Supporting Information

Identification and characterization of the actinomycin G gene cluster of *Streptomyces iakyrus*

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General materials and method

Streptomyces iakyrus (DSM 41873) was obtained from DSMZ (Germany). QIAprep Spin Miniprep kits (Qiagen) were used to prepare plasmids from *E. coli* strains. Wizard genomic DNA purification kits (Promega) were used to prepare the genomic DNA from *Streptomyces* strains. Restriction endonucleases, DNA ligase, DNA polymerase and alkaline phosphatase were purchased from various sources and used according to the manufacturers' recommendations. DNA fragments were purified using Wizard PCR preps DNA purification system (Promega). For degenerate primers, PCR reactions were performed in 20 μL of final volume with 5% DMSO and GoTaq DNA polymerase (1.5 unit, Promega). PCR conditions were preheated at 98 ^{o}C for 5 min, followed by 30 cycles of denaturation at 95 ^{o}C for 30s, annealing at 52 ^{o}C for 1 min and extension at 72 ^{o}C for 40 s, with 7 min infilling at 72^{o}C. The PCR products were purified and ligated into pGEM-T easy vector for the blue-white screening. For PCR screening, PCR reactions were performed in the same way as for degenerate PCR, except the annealing temperature was 58 ^{o}C. The PCR products in this work were subjected to DNA sequencing at Dundee Sequencing Centre (www.dnaseq.co.uk). Chemicals and solvents were purchased from Fisher Scientific (UK) and Sigma-Aldrich (UK) and used without further purification.

^{1}H NMR experiment was recorded on Varian Unity INOVA 600 MHz spectrometer in chloroform-d_1 solution. High resolution electrospray ionization mass spectra were obtained using a Thermo Scientific MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler, and Accela pump, C18 SunFire™ 150 × 4.6 mm Waters®). The following conditions were used: capillary voltage 45 V, capillary temperature 320 ^{o}C., auxiliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, mass range 100-2000 amu (maximum resolution 30 000).

Metabolite analysis

For metabolite analysis, spores of *Streptomyces iakyrus* wild type and its mutants were inoculated into ISP2 liquid medium (1% malt extract, 0.4% yeast extract, 0.4% D-glucose, pH = 7.0, 50 mL) in an Erlenmeyer flask (250 mL) and cultivated in a rotary incubator (180 rpm, Barnstead Lab-line MaxQ 5000) at 28^{o}C. After 5-day fermentation, the culture was extracted with ethyl acetate. The ethyl acetate fraction was removed under reduced pressure and the residue was dissolved in methanol (5 mL). The resulting crude extracts were submitted to high resolution LC-MS analysis (C18 Sunfire™ 150×4.6 mm Waters®; A: 0.1% formic acid in water, B: 0.1% formic acid in Methanol; gradient 0-20 min 0-100% B).

Before High-performance liquid chromatography (HPLC) analysis, the crude extracts were further purified by solid phase extraction (SPE). The crude extracts were dissolved in 30% aqueous methanol and loaded on SPE column (Chromabond® C18 ec 6 mL/500 mg, Macherey-Nagel). The column was then washed by 50% aqueous methanol. The fractions containing actinomycin Gs were eluted from column by 100% methanol. HPLC analysis was carried out using an Agilent 1100 HPLC system (Agilent Technologies). UV detection was set at 220, 280, 350, 400, and 420 nm.The purified fractions were separated on ACE®-321-1546 C18 column (150×4.6mm) with following program: A water, B acetonitrile; gradient 0-20 min 30-50% B, 20-25 min 50-51% B, 25-30 min 51% B, 30-35 min 51-54% B, 35-40 min 54% B, 40-60 min 54%-90% B; flow rate 1 mL/min. Actinomycin G_2 is $C_{61}H_{83}N_{12}O_{17}Cl$, with *m/z* of 1291.5729 $[M + H]^+$ or 1313.5540 $[M + Na]^+$, and an HPLC retention time of 18.8 min. Actinomycin G_3 is $C_{61}H_{84}N_{12}O_{18}$, with *m/z* of 1273.6068 $[M + H]^+$ or 1295.5881 $[M + Na]^+$, and an HPLC retention time of 6.3 min. Actinomycin G_4 is $C_{61}H_{84}N_{12}O_{17}$, with *m/z* of 1257.6145 $[M + H]^+$ or 1279.5979 $[M + Na]^+$, and an HPLC retention time of 16.5 min.

Actinomycin G_2 production, purification and analysis

S. iakyrus wild type was cultured in ISP2 medium (160 mL) as seed culture. After 5-day cultivation, the seed culture was then transformed to ISP2 medium (4 L) with the presence of Diaion HP-20 (50 g/L Resindion S.R.L., a subsidiary of Mitsubishi Chemical Co., Binasco, Italy). *S. iakyrus* was cultivated for 6 days at 28 ^{o}C in a rotary shaker (180 rpm). At the end of the fermentation the culture became greenish as previously reported.[1]

HP-20 resin was filtered under the reduced pressure and was extracted with methanol followed by MeOH-CH_2Cl_2 (1:1). The organic extracts were combined and concentrated under vacuum. The resulting crude extract was stored at 4 °C until used.

Fractionation of the crude extract [3.2 g] was performed using a modified Kupchan scheme[2] that results in four fractions; hexane [0.52 g], CH_2Cl_2 [0.4 g], MeOH / H_2O [1.3 g] and *sec*-BuOH [0.68 g].

The CH_2Cl_2 fraction was further purified by HPLC (column: C18, 250 × 10 mm Phenomenex®; A: H_2O, B: MeOH, gradient: 0-40 min, 60 - 100% B; flow rate: 1.25 mL/min) to afford the pure compound **5** [2 mg] which was identified as actinomycin G_2 by direct comparison with its physical and spectral data reported in literature[1]. UV detection was carried out at 240, 280, 350, 400, and 420 nm.

Construction of the *S. iakyrus* fosmid library and genomic scanning for α-KG halogenase gene

The fosmid library was prepared in pCC2FOS vector (Epicentre Biotechnologies) according to the manufacturer's protocol, with several modifications: the end-repair step was performed after size selection. Infected EPI100-T1R cells were grown in LB agar which was supplemented with 12.5 μg mL^{-1} of chloramphenicol. Cells were grown at 37 °C overnight resulting in a growth of 100 clones per plate. Each plate was subsequently mixed with sterilized water (500 μL) as a pool. 200 μL of the pool was denatured at 95 ^{o}C for 5 min and

subjected to centrifugation (13,300 rpm; 5 min). The supernatant was then used as DNA template for the PCR screening.

Two degenerate primers (Eurogentec, Table S2) were designed and used for the amplification of the α-KG halogenase gene fragment. PCR reactions were performed by using *S. iakyrus* genomic DNA as template.

The specific primers of the halogenase gene (AcmHaloG-F and AcmHaloG-R) were used for genomic scanning. The positive pools were re-plated on LB/agar/chloramphenicol plates, and about 200 colonies were subjected to colony PCR. Positive colonies were grown in liquid cultures overnight and induced to a high copy number according to the manufacturer's protocol (Epicentre), and the DNA was isolated by using the Qiagen Miniprep kit, with the following modifications: after the addition of neutralizing buffer, DNA was precipitated by the addition of isopropanol (0.6 volumes) and centrifugation (10 min, 13,300 rpm). The DNA was washed with 70% aqueous ethanol, air-dried, and resuspended in elution buffer (10 mM TrisCl, pH 8.5; Qiagen).

Sequence analysis

The fosmid 17D6 was subjected to 454 GS De Novo assembler (DNA Sequencing Centre, Department of Biochemistry, University of Cambridge). Fosmid sequence data were generated with the GS FLX system (454 Life Sciences). A library of double-stranded template DNA fragments was prepared from the purified fosmid DNA using the GS Rapid DNA Library Preparation Kit (Roche). The Shotgun GS Titanium emPCR Kit (Roche) was used for emulsion-based clonal amplification of a double-stranded template DNA library. The GS Titanium XLR70 Sequencing Kit (Roche) was used in combination with the GS Titanium PicoTiterPlate Kit (70 × 75; Roche) to determine the nucleotide sequence of the immobilized and clonally amplified DNA library. All kits were used according to the manufacturer's instructions and DNA sequencing was carried out at the University of Cambridge DNA

Sequencing Facility. The obtained sequences were assembled using GS /de novo /Assembler (Newbler 454 runAssembly software, version 2.3).

The genome scan sequencing of *S.iakyrus* and the sequencing assembly were provided by BGI-Shenzhen, China. The open reading frames (ORFs) of the actinomycin G biosynthetic gene cluster were deduced from the sequence by performing the FramePlot[3] and BLAST search tools on the server of the National Centre for Biotechnology Information, Maryland[4] (http://www.ncbi.nlm.nih.gov/). Amino acid sequence alignments were accomplished by the Jalview[5].

Generation of mutant strains

Gene disruptions were carried out according to a modified procedure of RED technology[6]. A 1.4 kb *aac(3)IV-oriT* replacement cassettes were amplified from pIJ773 using high fidelity polymerase according to the manufacture protocol. Specific primers used for recombination are list on table S2. The resulting PCR products were purified and electroporated into *E.coli* BW25113/pKD46/17D6. The mutagenized fosmids were introduced into wild type of *S.iakyrus* by conjugation from *E.coli* 12567/pUZ8002. Transconjugants resulting from double crossover homologous recombination were selected by apramycin. Correct constructions were verified by PCR and sequencing (Figure S6).

Complementation of the mutant strain

To complement the mutant strain, the genes of interest (*acmG9* and *acmG7*) were amplified by high fidelity PCR and cloned into the *NdeI/ Hind III* sites of pIJ10257[7], which can integrate into ΦBT1 phage site in *Streptomycete*s chromosomes. The resulting pIJ10257 derivatives were introduced into SIAΔ*acmG9* and SIAΔ*acmG7* by conjugation from *E.coli* 12567/pUZ8002, respectively. Transconjugants were selected by hygromycin B.

RNA analysis

GeneJET RNA Purification Kit (Thermo Fisher Scientific) was used to extract the total RNA from *Streptomyces iakyrus*. First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used to synthesize the corresponding cDNA library. All kits were used according to the manufacturer's instructions. PCR reactions were performed in 20 μL of final volume with 5% DMSO and GoTaq DNA polymerase (1.5 unit, Promega).

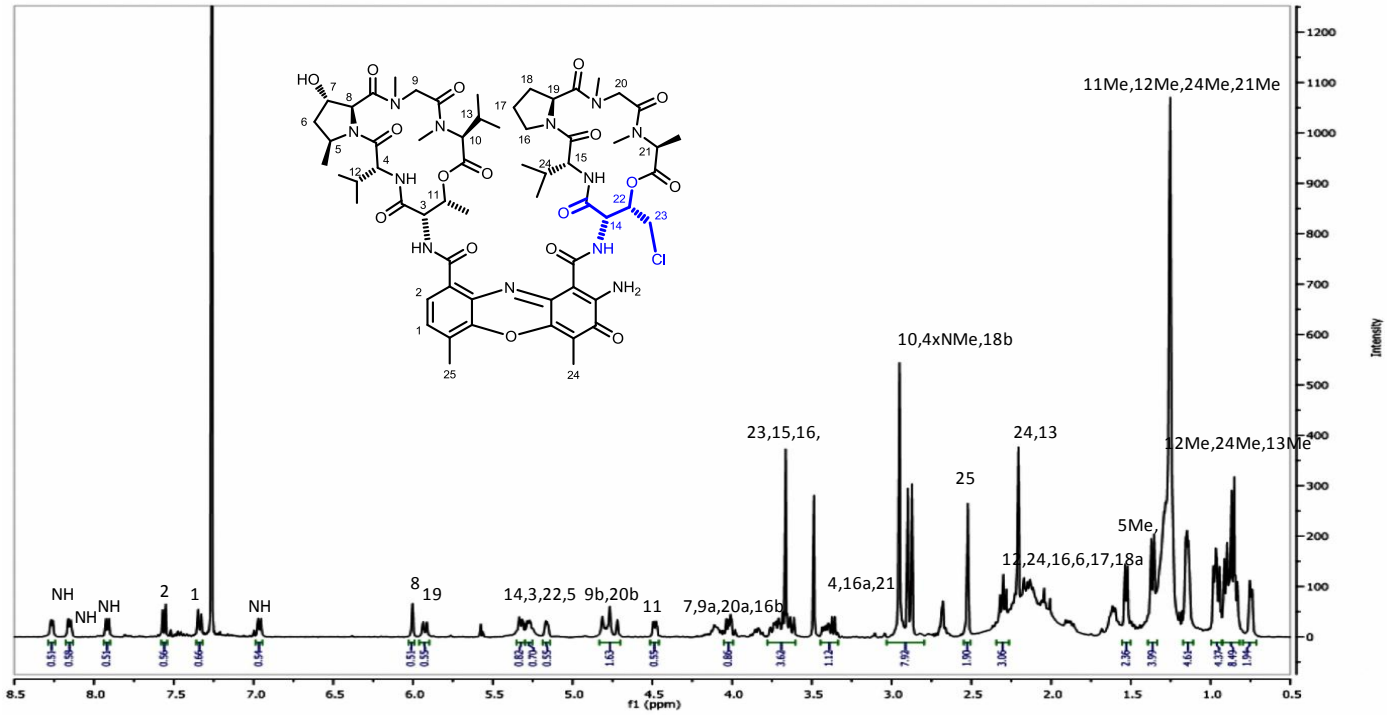


Figure S1. ^{1}H NMR spectrum of actinomycin G_2 **5** ($CDCl_3$, 600 MHz). The proton assignment of **5** was compared with the one reported previously[1].

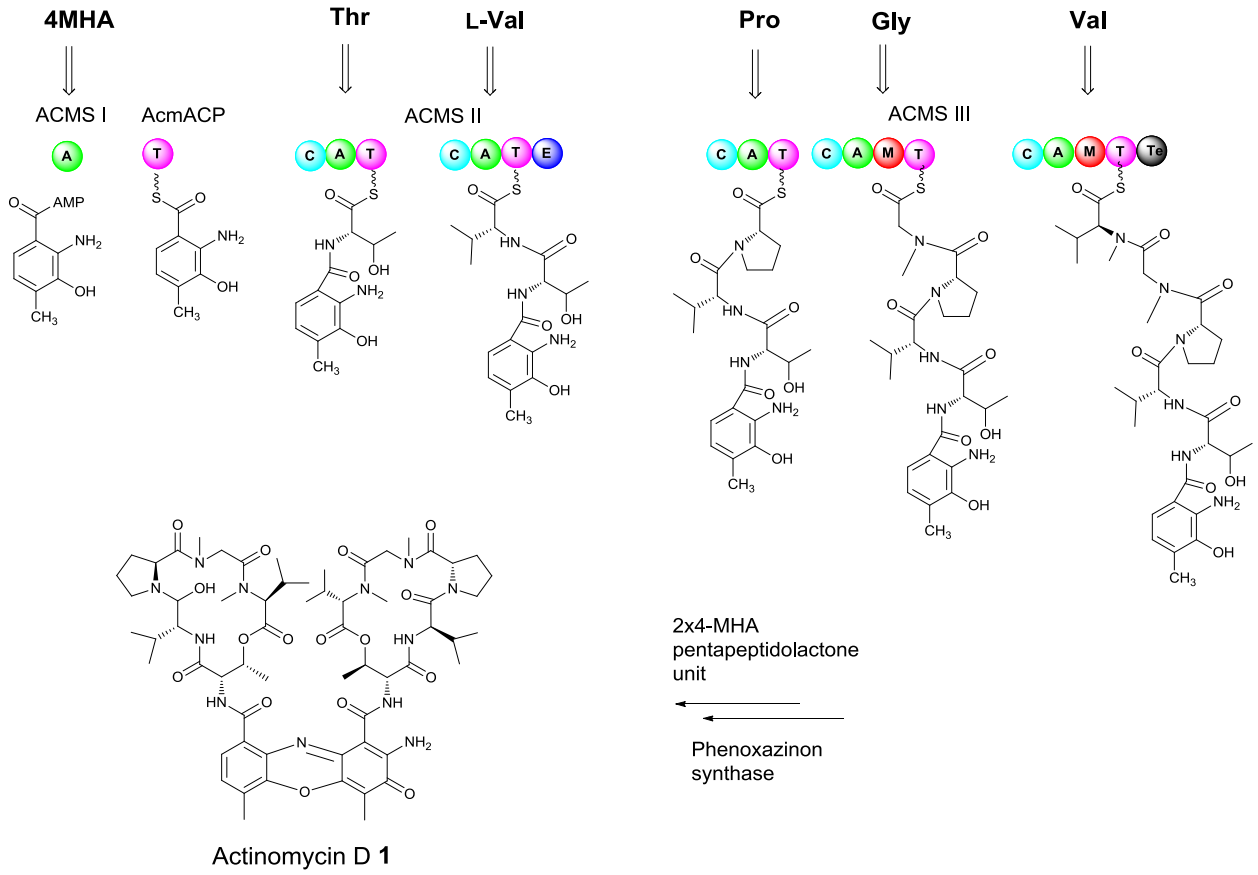
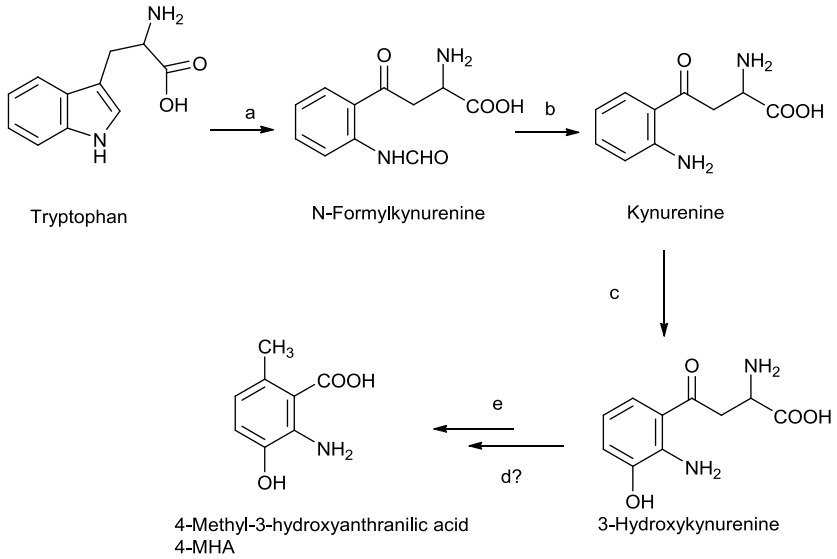


Figure S2. Proposed biosynthetic pathway of actinomycin D.



(a) tryptophan 2,3-dioxygenase (EC1.13.11.11); (b) kynurenine formamidase, (EC 3.5.1.9); (c) kynurenine 3-hydroxylase, (EC 1.14.13.9); (d) 3-Hydroxy-kynureninase/kynureninase (EC 3.7.1.3) ; (e) C-Methyltransferase

Figure S3. The precursor pathway of the biosynthesis of 4-methyl-3-hydroxyanthranilic acid (4-MHA) starting from L-tryptophan in the biosynthesis of actinomycins.

Table S1. Deduced function of ORFs in the biosynthetic gene cluster of actinomycin G

ORF	Residues	Proposed function	Sequence identity (similarity)	Accession number
AcmG12'	177	hypothetical protein	*Streptomyces ghanaensis* ATCC 14672 88% (92%)	ZP_06576141.1
AcmG11'	325	Ferredoxin, 2Fe-2S	*Streptomyces sviceus* ATCC 29083 71% (80%)	ZP_06914645.1
AcmG10'	423	fatty acid beta-hydroxylating cytochrome P450	*Streptomyces scabiei* 87.22 76% (82%)	YP_003493553.1
AcmG9'	80	Transposase (fragment)	*Streptosporangium roseum* DSM 4302191% (99%)	YP_003340278.1
AcmG8'	771	Excinuclease ABC subunit	*Streptosporangium roseum* DSM 43021 85% (95%)	YP_003343295.1
AcmG7'	219	hypothetical protein	*Streptomyces anulatus* 61% (75%)	ADG27360.1
AcmG6'	286	Tryptophan 2,3-dioxygenase	*Streptomyces anulatus* 65% (74%)	ADG27362.1
AcmG5'	346	o-methyltransferase	*Streptomyces anulatus* 66% (76%)	ADG27364.1
AcmG4'	213	LbmU	*Streptomyces anulatus* 53% (66%)	ADG27350.1
AcmG3'	293	siderophore-interacting protein	*Streptomyces anulatus* 56% (70%)	ADG27367.1
AcmG2'	4248	NPRS (C-A-T-C-A-ME-T-C-A-ME-T-TE)	*Streptomyces anulatus* ACMSIII 59%(69%)	ADG27359.1
AcmG1'	2642	NRPS (C-A-T-C-A-T-E)	*Streptomyces anulatus* ACMS II 60%(70%)	ADG27358.1
AcmG1	455	Long-chain-fatty-acid--CoA ligase	*Streptomyces anulatus* 61% (71%)	ADG27357.1
AcmG2	81	4-MHA acyl carrier protein	*Streptomyces anulatus* 48% (70%)	ADG27356.1
AcmG3	321	ABC transporter ATP-binding protein	*Streptomyces sp.* 69% (79%)	ZP_07281589.1
AcmG4	286	ABC-type multidrug transport system, permease component	*Salinispora tropica* CNB-440 54% (73%)	YP_001159975.1
AcmG5	269	TetR family, transcriptional regulator	*Streptomyces anulatus* 54% (61%)	ADG27349.1
AcmG6	371	bifunctional phosphoglucose/phosphomannose isomerase	*Streptomyces coelicoflavus* ZG0656 67% (78%)	EHN75033.1
AcmG7	247	3-oxoacyl-[acyl-carrier protein] reductase	*Streptomyces coelicoflavus* ZG0656 68% (79%)	EHN75032.1
AcmG8	427	CypC P450 hydroxylase	*Mycobacterium kansasii* ATCC 12478 47% (65%)	ZP_04751991.1
AcmG9	317	non-heme alpha-KG halogenase	*Syringae pv. syringae* 63% (76%)	AAD50521.1
AcmG10	2599	NRPS (C-A-T-C-A-T-E)	*Streptomyces anulatus* ACMSII 55% (67%)	ADG27358.1
AcmG11	4262	NRPS (C-A-T-C-A-ME-T-C-	*Streptomyces anulatus* ACMSIII 60% (71%)	ADG27359.1

		A-ME-T-TE)		
AcmG12	322	aryl hydrolase	*Streptomyces anulatus* 61% (69%)	ADG27361.1
AcmG13	416	kynureninase	*Streptomyces anulatus* 67% (77%)	ADG27363.1
AcmG14	446	Na/H antiporter	*Amycolatopsis orientalis* 60% (73%)	CAB45049.1
AcmG15	70	MbtH	*Streptomyces anulatus* 80% (94%)	ADG27355.1
AcmG16	211	hypothetical protein	*Streptomyces anulatus* 72%(81%)	ADG27353.1
AcmG17	304	hypothetical protein SchaN1_15380	*Streptomyces chartreusis* NRRL 12338 89% (94%)	ZP_09955267.1

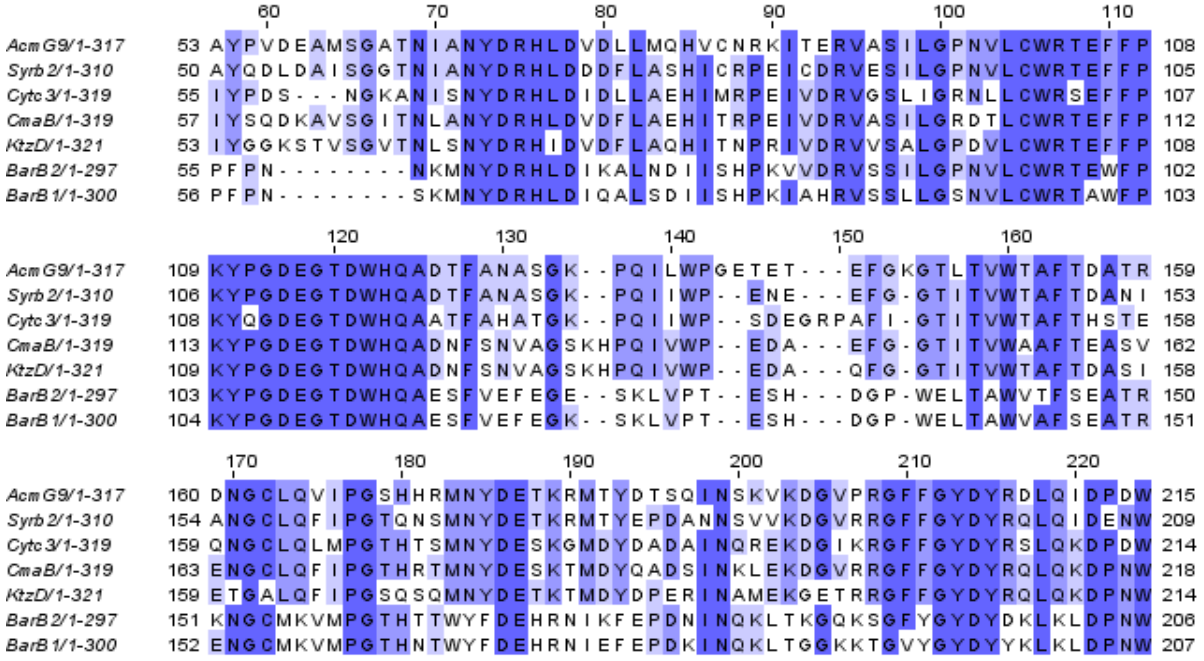


Figure S4. Sequence alignment of identified AcmG9 with known iron/αKG dependent halogenases showing the highly conserved domain. Syrb2 is involved in the syringomycin E biosynthesis (NCBI No: AAD50521)[8]. KtzD is involved in the kutznerides biosynthesis (NCBI No: ABV56584)[9]. CmaB is involved in the formation of coronamic acid (NCBI No. YP_001347497)[10]. BarB2 and BarB1 are involved in the barbamide biosynthesis (NCBI No: AAN32976)[11]. CytC is involved in the biosynthesis of cytotrienine (NCBI No: 3GJA_A)[12].

Table S2. Primers used for degenerate PCR, PCR-cloning,

Primer function and name	**Nucleotide sequence 5' → 3'**
SIAΔ*acmG9* - F	GCT CTC GGA AAC CGA GCT CGA CTT CTT CCG GAA GAA CGG ATT CCG GGG ATC CGT CGA CC
SIAΔ*acmG9* - R	CCT CCA GTC GGC CGG GGG TGC TGG GCC CGA CTC AGC TCA TCA TGT AGG CTG GAG CTG CTT C
SIAΔ*acmG9* - test - F	CGA GGA CGC ACC CGG AGA GA
SIAΔ*acmG9* - test - R	GGG TGC CCT CCT CCA GGC AG
AcmG8 null mutant	
SIAΔ*acmG8* - F	ATG CGC CTC CAG CCG GCC TGT GCC GCG CAC CCC TGC CTG ATT CCG GGG ATC CGT CGA CC
SIAΔ*acmG8* - R	TCA TCC GAG GAC GAC CGG CAG TTC GCG GAA GCG GGT CCAC TGT AGG CTG GAG CTG CTT C
SIAΔ*acmG8* - test-F	CGC GGC ACA CCG ATC TCC GAG TTC CTG AGC TG
SIAΔ*acmG8* - test-R	GTG CGG TTC GGC ATG GTC CTC TCA CGT GTT CTC G
AcmG7 null mutant	
SIAΔ*acmG7* - F	GGA CGC GGC CCC CGT ATG CCC GAC TGG AGG ACC ATG ATT CCG GGG ATC CGT CGA CC
SIAΔ*acmG7* - R	GAA AGG CTC TTC TCT CTC GAA CAG GGC GGT GAC CGC TACC TGT AGG CTG GAG CTG CTT C
SIAΔ*acmG7* - test - F	GAG GAA TTC ATA TGA CCG GGA CGC GGC CCC CGT ATG C
SIAΔ*acmG7* - test - R	GAG GAA ATC GGC ATA CCG GTC GAG GCG TGC GAA C
RT-PCR primers	
AcmG9-RT - F	CCG CGG AAC GCC GTC TTT GAC CTT GG
AcmG9-RT- R	CAC CGA ACG GGT CGC GTC CAT CCT GG
AcmG7-RT - F	GAG GAA TTC ATA TGA CCG GGA CGC GGC CCC CGT ATG C
AcmG7-RT - R	CGA CTC CGC TCG AGC TAC CAC ATG AAC TGG CCA CCA TCG ACG
Fosmid screening primers	
AcmHaloG - F	CG CGG AAC GCC GTC TTT GAC CTT GG
AcmHaloG - R	CAC CGA ACG GGT CGC GTC CAT CCT GG
Degenerate primers for targeting the halogenase gene fragment	
RadHalo - F	AAC TAC GAC MGS CAC YTS GAC
RadHalo - R	CTC GTC SGG CTY CAT GTT SGG GTC

Table S3. Strains and plasmids used in this work

Strain/Plasmid	**Relevant genotype/comments**	**Source/reference**
***Escherichia coli* strains**		
DH5α	F^- general cloning host	Gibro-BRL
ET12567	*dam*–, *dcm*–, *hsdS*–; Non-methylating host for transfer of DNA into *streptomyces* strains Cm^R	(Gust, 2002)[13]
BW25113	Host for REDIRECT PCR targeting system K12 derivative *:ΔaraBAD, ΔrhaBAD*	(Gust, 2002)[13]
Plasmid or fosmids		
pUZ8002	Supplies transfer functions for mobilization of *oriT*-containing vectors from *E. coli* to *Streptomyces*, *tra*, *neo*, RP4, Kan^R	(Gust, 2002)[13]
pIJ773	PCR template contains the [*aac(3)IV-oriT*] cassette used in REDIRECT PCR targeting system, Apr^R	(Gust, 2002)[13]
pKD46	Encodes λ-RED recombination machinery induced by arabinose, Amp^R	(Datsenko, 2000)[14]
pIJ10257	pMS81 derivative containing *ermEp**, integrates into the ΦBT1 *attB* site in *Streptomyces*; Hyg^R	[7]
pCC2FOS	Fosmid backbone for *S.iakyrus*; Cm^R	Epicentre Biotechnologies CopyControl™
17D6	Fosmid derivative containing *acmG2'-11 cat;* CM^R	This study
***Streptomyces iakyrus* strains**		
Streptomyces.iakyrus	Wild type, actinomycin G produducer	DSM 41873
SIAΔ*acmG9*	*S.iakyrus* Halogenase null mutant	This study
SIAΔ*acmG8*	*S.iakyrus* Cytochrome p450 hydroxylase null mutant	This study
SIAΔ*acmG7*	*S.iakyrus* Reductase null mutant	This study
SIAΔ*acmG9-acmG9*	Complemented *S.iakyrus* Halogenase null mutant	This study
SIAΔ*acmG7-acmG7*	Complemented *S.iakyrus* Reductase null mutant	This study

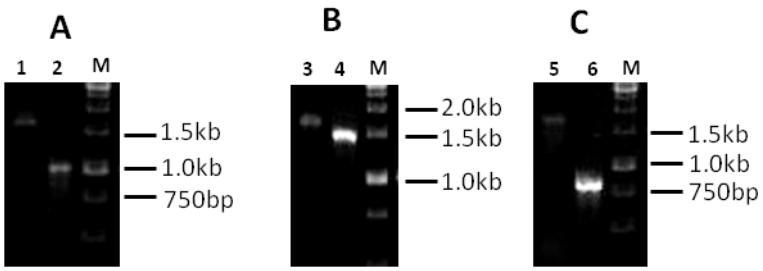
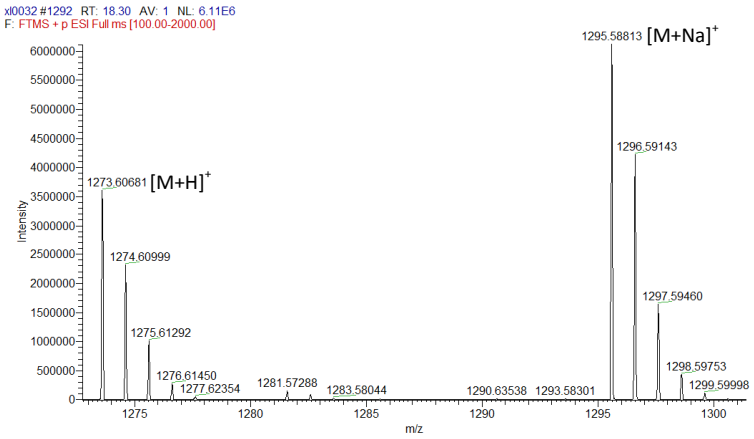


Figure S5. PCR analysis of gene disruptions in *S.iakyrus*. Total DNAs from mutants and wild-type were used as templates for PCR analysis. The specific primers for verification the combinations are provided in Table S2. (**A**) Inactivation of the non-heme α-KG halogenase gene *acmG9*: Lane **1**, PCR product of the mutant SIAΔ*acmG9*, indicating that *acmG9* was directly replaced by the *aacV(3)/oriT* cassette; Lane **2**, PCR product of the wild type *S. iakyrus*. (**B**) Inactivation of the P450 hydroxylase gene *acmG8*: Lane **3**, PCR product of the mutant SIAΔ*acmG8* indicating that *acmG8* was directly replaced by the *aacV(3)/oriT* cassette; Lane **4**, PCR product of the wild type *S. iakyrus*. (**C**) Inactivation of 3-oxoacyl-[acyl-carrier protein] reductase gene *acmG7*: Lane **5**, PCR product of the mutant SIAΔ*acmG7* indicating that *acmG7* was directly replaced by the *aacV(3)/oriT* cassette; Lane **6**, PCR product of wild type. Lane **M**, 1 kb DNA ladder (Fisher Thermo Scientific).

A



B

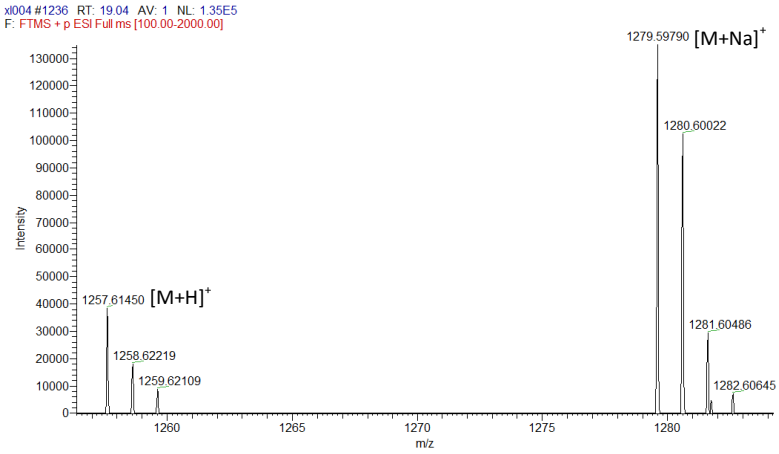


Figure S6. HRESI mass spectra of actinomycin G_3 6 (A) and G_4 7 (B)

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