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Biosynthesis of Antimycins with a Reconstituted 3-Formamidosalicylate Pharmacophore in *Escherichia coli*

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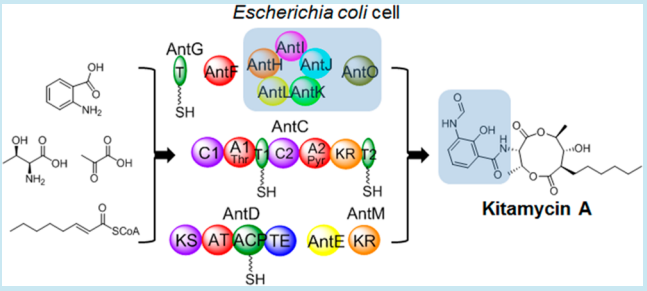
Supporting Information

ABSTRACT: Antimycins are a family of natural products generated from a hybrid nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) assembly line. Although they possess an array of useful biological activities, their structural complexity makes chemical synthesis challenging, and their biosynthesis has thus far been dependent on slow-growing source organisms. Here, we reconstituted the biosynthesis of antimycins in *Escherichia coli*, a versatile host that is robust and easy to manipulate genetically. Along with *Streptomyces* genetic studies, the heterologous expression of different combinations of *ant* genes enabled us to systematically confirm the functions of the modification enzymes, AntHIJKL and AntO, in the biosynthesis of the 3-formamidosalicylate pharmacophore of antimycins. Our *E. coli*-based antimycin production system can not only be used to engineer the increased production of these bioactive compounds, but it also paves the way for the facile generation of novel and diverse antimycin analogues through combinatorial biosynthesis.



KEYWORDS: *antimycin biosynthesis, nonribosomal peptide/polyketide hybrid, heterologous expression, 3-formamidosalicylate, multicomponent oxygenase, formyltransferase*

Antimycins are a family of depsipeptides consisting of a nine-membered dilactone ring substituted with one alkyl (C-7), one acyloxy (C-8), two methyl moieties (C-4 and C-9), and an amide linkage (C-3) connecting to a 3-formamidosalicylic acid (Figure 1). They are produced by various *Streptomyces* species, and over the past few years, a growing number of natural and modified antimycin-type compounds varying in the alkyl and acyl chains have been reported.[1−8] Additionally, several related antimycin-type depsipeptides with 12-, 15-, and 18-membered macrolactone rings were also recently isolated and characterized (Figure 1).[9,10] Notably, all of these compounds possess a common 3-formamidosalicylate unit, which previous studies have demonstrated to be essential for the antifungal, insecticidal, nematocidal, and piscicidal properties of antimycins.[1−3,11] While these bioactivities arise from the ability of antimycins to inhibit cytochrome c oxidoreductase in the mitochondrial electron transport chain,[12] other work has also shown antimycin-type compounds to be promising candidates for treating a variety of diseases, including asthma, cancer, Alzheimer's disease, and Parkinson's disease.[9,10,13−15] Understanding the enzymatic machinery and engineering efficient systems for antimycin biosynthesis can therefore expand the production of antimycin analogues with improved pharmaceutical properties.

We recently dissected the antimycin biosynthetic pathway by both genetic and enzymatic studies and identified the minimum set of enzymes (AntCDEFGM) needed for the generation of the antimycin dilactone scaffold. The molecular backbone is built from four distinct monomers: an aminobenzoate, a natural amino acid, an α-keto acid, and an acylmalonyl moiety through a hybrid NRPS-PKS assembly line-based mechanism (Figure 2).[16] However, the biosynthetic pathway toward 3-formamidosalicylic acid, the putative starter unit conserved in all antimycin-type depsipeptides but not found in any other natural products, was not fully elucidated (Figure 1). In addition, the production of antimycins has thus far been limited to slow-growing *Streptomyces* organisms that are challenging to genetically manipulate, which has impeded the facile understanding and engineering of the biosynthesis of antimycin-type depsipeptides. As an alternative, *E. coli* has recently emerged as a powerful microorganism for studying the biosynthesis of some natural products because of its fast growth rate, genetic tractability, and well-studied primary metabolism.[17−19] The reconstitution of NRPS-PKS activity in *E. coli* also provides immense opportunities for the biochemical understanding and protein engineering of these modular assembly lines in natural product synthesis and diversification.

In this work, we reconstituted the complete biosynthesis of antimycins in the heterologous host, *E. coli*. Using this

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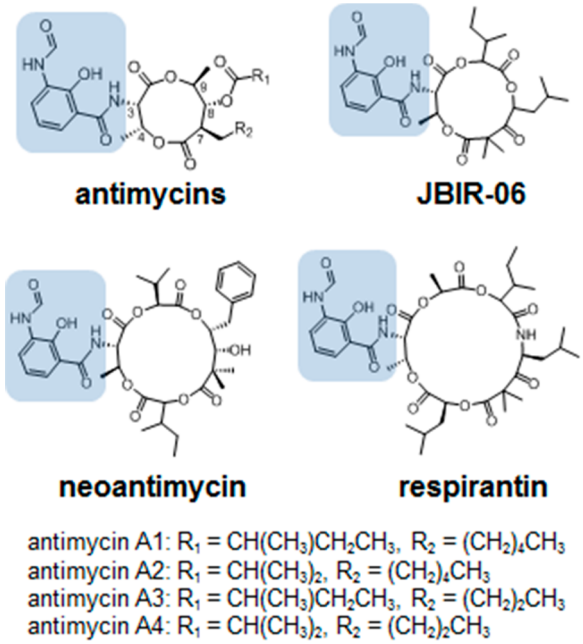

Figure 1. Structures of antimycin-type depsipeptides.

heterologous expression system in combination with performing a mutagenesis study in *Streptomyces*, we systematically confirmed the roles of two previously proposed modification enzymes, AntHIJKL and AntO, in the generation of the 3-formamidosalicylate moiety of antimycins from an anthranilate precursor (Figure 2).[7,16,20] Down the line, our flexible and robust *E. coli* expression system can be used to facilitate the rapid generation of novel antimycin analogues through combinatorial biosynthesis as well as to increase the yield of target antimycin analogues through metabolic engineering.

To produce the dilactone scaffold of antimycins in *E. coli*, we coexpressed six enzymes, AntCDEFGM, from either the *S. albus* J1074 or *S. ambofaciens* antimycin gene clusters[16,21] on three Duet vectors. Specifically, the dimodule NRPS-encoding *antC* and the crotonyl-CoA carboxylase/reductase homologue-encoding *antE* were cloned into pETDuet-1; the PKS-encoding *antD* and an operon consisting of *antGF*, which encodes a carrier protein and an acyl-ACP ligase homologue, respectively, were cloned into pCDFDuet-1; and the 3-oxoacyl-ACP reductase homologue-encoding *antM* was cloned into pCOLADuet-1. The three plasmids were cotransformed into the BAP1 strain of *E. coli*, which contains a chromosomal copy of the phosphopantetheinyl transferase Sfp to ensure the posttranslational modification of AntC, D, and G to their pantetheinylated forms.[22] Upon coexpression of *antCDEFGM* in this *E. coli* strain (JL1), trace amounts of antimycin-type compounds could be detected in the culture extracts by liquid chromatography-high resolution mass spectrometry (LC–HRMS) and HRMS/MS analysis (Figures 3 and S1, Supporting Information). Despite the relaxed substrate specificities of AntE and AntD,[16,23] **1**, which is derived from the substrate octenoyl-CoA, was the major observed product, indicating that *E. coli* has a different endogenous pool of fatty acyl-CoA intermediates compared to *Streptomyces* by which a suite of antimycins

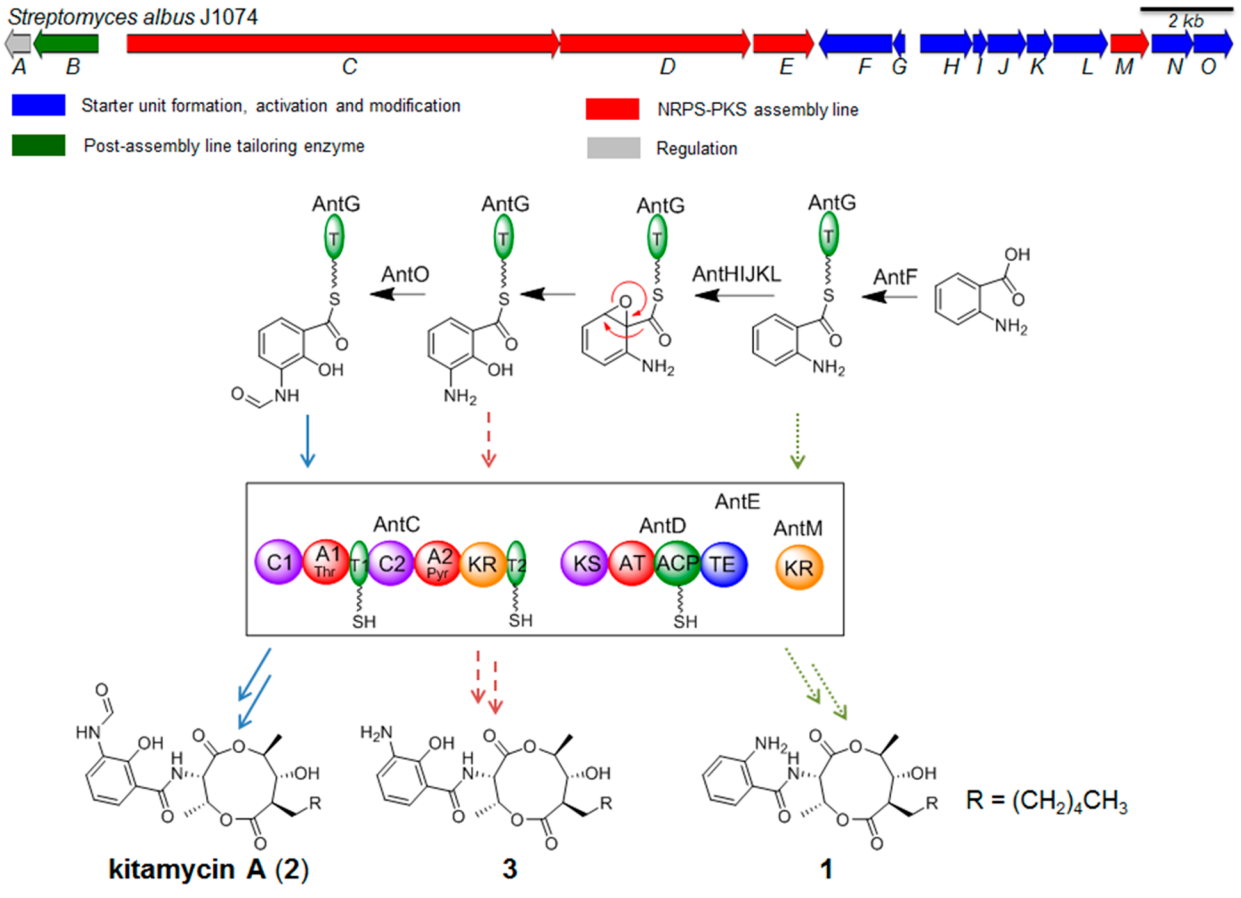

Figure 2. Schematic of the antimycin gene cluster encoded by *S. albus* and proposed biosynthetic and shunt pathways.

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Strain	Genes	Major products	Minor products
JL1	*antCDEFGM*	**1**	-
JL2	*antCDEFGHIJKLMNO*	**1**, **2**	-
JL3	*antCDEFGMO*	**1**	-
JL4	*antCDEFGHIJKLM*	**1**	**3**
JL5	*antCDEFGHIJKLNO*	-	-

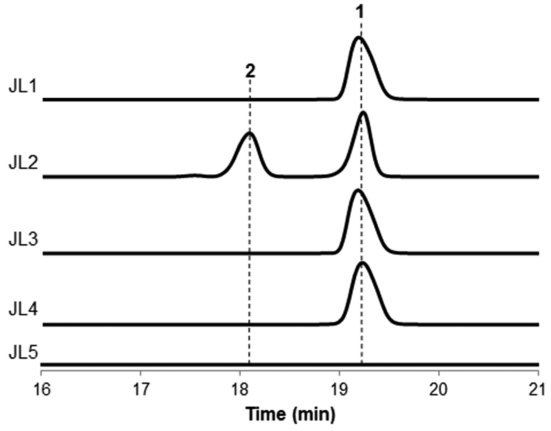

Figure 3. Biosynthesis of antimycins in *E. coli*. Table and extracted ion chromatograms (**1**, *m*/*z* 421.2333 $[M + H]^+$; **2**, *m*/*z* 465.2231 $[M + H]^+$; **3**, *m*/*z* 437.2282 $[M + H]^+$) showing compounds resulting from the heterologous expression of different combinations of antimycin biosynthetic genes in *E. coli*. Compound **3** was produced ~100-fold less than **1** and could thus not be detected at this scale. The calculated mass with a 10 ppm mass error tolerance was used.

varying at the C-7 position are typically produced.[6] As expected, the production of **1** was not observed in the extracts of cultures missing one or more of the six genes such as those of the *E. coli* strain JL5 (Figure 3). When anthranilic acid and octanoic acid were both fed at a final concentration of 1 mM to JL1 cultures at the time of protein induction, the titer of **1** was increased by approximately 140-fold to ~0.2 mg/L. These precursors were thus included in all subsequent experiments. Since previous mutagenesis studies in *Streptomyces* showed that a starter unit other than 3-formamidosalicylate decreased the efficiency of NRPS-PKS assembly line by more than 1000-fold (deoxy-isoantimycins were produced at ~10 μg/L),[16,20] the current production titer of **1** by JL1 suggests that this heterologous expression system is more robust than the native *Streptomyces* system in generating the active modular assembly line for antimycin synthesis.

With the ability to generate the antimycin core scaffold in *E. coli*, we next sought to use this highly tractable system to dissect the formation of the conserved 3-formamidosalicylate pharmacophore. To this end, we introduced the remaining genes from the antimycin biosynthetic gene cluster presumed to be important for the synthesis of this moiety into the strain JL1 to yield the new strain, JL2 (Figure 3). The newly coexpressed genes included *antHIJKL*, which encodes a multicomponent oxygenase homologue; *antN*, which encodes a putative tryptophan 2,3-dioxygenase; and *antO*, which encodes a lipase homologue (Figure 2). Although previous gene disruption experiments have demonstrated *antHIJKL* to be important for the biosynthesis of antimycins,[16,20] the possible roles of *antN* and *antO* in antimycin biosynthesis have never been studied before. Analysis of the culture extracts of JL2 by HPLC and LC−HRMS showed that the coexpression of *antCDEFGHIJKLMNO* not only resulted in the formation of **1**, but it also resulted in the formation of a product, **2**, in comparable yield (Figures 3, 4A, and S5, Supporting Information). HRMS/

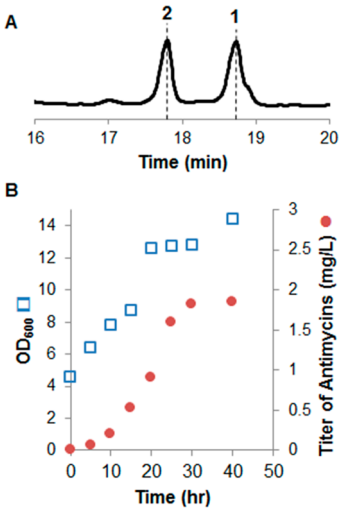

Figure 4. Fed-batch fermentation production of antimycins by *E. coli* expressing AntCDEFGHIJKLMNO. (A) HPLC analysis (330 nm) showing the production of **1** and **2**. (B) The maximum titer achieved was ~2 mg/L at OD_{600} ≈ 13, 30 h after IPTG induction.

MS analysis suggested **2** to be the antimycin-type compound, kitamycin A, with a completely reconstituted 3-formamidosalicylate moiety (Figures 3 and S2, Supporting Information).[8] To further confirm the identity of this compound, we purified **2** from *E. coli* culture extracts and incubated it with isobutyryl-CoA and AntB, the acyltransferase that catalyzes the C-8 acyloxy formation of antimycins.[24] This enzymatic assay resulted in the conversion of **2** into antimycin A2 with a retention time, UV spectrum, and mass fragmentation patterns precisely matching those of the standard, thus supporting the successful reconstitution of 3-formamidosalicylate biosynthesis in *E. coli* (Figures 5, S4, and S5, Supporting Information). To

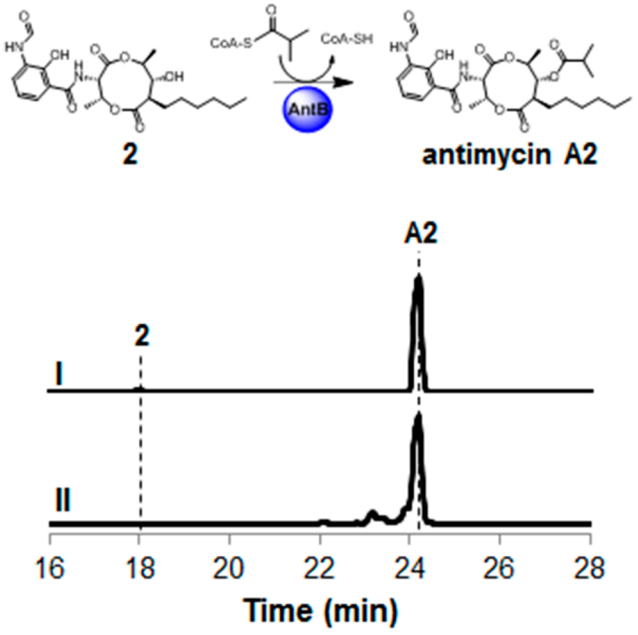

Figure 5. Extracted ion chromatograms (**2**, *m*/*z* 465.2231 $[M + H]^+$; antimycin A2, *m*/*z* 535.2650 $[M + H]^+$) for the *in vitro* generation of antimycin A2 from **2** and isobutyryl-CoA. The product from the AntB-catalyzed reaction (I) was compared to the antimycin A2 standard (II). The calculated mass with a 10 ppm mass error tolerance was used.

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demonstrate the potential scalability of this heterologous antimycin production system, a fed-batch fermentation of this antimycin-producing JL2 strain was then performed in F1 medium supplemented with 1 mM anthranilic acid, octanoic acid, and threonine. Upon the coexpression of the 13 *ant* genes, an antimycin titer of approximately 2 mg/L was achieved when the OD_{600} plateaued at ~13 (Figure 4). It is anticipated that this titer can be further increased by the manipulation of protein expression levels and cultivation conditions.

After fully reconstituting the biosynthesis of antimycins in *E. coli*, we set out to delineate the functions of the individual enzymes in the generation of the 3-formamidosalicylate group by systematically expressing different combinations of *antHIJKL*, *antN*, and *antO* in JL1. A strain containing all of the genes, except *antN*, was first constructed. LC−HRMS analysis of the extracts of this culture showed a profile very similar to that of JL2 with the production of both **1** and **2** (Figure S6, Supporting Information), demonstrating that AntN is not required for the formation of the 3-formamidosalicylate moiety from anthranilate. Rather, it is known that anthranilate can be derived from tryptophan under the tandem action of a tryptophan 2,3-dioxygenase, kynureninase, and deformylase, and an operon containing these three genes is widespread in *Streptomyces*, including those that do not produce antimycins.[25] An additional copy of a tryptophan 2,3-dioxygenase encoded by *antN* in the antimycin biosynthetic gene cluster might therefore increase the flux in generating anthranilate from tryptophan, and similar strategies to increase substrate availability have been observed in biosynthesis of other natural products.[26,27]

Subsequently, strains lacking either *antHIJKL* (JL3) or *antO* (JL4) were constructed. LC−HRMS analysis of the culture extracts showed that the production of **2** was completely abolished in both of these strains (Figure 3), confirming the essential roles of AntHIJKL and AntO in the synthesis of the 3-formamidosalicylate moiety. Careful analysis of the product profile of JL3 and JL4 provided additional insight into the reaction timing of these two enzymes. Analysis of the culture extracts of JL4 showed the formation of **1** as well as trace amounts of **3**, an antimycin-type compound proposed to contain a 3-aminosalicylate moiety resulting from the action of AntHIJKL (Figures 2 and S3, Supporting Information). AntHIJKL has high sequence similarity to PaaABCDE, a multicomponent oxygenase that catalyzes the epoxidation of the aromatic ring of phenylacetyl-CoA.[28−30] AntHIJKL presumably catalyzes the formation of the 3-aminosalicyloyl-*S*-AntG from anthraniloyl-*S*-AntG by an epoxidation and a 1,2-shift of the thioester group, which has been suggested by feeding experiments with isotope- and fluorine-labeled precursors and gene disruption experiments in *Streptomyces*.[16,20] We here have reconstituted the oxidation activity of AntHIJKL in *E. coli* for the first time. Analysis of the culture extracts of JL3 then showed the formation of **1** as the major product without the presence of any deoxy-isoantimycin analogues, suggesting that oxygenation by AntHIJKL likely precedes the *N*-formylation by AntO (Figure 2). It is further proposed that *N*-formylation of the 3-aminosalicyloyl moiety occurs on the assembly line, as no *N*-formylated antimycin-type compounds were detected upon the feeding of **1** and **3** to *E. coli* cultures overexpressing *antO* or upon the incubation of **1** and **3** with the cell lysate of these cultures (Figures S7−S9, Supporting Information).

To further probe the function of AntO in antimycin biosynthesis, we carried out a gene disruption and complementation experiment in the antimycin-producing organism, *S. albus* S4. The *antO* gene was replaced with an antibiotic resistance marker through double crossover, which completely abolished the production of antimycins that could be identified from the cultures of wild-type strain (Figure 6). Antimycin

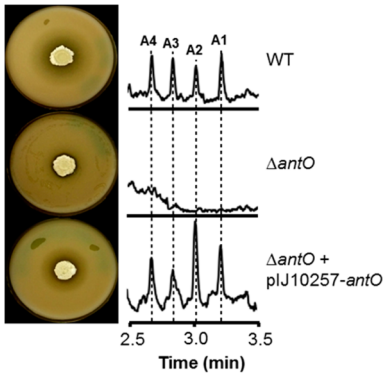

Figure 6. Bioactivity of *S. albus* S4 strains against *Candida albicans* (left) and the corresponding total ion chromatograms illustrating that AntO is required for the production of antimycin A1−A4 (right).

production resumed when *antO* was reintroduced into the Δ*antO* mutant on a plasmid (Figure 6). The results of this metabolite analysis are in good alignment with those of the bioassays in which the Δ*antO* mutant displayed a marked decrease in the ability to inhibit the growth of *Candida albicans* compared to the wild-type strain, and complementation of this mutant with the *antO* gene restored bioactivity against *C. albicans* to the wild-type level (Figure 6). Careful analysis of the Δ*antO* mutant cultures by LC−HRMS also revealed the production of trace amounts of putative deformylated antimycins (Figure S10, Supporting Information), which is consistent with the above proposal that the *N*-formylation of the 3-aminosalicylate moiety occurs on the assembly line, likely in the early stage as shown in Figure 2, and the disruption of the *N*-formylation significantly impairs the NRPS-PKS assembly line. Because AntO also shows homology to the deformylase involved in the conversion of tryptophan to anthranilate, this possible role of AntO was further assessed by feeding anthranilic acid to the cultures of the Δ*antO* mutant. However, the titer of the putative deformylated antimycins did not increase upon the exogenous addition of anthranilic acid (Figure S10, Supporting Information), eliminating the possibility that the low product titer is due to a limited supply of anthranilate precursors being generated through the additional function of AntO in antimycin biosynthesis. These results unequivocally confirmed the role of AntO as a formyltransferase that installs a formyl group on the 3-aminosalicylate moiety.

In summary, we have established a versatile system for the biosynthesis of antimycins in *E. coli*. The heterologous expression and mutagenesis studies reported here have led to a working hypothesis for the biosynthetic pathway of the 3-formamidosalicylate pharmacophore conserved in all antimycin-type depsipeptides. In particular, the substrate, anthranilic acid, is first activated by AntF and loaded onto AntG. The resulting anthraniloyl-*S*-AntG is then subjected to oxidation and rearrangement reactions catalyzed by AntHIJKL and a formylation reaction promoted by AntO to generate the 3-formamidosalicylate starter unit for the hybrid NRPS-PKS assembly line (Figure 2). To our surprise, the *E. coli* production

titer of **2**, which contains the natural starter unit, was not significantly increased compared to that of **1**, which is generated from a disrupted modular assembly line with an unnatural starter unit (Figure 4A). Since the overexpression of *antO* in *E. coli* yielded more than 15 mg/L of soluble protein, the low titer of **2** in this heterologous host is possibly due to the low activity of the multicomponent oxygenase, AntHIJKL, and the reconstitution of oxygenase activity *in vitro* using purified proteins from *E. coli* has not yet been successful (Figure S7, Supporting Information). Screening additional genes homologous to *antHIJKL* from the dozens of gene clusters identified for antimycin-type depsipeptides may yield new enzyme candidates with improved efficiency in *E. coli* and lead to much higher titers of antimycins.[16,23] Nonetheless, our work not only shows additional promise in using *E. coli* as a heterologous host for the production of natural products synthesized through NRPS/PKS enzymes, but it also paves the way for the rapid generation of new antimycin analogues through combinatorial biosynthesis.

METHODS

Bacterial Strains, Plasmids, and Growth Conditions. Bacterial strains and plasmids used in this study are described in Tables S1 and S2 (Supporting Information), respectively. Lysogeny broth (LB) or agar[31] was used for the propagation of *E. coli* strains at 37 °C unless specified otherwise. Mannitol-soya flour media (SFM)[32] was used to maintain *Streptomyces* strains. Growth media was supplemented with antibiotics as required at the following concentrations unless otherwise indicated: apramycin (50 μg/mL), carbenicillin (100 μg/mL), hygromycin B (50 μg/mL), kanamycin (50 μg/mL), nalidixic acid (50 μg/mL), and spectinomycin (50 μg/mL).

Construction of Plasmids Used for Heterologous Expression of Antimycins. Oligonucleotide primers used in this study are listed in Table S3 (Supporting Information). *S. albus* J1074 genomic DNA was used to PCR amplify *antC, D, GF, HIJKL*, and *M/MNO/MO*, and *S. ambofaciens* genomic DNA was used to PCR amplify *antE*. The *antC* gene was digested from pET30-*antC*[16] using NdeI/XhoI (Thermo Scientific) and ligated into MCS1 of pETDuet-1 (Novagen) using Quick T4 DNA ligase (New England Biolabs). The resulting plasmid was then digested with *Pst*I/*Hin*dIII for the ligation of *antE* (PCR product generated using antEam-Duet-F/R primers) into MCS2 to yield the final pETDuet-*antC-antE* construct. The *antD* gene (PCR product generated using antD-Duet-F/R primers) was cloned into MCS1 of pCDFDuet-1 using the *Eco*RI/*Hin*dIII restriction sites, and *antGF* (PCR product generated using AntG_NdeI/antGF-Duet-R primers) was subsequently cloned into MCS2 of the resulting plasmid using the NdeI/XhoI restriction sites to yield the final pCDFDuet-*antD-antGF* construct. The *antM*, *MNO*, or *MO* genes (PCR products generated using antM-Duet-F/R, antM-Duet-F/antNO-Duet-R, or antM-Duet-F/antMO-F2 + antMO-F2/antNO-Duet-R primers, respectively) were cloned into MCS2 of pCOLADuet-1 using the NdeI/XhoI restriction sites to form pCOLADuet-*antM/MNO/MO*. These plasmids were then further digested with *Pst*I/*Hin*dIII for the introduction of *antHIJKL* (PCR product generated using antHIJKL-Duet-F/R primers) into MCS1 to form pCOLADuet-*antHIJKL-antM/MNO/MO*. Plasmids were isolated using a QIAprep Spin Miniprep Kit (Qiagen) and confirmed by DNA sequencing (UC Berkeley DNA Sequencing Facility).

Biosynthesis of Antimycins in *E. coli*. The plasmids, pETDuet-*antC-antE*, pCDFDuet-*antD-antGF*, and pCOLA-Duet-(*antHIJKL*)-*antM*(*N*)(*O*), were electroporated into *E. coli* BAP1, and transformants were selected on LB agar plates supplemented with the appropriate antibiotics. Single colonies were inoculated into 3 mL of LB with antibiotics and grown overnight at 37 °C as a seed culture, of which 0.25 mL was used to inoculate 25 mL of fresh LB medium with antibiotics. The cultures were then grown at 37 °C to $OD_{600} \approx 0.4-0.6$ before induction with 0.12 mM IPTG and the addition of anthranilic acid and octanoic acid at a final concentration of 1 mM. After induction, the temperature was dropped to 20 °C, and compound production was allowed to proceed for approximately 2 days.

The F1 fed-batch fermentation was conducted with a DASGIP Parallel Bioreactor System, and methods for the fermentation and medium composition were adopted from methods described previously by Pfeifer et al. and Zhang et al.[19,33] A starter culture was grown overnight at 37 °C in 3 mL of LB medium supplemented with the appropriate antibiotics, and 1 mL of this culture was used to inoculate 50 mL of LB medium with antibiotics. The culture was then grown overnight at 30 °C before being centrifuged and resuspended in 20 mL of F1 medium, of which 8 mL was used to inoculate a 1 L vessel containing 800 mL of F1 medium supplemented with 150 μg/mL carbenicillin, 100 μg/mL spectinomycin, and 75 μg/mL kanamycin. The fermentation was started at 37 °C, and the pH was maintained at 7.0 throughout the experiment with concentrated HCl and half-concentrated NH_4OH. Aeration was controlled at 0.2 L/min, and agitation was maintained at 600 rpm. When the OD_{600} reached ∼4−5, the temperature of the fermentation was reduced to 20 °C, followed by the addition of 0.5 mM IPTG, 1 mM anthranilic acid, 1 mM octanoic acid, and 1 mM threonine. At the same time, 0.1 mL/min of feed medium began to be delivered to the fermenter. At each time point, a 1 mL aliquot was removed from the culture for compound extraction and analysis.

HPLC and LC−MS Analysis of Antimycin Production. *E. coli* culture samples were pelleted by centrifugation (4000 × *g* for 10 min), and the supernatant was extracted with two volumes of ethyl acetate. The solvent was removed by rotary evaporation (or centrifugal evaporation for 1 mL samples), and the residue was redissolved in methanol (400 μL for 25 mL cultures and 100 μL for 1 mL samples) for LC−HRMS, HRMS/MS, and HPLC analysis (20 μL injection). LC−HRMS analysis was performed on an Agilent Technologies 6520 Accurate-Mass Q-TOF LC-MS instrument with an Agilent Eclipse Plus C18 column (4.6 × 100 mm). A linear gradient of 25−95% CH_3CN (v/v) over 20 min followed by an additional 10 min of 95% CH_3CN (v/v) in H_2O with 0.1% formic acid (v/v) at a flow rate of 0.5 mL/min was used. A collision energy of 20 V was used for all HRMS/MS experiments. Antimycins were quantified by generating a standard curve with antimycin standards and using the Agilent MassHunter Qualitative Analysis software to determine the extracted ion chromatogram peak areas. HPLC analysis was performed on an Agilent Technologies 1260 LC system with DAD using the same column and program as described above but with trifluoroacetic acid in place of formic acid.

Enzymatic Synthesis of Antimycin A2. AntB was expressed and purified as described previously.[24] Compound **2** was purified by reverse-phase high-performance liquid chromatography (RP-HPLC, Agilent 1260 HPLC with DAD)

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using an Agilent Eclipse Plus C18 column (4.6 × 100 mm) with a linear gradient of 25−95% CH_3CN (v/v) in H_2O over 20 min at a flow rate of 0.5 mL/min. Fractions containing antimycins were collected manually and concentrated under vacuum. Assays were then performed in 100 μL of 50 mM HEPES (pH 8.0) containing **2**, 2 mM isobutyryl CoA, and 20 μM AntB. After a 2 h incubation period at room temperature, the reactions were extracted with two volumes of ethyl acetate, and the organic layer was dried under vacuum. The residue was then redissolved in methanol (50 μL) for HPLC, LC−HRMS, and HRMS/MS analysis as described above. The antimycin A2 standard was obtained from Sigma and used for comparison with the product of the enzymatic assay.

Formylation Studies with *E. coli* Overexpressing AntO. The *antO* gene was PCR amplified from *S. albus* J1074 genomic DNA using primers AntO_PET30F/R and cloned into pET-30 XA/LIC (Novagen) following standard protocols. The resulting pET30-*antO* plasmid was transformed into BL21 Gold, and single colonies were inoculated into 3 mL of LB + kanamycin and grown overnight at 37 °C as a seed culture, of which 0.25 mL was used to inoculate 25 mL of fresh LB + kanamycin. The cultures were then grown at 37 °C to $OD_{600} \approx 0.4-0.6$ and induced with 0.12 mM IPTG. AntO was expressed at 20 °C for 2 h after which compounds **1** and **3**, which were partially purified by running the culture extracts of 250 mL of JL5 through Sep-Pak C18 Plus cartridges (Waters) and eluting with 95% CH_3CN, were added to the cultures. Expression of AntO at 20 °C was resumed for approximately 2 days before extraction and product analysis as described above. For assays with *E. coli* lysate, AntO was expressed in BL21 Gold for 16−20 h after which the cells from 350 mL of culture were pelleted by centrifugation (6000 × *g* for 15 min), resuspended in 20 mL of lysis buffer (20 mM HEPES, pH 8, 0.5 M NaCl), and lysed by homogenization on ice. Cellular debris was removed by ultracentrifugation (15 000 × *g* for 1 h), and the soluble fraction was incubated with **1** and **3** purified from an equivalent amount of culture. After overnight incubation at room temperature, the lysate was subjected to extraction and product analysis as described above.

Construction of *S. albus* S4 Strains. The *antO* null mutant strain (Δ*antO*) was constructed using λ-RED based PCR-targeting mutagenesis.[34] A disruption cassette consisting of the RK2 conjugal origin of transfer and the apramycin resistance gene *aac*(3)*IV* from pIJ773,[34] was generated by PCR using BioTaq Polymerase (Bioline) and primers RFS202 and RFS203, which contained 39 bp of homology that included the start and stop codons of *antO*, respectively. The resulting PCR product was gel purified and electroporated into *E. coli* BW25113/pIJ790 harboring cosmid 213, which encodes the entire antimycin gene cluster.[35] Apramycin-resistant transformants were screened for a polymorphic NotI restriction fragment. The mutated cosmid was electroporated into *E. coli* ET12567/pUZ8002 and subsequently transferred to *S. albus* S4 by intergenera conjugation as previously described.[32] Transconjugants were selected for resistance to apramycin and sensitivity to kanamycin. The integrity of the Δ*antO* mutant strain was verified by PCR using primers RFS254 and RFS255.

The plasmid pIJ10257-*antO*, which was used to complement the Δ*antO* mutant, was constructed as follows. The AntO coding sequence was amplified using Phusion polymerase (New England Biolabs) and primers RFS420 and RFS421, which contained recognition sequences for the NdeI and *Hin*dIII restriction enzymes, respectively. The resulting PCR product was gel purified, digested with NdeI and *Hin*dIII (New England Biolabs), and ligated to pIJ10257[36] cut with the same enzymes using T4 DNA ligase (New England Biolabs). The resulting plasmid, pIJ10257-*antO*, was introduced into *S. albus* S4 Δ*antO* by intergeneric conjugation, and transconjugants were selected for by resistance to hygromycin B.

Analysis of *S. albus* S4 Strains. Fifty milliliters of liquid SFM contained in 250 mL shake flasks were inoculated with 70 μL of spores and mycelia fragments from wild-type *S. albus*, and mutant strains were cultivated at 30 °C as previously described.[21] After 5 days of incubation, cultures were clarified by centrifugation (4000 × *g* for 10 min). The supernatant of two independently grown cultures was combined and extracted with ∼3 g of XAD16 resin (Sigma) for 3 h at room temperature with occasional agitation. After extraction, the resin was collected by gravity sedimentation and washed 3 × 20 min with water (30 mL). *S. albus* S4 metabolites were eluted from XAD16 resin with 5 mL of methanol. Immediately prior to LC−MS analysis, samples were centrifuged at 16 000 × *g* for 10 min to remove insoluble material. Only the supernatant (2 μL) was injected into a Bruker MaXis Impact TOF mass spectrometer equipped with a Dionex Ultimate 3000 HPLC. Calibration was achieved with a postrun injection of sodium formate. Mass spectra were acquired using electrospray ionization running in positive ion mode. Compounds were separated on a Waters Acquity UPLC Peptide CSH C18 column (1.7 μm, 2.1 × 100 mm) using the following gradient: (solvent A, 0.1% formic acid in water (v/v); solvent B, 0.1% formic acid in CH_3CN (v/v); flow rate, 0.7 mL/min) 0.0−1.5 min, 1% B; 1.5−3.5 min, 75−95% B; 3.5−4.5 min, 95% B; 4.5−5.0 min, 95−1% B; 5.0−5.5 min, 1% B. For anthranilate feeding studies, cultures were inoculated and cultivated as above but were supplemented with 50 μL of 1 M ethanolic anthranilate (final concentration 1 mM). The ability of *S. albus* strains to inhibit the growth of *Candida albicans* was performed exactly as described previously.[21]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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