In contrast, the engineered strain C8-pRpsL produced a variety of lysocins, and the number of lysocin peaks on HPLC varied from 7 to 19 depending on the media (Table S3). In YME medium and M813m medium, promoter *pRpsL* led to production of 15 and 13 lysocin compounds, respectively, while production of the compounds was completely abolished in the deletion mutant C8-pRpsL/ΔORF2698 (Figure 4A,B). In LBM, PSE, and YPG medium, promoter *pRpsL* activated 7, 7, and 11 lysocin compounds, respectively (Figure 4C,D). The wild type could produce a negligible amount of compounds **2** and **4** in NB medium, and promoter *pRpsL* markedly enhanced the yield of these two compounds and also activated nine new lysocin compounds that were absent in the wild type (Figure 4D). Among the media, YME and M813m could afford the most of the lysocin compounds with a high yield (Table S3). As a result, these two media were selected in following studies. Together, the results unequivocally showed that promoter *pRpsL* activated the constitutive gene expression and lysocin biosynthesis in various media, probably due to a bypass of the transcriptional controls of the native promoter. Promoter *pRpsL* not only significantly enhanced the yield of lysocins but also resulted in the production of a large number of new lysocin congeners.

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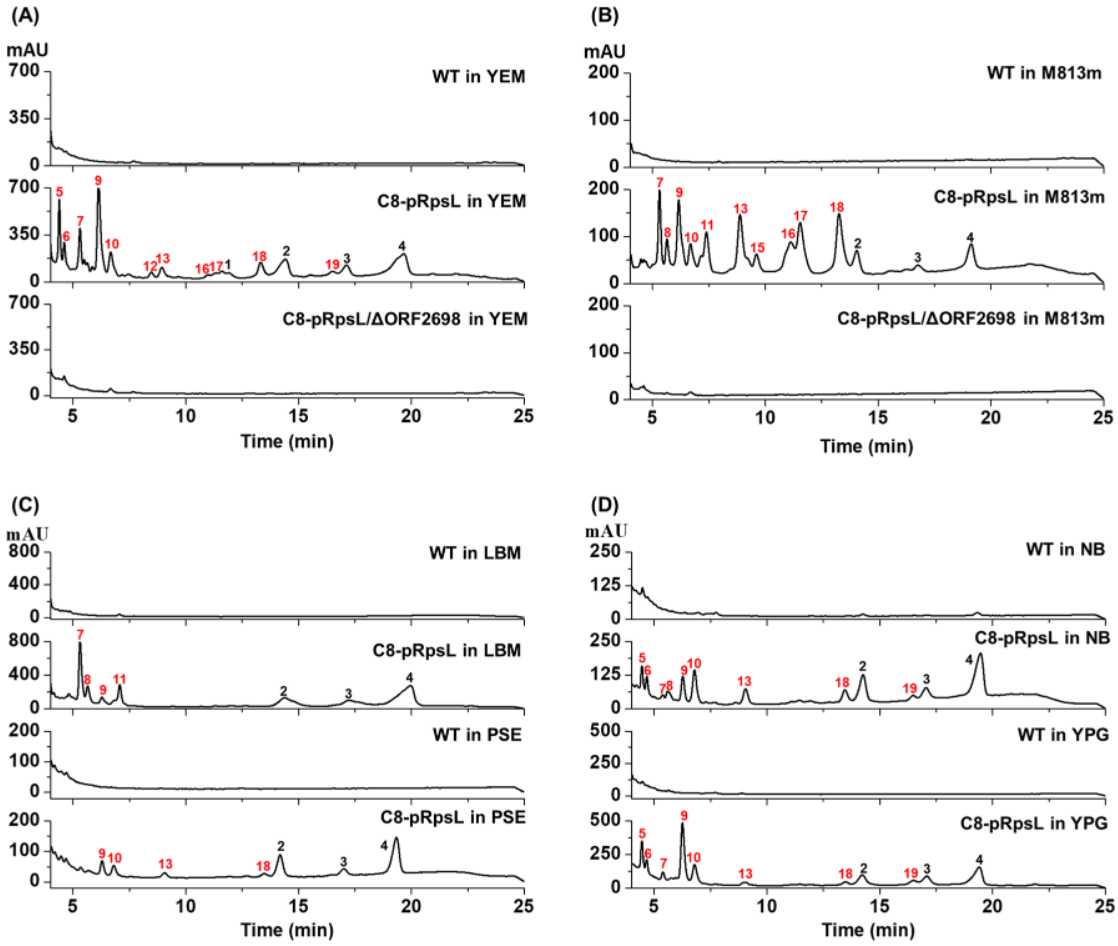

Figure 4. HPLC analysis of lysocin production in the engineered strain grown in various culture media that do not support lysocin production in the wild type. All cultures were grown for 72 h. (A) WT, C8-pRpsL, and C8-pRpsL/ΔORF2698 in YME liquid medium; (B) WT, C8-pRpsL, and C8-pRpsL/ΔORF2698 in M813m liquid medium; (C) WT and C8-pRpsL in LBM liquid medium and in PSE liquid medium; (D) WT and C8-pRpsL in NB liquid medium and in YPG liquid medium.

Analysis of the New Lysocin Compounds. Nine lysocin compounds were reported from *Lysobacter* sp. RH2180-5.[8] In the engineered *Lysobacter* sp. 3655, we found at least 19 lysocin-like compounds were produced in various cultures (Table S3). Among them, compounds **1**−**4** had been reported as described above. Compounds **5** and **6** did not give a clear signal in MS analysis (Figure S11). The remaining 13 compounds appeared to be new lysocin-related products based on the results from gene mutation and complementaion. The mass of **7**−**10** was much smaller than that of the reported lysocins, whereas the mass of **11**−**19** was in the similar range of the reported lysocins (Table S4, Figures S12−S13). To get more structural information, we purified compound **7** and analyzed it by HR-ESI-MS/MS. Compound **7** gave *m*/*z* 625.8305 for $[[M + 2H]^{2+}$ and *m*/*z* 1250.6534 for $[M + H]^{+}$, and its MS/MS fragmentation pattern of the a-, b-, and y-series of ions was consistent with a linear decapeptide derivative of lysocins (Figure S14). This compound only contains the portion of the third through twelfth amino acid residues of lysocin E, without L-Thr_1 and D-Arg_2, as well as the 3-hydroxy fatty acyl chain, which are common to all previously reported lysocins (Figure 5A).

Possible Origin of Compound 7. The composition and sequence of the 10 amino acid residues of **7** are identical to that of the third through twelfth residues of compounds **2**−**4**, indicating that **7** may be a degradation product of the known lysocins or an aberrent biosynthetic product of the lysocin BGC due to a NRPS module-skipping, as seen in other *Lysobacter* NPRS.[22] To determine the origin of compound **7**, we performed a site-directed mutagenesis (K508E) at one of the active sites within the first adenylation domain (A_1) of the NRPS gene *orf2698*. This lysine residue (K508) is conserved in A domains and has been shown to be critical to the adenylate-forming half reaction.[23] The strain containing K508E mutation in A_1 was generated and verified (Figure S15), and the lysocin production was totally abolished (Figure 5B). Since the likelihood that the K508E single residue change in the first NRPS module would affect the enzymatic activity of the other 11 NRPS modules is low, the result suggests that **7** is most likely a degradated product of regular lysocins, rather than an aberrent biosynthetic product through module-skipping of the first two NRPS modules. Moreover, an examination of the change of the profile of lysocin congeners at different growth times showed that **2**−**4** decreased, as the culture time increased. Conversely, **7** and **9** gradually increased, as the culture time increased (Figure 5C,D and Figure S16). A prolonged culture of 144 h reduced the "full-length" lysocins to 20% of that of 24 h, while it increased the "partial-length" lysocins by 4−20-fold when compared to that at 24 h. Together, the results support that **7** is a degradation

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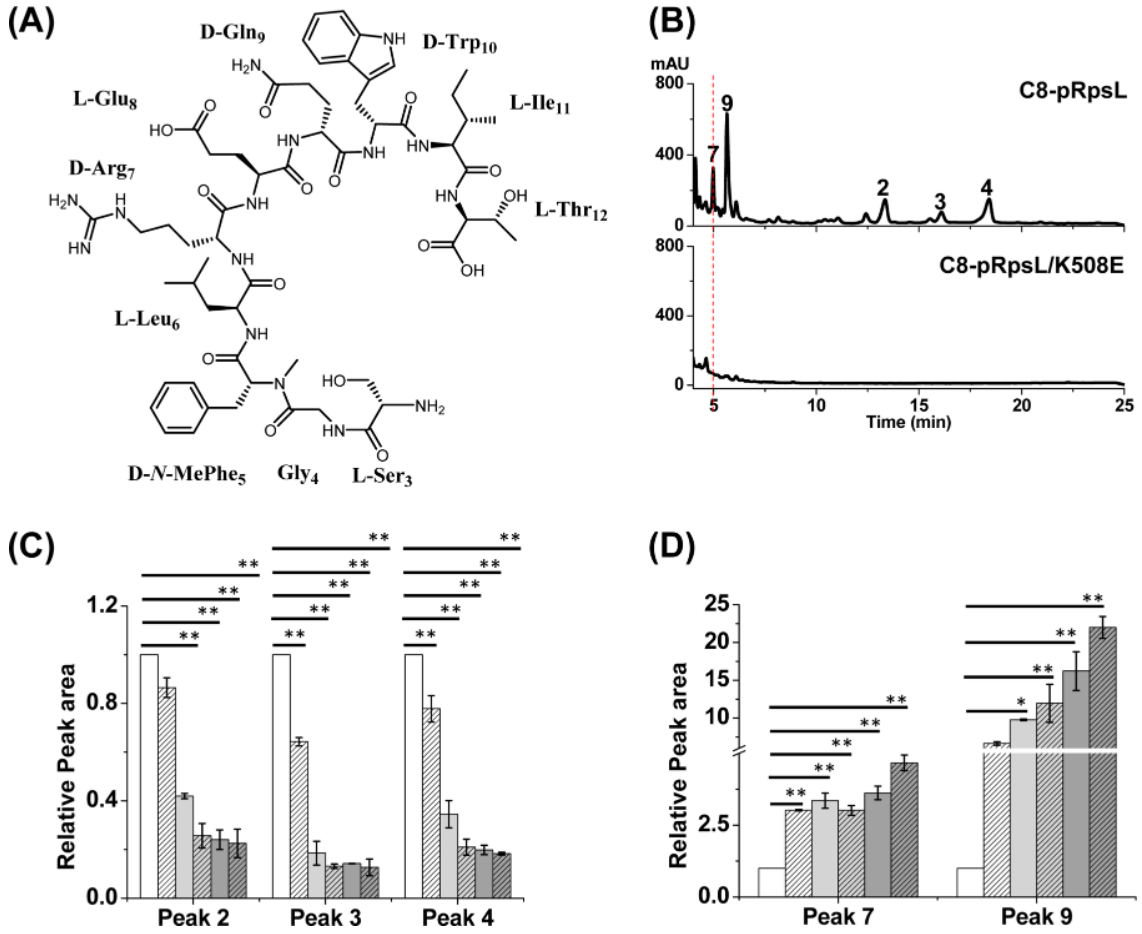

Figure 5. Possible origin of compound 7. (A) Proposed structure of 7. (B) HPLC analysis of lysocin production in the engineered strain C8-pRpsL and the point-mutated strain C8-pRpsL/K508E that were cultured in YME liquid medium for 72 h. (C) Relative amounts of lysocins **2**–**4** in strain C8-pRpsL at different culture times (24–144 h). (D) Relative amounts of compounds 7 and **9** in strain C8-pRpsL at different culture times (24–144 h). For each of the compounds, the first bar (white) for 24 h, the second bar (white with stripes) for 48 h, the third bar (light gray) for 72 h, the fourth bar (light gray with stripes) for 96 h, the fifth bar (dark gray) for 120 h, and the final bar (dark gray with stripes) for 144 h. Data are presented as averages of three independent experiments each conducted in triplicate. *, $P < 0.05$; **, $P < 0.01$.

product of **2**–**4**, whereas the other putative "partial-length" compounds (**8**–**10**) also are likely derived from a degradation/modification of the full-length lysocins. It is worth further investigations into the degradation enzymes and their potential relevance to drug resistance against cyclic lipodepsipeptide antibiotics, such as lysocins, WAP-8294A, and WBP-29479A1, which is essentially unknown. Considering the potent antibacterial activity of lysocins, the degradation may be a mechanism of self-protection of *Lysobacter* sp. 3655. Indeed, the antibiotic activity tests showed that the "partial-length" compounds had no activity against *B. subtilis*, while compound **2** (lysocin E) exhibited the highest activity among the known lysocins (**2**–**4**) (Figure S17). Interestingly, the remaining "full-length" compounds (**11**–**19**) generally showed low activity, except **18** and **19** (Figure S17). Since the overall yield of **2**–**4** was significantly enhanced in the enginneered strain C8-pRpsL, the convertion of **2**–**4** to **7** and other low-activity compounds may be a necessity to attenuate the potent antibiotic effect against the producer *Lysobacter* sp. 3655. However, no putative peptidase/esterase gene was found within or around the lysocin BGC (Table S2), indicating that the degradation process is likely due to peptidases/esterases *in trans*.

Summary and Concluding Remarks. This work showed that *Lysobacter* sp. 3655 is a producer of lysocins and experimentally identified the lysocin biosynthetic gene cluster for the first time. The studies also identified several powerful promoters useful in constitutive expression of cryptic genes in *Lysobacter* sp. 3655, as demonstrated in the significant increase of the gene transcription as well as the natural product production of both the known lysocins and the large number of new lysocin-related compounds. Further spectroscopic analyses are needed to fully establish the structures. In the wild type, only a few lysocin congeners were produced and only under certain conditions, such as GBS solid medium. The presence of the compounds in the wild-type cultures was barely detectable by common analytical HPLC. Using the promoter engineering approach, we demonstrated that the engineered strains could produce a large number of lysocin compounds in a markedly enhanced yield in every tested media. These studies showed the feasibility of using the engineered strains to study the mechanism for biosynthesis and resistance, as well as production of new lysocins through synthetic biology approaches.

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METHODS

Bacterial Strains, Plasmids, and Growth Conditions. Bacterial strains and plasmids used in this study are shown in Table S5. Luria−Bertani (LB) broth medium was used for the growth of *Lysobacter* sp. 3655. Four different media with liquid and solid (1.5% agar) fermentations, including two eutrophic media of GBS and PSE, as well as two oligotrophic media of 0.1TSB and R2A, were initially used to explore the antibiotic-producing potential of strain 3655. GBS solid medium was also used for the RNA extraction. Besides, YME, M813m, LBM, PSE, NB, and YPG media were further used to evaluate the lysocin-producing potential of promoter engineered strains. The compositions of the above-mentioned media are as follows: GBS (2% glucose, 0.5% beef extract, 1.6% soya-bean oil, 0.1% NaCl, 0.1% $CaCO_3$, pH 8.5); PSE (1% glycerol, 1% tryptone, 1% bactopeptone, 0.15% K_2HPO_4, 0.15% $MgSO_4$); 0.1TSB (0.15% tryptone, 0.05% soybean peptone, 0.05% NaCl, pH 7.0); R2A (0.05% glucose, 0.05% soluble starch, 0.05% proteose peptone, 0.05% casamino acids, 0.05% yeast extract, 0.03% NaBr, 0.03% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$); YME (0.4% glucose, 1% malt extract, 0.4% yeast extract); M813m (0.4% glucose, 0.3% K_2HPO_4, 0.138% $NaH_2PO_4{\cdot}H_2O$, 0.1% NH_4Cl, 0.0144% $MgSO_4$, 0.015% KCl, 0.00111% $CaCl_2$, 0.000278% $FeSO_4{\cdot}7H_2O$); LBM (1% tryptone, 0.5% yeast extract, 1% NaCl, 5% maltose); NB (0.3% beef extract, 0.1% yeast extract, 0.5% peptone, 1% sucrose); and YPG (0.7% glucose, 0.7% bactopeptone, 0.7% yeast extract). Plasmid pJQ200SK was used for gene deletion in *Lysobacter* sp. 3655.[24] *B. subtilis* was used as the indicator bacterium for the activity-guided isolation and antibiotic potential of lysocin compounds.[7] *Escherichia coli* strain XL-1 Blue was cultured at 37 °C in LB medium supplemented with gentamicin (Gm, 50 μg/mL) to propagate plasmids. *E. coli* strain S17-1 was used for intergeneric conjugation.

DNA Manipulation and *Lysobacter* Transformation. Chromosomal DNA and plasmids were isolated from *Lysobacter* sp. 3655 or *E. coli* according to the standard techniques using phenol−chloroform extraction followed by ethanol precipitation. Database searching and sequence analysis were performed using the online program PSI-BLAST. For *Lysobacter* transformation, plasmids were first introduced into the *E. coli* S17-1 and then transferred to *Lysobacter* sp. 3655 by intergeneric conjugation. The transformants were spread on LB plates with kanamycin (Km, 100 μg/mL) and Gentamicin (Gm, 150 μg/mL). Kanamycin was used to inhibit the growth of *E. coli* S17-1 after conjugating with *Lysobacter* sp. 3655, since stain 3655 is intrinsically kanamycin resistance. Gentamicin was used to screen the single crossover mutant since plasmid pJQ200SK has the Gentamicin resistance gene. After growing at 30 °C for 72 h, the single crossover colonies were plated on LB plates containing 10% (w/v) sucrose and Km (100 μg/mL), 30 °C for 72 h. Plasmid pJQ200SK contains *SacB* encoding levansucrase, which can degrade sucrose to glucose and fructose, and then catalyze fructose to fructosan. Fructosan is harmful to the bacterial growth, resulting in growth only for colonies that have lost the plasmid due to a second crossover, and any remaining single crossover mutants containing *SacB* would not grow in the presence of sucrose. Then individual transformants were transferred into LB plates supplemented with Km (100 μg/mL) or Km (100 μg/mL) + Gm (150 μg/mL) to further select for the double crossover mutants. The Km resistant and Gm sensitive strains, which represented the putative double crossover mutants, were confirmed by PCR verification.

Primers and PCR. All primers used in this study are listed in Table S6. PCRs were carried out using Phanta Max Super-Fidelity DNA polymerase (Vazyme) or r*Taq* DNA polymerase (Takara). For Phanta DNA polymerase, an initial denaturation at 95 °C for 3 min was followed by 30 cycles of amplification (95 °C for 15 s, 60 °C for 15 s, 72 °C for 1 min), and additional 5 min at 72 °C. For r*Taq* DNA polymerase, an initial denaturation at 95 °C for 5 min was followed by 30 cycles of amplification (95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min), and additional 10 min at 72 °C. Considering different DNA templates and primers, the annealing temperature and the elongation time were changed in some cases.

Fermentation and Preparation of Various Crude Extracts. Various strains of *Lysobacter* sp. 3655 were grown in 1 mL LB medium at 30 °C for 24 h with shaking, and then 1% cell culture was transferred to various solid (100 mL) or liquid (25 mL) media, respectively. The solid or liquid fermentations were incubated at 30 °C without or with shaking at 200 rpm for 72 h, respectively. The cultures were first treated with ethyl acetate (EtOAc) to remove lipid soluble components, and then exhaustively extracted with water-saturated butanol (0.05% TFA) to obtain various crude extracts.

Various extracts were analyzed by HPLC or LC-MS using the MeCN−H_2O eluting system. The mobile phase comprised solvents A (H_2O, 0.1% TFA) and B (MeCN, 0.1% TFA). The analysis program was as follows: 25% to 35% B in A for 0 to 3 min, slowly increased to 36% B at 6 min, then to 48% B at 23 min, and back to 25% B at 24 min. The flow rate was 2.0 mL/min with the UV detection of 220 nm.

Antibacterial Bioassay. *B. subtilis* was incubated in 3 mL LB medium at 37 °C for 6 h with shaking, and then diluted using LB medium to OD_{600} = 0.3. The culture was mixed with LB agar (1:1000 ratio), and the mixture was poured into a plate for solidification. Various culture extracts, isolated fractions, and purified lysocin compounds were diluted with methanol to the concentrations of 200 mg/mL, 100 mg/mL, and 2 mg/mL, respectively, which were added to each of the sterilized small round filter-papers with the amount of 5 μL. After the solvent naturally dried, the filter-papers were placed on the surface of the LB dishes. The dishes were incubated at 37 °C until clear inhibition zones appeared.

Bioassay and MS Guided Isolation of Lysocin Compounds. Due to the significant antibacterial activity of the crude extract of GBS solid medium (Figure S1A), this medium was chosen to be further used for the large-scale fermentation of strain 3655, which was cultured in 100× Petri dishes (2 L per batch, total 6 L). The crude extract (9.742 g) was first isolated via column chromatography over RP-C18 eluting with a MeOH−H_2O gradient (from 1:9 to 1:0) and final MeOH with 0.2% TFA to obtain seven fractions [Fractions (Frs.) 1−7] (Figure S1B). Based on the antibacterial and LC-MS data (Figure S1B−C), Fr. 6 and 7 were combined and then separated via CC over Sephadex LH-20 (MeOH) to yield seven subfractions (Fr. 6 +7 − 1 to 6 + 7 − 7). Finally, the bioactive Fr. 6 +7 − 3∼5 were combined and further purified via HPLC to obtain compounds **1** (1 mg, t_R 11.9 min), **2** (6 mg, t_R 14.1 min), **3** (2 mg, t_R 16.8 min), and **4** (2 mg, t_R 19.1 min) (Figure S1D).

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Generation of the Engineered Strains C8-pGAPDH and C8-pRpsL. To construct the engineered strains, two adjacent DNA fragments and the promoter region of *gapdh* or *rpsL* were amplified using relevant primers listed in Table S6. The three overlap extension fragments were fused using overlapping PCR strategy.[25] The fusion fragment containing the promoter flanked on each side by the sequence homologues to the specific recombination site of *orf2697* was digested by *Spe*I/*Sac*I and ligated into the same sites of plasmid pJQ200SK to generate the recombination plasmid pJQ200SK::C8-pGAPDH or pJQ200SK::C8-pRpsL.Then, the plasmid was introduced into 3655 by intergeneric conjugation. After antibiotic and sucrose screening, the transformants were used for PCR verification (Figure S8) by relevant primers listed in Table S6.

Construction of Deletion Mutant of *orf2698* and Its Complemented Strain. The DNA fragments corresponding to the upstream and downstream region of part of *orf2698* was amplified by using primers ORF2698UF/UR and ORF2698DF/DR. Then, the upstream region was treated with *Xho*I and *Bam*HI, the downstream region was treated with *Bam*HI and *Spe*I, and the two DNA fragments were ligated into the *Xho*I/*Spe*I sites of plasmid pJQ200SK to generate the recombination plasmid pJQ200SK::ORF2698. The plasmid was introduced into 3655 or C8-pRpsL strain by intergeneric conjugation. After antibiotic and sucrose screening, the transformants were used for PCR verification by primers ORF2698VFO/VRO and ORF2698VFI/VRI (Figure S9).

For complementation analysis, the DNA fragment containing the upstream region, downstream region and the deletion part of *orf2698* was amplified using primers ORF2698VFO/VRO. Then, the fragment was treated with *Apa*I/*Sma*I and ligated into the same sites of plasmid pJQ200SK to generate the recombination plasmid pJQ200SK::ORF2698C. The recombination plasmid was transferred into C8-pRpsL/ΔORF2698 strain by intergeneric conjugation. After antibiotic and sucrose screening, the transformants were used for PCR verification by primers ORF2698VFO/VRO and ORF2698VFI/VRI (Figure S10).

Site-Directed Mutagenesis of orf2698. Site-directed mutagenesis was performed using the Easy Mutagenesis System (TransGen Biotech). Briefly, the target fragment was amplified by primers A1-F/R, and then ligated into the *Xho*I/*Spe*I sites of plasmid pJQ200SK to generate the plasmid pJQ200SK::A1, the fragment was mutanted using the Easy Mutagenesis System (TransGen Biotech). The resultant plasmid pJQ200SK::A1K508E was verified by *Xho*I/*Spe*I and *Xho*I/*Sac*I digestion (Figure S15A,B). Then, the plasmid was transferred into C8-pRpsL strain by intergeneric conjugation. After antibiotic and sucrose screening, the transformants were confirmed by PCR and enzyme digestion (Figure S15C).

RNA Isolation and qRT-PCR. RNA was isolated from 3655, C8-pGAPDH, and C8-pRpsL strains cultured in GBS solid medium for 48 h, as described previously.[26] RNA was reverse transcribed to complementary DNA by using SuperScript II RT reagent kit (Invitrogen) after treated with DNase I (Thermo Scientific). qRT-PCR was carried out in CFX Connect Real-Time PCR Detection System (BIO-RAD Laboratories, Inc.) using UltraSYBR mixture (CWBIO) with primers listed in Table S6. The conditions are used as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The 16S rRNA was used as an internal control. The relative transcriptional levels of tested genes were normalized to 16S rRNA and determined by using the $2^{-\triangle\triangle CT}$ method.[27] The values were presented as fold change in comparison with the relative expression levels for each gene at the first test time point in the wild-type strain. Data are presented as the averages of three independent experiments conducted in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00067.

Details of primers, plasmid constructs, generation and verification of various strains, and spectroscopic data of lysocins (PDF)

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Author Contributions

L.D. and F.L. conceived and designed experiments. L.Y., F.D., X.C., Y.Z., and M.M. carried out experiments. L.D., L.Y., and F.D. analyzed data. L.D., L.Y., and F.D. wrote the manuscript. L.Y. and F.D. contributed equally.

Notes

The authors declare no competing financial interest.

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