Supporting Information

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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Table S1. ^{13}C (125 MHz) NMR data of lysocin E (DMSO-d_6, δ: ppm). The numbers and labels of the carbons correspond to that in the structure of lysocin E in Figure 1.

Position	δ_C (type)	Position	δ_C (type)	Position	δ_C (type)
	3-hydroxy-5-methylhexanoic acid		***N*-MePhe$_5$**		**Gln$_9$**
1	172.1, CO	*1*	169.6, CO	CO	171.7, C
2	43.1, CH_2	*2*	51.0, CH	*α*	51.8, CH
3	65.5, CH	*3*	33.8, CH_2	*β*	29.0, CH_2
4	46.1, CH_2	*4*	138.5, C	*γ*	30.2, CH_2
5	23.9, CH	*5,9*	129.1, CH	*γ*-$CONH_2$	173.6, C
6	23.3, CH_3	*6,8*	128.4, CH		**Trp$_{10}$**
7	21.8, CH_3	*7*	126.3, CH	*1*	170.7, CO
	Thr$_1$	*N*-CH_3	35.9, CH_3	*2*	52.6, CH
CO	168.1, C		**Leu$_6$**	*3*	28.4, CH_2
α	55.4, CH	CO	171.1, C	*4*	109.3, C
β	71.8, CH	*α*	51.4, CH	*4a*	127.3, C
γ	16.6, CH_3	*β*	40.2, CH_2	*5*	118.0, CH
	Arg$_2$	*γ*	24.2, CH	*6*	118.3, CH
CO	170.9, C	*δ*	22.9, CH_3	*7*	120.9, CH
α	51.5, CH	*δ'*	20.7, CH_3	*8*	111.2, CH
β	30.0, CH_2		**Arg$_7$**	*8a*	136.0, C
γ	24.8, CH_2	CO	172.9, C	9	123.0, CH
δ	40.3, CH_2	*α*	58.7, CH		**Ile$_{11}$**
NH=C	156.6, C	*β*	30.0, CH_2	CO	170.5, C
	Ser$_3$	*γ*	24.2, CH_2	*α*	58.1, CH
CO	170.0, C	*δ*	40.3, CH_2	*β*	36.1, CH
α	54.5, CH	NH=C	156.6, C	*β*-CH_3	10.7, CH_3
β	62.3, CH_2		**Glu$_8$**	*γ*	15.0, CH_2
	Gly$_4$	CO	170.5, C	*δ*	24.7, CH_3
CO	169.0, C	*α*	51.7, CH		**Thr$_{12}$**
α	41.7, CH_2	*β*	29.4, CH_2	CO	169.2, C
		γ	31.3, CH_2	*α*	58.2, CH
		γ-COOH	173.9, C	*β*	66.5, CH
				γ	20.0, CH_3

Table S2. Deduced function of ORFs in and around the lysocin gene cluster (Cluster-8) of *Lysobacter* sp. 3655.

ORF	Size (aa)	Annotation	%identity/ similarity	Accession number
orf2680	208	protein involved in polysaccharide export	89/92	SDY56278.1
orf2681	46	No significant similarity found		
orf2682	504	undecaprenyl-phosphate glucose phosphotransferase	99/99	WP_074868051.1
orf2683	279	porin family protein	96/97	WP_096378230.1
orf2684	257	hypothetical protein	93/94	WP_074868058.1
orf2685	207	sigma-70 family RNA polymerase sigma factor	99/100	WP_096378236.1
orf2686	246	hypothetical protein	99/99	WP_074868061.1
orf2687	253	acyl-CoA desaturase	99/99	WP_096378242.1
orf2688	435	FAD-dependent oxidoreductase	99/99	WP_074868066.1
orf2689	264	DUF1365 domain-containing protein	97/97	WP_074868068.1
orf2690	429	class I SAM-dependent methyltransferase	99/99	WP_096378248.1
orf2691	180	DUF2878 domain-containing protein	99/99	WP_096378251.1
orf2692	268	DUF1295 domain-containing protein	99/99	WP_096378254.1
orf2693	363	class I SAM-dependent methyltransferase	99/99	WP_083382572.1
orf2694	161	hypothetical protein	98/98	WP_074868081.1
orf2695	328	ATP-binding protein	99/100	WP_074870226.1
orf2696	135	No significant similarity found		
orf2697	586	ABC transporter ATP-binding protein	99/99	WP_074870228.1
orf2698	9232	non-ribosomal peptide synthetase	99/99	WP_083383100.1
orf2699	6471	non-ribosomal peptide synthetase	99/99	WP_074873721.1
orf2700	76	MbtH family protein	99/99	WP_074873762.1
orf2701	108	copper amine oxidase	78/89	ODU52305.1
orf2702	609	NADH:ubiquinone oxidoreductase, Na(+)-translocating, F subunit	98/98	SDX37479.1
orf2703	230	hypothetical protein	98/99	WP_074868090.1
orf2704	902	TonB-dependent receptor	99/99	WP_074868093.1
orf2705	244	Histidine utilization repressor	99/99	WP_096378293.1
orf2706	449	DUF239 domain-containing protein	99/99	WP_083382573.1
orf2707	124	No significant similarity found		
orf2708	293	alpha/beta hydrolase	99/100	WP_083382643.1
orf2709	214	glutathione S-transferase	96/98	WP_096378319.1
orf2710	86	No significant similarity found		
orf2711	146	No significant similarity found		
orf2712	663	sulfatase	99/99	WP_083382576.1
orf2713	623	ABC transporter ATP-binding protein	99/99	WP_096378331.1

Table S3. Lysocin compounds produced by C8-pRpsL engineered strain in various media

Medium	**Compounds**	Most abundant **compounds**（>100 mAU）
GBS solid	**1-10, 12, 13, 16-19**	**2, 4, 5, 7, 9**
YME	**1-7, 9, 10, 12, 13, 16-19**	**2, 4-7, 9, 10, 18**
M813m	**2-4, 7-11, 13, 15-18**	**7, 9, 13, 17, 18**
LBM	**2-4, 7-9, 11**	**2, 4, 7, 11**
PSE	**2-4, 9, 10, 13, 18**	**4**
NB	**2-10, 13, 18, 19**	**2, 4, 9, 10**
YPG	**2-7, 9, 10, 13, 18, 19**	**4, 5, 9, 10**

Table S4. Summary of the mass of the new compounds produced in the engineered strain C8-pRpsL.

Compound	*m/z* $(M+H)^+$	**Compound**	*m/z* $(M+H)^+$
5	Not determined	**13**	1535.40
6	Not determined	**14**	1650.87
7	1250.27	**15**	1649.60
8	1163.40	**16**	1664.40
9	1149.40	**17**	1548.67
10	1062.53	**18**	1564.67
11	1636.67	**19**	1632.53
12	1449.27		

Table S5. Bacterial strains and plasmids used in this study

Bacterial strains/plasmids	Relevant characteristics[a]	Source/references
***Lysobacter* sp. 3655**		
3655	Wild-type, Km^r	DSM
ΔORF2698	The deletion mutant of part of *orf2698* in 3655 (the deletion part was from +8746 to +12018 with respect to the start codon of *orf2698*)	This study
C8-pGAPDH	3655 containing the promoter *pGAPDH* in front of cluster-8	This study
C8-pRpsL	3655 containing the promoter *pRpsL* in front of cluster-8	This study
C8-pRpsL/ΔORF2698	The deletion mutant of part of *orf2698* in C8-pRpsL	This study
C8-pRpsL/ORF2698C	The complementary strain of C8-pRpsL/ΔORF2698	This study
C8-pRpsL/A1K508E	C8-pRpsL containing site mutation of K508E in A1 domain	This study
Other bacteria		
Bacillus subtilis	Indicator strain of lysocin	1
Escherichia coli strain		
XL-1 Blue	Host strain for molecular cloning	2
S17-1	Strain for conjugation with *Lysobacter*	3
Plasmids		
pJQ200SK	Cloning vector, Gm^r	4
pJQ200SK::ORF2698	Plasmid for the deletion of part of *orf2698*, Gm^r	This study
pJQ200SK::ORF2698C	Plasmid for the complementary of *orf2698*, Gm^r	This study
pJQ200SK::pGAPDH	Plasmid for the insertion of promoter *pGAPDH* in front of cluster 8, Gm^r	This study
pJQ200SK::pRpsL	Plasmid for the insertion of promoter *pRpsL* in front of cluster 8, Gm^r	This study
pJQ200SK::A1K508E	Plasmid for the site mutation of K508E in A1 domain, Gm^r	This study

[a] Km^r, kanamycin resistant; Gm^r, gentamicin resistant

Table S6. Primers used in this study.

Primer	Sequence (5'-3')	Purpose
For gene *orf2698* deletion, complementation and confirmation		
ORF2698UF	CCGCTCGAGGCCTTCACCAGCCACCATT (*Xho*I)	To amplify the upstream and downstream homolog fragment of *orf2698*
ORF2698UR	CGGGATCCGAAGTTCTCGAACACCACCAAG (*Bam*HI)	
ORF2698DF	CGGGATCCTTCAACGAACGCCACGAC (*Bam*HI)	
ORF2698DR	GGACTAGTCGGAGGTGTAGATCACATAGG (*Spe*I)	
ORF2698VFO	CCGACCTGGACGAACCGA	To amplify the complementary fragment of *orf2698*; To confirm the deletion mutant of *orf2698*
ORF2698VRO	CGGGTCCAGCGGCAAGTA	
ORF2698VFI	CGAACGCATGAGCCTGAAG	
ORF2698VRI	TCGGTATCCAGCGGTAGGT	
For promoter exchange and confirmation		
C8-UF	GGACTAGTTGAGGCTCCGCCAAGACG (*Spe*I)	To amplify the promoters of *gapdh* and *rpsL* and their adjacent fragments
C8-DR	CGAGCTCCCTTCGCTGGGCTGGTAG (*Sac*I)	
C8-pGAPDH-UR	GCAAGGGGGACTCCAGGAGCGGACTCCGGAAAGCGT	
C8-pGAPDH-F	ACGCTTTCCGGAGTCCGCTCCTGGAGTCCCCCTTGC	
C8-pGAPDH-R	GGACGAGGGACTGCTCATTGACATAACTCCTGCGGC	
C8-pGAPDH-DF	GCCGCAGGAGTTATGTCAATGAGCAGTCCCTCGTCC	
C8-pRpsL-UR	CCTTGCCTGGAACTTCGCGCGGACTCCGGAAAGCGT	
C8-pRpsL-F	ACGCTTTCCGGAGTCCGCGCGAAGTTCCAGGCAAGG	
C8-pRpsL-R	GGACGAGGGACTGCTCATCTGGAACTCTTGTTTAAGGGC	
C8-pRpsL-DF	GCCCTTAAACAAGAGTTCCAGATGAGCAGTCCCTCGTCC	
For qRT-PCR		
ORF2697-real-F	AGACCGTGAACCTGCTGAC	To amplify lysocin biosynthetic related genes
ORF2697-real-R	GGAATCATCAAGGCGAAGGC	
ORF2698-real-F	TGTTCCACTCCTACGCCTTC	
ORF2698-real-R	TCTGGTTGAGCACGGTGAC	
ORF2699-real-F	GCTGGATGTGTCCGAGTTG	
ORF2699-real-R	TGTGGTGGCTGGTGAAGAT	
ORF2900-real-F	ACACCAACGGCACCTTCCT	
ORF2900-real-R	CGATGTAGTCCAGGCATTCTTG	
3655-16S-real-F	TCCACGCCCTAAACGATG	To amplify 16S rRNA gene
3655-16S-real-R	TTGCAGCCCTCTGTCCCT	
GAPDH-real-F	CCAAGAAGGTGGTGCTGTC	To amplify house-keeping genes of *Lysobacter* sp. 3655
GAPDH-real-R	CCAGGCAGTTGGTCGTG	
GyrB-real-F	CCTCAACTCGGGCGTCAA	
GyrB-real-R	CGGTGATGCCGTTCTGCT	
pyK-real-F	CGCCAGTTCAACCACGAC	
pyK-real-R	GGATTCGGTCATCGCCAC	
RpoA-real-F	CGTGCTGGAAGTCCTGCT	
RpoA-real-R	CCTTGGTCAGGTGGCAGA	

RpoB-real-F	TCGGAAGTCACGCACAAGC	
RpoB-real-R	CACCGCCAGCGAGTTGAT	
RpsL-real-F	CCCCGAAGAAGCCGAACT	
RpsL-real-R	ACGACCGAGTGCTCCTGC	
For gene site-directed mutagenesis and confirmation		
A1-F	CCGCTCGAGTGTTCCACTCCTACGCCTTC(*Xho*I)	To amplify, mutant and confirm the *orf2698* A1-domain K508E
A1-R	GGACTAGTGCAGCACCACGGTATTGAC(*Spe*I)	
A1-K508E-F	CTGACCGCCAACGGCGAGCTCGACCG	
A1-K508E-R	CGCCGTTGGCGGTCAGCGGCAGCGAG	
A1-VR	GCATCAGCAGCAGCAATACG	

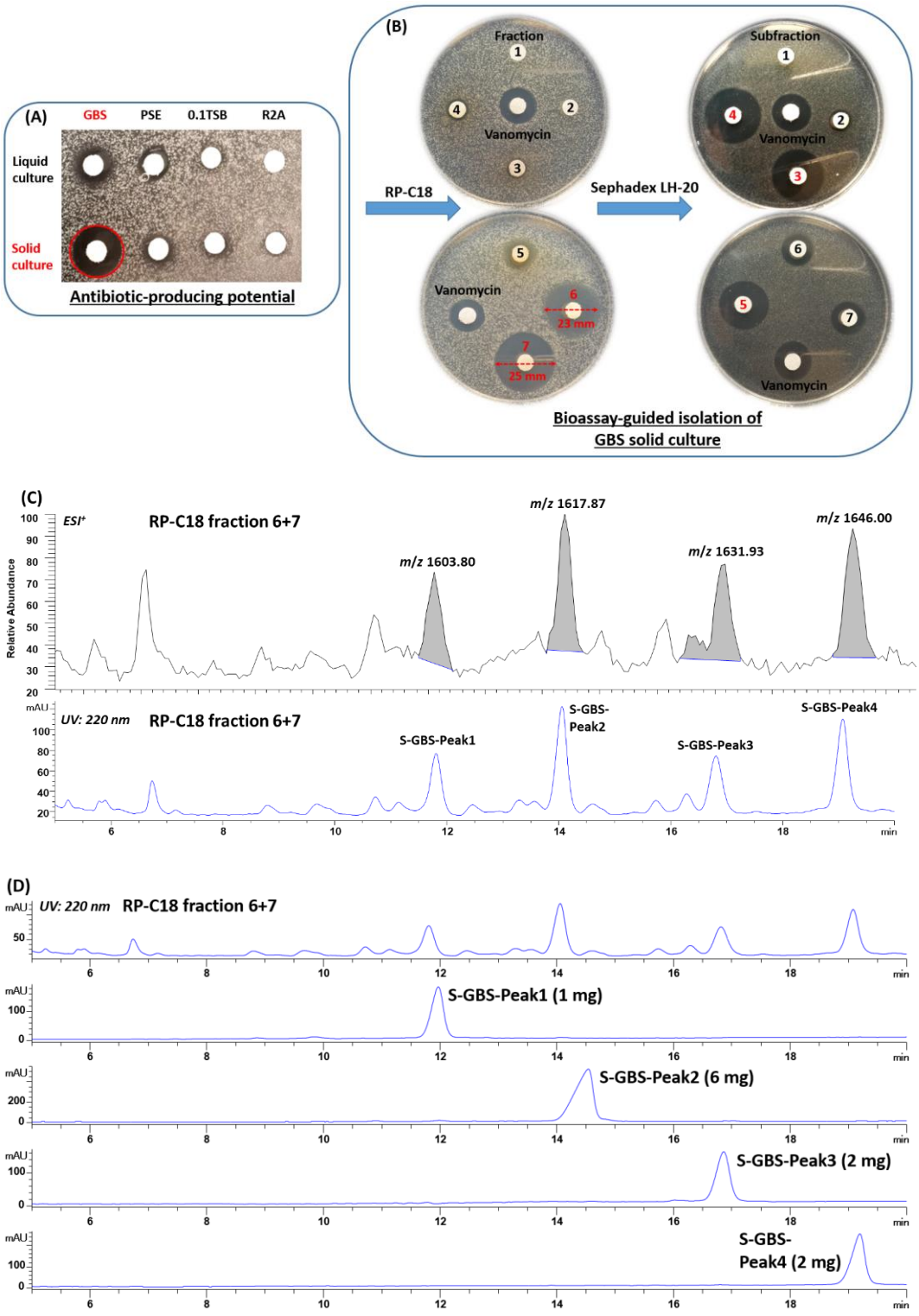
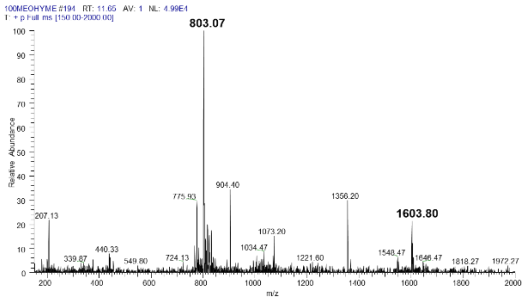


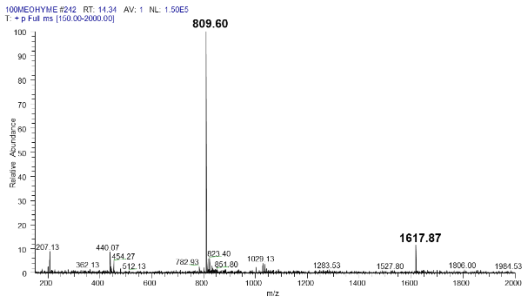
Fig S1. Identification of antibiotic compounds from *Lysobacter* sp. 3655. (A) Antibiotic activity of crude extracts from strain 3655 grown in various media, with *Bacillus subtilis* as the testing organism. (B) Bioassay-guided isolation of the active fractions

from the extracts of the GBS solid culture. (C) LC-MS analysis and (D) purification of the main active compounds (**1-4**).

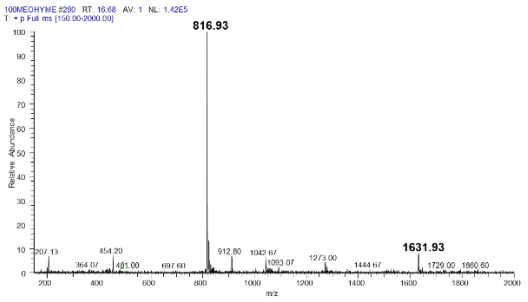
(A) Compound **1**



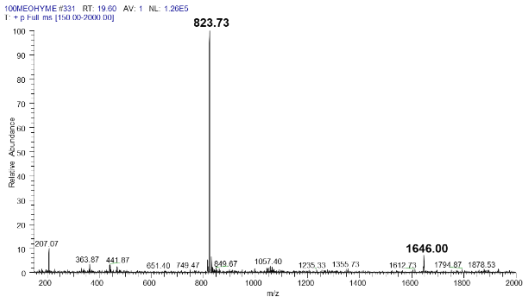
(B) Compound **2**



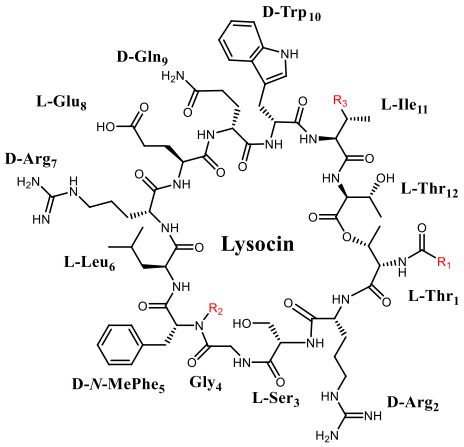
(C) Compound **3**



(D) Compound **4**



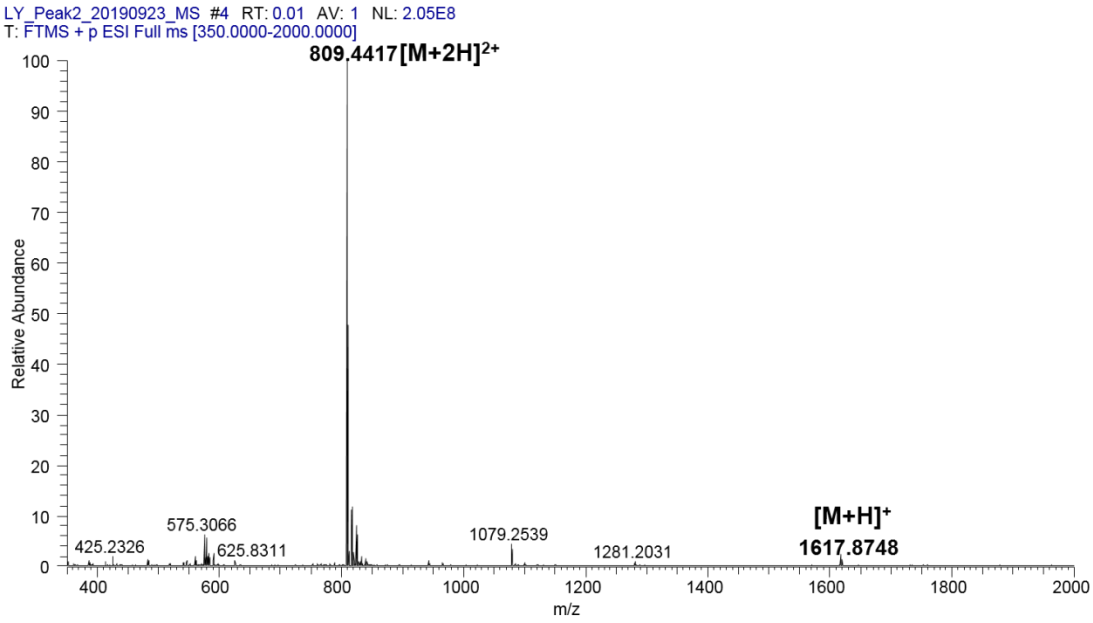
(E)



Compound	R1	R2	R3	Calculated mass
A	$C_6H_{13}O$	H	CH_3CH_2	1602.8518
B	$C_6H_{13}O$	CH_3	CH_3	1602.8518
C	$C_8H_{17}O$	H	CH_3CH_2	1630.8831
D	$C_8H_{17}O$	H	CH_3CH_2	1630.8831
E	$CH_3(CH_3)CHCH_2CHOHCH_2$	CH_3	CH_3CH_2	1616.8675
F	$C_7H_{15}O$	CH_3	CH_3CH_2	1630.8831
G	$C_7H_{15}O$	CH_3	CH_3CH_2	1630.8831
H	$C_8H_{17}O$	CH_3	CH_3CH_2	1644.8988
I	$CH_3(CH_3)CH(CH_2)_3CHOHCH_2$	CH_3	CH_3CH_2	1644.8988

Fig S2. Mass spectrum of compound **1-4** (A-D). The structure and calculated mass (E) of lysocins A-I are included to show that the mass of compoud **2** is consistent with that of lysocin E, and the mass of compounds **1**, **3**, and **4** is with that of lysocin A/B, C/D/F/G, and H/I, respectively.

(A)



(B)

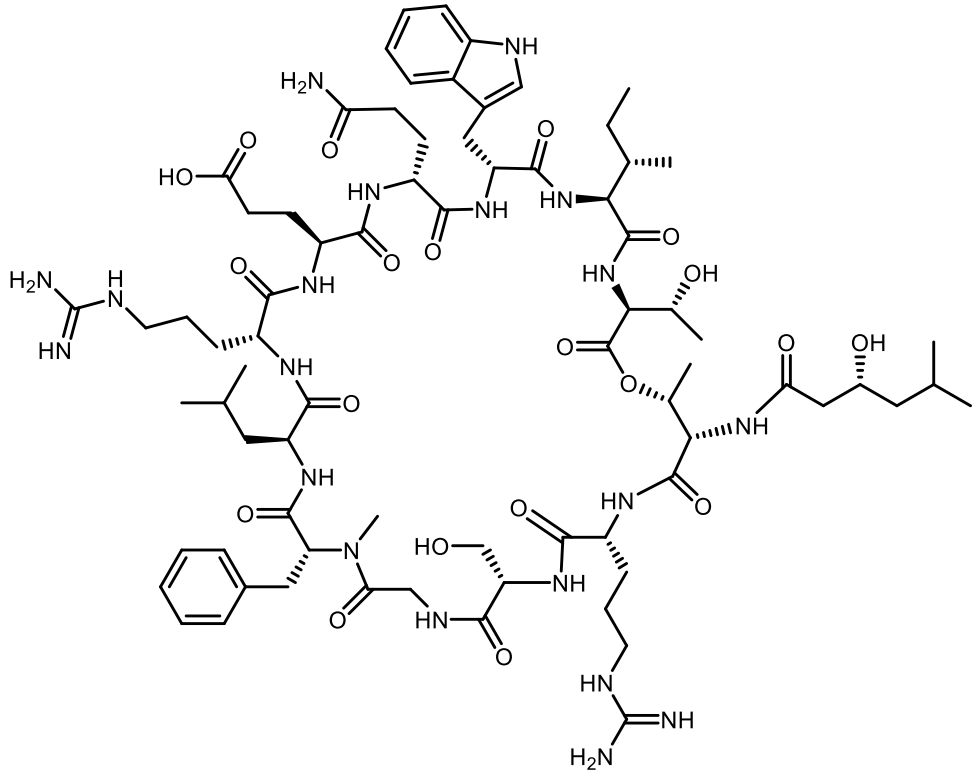
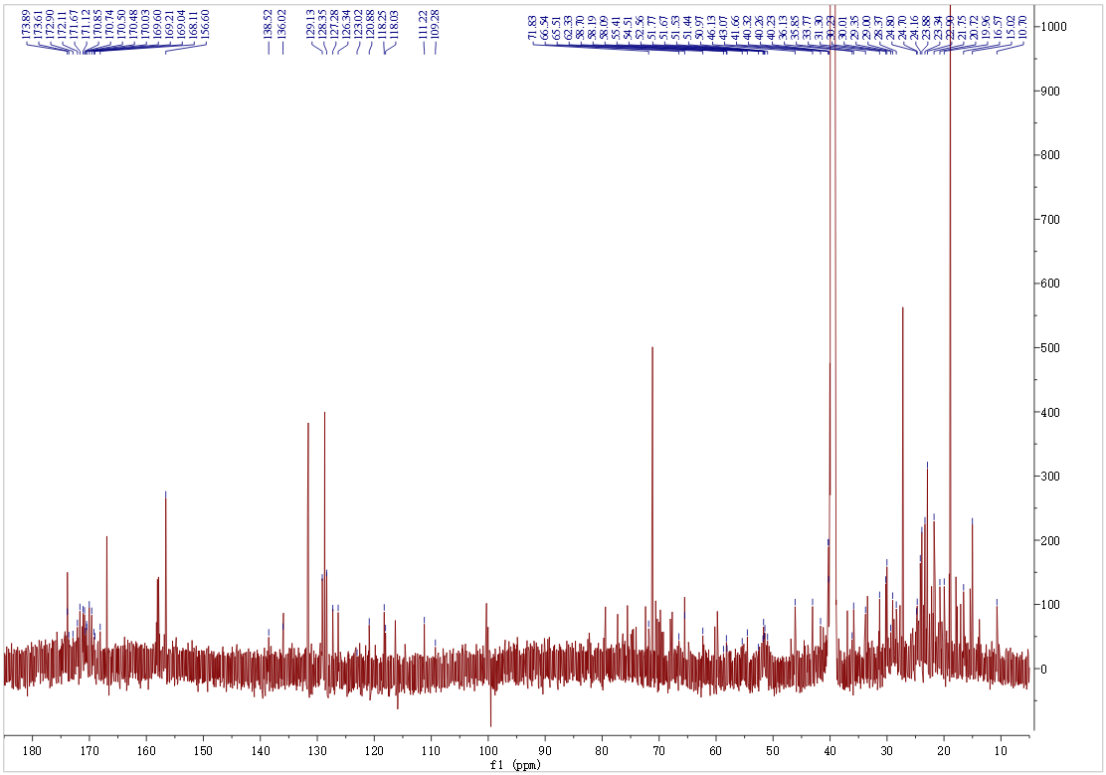


Fig S3. HR-ESI-MS spectrum of compound **2** (A) and the structure of lysocin E (calculated mass 1616.8675) (B).

(A)



(B)

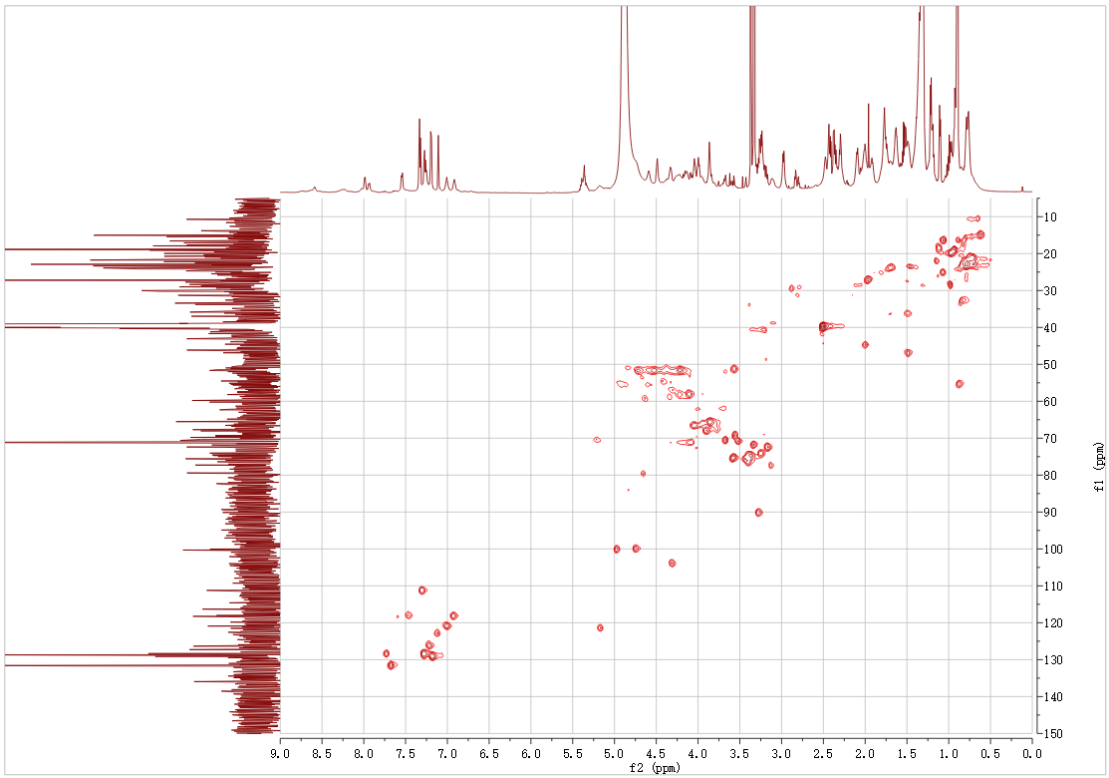


Fig S4. ^{13}C NMR (A) and HSQC (B) spectra of compound **2**.

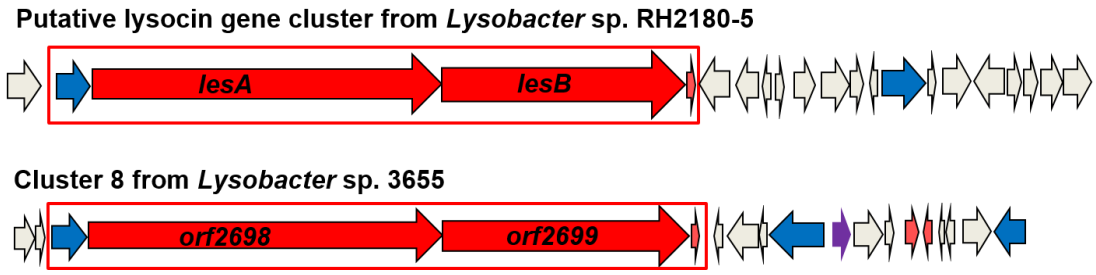


Fig S5. Comparison of the putative lysocin gene cluster (*les*) identified *in silico* from *Lysobacter* sp. RH2180-5[5] with Cluster-8 from *Lysobacter* sp. 3655.

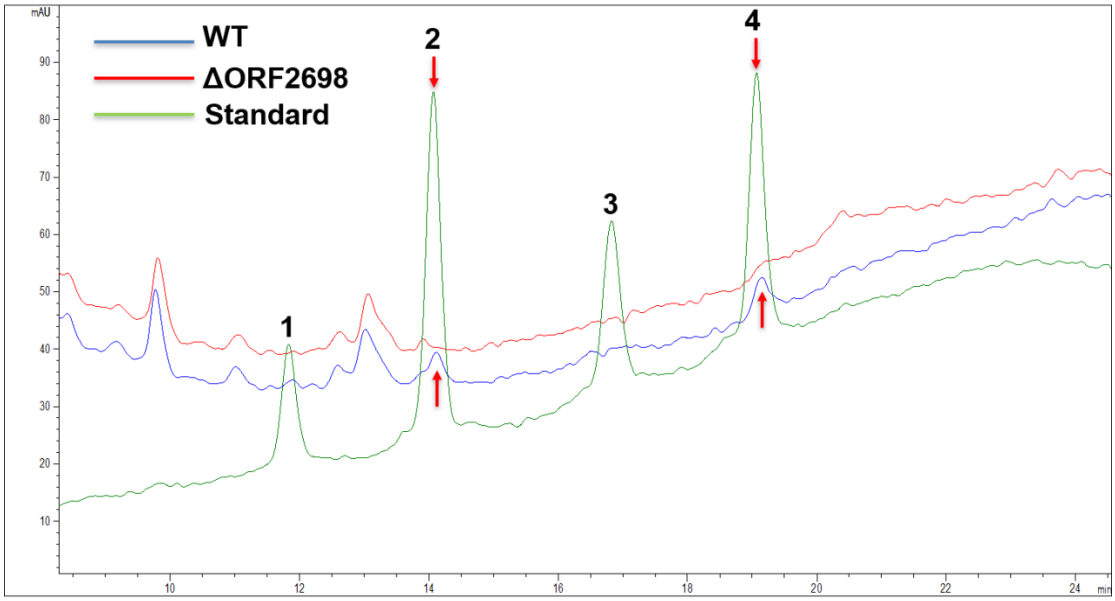


Fig S6. HPLC analysis of lysocin production in the wild type and the NRPS *orf2698* (*lesA*) deletion mutant of the wild type. The strains were cultured in GBS solid medium for 72 h. WT, *Lysobacter* sp. 3655 wild type; ΔORF2698, *orf2698* deletion mutant of *Lysobacter* sp. 3655. Standard lysocins were included as references.

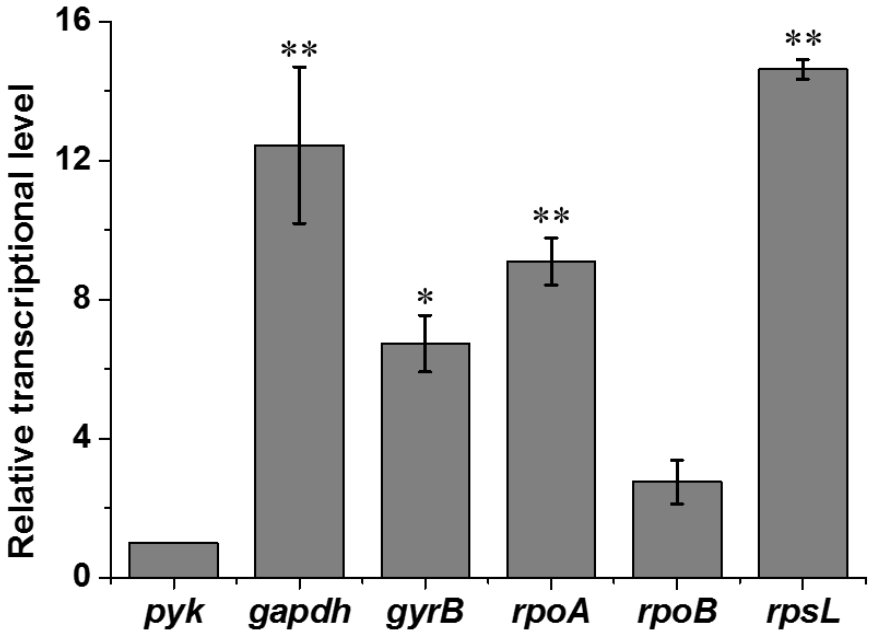


Fig S7. The relative transcription level of selected housekeeping genes in *Lysobacter* sp. 3655. *pyK*, pyruvate kinase encoding gene; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase encoding gene; *gyrB*, DNA gyrase subunit B encoding gene; *rpoA*, RNA polymerase subunit α encoding gene; *rpoB*, RNA polymerase subunit β encoding gene; *rpsL*, 30s ribosomal protein S12 encoding gene. Data are presented as averages of three independent experiments, with each conducted in triplicate. *, $P< 0.05$; **, $P < 0.01$.

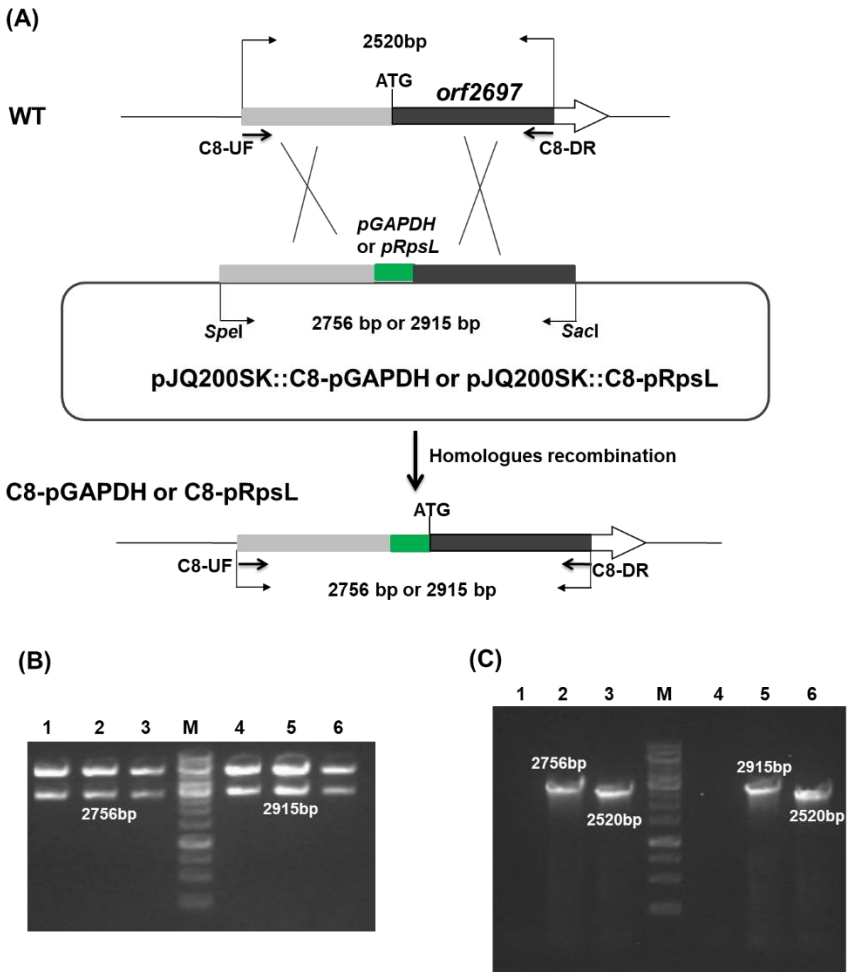


Fig S8. Generation of the engineered strains C8-pGAPDH and C8-pRpsL of *Lysobacter* sp. 3655. (A) Schematic illustration of the insertion of promoter *pGAPDH* or promoter *pRpsL* in front of the core genes of Cluster-8 in *Lysobacter* sp. 3655. (B) Verification of plasmid pJQ200SK::C8-pGAPDH and pJQ200SK::C8-pRpsL. Lanes 1-3: pJQ200SK::C8-pGAPDH treated with *Spe*I/*Sac*I, and the product of 2756 bp was expected; Lanes 4-6: pJQ200SK::C8-pRpsL treated with *Spe*I/*Sac*I, and the product of 2915 bp was expected; M: DNA marker. (C) PCR verification of the engineered strains C8-pGAPDH and C8-pRpsL using primers C8-UF/DR. Lanes 1 and 4: negative control, with H_2O as the template; lane 2, C8-pGAPDH strain, and the product of 2756 bp expected; lanes 3 and 6: positive control, with genomic DNA of WT as the template, and the product of 2520 bp expected; lane 5: C8-pRpsL strain, and the product of 2915 bp expected; M: DNA marker.

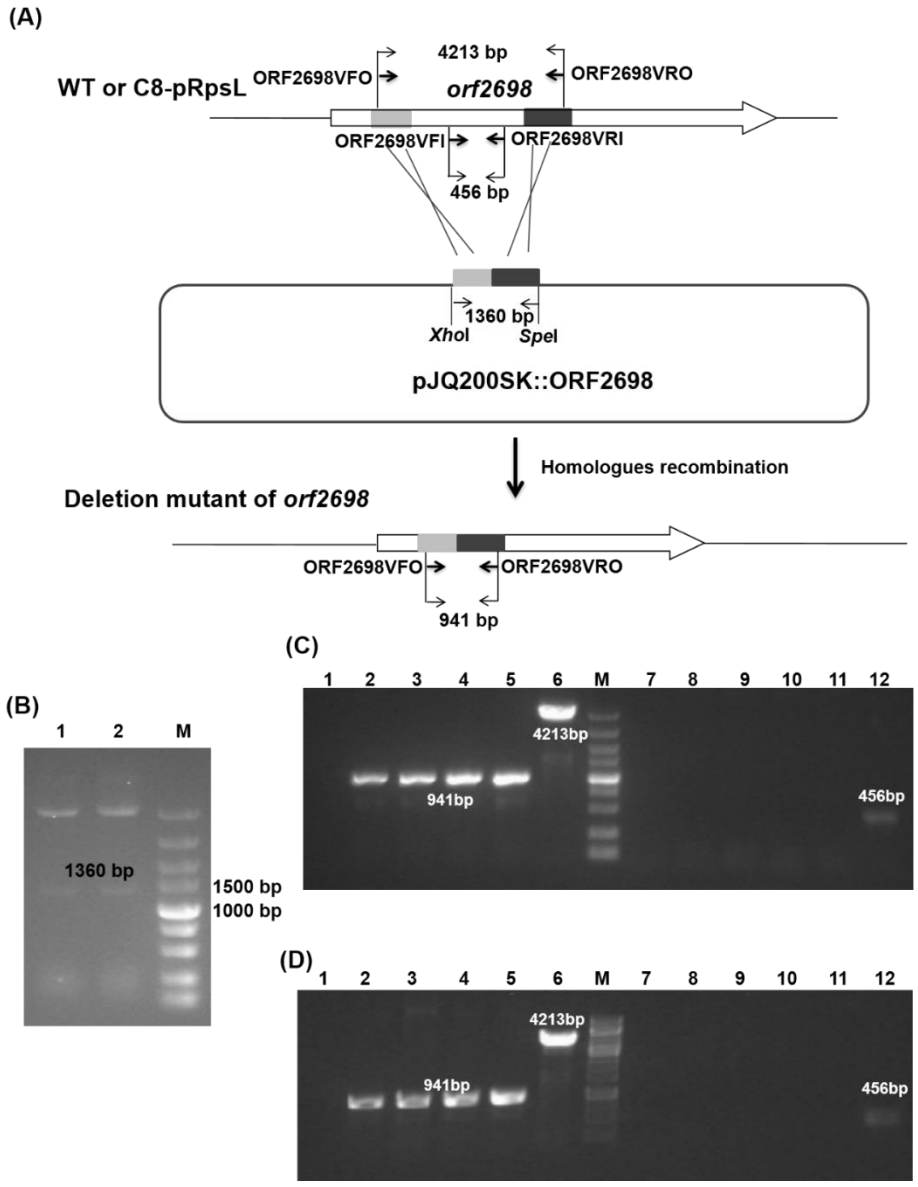


Fig S9. Generation of the *orf2698* (*lesA*) deletion mutant of *Lysobacter* sp. 3655. (A) Schematic illustration of deleting *orf2698* in the wild type and in the engineered strain C8-pRpsL. (B) Verification of plasmid pJQ200SK::ORF2698. Lanes 1 and 2: pJQ200SK::ORF2698 treated with *Xho*I/*Spe*I, and product of 1360 bp was expected; M: DNA marker. (C) PCR verification of mutant ΔORF2698, derived the wild type. (D) PCR verification of mutant C8-pRpsL/ΔORF2698, derived from strain C8-pRpsL. Lanes 1 and 7: negative control, H_2O as the template; lanes 2-5: the deletion mutant, the product of 941 bp expected with ORF2698VFO/VRO as primers; lane 6: positive control, the product of 4213 bp expected with genomic DNA of the wild type as template and ORF2698VFO/VRO as primers; lanes 8-11: the deletion mutant, no

product expected with ORF2698VFI/VRI as primers; lane 12: positive control, the product of 456 bp expected with genomic DNA of the wild type as template and ORF2698VFI/VRI as primers; M: DNA marker.

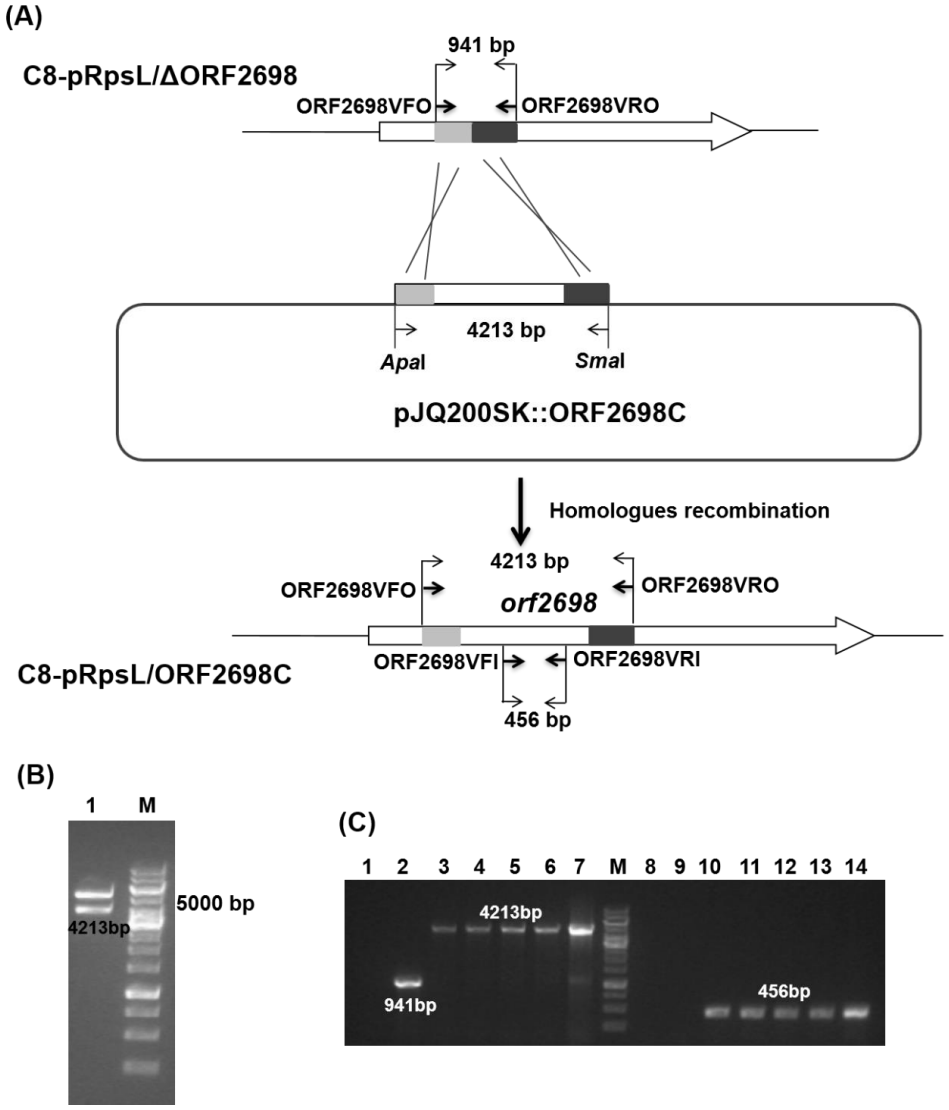


Fig S10. Generation of the *orf2698* complementary strain, C8-pRpsL/ORF2698C, of the deletion mutant, C8-pRpsL/ΔORF2698. (A) Schematic illustration of the *orf2698* complementation in the mutant. (B) Verification of plasmid pJQ200SK::ORF2698C. Lane 1: plasmid treated with *Apa*I/*Sma*I, and the product of 4213 bp was expected; M: DNA marker. (C) PCR verification of complementary strain C8-pRpsL/ORF2698C. Lanes 1 and 8: negative control, with H_2O as the template; lane 2: mutant C8-pRpsL/ΔORF2698, the product of 941 bp expected with ORF2698VFO/VRO as primers; lanes 3-6: complementary strain C8-pRpsL/ORF2698C, the product of 4213 bp expected with ORF2698VFO/VRO as primers; lane 7: positive control, the product of 4213 bp expected with genomic DNA of the wild type as template and ORF2698VFO/VRO as primers; lane 9: mutant C8-pRpsL/ΔORF2698, no product expected with

ORF2698VFI/VRI as primers; lanes 10-13: complementary strain C8-pRpsL/ORF2698C, the product of 456 bp expected with ORF2698VFI/VRI as primers; lane 14: positive control, the product of 456 bp expected with genomic DNA of the wild type as template and ORF2698VFI/VRI as primers; M: DNA marker.

(A) compound **5** (B) compound **6**

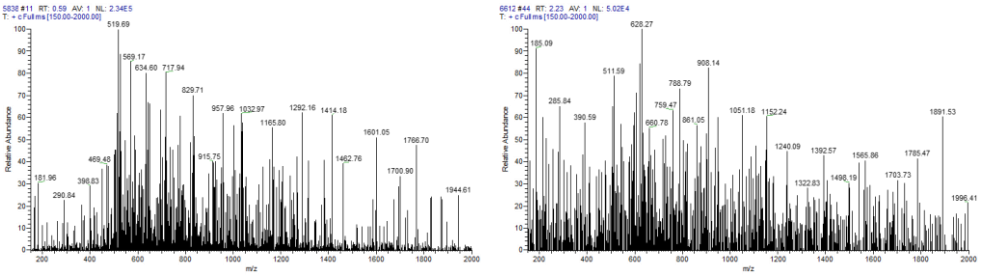
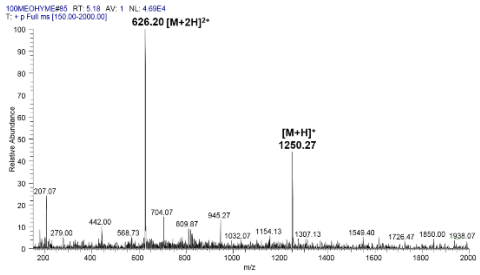
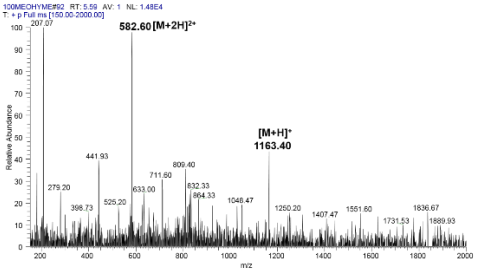


Fig S11. MS analysis of compound **5** and **6**.

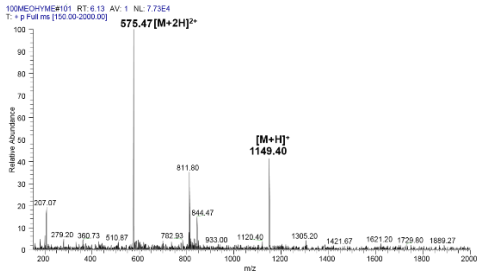
(A) compound **7**



(B) compound **8**



(C) compound **9**



(D) compound **10**

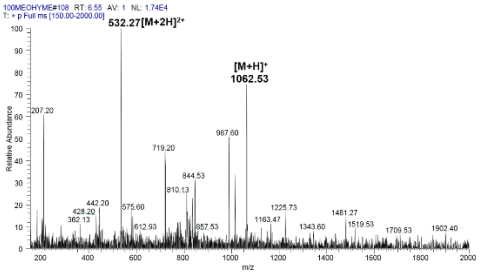
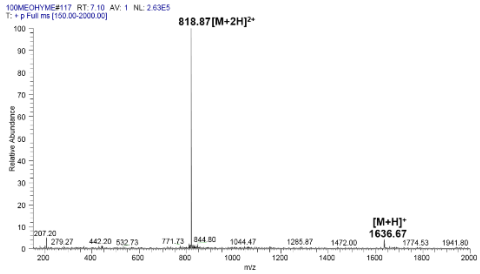
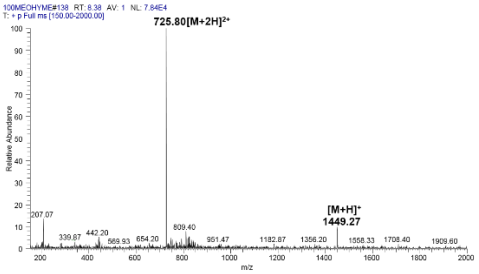


Fig S12. MS spectrum of compounds **7-10**, which appeared to contain a "partial" structure of the "full-length" lysocins (**1-4**).

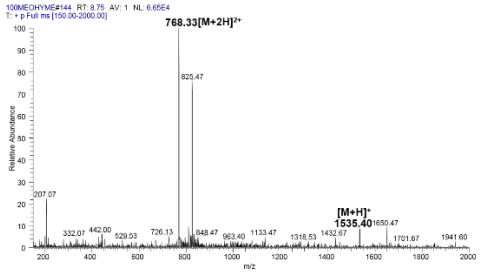
(A) compound **11**



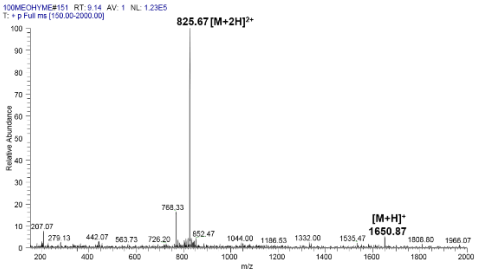
(B) compound **12**



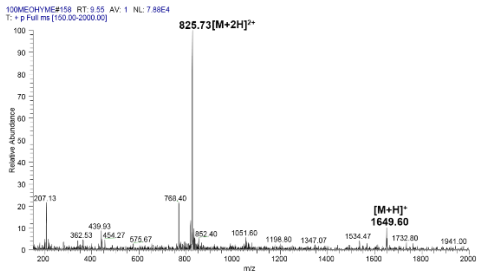
(C) compound **13**



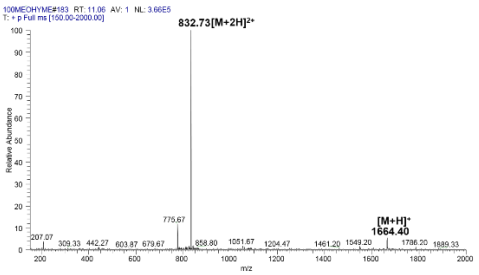
(D) compound **14**



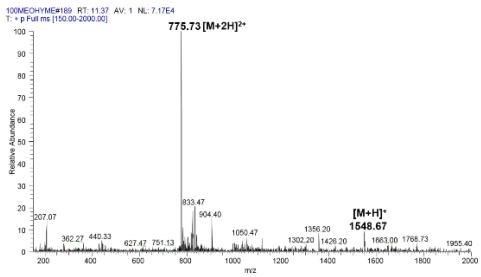
(E) compound **15**



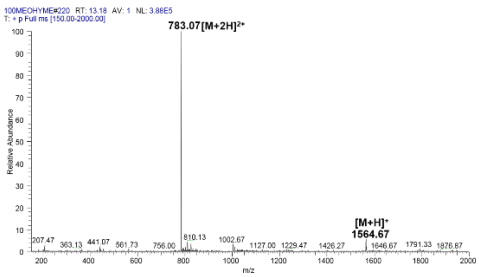
(F) compound **16**



(G) compound **17**



(H) compound **18**



(I) compound **19**

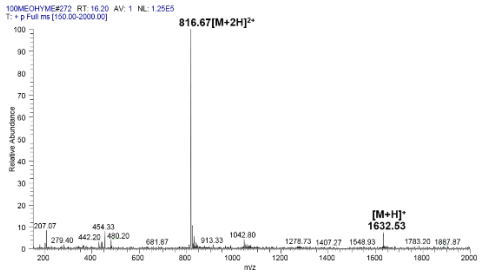


Fig S13. MS spectrum of compounds **11-19**.

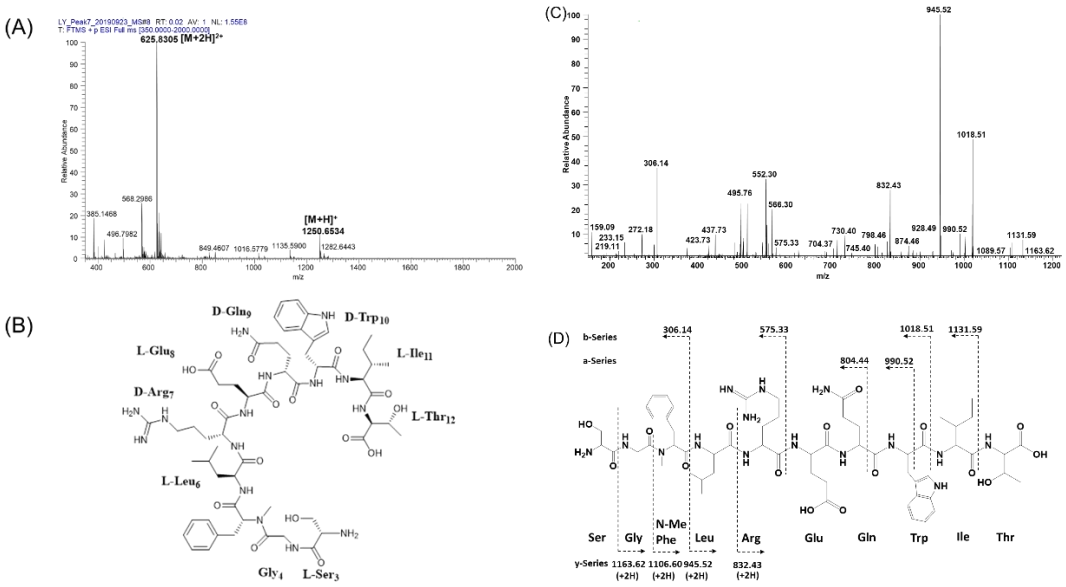


Fig S14. HR-ESI-MS/MS analysis of compound **7**. (A) Mass spectrum of **7**. (B) proposed structure of **7**, with a calculated mass of 1249.6455. (C) MS/MS fragmentation of **7**. (D) Assigned characteristic product ions of the a-, b-, and y-series of the amino acid fragmentation.

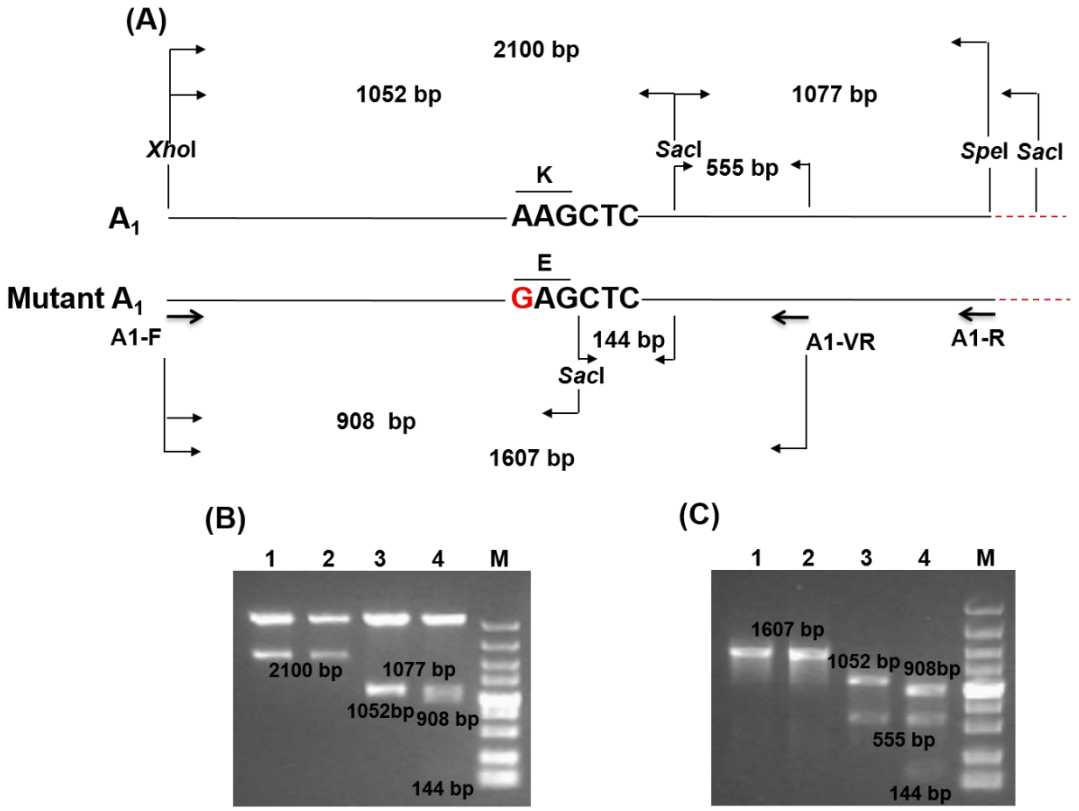


Fig S15. Generation of K508E mutant of A_1. (A) Schematic illustration of the K508E mutagenesis of A_1. The red dotted lines represent sequences from the plasmid. (B) Verification of the mutant plasmid pJQ200SK::A1K508E. Lane 1: non-mutant plasmid pJQ200SK::A1 treated with *Xho*I/*Spe*I, and the product of 2100 bp was expected; lane 2: mutant plasmid pJQ200SK::A1K508E treated with *Xho*I/*Spe*I, and the product of 2100 bp was expected; lane 3: non-mutant plasmid pJQ200SK::A1 treated with *Xho*I/*Sac*I, and the products of 1077 bp and 1052 bp were expected; lane 4: mutant plasmid pJQ200SK::A1K508E treated with *Xho*I/*Sac*I, and the products of 1077 bp, 908 bp, and 144 bp were expected; M: DNA marker. (C) PCR verification of strain C8-pRpsL/K508E with site-directed mutagenesis. Lane 1: PCR product of C8-pRpsL, 1607 bp expected with A1-F/VR as primers; lane 2: PCR product of C8-pRpsL/K508E, 1607 bp expected with A1-F/VR as primers; lane 3: PCR product of C8-pRpsL digested with *Sac*I, 1052 bp and 555 bp expected; lane 4: PCR product of C8-pRpsL/K508E digested with *Sac*I, 908 bp, 555 bp, and 144 bp expected; M: DNA marker.

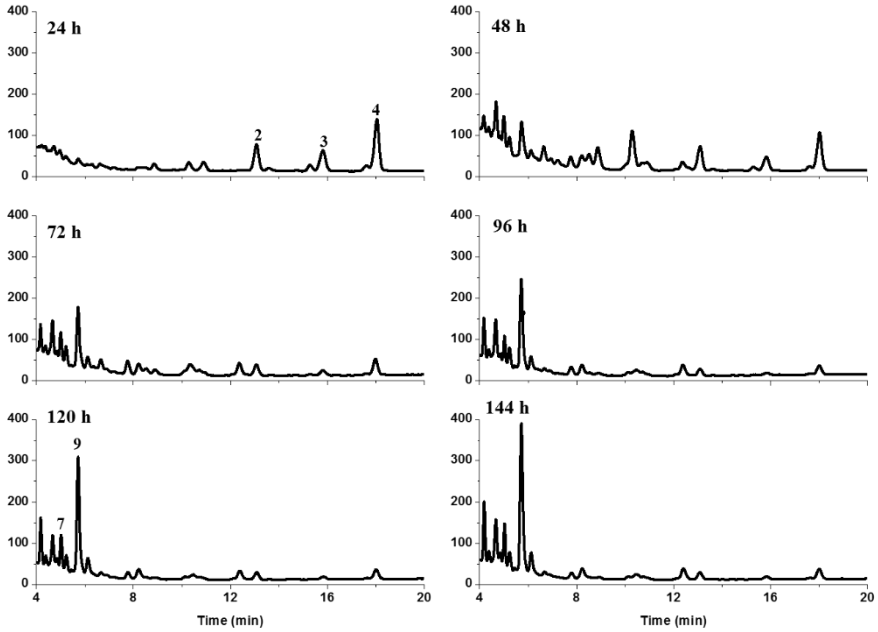


Fig S16. Changes in the profile of lysocin congeners produced in the engineered strain C8-pRpsL at different growth times. Strain C8-pRpsL was cultured in YME medium, and the profile of lysocin compounds was evaluated by HPLC every 24 h.

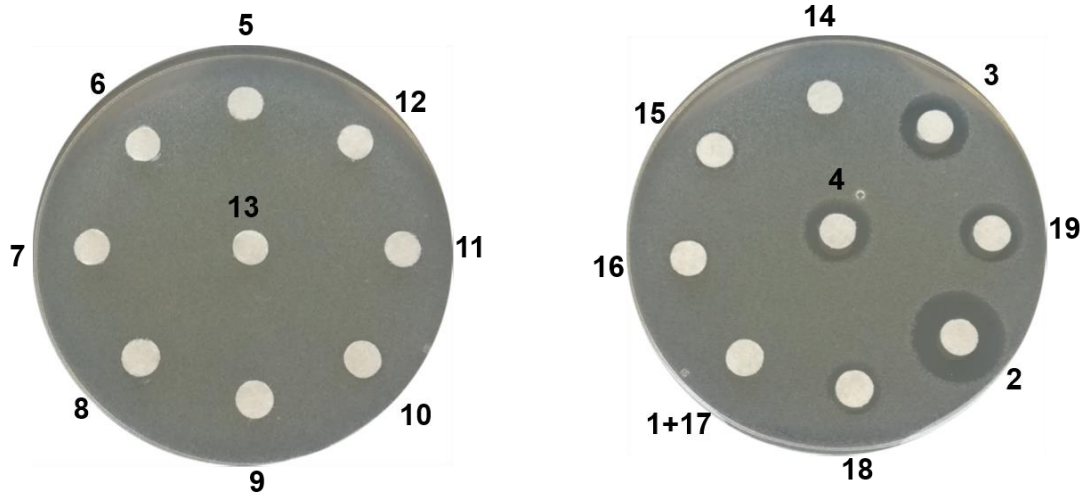


Fig S17. The antibacterial activity of lysocin compounds. The numbers represent the corresponding lysocin congeners listed in Table S3-S4. *B. subtilis* was the indicator organism for the antibiotic activity.

Reference

1. Zhang, W., Li, Y., Qian, G., Wang, Y., Chen, H., Li, Y., Liu, F., Shen, Y., and Du, L. (2011) Identification and characterization of the anti-methicillin-resistant *Staphylococcus aureus* WAP-8294A2 biosynthetic gene cluster from *Lysobacter enzymogenes* OH11. *Antimicrob. Agents Chemother. 55,* 5581–5589.
2. Bullock, W.O., Fernandez, J.M., and Short, J.M. (1987) XL1-blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. *Biotechniques 5*, 376–378.
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4. Quandt, J., and Hynes, M. F. (1993) Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene 127,* 15–21.
5. Panthee, S., Hamamoto, H., Suzuki, Y., and Sekimizu, K. (2017) *In silico* identification of lysocin biosynthetic gene cluster from *Lysobacter* sp. RH2180-5. *J. Antibiot. 70,* 204–207.