Supporting Information

Cycloheximide and Actiphenol Production in Streptomyces sp. YIM56141 Governed by Single Biosynthetic Machinery Featuring an Acyltransferase-less Type I Polyketide Synthase

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Materials and general experimental methods

**Bacterial strains, plasmids, and media.** *Escherichia coli* DH5α was used for routine cloning, *E. coli* XL1 Blue MR and Gigapack III XL (Stratagene, La Jolla, CA) for genomic library construction, *E. coli* BW25113/pIJ790 for PCR-targeting and λ-RED-mediated mutagenesis,1 *E. coli* ET12567/pUZ80022 for *E. coli-Streptomyces* sp. YIM56141 conjugation, and *E. coli* BL21(DE3) for heterologous expression. *S. sp.* YIM56141 has been described previously.3 The pGEM-T easy vector (Promega, Madison, WI), SuperCos1 (Stratagene, La Jolla, CA), and pUC18 (Thermo-Fisher Scientific, Pittsburgh, PA) were from commercial sources. The plasmids pIJ773,1 pIJ790,1 pBS3080,4 and pBS90105 were described previously. All other plasmids and strains used in this study are summarized in Table S2. Ampicillin (100 μg/ml), apramycin (50 μg/ml), and chloramphenicol (25 μg/ml) were used for selection in *E. coli*. Apramycin (50 μg/ml) and kanamycin (200 μg/ml) were used for selection of *S. sp.* YIM56141 recombinants. Trimethoprim (50 μg/ml) was used to select against the *E. coli* conjugal donors after *E. coli*-S. sp.* YIM56141 conjugation. *S. sp.* YIM56141 wild-type and mutant strains were routinely grown at 28°C in TSB liquid medium. ISP4 solid medium was used to prepare spores, and IWL4 solid medium (ISP4 medium supplemented with 0.05% yeast extract, 0.1% tryptone, and MgCl2 to a final concentration of 20 mM) was used to plate out conjugation mixtures. For cycloheximide (1), actiphenol (2) and congener production, fresh spores of *S. sp.* YIM56141 and recombinant strains were cultured in 250-ml baffled flasks containing 50 ml of TSB medium for 40 hrs at 28°C and 250 rpm. Then, 0.5 ml of the seed cultures were inoculated into 250-ml baffled flasks containing 50 ml of the production medium (malt extract 5.0 g, dextrose 2.0 g, yeast extract 2.0 g, pH 7.0) and fermented at 28°C, 250 rpm for 5 days.

**Instrumentation.** Unless otherwise noted, PCR experiments were performed with LA Taq polymerase (Takara Bio Inc, Shiga, Japan) and run in an Eppendorf Gradient Thermocycler (Brinkman Instruments, Westbury, NY). High performance liquid chromatography (HPLC) was performed with an Apollo C18 column (5 μm, 4.6 x 250 mm; Grace Davison Discovery Sciences, Deerfield, IL) (analytical) or an Alltima C18 column (5 μm, 10 x 250 mm; Grace Davison Discovery Sciences, Deerfield, IL) (semi-prep) on a Varian Liquid Chromatography System (Varian, Walnut Creek, CA) consisting of Varian ProStar 210 pumps and a ProStar 330 photodiode array detector. Electrospray ionization-mass (ESI-MS) spectra were acquired on an IonSpec HiResMALDI FT-Mass spectrometer with a 7 tesla superconducting magnet. 1H and 13C NMR spectra were recorded at 25°C on a Varian Unity Inova 500 instrument operating at 500 MHz for 1H and 125 MHz for 13C nuclei. UV-Vis spectrophotometry was performed on a NanoDrop (Thermo-Fisher Scientific).

**DNA isolation, manipulation, and development of a genetic system for S. sp. YIM56141.**

DNA isolations and manipulations in *E. coli* and *Streptomyces* were carried out according to standard procedures.2 For Southern analysis, digoxigenin labeling of DNA probes, hybridization, and detection were performed according to the protocols provided by the manufacturer (Roche Diagnostics Corp., Indianapolis, IN, USA). *E. coli*-S. sp.* YIM56141 conjugation was carried out using standard literature conditions with the following minor modifications: (i) *S. sp.* YIM56141 spores in 2 x YT medium were first incubated at 50°C for 10 min followed by 3 hrs at 28°C before mixing with the *E. coli* ET12567/pUZ8002 donors and (ii) the resultant mixed culture was grown on IWL4 medium. The plates were incubated for 18 hrs at 28°C before they were overlaid with 1 ml H2O containing final concentrations of 25 μg/ml trimethoprim (to select against *E. coli*) and 50 μg/ml apramycin (to select for exconjugants). The plates were incubated for 3-6 days at 28°C. The resultant colonies were replica-plated to isolate
the apramycin-resistant and kanamycin-sensitive clones that had undergone double crossover homologous recombination to replace the targeted gene.

**Cloning, sequencing, and annotation of the chx gene cluster**

A cosmid library of *S. sp.* YIM56141 chromosomal DNA was constructed by partial digestion with *Sau3AI*, dephosphorylation, and ligation into the *BamHI* site of *SuperCos1*. The ligation mixture was packaged with Gigapack III XL packaging extract and transduced into *E. coli* XL1 Blue MR cells. Based on the studies of the *mgS* biosynthetic gene cluster, *mgS*, *mgS*I, and *mgS*E were proposed to participate in the formation of glutarimide moiety. Using degenerate primers designed according to known *mgS*, *mgS*I and *mgS*E genes, a fragment of partial putative *chx*E, *chx*D and *chx*E was amplified by PCR from *S. sp.* YIM56141 using primers p8 and p10 (Table S1) and sequenced. A 1.67-kb fragment of *chx*D and *chx*E was then amplified by PCR from *S. sp.* YIM56141 using primers p9 and p10 (Table S1) and digoxigenin labeled as a probe. The genomic library was screened by colony hybridization with the probe, and positive clones were confirmed by end sequencing. Two overlapping cosmids (pBS19001 and pBS19002) (Table S2) were chosen for sequencing. A subclone library was constructed by inserting cosmid fragments of between 1-5 kb into pUC18, which were then sequenced by ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). ORF assignments, sequence alignments, and identity and similarity calculations were made with the assistance of ORF finder, glimmer, and BLAST from NCBI websites, respectively.

**λ-RED-mediated PCR-targeting gene inactivation and complementation**

*Construction of the mutants.* Seven genes (*chx-orf*1, *chxE*, *chxG*, *chxH*, *chxl*, *chxJ*, *chx-orf1*), located on pBS19001 and pBS19002 for the chx cluster, were inactivated in *S. sp.* YIM56141 by following the λ-RED-mediated PCR-targeting mutagenesis method. Thus, each of the targeted genes was replaced by the *aac(3)V-oriT* cassette from pJ773 via λ-RED-mediated PCR-targeting strategy, using *orf*(1-*delF* and *orf*(1-*delR*, *orf1-delF* and *orf1-delR*, *chxE-delR*, *chxJ-delF* and *chxJ-delR*, *chxl-delF* and *chxl-delR*, *chxG-delF* and *chxG-delR*, and *chxH-delF* and *chxH-delR* (Table S1), to yield pBS19003, pBS19004, pBS19005, pBS19006, pBS19008, pBS19010 and pBS19012 (Table S2), respectively. Each of the mutant constructs was confirmed by PCR using primer pairs *orf*(1-*CF* and *orf*(1-*CR*, *orf1-CF* and *orf1-CR*, *chxE-CF* and *chxE-CR*, *chxJ-CF* and *chxJ-CR*, *chxl-CF* and *chxl-CR*, *chxG-CF* and *chxG-CR*, and *chxH-CF* and *chxH-CR* (Table S1), respectively. These mutated cosmids were subsequently introduced into the *S. sp.* YIM56141 wild-type strain by intergeneric conjugation between *S. sp.* YIM56141-*E. coli* ET12567/pUZ8002. Exconjugants with the apramycin-resistant and kanamycin-sensitive phenotypes were selected as the desired double crossover mutants. Their genotypes were finally confirmed by Southern analysis (the primers used to prepare the probes for Southern analysis can be found in Table S1) (Figures S1, S2, and S4-S8). The correct mutant strains were named SB19001 (Δchx-orf(1)), SB19002(Δchx-orf1), SB19003 (ΔchxE), SB19004 (ΔchxJ), SB19006 (Δchxl), SB19008 (ΔchxG), and SB19010 (ΔchxH), respectively (Table S2).

*Complementation of mutants.* For complementation, a derivative of pBS9015 was first constructed and named pBS19015, which contained φC31 attachment site for site-specific integration, ampicillin and thiostrepton resistance genes for selection, and multiple cloning sites behind the *ErmE* promoter to clone the targeted gene whose expression will be ensured under *ErmE* (Table S2). Thus, each of the *chxJ*, *chxl*, *chxG*, and *chxH* genes was first amplified with primers (see Table S1) containing *BamHI* and *XbaI* sites. The resulting PCR fragments were then isolated, digested with *BamHI* and *XbaI*, and ligated into the same sites of pBS19015 to
yield expression plasmids pBS19007 (chxJ), pBS19009 (chxI), pBS19011 (chxG), and pBS19013 (chxH) (Table S2). Introduction of pBS19007, pBS19009, pBS19011, and pBS19013 into SB19004, SB19006, SB19008, and SB19010 by conjugation finally afforded the complementation strains SB19005, SB19007, SB19009, and SB1911, respectively (Table S2).

HPLC analysis of fermentation extracts

Culture broths of S. sp. YIM56141 wild-type or recombinant strains were extracted with EtOAc and analyzed by a Varian liquid chromatograph system with an Apollo C18 column (5 μm, 250 × 4.6 mm). The column was equilibrated with 100% solvent A (H2O) and developed with a 30 min linear gradient from 0% B (acetonitrile) to 100% B and then kept 100% B for 5 min at a flow rate of 1 ml/min and UV detection at 210 nm using a Varian Prostar 330 PDA detector.

Isolation of dehydrocycloheximide (10)

A 5-L fermentation culture (10 x 500 ml in 2-L baffled Erlenmeyer flasks) of mutant SB19010 (∆chxH) was centrifuged at 3500 x g for 30 min, and the resulting supernatant was extracted with EtOAc twice. The EtOAc extract was evaporated to dryness, and the residue was dissolved in a mixture of CHCl3-CH3OH (1:1) and mixed with an appropriate amount of silica gel. The sample was applied to a normal phase silica gel column and subsequently eluted with CHCl3-CH3OH (10:0-8:2) to afford six fractions, each of which was analyzed by HPLC. The fractions that contained 10 were combined, and the compound was finally purified by Varian liquid chromatograph system with an Alltima-C18 column (5 μm, 10 mm x 25 cm). The column was equilibrated with 60% solvent A (H2O) and 40% solvent B (acetonitrile) and developed with 40% B for 7 min then change to 55% B for 5 min, finally kept 40% B for 6 min at a flow rate of 3 mL min⁻¹ and UV detection at 280 nm using a Varian Prostar 330 PDA detector. Structural characterization of 10 can be found in Figure S9 and Table S4.

Cloning and expression of chxH and overproduction and purification of ChxH

The 789bp chxH gene was PCR-amplified from cosmid pBS19002 with primers ChxH-RSF-F and Chx-RSF-R. The PCR reaction was carried out using the Expand High Fidelity PCR System (Roche Diagnostics). Reactions (20 μL) contained 2 μL 10x PCR buffer with MgCl₂, 2 μl dNTPs (2.5 mM), 1 μl sense primer (10 μM), 1 μl anti-sense primer (10 μM), 1 μl dimethylsulfoxide (DMSO), 1 μl pBS19002 DNA (20 ng), 0.3 μl Taq (3.5 U/μl), and 11.7 μl H2O. The following PCR program was used: 95°C 4 min, 30 cycles of 95°C, 30 s, 54°C, 40 s, 72°C, 1 min, and a final extension cycle at 72°C, 10 min. Then PCR product with the expected size was recovered from the agarose gel with a Qiagen gel extraction kit. The fragment was introduced into pBS3080 as described previously to yield plasmid pBS19014. The plasmid pBS19014 was introduced to E. coli BL21 (DE3) to yield SB19012, which was confirmed by DNA sequencing. For chxH expression, a starter culture of SB19012 was grown overnight at 37°C with shaking at 250 rpm in 5 ml of LB media containing 50 μg/l of kanamycin. The starter culture was inoculated into 500 ml of LB media containing 50 μg/l of kanamycin, and the culture was grown at 37°C with shaking at 250 rpm until it reached an A600 of 0.6, at which time the culture was cooled to 18°C and IPTG was added to a final concentration of 0.25 mM; the induced culture was left shaking overnight. The cells were harvested by centrifugation and resuspended in lysis buffer (100 mM Tris pH 8.0, 15 mM imidazole, 300 mM NaCl, 10 % glycerol). The cells were then lysed by sonication and the resulting lysate was clarified by centrifugation at 17000 g for 30 min. The lysate was loaded onto a 5 ml HisTrapFF column (GE lifesciences), washed with two column volumes of wash buffer (100 mM Tris pH 8.0, 15 mM imidazole, 300 mM NaCl), and eluted with 50% elution buffer (100 mM Tris pH 8.0, 500 mM imidazole, 300 mM NaCl, 10 %
glycerol). Fractions containing ChxH were pooled and buffer exchanged using a PD10 column (GE lifesciences) with 100 mM NaH₂PO₄ (pH 7.2), 10% glycerol. Protein concentrations were determined using the Bradford method. The resulting protein was near homogeneity based on SDS-PAGE analysis (Figure S11) and stored at -80°C.

**In vitro assay and kinetic analysis of ChxH activity**

The activity of ChxH was examined at different time points, pH values (pH 5.8-8.0), and concentrations of DMSO. The assay buffer consisted of 100 mM sodium phosphate, 1 mM TCEP, 0.5 mM substrate (suspended in DMSO), 0.5 mM NADPH in a final volume of 100 μl, and the reaction was initiated by addition of ChxH to 0.2 μM, which were allowed to proceed for 10 min at 24°C. Then the reactions were quenched by adding 200 μl MeOH and centrifuged at 13,000g for 10 min, and 100 μl of supernatant was subjected to HPLC analysis. Analytical HPLC was conducted using a Alltima C18 column (250 × 4.6 mm, 5 μm) and eluted with a linear gradient of 60 % water and 40 % acetonitrile for 7 min, then to 45 % water and 55 % acetonitrile for 5 min, followed by 6 min at 60 % water and 40 % acetonitrile at a flow rate of 1.0 mL/min with UV detection at 215 nm. The optimal pH and DMSO concentration was found to be pH 7.2 and 5 % respectively (Figure S11).

The kinetics of ChxH was measured by the decrease or increase in absorbance at 340 nm using an extinction coefficient of 6220 M⁻¹cm⁻¹ for NADPH and NADH. Recordings were carried out with a NanoDrop instrument (Fisher Scientific). Different substrate concentrations (10 - 750 μM of 7) and NADPH concentrations (10 - 500 μM) were used when measuring the initial velocity. The reactions were started by the addition of ChxH (8.5 nM) and allowed to proceed for 20 min at 24°C. All assays were performed in duplicate, the K_M and V_max values were calculated from curve fitting to the Michaelis-Menten equation: y = V_max × [S]/(K_M + [S]) using GraphPad Prism 5.00 (GraphPad Software, San Diego California USA).

**References**

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Table S1. Summary of primers used in this study

For gene inactivation by A-R-mediated PCR-targeting mutagenesis

| Plasmid | Oligonucleotide | Sequence (bold-letters represent DNA sequence flanking the aac(3′)/aphI cassette from pJJ773) |
|---------|-----------------|------------------------------------------------------------------------------------------------|
| pBS19003 | orf1(1)-delR | 5′-TCTCTCGCAAGACCGAGACTCACTGGGATCCCTGATGCC-3′ |
| pBS19004 | orf1-delF | 5′-CTGTGACGAGGTGTGCTGAGATAATGAGAGAACGAC-3′ |
| pBS19005 | chx1-5F | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |
| pBS19006 | chx1-delF | 5′-TACCGTGATGGGTAACAGTTGGATTGTCAGTGG-3′ |
| pBS19008 | chx1-5F | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |
| pBS19010 | chx1-5F | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |
| pBS19012 | chx1-5F | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |

For amplification of the putative chxC, chxD and chxE genes from S. yimelus141 to prepare the probe to clone the chx cluster

| Plasmid | Oligonucleotide | Sequence |
|---------|-----------------|----------|
| p8 | 5′-CGAGTCAAGACCAGCAGAC-3′ |
| p9 | 5′-GGAGTACAGGCGCATACGCC-3′ |
| p10 | 5′-GTCCTTCTTGCGGATCCCATGAC-3′ |

For amplification of mutated plasmids

| Plasmid | Oligonucleotide | Sequence |
|---------|-----------------|----------|
| pBS19003 | orf1(1)-CF | 5′-CCCCGTGCGCGGATCCCTGAC-3′ |
| pBS19004 | orf1-CR | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |
| pBS19005 | chx1-5F | 5′-CTCGAGTCCCGAGACGAGATGGAAC-3′ |
| pBS19006 | chx1-CF | 5′-TCTCTCGCAAGACCGAGACTCACTGGGATCCCTGATGCC-3′ |
| pBS19008 | chx1-5F | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |
| pBS19010 | chx1-CF | 5′-TCTCTCGCAAGACCGAGACTCACTGGGATCCCTGATGCC-3′ |
| pBS19012 | chx1-CF | 5′-TCTCTCGCAAGACCGAGACTCACTGGGATCCCTGATGCC-3′ |

For amplification of probes to confirm each mutant by Southern analysis

| Strains | Oligonucleotide | Sequence |
|---------|-----------------|----------|
| SB19001 | ps1-F | 5′-GTCCTTCTTGCGGATCCCATGAC-3′ |
| SB19002 | ps1-R | 5′-GTCCTTCTTGCGGATCCCATGAC-3′ |
| SB19006 | pS8-S | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |
| SB19004 | pSF1 | 5′-GTCCTTCTTGCGGATCCCATGAC-3′ |
| SB19006 | pSRE | 5′-GTCCTTCTTGCGGATCCCATGAC-3′ |
| SB19008 | pSF2 | 5′-GTCCTTCTTGCGGATCCCATGAC-3′ |
| SB19010 | pS8-H | 5′-GTCCTTCTTGCGGATCCCATGAC-3′ |

For amplification of probes to confirm each of the expression plasmids for complementation

| Plasmid | Oligonucleotide | Sequence (the BamHI and XbaI sites highlighted in bold) |
|---------|-----------------|----------------------------------------------------------------|
| pBS19007 | chxJ-S | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |
| pBS19009 | chxJ-A | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |
| pBS19011 | chxK-S | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |
| pBS19013 | chxK-A | 5′-GGATCCATGATCGAGTCTCCCGAGACGAGATGGAAC-3′ |

For amplification of genes for heterologous expression

| Plasmid | Oligonucleotide | Sequence (highlighted in bold are sequences used for ligation independent cloning) |
|---------|-----------------|----------------------------------------------------------------|
| pBS19014 | chxH-RSf-F | 5′-GTCCTTCTTGCGGATCCCATGAC-3′ |
| pBS19014 | chxH-RSf-R | 5′-GTCCTTCTTGCGGATCCCATGAC-3′ |
Table S2. Summary of plasmids and strains used in this study

| Plasmids/Strains | Relevant characteristics |
|------------------|--------------------------|
| **Plasmids**     |                          |
| pBS19001         | SuperCos1-based cosmid that harbors downstream part of the *chx* cluster |
| pBS19002         | SuperCos1-based cosmid that harbors upstream part of the *chx* cluster |
| pBS19003         | pSB19002-based cosmid in which *chx-orf(-1)* was replaced with the *acc(3)IV-oriT* cassette |
| pBS19004         | pSB19001-based cosmid in which *chx-orf1* was replaced with the *acc(3)IV-oriT* cassette |
| pBS19005         | pSB19002-based cosmid in which *chxE* was replaced with the *acc(3)IV-oriT* cassette |
| pBS19006         | pSB19001-based cosmid in which *chxJ* was replaced with the *acc(3)IV-oriT* cassette |
| pBS19007         | complementation plasmid for Δ*chxJ* mutant |
| pBS19008         | pBS19001-based cosmid in which *chxI* was replaced with the *acc(3)IV-oriT* cassette |
| pBS19009         | complementation plasmid for Δ*chxI* mutant |
| pBS19010         | pBS19001-based cosmid in which *chxG* was replaced with the *acc(3)IV-oriT* cassette |
| pBS19011         | complementation plasmid for Δ*chxG* mutant |
| pBS19012         | pBS19001-based cosmid in which *chxH* was replaced with the *acc(3)IV-oriT* cassette |
| pBS19013         | complementation plasmid for Δ*chxH* mutant |
| pBS19014         | *chxH* expression plasmid for *E. coli* derived from pBS3080 |
| pBS19015         | a derivative of pBS9010 that contains the *ErmE* promoter and can be selected with ampicillin and thiostrepton |

| S. sp. strains   |                          |
|------------------|--------------------------|
| YIM56141         | Wild-type, cycloheximide (CHX) and actiphenol (APN) producing |
| SB19001          | Δ*chx-orf(-1)* mutant, CHX and APN producing |
| SB19002          | Δ*chx-orf1* mutant, CHX and APN producing |
| SB19003          | Δ*chxE* mutant, CHX and APN non-producing |
| SB19004          | Δ*chxJ* mutant, CHX and APN non-producing |
| SB19005          | complementation strain of Δ*chxJ* mutant, CHX and APN producing |
| SB19006          | Δ*chxI* mutant, CHX and APN non-producing |
| SB19007          | complementation strain of Δ*chxI* mutant, CHX and APN producing |
| SB19008          | Δ*chxG* mutant, CHX non-producing but APN producing |
| SB19009          | complementation strain of Δ*chxG* mutant, CHX and APN producing |
| SB19010          | Δ*chxH* mutant, CHX non-producing but APN producing |
| SB19011          | complementation strain of Δ*chxH* mutant, CHX and APN producing |
| SB19012          | *E. coli* BL21(DE3) expressing *chxH* from pBS19014 |
Table S3. Deduced functions of the genes within the chx cluster\textsuperscript{a}

| Gene | amino acids | Proposed function | Sequence homologs | Identity/similarity (%) |
|------|-------------|-------------------|------------------|-------------------------|
| orf(-3)-orf(-1) | | Beyond the upstream boundary | | |
| chxA | 1123 | SARP family pathway specific regulatory protein | MgsA (ACY01386) | 46/58 |
| chxB | 291 | malonyl-CoA:ACP transacylase | SmdF (EFL22142) | 68/79 |
| chxC | 85 | acyl carrier protein | MgsC (ACY01388) | 44/67 |
| chxD | 678 | amidotransferase | MgsD (ACY01389) | 57/70 |
| chxE | 6918 | AT-less type-I PKS | Smdl (EFL22140) | 61/70 |
| chxF | 319 | LysR family transcriptional regulator | Pa01 (AAG03607) | 32/51 |
| chxG | 364 | putative enoylreductase | MorB (AAC43569) | 35/43 |
| chxH | 262 | ketoreductase | FabG (ACB02286) | 34/50 |
| chxI | 397 | cytochrome P450 | MgsK (ACY01396) | 43/57 |
| chxJ | 1148 | carboxylic acid reductase | CAR (AAR91681) | 59/72 |
| Orf1)-orf5 | | | | |

\textsuperscript{a}The sequence has been deposited in GenBank with the accession number JX014302.

Table S4. \textsuperscript{1}H (500 MHz) and \textsuperscript{13}C NMR (125 MHz) data of 10 in CDCl\textsubscript{3}\textsuperscript{a}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{structure}
\end{figure}

| position | \(\delta_C\), type | \(\delta_H\) (J in Hz) |
|----------|------------------|----------------------|
| 2        | 172.0, C         |                      |
| 3        | 37.5, CH\textsubscript{2} | 2.80 m\textsuperscript{*}, 2.37 m\textsuperscript{*} |
| 4        | 26.1, CH         | 2.76 m\textsuperscript{*} |
| 5        | 37.5, CH\textsubscript{2} | 2.80 m\textsuperscript{*}, 2.37 m\textsuperscript{*} |
| 6        | 171.9, C         |                      |
| 7        | 41.2, CH\textsubscript{2} | 2.52 dd (16.8, 6.8), 2.45 dd (16.8, 6.8) |
| 8        | 198.2, C         |                      |
| 9        | 105.7, C         |                      |
| 10       | 185.0, C         |                      |
| 11       | 34.1, CH         | 2.53 m\textsuperscript{*} |
| 12       | 36.9, CH\textsubscript{2} | 2.34 m\textsuperscript{*}, 1.49 m |
| 13       | 24.5, CH         | 1.86 m\textsuperscript{*} |
| 14       | 32.6, CH\textsubscript{2} | 1.85 m\textsuperscript{*} |
| 15       | 19.2, CH\textsubscript{3} | 1.23 d (7.5) |
| 16       | 21.4, CH\textsubscript{3} | 1.02 d (6.5) |
| NH       |                  | 8.35 s               |
| OH       |                  | 15.68 s              |

\textsuperscript{a}Assignments based on COSY, HSQC, and HMBC.

\textsuperscript{*}Signals overlapped with others.
Table S5. Summary of the apparent kinetic parameters for ChxH

![Chemical structures](image)

| variable substrate | constant substrate | $K_M$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_M$ (μM$^{-1}$ min$^{-1}$) |
|--------------------|--------------------|------------|------------------------|-------------------------------------|
| 10                 | NADPH              | 44 ± 4     | 599 ± 69               | 14 ± 2                              |
| NADPH              | 10                 | 34 ± 4     | 17 ± 3                 |                                     |
| 10                 | NADH               | 139 ± 23   | 20 ± 3                 | 0.15 ± 0.03                         |
| NADH               | 10                 | 75 ± 8     | 0.27 ± 0.05            |                                     |
| 1                  | NADP$^+$           | 99 ± 7     | 387 ± 23               | 3.9 ± 0.4                           |
| NADP$^+$           | 1                  | 76 ± 5     | 5.1 ± 0.4              |                                     |
| 1                  | NAD$^+$            | 162 ± 32   | 13 ± 3                 | 0.08 ± 0.02                         |
| NAD$^+$            | 1                  | 223 ± 37   | 0.06 ± 0.01            |                                     |
**Figure S1.** (I) Construction of the Δorf(-1) mutant strain SB19001 and restriction maps of S. sp. YIM56141 wild-type and SB19001 strains showing fragment sizes upon BamHI digestion. (II) Southern analysis of S. sp. YIM56141 wild-type (lane 2) and SB19001 (lane 3) genomic DNA digested with BamHI using the 0.43-kb PCR-amplified fragment as a probe.

**Figure S2.** (I) Construction of the Δorf1 mutant strain SB19002 and restriction maps of S. sp. YIM56141 wild-type and SB19002 strains showing fragment sizes upon Ncol and Scal digestion. (II) Southern analysis of S. sp. YIM56141 wild-type (lane 2) and SB19002 (lane 3) genomic DNA digested with Ncol and Scal using the 0.31-kb PCR-amplified fragment as a probe.
Figure S3. Architectural similarity between the first six modules of the AT-less type I PKSs from the biosynthetic machineries of selected glutarimide-containing polyketides: (A) 5, (B) 6, (C) 4, and (D) 1. The aberrant methyl group at C-11 of 1 that is installed by ChxE module-6, is shared in 4, 5, and 6 (highlighted with an oval).
Figure S4. (I) Construction of the ΔchxE mutant strain SB19003 and restriction maps of S. sp. YIM56141 wild-type and SB19003 strains showing fragment sizes upon Ncol digestion. (II) Southern analysis of S. sp. YIM56141 wild-type (lane 2) and SB19003 (lane 3) genomic DNA digested with Ncol using the 0.34-kb PCR-amplified fragment as a probe.

Figure S5. (I) Construction of the ΔchxJ mutant strain SB19004 and restriction maps of S. sp. YIM56141 wild-type and SB19004 strains showing fragment sizes upon SspI and KpnI digestion. (II) Southern analysis of S. sp. YIM56141 wild-type (lane 2) and SB19004 (lane 3) genomic DNA digested with SspI and KpnI using the 0.30-kb PCR-amplified fragment as a probe.
Figure S6. (I) Construction of the Δchxl mutant strain SB19006 and restriction maps of S. sp. YIM56141 wild-type and SB19005 strains showing fragment sizes upon Scal and NotI digestion. (II) Southern analysis of S. sp. YIM56141 wild-type (lane 2) and SB19006 (lane 3) genomic DNA digested with Scal and NotI using the 0.35-kb PCR-amplified fragment as a probe.

Figure S7. (I) Construction of the ΔchxG mutant strain SB19008 and restriction maps of S. sp. YIM56141 wild-type and SB19008 strains showing fragment sizes upon Scal and Ncol digestion. (II) Southern analysis of S. sp. YIM56141 wild-type (lane 3) and SB19008 (lane 2) genomic DNA digested with Scal and Ncol using the 0.35-kb PCR-amplified fragment as a probe.
Figure S8. (I) Construction of the ΔchxH mutant strain SB19010 and restriction maps of S. sp. YIM56141 wild-type and SB19010 strains showing fragment sizes upon SalI digestion. (II) Southern analysis of S. sp. YIM56141 wild-type (lane 2) and SB19010 (lane 3) genomic DNA digested with SalI using the 0.35-kb PCR-amplified fragment as a probe.
(A) $^1$H

(B) $^{13}$C
(C) HSQC

(D) HMBC
Figure S9. NMR spectra of 10 in CDCl₃: (A) ¹H, (B) ¹³C, (C) HSQC, (D) HMBC, and (E) COSY.
Figure S10. Sequence alignment of ChxG and other members of the OYE family: (A) Sequence alignment and (B) sequence identity matrix. Accession numbers and source organisms: ChxG (AFO59868, Streptomyces sp. YIM 56141); Sc (CAB93057, Streptomyces coelicolor A3(2)); XenB (WP_020191195, Pseudomonas putida); MR (AAC43569, Pseudomonas putida); PETNR (AAB38683, Enterobacter cloacae); Sye1 (AAN55488, Shewanella oneidensis MR-1); Bm NemA (AAU46718, Burkholderia mallei ATCC 23344); GeoOYE (BAD76617, Geobacillus kaustophilus HTA426); YqjM (CAB14314, Bacillus subtilis subsp. subtilis str. 168); and TOYE (ABY93685, Thermoanaerobacter pseudethanolicus ATCC 33223).
Figure S11. SDS-PAGE analysis of purified ChxH protein. Marker (M), and numbers above lanes represents the number of μg of ChxH loaded onto the gel.

Figure S12. In vitro assay of ChxH for the reduction of 10 (♦) to 1 (●) as followed by HPLC analysis. (A) Time course for the reduction of 10 to 1 at varying time: (I) 2 min., (II) 5 min., (III) 10 min., (IV) 15 min., and (V) 30 min. (B) Relative ChxH activity for the reduction of 10 to 1 at varying pH: (I) pH 5.8, (II) pH 6.4, (III) pH 7.2, (IV) pH 7.6, (V) pH 8.0, (VI) pH 8.5, and (VII) pH 9.5.
Figure 13. Pseudo-first order kinetic studies of ChxH under steady state conditions shown that plots of initial velocity versus the concentration of substrates or cofactors all displayed Michaelis-Menten kinetics. ChxH was assayed in both forward direction (10-to-1), using NADPH or NADH, or reverse direction (1-to-10), using NDAP⁺ or NAD⁺. Plots with substrate held at constant concentration: (I) NADPH, (II) 10, (III) NADP⁺, (IV) 1, (V) NADH, (VI) 10, (VII) NAD⁺, and (VIII) 1.