

Supplemental Data

Table S1. Strains, plasmids and cosmids used in this study

Strain/ Plasmid / Cosmid	Relevant characteristics*	Reference or source
<i>S. cacaoi</i> strains		
WT	Wild-type of polyoxin producing strain	(1)
CY1	Non-producer for polyoxin generated through disruption of <i>polA</i> by <i>aac(3)IV</i>	This study
CY1/695	CY1 mutant containing pJTU695	This study
CY1/2179	CY1 mutant containing pJTU2179	This study
CY1/2198	CY1 mutant containing pJTU2198	This study
CY9	<i>orf1</i> disruption mutant	This study
CY11	<i>orf3</i> and <i>orf4</i> double mutant	This study
CY14	Non-producer for polyoxin generated through disruption of <i>polO</i> by <i>aac(3)IV</i>	This study
<i>S.aureochromogenes</i>	polyoxin producing strain	CGMCC
<i>S. lividans</i> TK24	A derivative of <i>S. lividans</i> 1326	(2)
<i>E. coli</i> strains		
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) φ80d <i>lacZ</i> Δ M15 Δ <i>lacX74 deoR recA1 endA1 araΔ139 D(ara, leu)1697 galU galKλ⁻ rpsL nupG</i>	GIBCO BRL
ET12567 (pUZ8002)	<i>dam dcm hsdS</i> pUZ8002	(3)
EPI300-T1 ^R	Host cell for construction of genomic cosmid library	EPICENTER
BL21Gold(DE3)pLysE	F ⁻ , <i>ompT, hsdS_B (r_B⁻ m_B⁻) ,gal ,dcm</i> (DE3), pLysE (Cm ^R)	Stratagene
<i>Trichosporon cutaneum</i>	Indicator fungi used for bioassay of polyoxin	CGMCC
Plasmids		
pIJ2925	<i>bla, lacZ</i>	(2)
pBlueScriptII SK(+)	<i>bla, lacZ, orif1</i>	Stratagene
pMD18-T	pUC18 derivative T-vector	TaKaRa
pSET152	<i>aa (3)I V, lacZ, rep^{pMB1*}</i> attΦC31, ori T	(4)
pOJ446	<i>aa (3)I V, SCP2, rep^{pMB1*}</i> , attΦC31, ori T	(4)
pJTU1278	pHZ1358 derivative, <i>bla, lacZ, tsr</i> , ori T	(He <i>et al.</i> unpublished)
pJTU1289	pJTU1278 derivative, <i>bla, lacZ, tsr</i>	(He <i>et al.</i> unpublished)
pHZ1070	pIJ2925 derivative carrying <i>aac(3)IV</i>	(5)
pET28a	Kan, <i>rep^{pMB1}</i> , T7 promoter	Novagen
pJTU695	<i>tsr, bla, oriT, ori, pI J101</i> derivative, <i>PerME*</i>	(6)
pJTU2152	pMD18-T derivative carrying 723-bp PCR fragment from <i>S. aureochromogenes</i>	This study
pJTU2158	pIJ2925 derivative carrying ca. 3.0-kb sequenced PvuII fragment including <i>polA</i> from cosmid	This study
pJTU2161	pJTU1278 derivative with insertion of engineered ca.3.0-kb XbaI-EcoRI fragment including <i>polA</i> amplified by PCR	This study

pJTU2165	pJTU2161 derivative with insertion of engineered BamHI fragment bearing <i>aac(3)IV</i> from pHZ1070	This study
pJTU2173	pIJ2925 derivative with insertion of <i>nikO</i> structural gene	This study
pJTU2810	pBlueScriptII SK(+) derivative with insertion of <i>nikO</i> structural gene	This study
pJTU2178	pET28a derivative containing complete structural gene of <i>nikO</i>	This study
pJTU2179	pJTU695 containing complete structural gene of <i>nikO</i>	This study
pJTU2197	pET28a containing complete structural gene of <i>polA</i>	This study
pJTU2198	pJTU695 containing complete structural gene of <i>polA</i>	This study
pJTU2896	pMD18-T with insertion of <i>polO</i> structural gene	This study
pJTU2860	pJTU1278 derivative with insertion of engineered <i>ca.</i> 4.5-kb XbaI-EcoRI fragment including <i>orf3</i> and <i>orf4</i> amplified by PCR	This study
pJTU2861	pJTU2860 derivative with <i>aac(3)IV</i> from pHZ1070 replaced <i>ca.</i> 1.1-kb corresponding region in <i>orf3</i> and <i>orf4</i>	This study
pJTU2855	pJTU1289 derivative with insertion of engineered <i>ca.</i> 4.3-kb XbaI-EcoRI fragment including <i>orf1</i> amplified by PCR	This study
pJTU2866	pJTU2855 derivative with insertion of <i>aac(3)IV</i> + <i>oriT</i> cassette	This study
pJTU2900	pBlueScriptII SK(+) derivative with insertion of <i>ca.</i> 4.1-kb PvuII fragment including <i>orf1</i> from 5A7 in its EcoRV site	This study
pJTU2940	A HindIII-SpeI engineered fragment inserted into HindII-XbaI site of pJTU1289	This study
pJTU2941	pJTU2940 derivative with insertion of <i>aac(3)IV</i> + <i>oriT</i> cassette	This study
Cosmid		
8B9	A positive cosmid used to clone the <i>ca.</i> 4-kb PvuII fragment harboring <i>polA</i>	This study
5A7	A positive cosmid selected for sequencing	This study
m5A7	A derivative of cosmid 5A7 with SCP2 replicon replaced by <i>int</i> and <i>atp</i> from pSET152	This study

oriT, origin of transfer of plasmid RK2; *tsr*, thiostrepton resistance gene; *aac(3)IV*, apramycin resistance gene; Cm^R, chloramphenicol resistance gene; *neo*, Neomycin resistance gene; Kan, kanamycin resistance gene; CGMCC, China General Microbiological Culture Collection Center; rep^{pMB1*}, mutated rep^{pMB1}.

Table S2. PCR primers used in this study

Primers	Sequence
NikOTF	(5'-TCCACGGTTCGCTCTACCTG-3')
NikOTR	(5'-AGGGCTGGCTGTGCTGAA-3')
PolAF	(5'-GTCCGGTGCGCCATGTCCT-3')
PolAR	(5'-GGTGGGCCGTGATCGAGTC-3')
5a7mF	(5'-GCTCTAGATGGGTGGGTACACGACG-3')
5a7mR	(5'-GGAATTCGCATCAGGTCGGAGACGC-3')
Podf	(5'-GCTCTAGACTGTTTCGCTGGCTGGACC-3')
Podr	(5'-GGAATTCGAAACTGACCTACGGCGTC-3')
PiomF	(5'-CCAGATGGTCGCCGAACAGA-3')
PiomR	(5'-GGGATAGAGGTGGTGCAGGTG-3')
NikO-EXF	(5'-CCCATATGCAGGACCGTTGGCGAG-3')
NikO-EXR	(5'-GGAATTCCTCAGGCCGGTCCACACC-3')
polAeF	(5'-CCATATGCTCGAAGTCAACGGTG-3')
polAer	(5'-GGAATTCGCCTGACGGGCCCTCTCA-3')
polOexF	(5'-ccatATGATCACCTGTGGCCTGAAA-3')
polOexR	(5'-ggaattcGTGCGGTGCTCACCCGGT-3')
polO IDF	(5'-ACCTGTGGCCTGAAACTGA)
polO IDR	5'-GTCCCAGACGAGCACGAAG-3')
polOtgfF	(5'-CGCGAACTCGCTGGTGCAGTAGGTGCTCA GCACGTGTCCTGTAGGCTGGAGCTGCTTC-3')
polOtgfR	(5'-GTCCTGAGCGTGGAGATGGAGAACTCGC CAACAACCCGATTCCGGGGATCCGTCGACC-3')
cyclonXF	(5'-GGAATTCGACCTGACCCTGCTGACCACC-3')
cyclonER	(5'-GCTCTAGACGTCCGTACCCATGAAGATGCC-3')
CycaprF	(5'-ATTCCGGGGATCCGTCGACCGAGATGA TCCTTCCGCTGGTGGTCCCTCGACATCAGCGAC-3')
CycaprR	(5'-TGTAGGCTGGAGCTGCTTCCGGGACCCGGTGG AGACCGGCCATCAGCTCGATCTGCCA-3')
cycIDF	(5'-ACCTCACCCATGCCTTCCGTC-3')
cycIDR	(5'-CGCTCGTCCCGGTGTCGTCATAC-3')
5A7-TgtF	(5'-CACCCACTGGTGGATCGTGGAGGACGACA GCTATCTCGGCTATTCCAGAAGTAGTGAGG-3')
5A7-TgtR	(5'-GAGCAGCCAGGAGGTCGTGACGCCGTAG GTCAGTTTCAGCTGGATGCCGACGGATTTG-3')
5A7-tgtIDF	(5'-GACCTCCATACGGTTGCG-3')
5A7-tgtIDR	(5'-CGTCGTGGCGTCTTCAC-3')

Constructs for determination of the boundaries of the *pol* gene cluster

For construction of m5A7-kan, a kanamycin resistance gene cassette was amplified using SuperCos 1 as template with primers, 5A7-tgtF and 5A7-TtgtR, *neo* cassette was recombined into m5A7 to yield m5A7-kan by utilizing Redirect technology (7).

For construction of the *orf1* disruption vector, a ca. 4.3-kb fragment was amplified with primers cyclonXF and cyclonER, and this EcoRI-XbaI fragment was cloned into the corresponding sites of pJTU1289 to give pJTU2855. With primers, CycaprF and CycaprR, a *aac(3)IV + oriT* cassette was amplified and recombined into pJTU2855 to yield pJTU2866 by utilizing Redirect technology (7).

Figure S1. Detection of *nikO* Homologues in Two Polyoxin-producing Strains by Southern Blot Analysis

1. *S. aureochromogenes*, 2. *S. cacaoi* var. *asoensis*.

Genomic DNA of both strains was digested with BamHI, and 0.7-kb amplified fragment from *nikO* was used as probe.

Figure S2. Putative Fragment Structures of Polyoxin H

Figure S3. Determination of the Left Boundary of the *pol* Gene Cluster with PCR-targeting Technology

(A) Representation map for construction of cosimid m5A7-kan.

(B) Confirmation of m5A7 in *S. lividans* TK24, M 1kb ladder; 1 PCR product using genomic DNA of *S. lividans* TK24 containing m5A7 as negative control; 2 PCR product using genomic DNA of *S. lividans* TK24 containing m5A7-kan.

(C) Bioassay of *S. lividans*/m5A7-kan. 1 *S. lividans* TK24 containing m5A7; 2 *S. lividans* TK24 containing m5A7-kan.

(D) HPLC analysis of *S. lividans* TK24 containing m5A7-kan. TK24/m5A7, *S. lividans* TK24 containing m5A7; TK24/m5A7-kan, *S. lividans* TK24 containing m5A7-kan.

Figure S4. Targeted Inactivation of *orf1*

- (A) Representation map for construction of CY9 mutant. FRT, FLP recognition site.
- (B) Identification of CY9 mutant by PCR approach. M, 1-kb ladder; 1, WT; 2-3, CY9 mutants;
- (C) Bioassay for the CY9 mutants. 1, WT; 2-3, CY9 mutants.

Figure S5. Analysis of the Related Mutants by MS and MS/MS

Positive mode was used for detection of polyoxin, and polyoxin A standard generates $[M+H]^+$ ion at m/z of 617.4, which was predominantly fragmented into 599.2 and 538.2.

Figure S6. Confirmation of CY1 Mutants by Southern Blot.

The genomic DNA of *S. cacaoi* (mutants and wild type) was digested with PvuII and a 723-bp *polA* fragment labeled with α - $[^{32}P]$ -dCTP was used as probe. WT *S. cacaoi* generated positive signal at *ca.* 3.0 kb, while CY1 mutants produced that at *ca.* 4.5 kb.

Figure S7. Complementation of CY1 Mutants

- (A) MS and MS/MS analysis of polyoxin A produced by CY1/*polA*.
- (B) MS and MS/MS analysis of polyoxin K produced by CY1/*nikO*. Polyoxin K standard give $[M+H]^+$ ion at m/z of 587.5, which was further predominantly fragmented into 397.1, 442.0, 460.1 etc.

Figure S8. MS and MS/MS Analysis of the NikO-catalyzed Product Using UMP as Substrate.

NikO-catalyzed product, 3'-EUMP, produced $[M-H]^-$ ion at m/z of 393.3, which was fragmented into $[M-H]^-$ ion at m/z 305.0, 322.9, 364.9 etc.

Figure S9. MS Analysis of AHV and PolO-catalyzed Product

AHV (α -amino- δ -hydroxyvaleric acid); AHV standard;
ACV (α -amino- δ -carbamoylhydroxyvaleric acid), ACV standard,
PolO, PolO-catalyzed product.

ACV and the PolO-catalyzed product, produce the characteristic $[M+H]^+$ ion at 177.0, which was fragmented into 70.3 and 116.1, while AHV standard gives the $[M+H]^+$ ion at 134.0 that is fragmented into 70.3, 71.3, 88.2 and 116.1.

Supplemental References

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