Acyltransferase Domain Substitutions in Erythromycin Polyketide Synthase Yield Novel Erythromycin Derivatives

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The methylmalonyl coenzyme A (methylmalonyl-CoA)-specific acyltransferase (AT) domains of modules 1 and 2 of the 6-deoxyerythronolide B synthase (DEBS) of Saccharopolyspora erythraea ER720 were replaced with three heterologous AT domains that are believed, based on sequence comparisons, to be specific for malonyl-CoA. The three substituted AT domains were “Hyg” AT2 from module 2 of a type I polyketide synthase (PKS)-like gene cluster isolated from the rapamycin producer Streptomyces hygroscopicus ATCC 29253, “Ven” AT isolated from a PKS-like gene cluster of the pikromycin producer Streptomyces venezuelae ATCC 15439, and RAPS AT14 from module 14 of the rapamycin PKS gene cluster of S. hygroscopicus ATCC 29253. These changes led to the production of novel erythromycin derivatives by the engineered strains of S. erythraea ER720. Specifically, 12-desmethyl-12-deoxyerythromycin A, which lacks the methyl group at C-12 of the macrolactone ring, was produced by the strains in which the resident AT1 domain was replaced, and 10-desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A, both of which lack the methyl group at C-10 of the macrolactone ring, were produced by the recombinant strains in which the resident AT2 domain was replaced. All of the novel erythromycin derivatives exhibited antibiotic activity against Staphylococcus aureus. The production of the erythromycin derivatives through AT replacements confirms the computer predicted substrate specificities of “Hyg” AT2 and “Ven” AT and the substrate specificity of RAPS AT14 deduced from the structure of rapamycin. Moreover, these experiments demonstrate that at least some AT domains of the complete 6-deoxyerythronolide B synthase of S. erythraea can be replaced by functionally related domains from different organisms to make novel, bioactive compounds.

Erythromycin A is a clinically useful, broad-spectrum macrolide antibiotic produced by the gram-positive bacterium Saccharopolyspora erythraea. Erythromycin belongs to a class of natural products called complex polyketides which includes many important compounds, such as the antihelminthic agent avermectin and the immunosuppressants FK506 and rapamycin. The central feature of these compounds is a polyketide-derived macrocyclic lactone ring that is synthesized by an ordered condensation of acyl thioesters. These rings are typically built from carbon chains of specific lengths, and they exhibit different degrees of reduction and substitution around the ring (8, 29, 32). The production of polyketides has long been recognized to resemble fatty acid biosynthesis (25, 43), but with important differences (15). Unlike a fatty acid synthase, which usually starts fatty acid synthesis with acetyl coenzyme A (acetyl-CoA) and extends the chain by two carbons without side chains, using malonyl-CoA as the extender unit, a complex polyketide synthase (PKS) is programmed to make distinct choices at each step of carbon chain assembly. These include choice of the starter from a number of possibilities, such as acetyl-CoA (oleandomycin), propionyl-CoA (erythromycin), and isobutyryl-CoA (avermectin), choice of either unbranched or branched chain extender (e.g., malonyl-CoA [rapamycin], methylmalonyl-CoA [erythromycin], and ethylmalonyl CoA [tylosin]), and determination of the degree of reduction of the β-keto group that is generated after each condensation (ranging from no reduction through ketoreduction only, ketoreduction and dehydration, or ketoreduction, dehydration, and enoyl reduction).

Polyketide programming is built into the structure of the PKS, which is determined at the genetic level. The genes (and enzymatic domains) of a complex PKS are organized in modules (8, 29, 32, 37). Each module encodes all of the functions necessary for a single, specific condensation event and the attendant processing of the newly formed β-keto group. Each module carries a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain. In addition, β-ketoreductase (KR), dehydratase, and enoyl reductase (ER) domains may also be present. The KS, AT, and ACP domains are responsible for chain extension. The AT domain is believed to determine which extender is incorporated at each step of polyketide chain growth (10, 12, 18), while the KS domain is responsible for catalyzing the actual condensation reaction. The ACP domains tether the growing polyketide chain to the PKS between condensations and also accept extender units from the AT domains in preparation for a condensation.

The macrolactone ring of erythromycin is synthesized by the erythromycin PKS, 6-deoxyerythronolide B synthase (DEBS) (Fig. 1). DEBS contains (i) six modules to program the six successive condensations between the starter propionyl-CoA and (ii) the six methylmalonyl-CoA extender units to build the 14-membered, 6-deoxyerythronolide B (6-dEB) ring (Fig. 1). The precise order of the elongation steps is colinear with the genetic order of the six modules: module 1 determines the first condensation, module 2 determines the second, module 3 determines the third, and so on until the sixth condensation step has occurred (8). In the case of 6-dEB, the extender unit for
each condensation is methylmalonyl-CoA, which gives rise to the characteristic pattern of alternating methyl groups around the 6-dEB ring. Although only the (2S)-stereoisomer of methylmalonyl-CoA is employed in the synthesis of erythromycin (23), the opposite (2R) stereochemistry is found in three of the six methyl side chains. Whether the epimerizations take place during or following the condensation reactions remains to be determined.

Novel derivatives of erythromycin have been most frequently generated by chemical modifications to the antibiotic. However, in recent years derivatives have been made by genetically altered erythromycin-producing strains. 2-Norerythromycin A and its congeners 2-norerythromycin B, C, and D were the first examples of use of recombinant DNA techniques to produce novel structures of erythromycin (24). Characterization of the PKS genes responsible for the biosynthesis of erythromycin in S. erythraea led to the generation of derivatives through directed manipulation of the eryA genes (10). The production of these novel compounds illustrated that recombinant DNA technology can be effectively used to generate erythromycin derivatives that are difficult or impossible to make chemically.

With the recent evidence that AT domains determine the extender used for each condensation step during polyketide chain growth (26), a series of novel erythromycins carrying side chain alterations were generated by genetically engineering DEBS with heterologous malonyl-CoA-incorporating AT domains. Here, we report that biosynthesis of the carbon backbone of the erythromycin macrolactone ring can be reprogrammed through AT replacements in DEBS1, giving rise to new erythromycin derivatives that lack methyl groups at either C-10 or C-12 of the macrolactone ring.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Actinomycete strains and plasmids used in this study are listed in Table 1. Plasmid-containing Escherichia coli strains were grown in Luria broth (30) supplemented with ampicillin (150 µg/ml). For growth of S. erythraea strains in liquid cultures, either SCGP (44) or SCM (20 g of soytone, 15 g of soluble starch, 10.5 g of morpholinepropanesulfonic acid [MOPS], 1.5 g of yeast extract, and 0.1 g of CaCl₂ per liter of distilled H₂O) medium was used. For the growth of S. erythraea strains on plates, R3M medium was used. One liter of R3M medium contains 4 g of yeast extract, 4 g of Casamino Acids, 4 g of Bacto Tryptone, 0.25 g of K₂SO₄, 0.6 g of Tris base, 3.2 g of Tris-HCl, 2.5 ml of 1 M NaOH, 103 g of sucrose, 22 g of agar, and 940 ml of
**Plasmid**

- pNJ1: Actinomycete-*E. coli* cosmid containing rep-pVI
- pAL58: pNJ1 cosmid containing part of a “Hyg” PKS gene cluster isolated from *S. hygroscopicus* ATCC 29253
- pWHM3: pNJ1 shuttle vector containing rep-pUL702; replicates poorly in *S. erythraea*.

**DNA manipulations**

- Standard molecular biology techniques were performed as described previously (30).
- For DNA sequencing, denatured plasmid DNA or single-stranded DNAs prepared from subclones of pUC18/19 or M13mp18/19 (Bethesda Research Laboratories) were used as the templates. Nucleotide sequences were determined by the dideoxyribonucleotide chain termination method (31), using either the fmol DNA cycle sequencing system (Promega Corp., Madison, Wis.) or Sequenase version 2.0 with 7-deaza-dGTP (U.S. Biochemical, Cleveland, Ohio) according to the manufacturer's instructions. The sequences were analyzed by using the Genetics Computer Group (Madison, Wis.) sequence analysis programs (6). Database searches for sequence homology to the deduced amino acid sequences were performed by using BLAST (1). Amino acid sequences were also performed by using PILEUP and DENDROGRAPH programs (4).

**Cloning the DNA encoding “Ven” AT**

A genomic library of *Streptomyces venezuelae* ATCC 15439 was constructed in plasmid pNJ1 (38) and screened by PCR cloning of the heterologous AT domains and 72°C for 2 min, with a 5-min extension at 72°C for the last cycle. For the PCR-amplified flanking regions of DEBS AT1, the upstream fragment (corresponding to the segment nucleotides (nt) 2781 to 3825; GenBank accession no. M63676) was subcloned into the EcoRI/BamHI sites of pUC18 to give pUC18/“Hyg” AT2 and pUC18/“Ven” AT or into the EcoRI/HindIII sites of pUC19 to give pUC19/RapAT14. For the PCR-amplified flanking regions of DEBS AT2, the upstream fragment (corresponding to the segment nt 4866 to 5912) was subcloned into the BamHI/HindIII sites of pUC19 to give pUC19/EryAT1/downstream (see Fig. 3B). For the PCR-amplified flanking regions of DEBS AT2, the upstream fragment (corresponding to the segment nt 7090 to 8255) was subcloned into the HindIII/PstI sites of pUC19 to give pUC19/EryAT2/upstream: the downstream fragment (corresponding to the segment nt 9282 to 10368) was subcloned into the PstI/EcoRI sites of pUC19 to give pUC19/EryAT2/downstream (see Fig. 3B). The fidelity of these PCR-amplified fragments was confirmed by sequencing both DNA strands of the subclones.

**Genetic manipulation of *S. erythraea* ER720**

Preparation of protoplasts of *S. erythraea* ER720, transformation of the protoplasts with integrative pWHM3 derivatives (Table 1), and resolution of the integrants were carried out according to previously described procedures (41, 44). Protoplasts were independently transformed with the five pWHM3-derived AT replacement plasmids. To select stable transformants (integrants) via homologous recombination, colonies arising from the transformation plates were restreaked onto R3M plates to ensure Thio r and to isolate chromosomal DNA for confirmation of the integration event by Southern analysis. The con- firming integrants were next grown in SGGP without thiostrepton for 4 days and then plated onto nonselective R3M plates for sporulation. Spore suspensions were plated on R3M plates to obtain individual colonies, which were then screened for sensitivity to thiostrepton, indicating the loss of the plasmid sequence from the chromosome through a second homologous recombination event. Southern analysis of the thiostrepton-sensitive colonies confirmed the desired genotypes of the resolvants.

**Characterization of erythromycin derivatives produced by recombinant *S. erythraea* strains**

For thin-layer chromatography (TLC) analysis, cells were grown in shake flasks in either SGGP or SCM for 4 to 5 days at 30°C and then removed by centrifugation. The resulting supernatant was adjusted to pH 9.0 by the addition of NaOH and extracted twice with an equal volume of ethyl acetate. The organic phase, which contained the desired macrocyclics, was dried in

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**TABLE 1. Actinomycete strains and plasmids used**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. erythraea</em> ER720</td>
<td>Wild type, erythromycin producer</td>
<td>7</td>
</tr>
<tr>
<td><em>S. venezuelae</em> ATCC 15439</td>
<td>Wild type, pikromycin producer</td>
<td>ATCC*</td>
</tr>
<tr>
<td><em>S. hygroscopicus</em> ATCC 29253</td>
<td>Wild type, rapamycin producer</td>
<td>ATCC</td>
</tr>
<tr>
<td><em>S. erythraea</em> EryAT1/*Hyg” AT2</td>
<td><em>S. erythraea</em> ER720 with DEBS AT1 replaced by “Hyg” AT2</td>
<td>This study</td>
</tr>
<tr>
<td>EryAT2/*Hyg” AT2</td>
<td><em>S. erythraea</em> ER720 with DEBS AT2 replaced by “Hyg” AT2</td>
<td>This study</td>
</tr>
<tr>
<td>EryAT1/*Ven” AT</td>
<td><em>S. erythraea</em> ER720 with DEBS AT1 replaced by “Ven” AT</td>
<td>This study</td>
</tr>
<tr>
<td>EryAT1/RapAT14</td>
<td><em>S. erythraea</em> ER720 with DEBS AT1 replaced by RAPS AT14</td>
<td>This study</td>
</tr>
<tr>
<td>EryAT2/RapAT14</td>
<td><em>S. erythraea</em> ER720 with DEBS AT2 replaced by RAPS AT14</td>
<td>This study</td>
</tr>
</tbody>
</table>

* ATCC, American Type Culture Collection, Rockville, Md.

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H2O. After autoclaving at 121°C for 20 min, the following solutions were added: 5,000×-concentrated tris elements solution, 0.2 ml (14); 50% glucose solution, 20 ml; 2.5× MgCl2 solution, 20 ml; 0.5% KH2PO4 solution, 5 ml; and 2.5 M CaCl2 solution, 20 ml. For selection of thiostrepton-resistant (Thio r) *S. erythraea* strains, 25 μg of thiostrepton/ml was used for growth on plates and 10 μg/ml was used in liquid culture.

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**Characterization of erythromycin derivatives produced by recombinant *S. erythraea* strains**

For thin-layer chromatography (TLC) analysis, cells were grown in shake flasks in either SGGP or SCM for 4 to 5 days at 30°C and then removed by centrifugation. The resulting supernatant was adjusted to pH 9.0 by the addition of NaOH and extracted twice with an equal volume of ethyl acetate. The organic phase, which contained the desired macrocyclics, was dried in...
a SpeedVac and redissolved in ethyl acetate to give an appropriate concentration for spotting onto a TLC plate (Merck 60F-254 Silica Gel). Migration was allowed for approximately 1.5 h, using isopropyl ether-methanol-NH4OH (75:35:2), and the erythromycin derivatives were visualized with an anisaldehyde-sulfuric acid-ethanol (1:1:9) spray.

For TLC-bioautography, the TLC plate was developed as described above, air dried, and placed in a sterile bioassay dish (245 by 245 by 25 mm). The plate was then covered with 150 ml of antibiotic medium 11 (Difco) containing 0.1 ml of an overnight culture of *Staphylococcus aureus* as an indicator strain and incubated overnight at 37°C to visualize zones of inhibition.

For mass spectrometry (MS), ethyl acetate extracts were separated by either high-pressure liquid chromatography (HPLC) or TLC. After TLC separation, the region of interest was scraped from the plate and extracted once with ethyl acetate-methanol (1:1), followed by two extractions with ethyl acetate. The combined organic phases were then dried in a SpeedVac and submitted for MS analysis. For HPLC separation, the sample was run through a Prodigy ODS (2) column (5 µm, 50 by 2 mm) on a Hewlett-Packard 1050 liquid chromatograph, using a gradient elution of 5 mM ammonium acetate and methanol at the flow rate of 0.3 ml/min. MS analysis was routinely performed with a Finnigan-MAT 7000 mass spectrometer equipped with an atmospheric pressure chemical ionization source. Electrospray MS was performed with a Finnigan-MAT 752-7000 mass spectrometer equipped with a Finnigan atmospheric pressure ionization source.

To isolate 10- and 12-desmethylerythromycins for nuclear magnetic resonance (NMR) structural studies, 30 liters of SCM was prepared in 42-liter LH Fermentation Series 2000 stainless steel stirred vessels and sterilized at 121°C and 15 lb/m² for 1 h. Antifoam (XFO-371; Ivanhoe Chemical Co., Mundelein, Ill.) was added initially at 0.01% and then was available on demand. The fermentors were inoculated with 1.5 liters of second-passage seed growth of either the EryAT2/“Hyg” AT2 or EryAT1/“Hyg” AT2 strain of *S. erythraea*. The temperature was controlled at 32°C. The agitation rate was 260 rpm, and the air flow was 1.3 vol/vol/min. The head pressure was maintained at 6 lb/m², and the pH was controlled at 7.3 with 5 M propionic acid. The fermentations were terminated between 100 and 120 h. The resulting fermentation broths were then adjusted to pH 9 with NH₄OH and extracted twice with equal volumes of CH₂Cl₂.

For the 10-desmethylerythromycin sample, the concentrated crude material was twice extracted with an equal volume of 0.05 M aqueous potassium phosphate (pH 4.25). The pooled aqueous phase was then adjusted to pH 9 with NH₄OH and reextracted twice with an equal volume of ethyl acetate. After concentration of the organic phase, the aqueous back extraction-organic reextraction sequence was repeated. The oily residue remaining after concentration of the final pooled ethyl acetate extracts was then digested in 5 ml each of the upper and lower phases of a solvent system consisting of *n*-hexane, ethyl acetate, and 0.02 M aqueous potassium phosphate (pH 6.6), 6:4:5 (vol/vol/vol), and was chromatographed on a custom droplet countercurrent chromatography instrument (100 vertical columns; 0.4-cm diameter by 24-cm length) (16), using the lower phase as the mobile phase. Fractions were analyzed according to bioactivity against *S. aureus* and by 1H NMR. Bioactive fractions were pooled and subjected to a series of five countercurrent chromatographic separations in an Ito multilayer Planet Coil centrifuge. The solvent systems used at each step were as follows: (i) trichloroethylene–chloroform–methanol–0.02 M aqueous potassium phosphate (pH 6.4), 3:1:3:3 (vol/vol/vol/vol), with the lower phase mobile; (ii) *n*-hexane–ethanol–0.02 M aqueous potassium phosphate (pH 7), 1:1:1 (vol/vol/vol), with the upper phase mobile; (iii) *n*-hexane–ethyl acetate–0.02 M aqueous potassium phosphate (pH 8.1), 1:1:1 (vol/vol/vol), with the upper phase mobile; (iv) *n*-butanol–acetone–0.02 M aqueous potassium phosphate (pH 6.5), 8:1:10 (vol/vol/vol), with the upper phase mobile; and (v) *n*-hexane–ethyl acetate–0.02 M aqueous potassium phosphate (pH 7), 1:1:1 (vol/vol/vol), with the upper phase mobile.

For the 12-desmethylerythromycin sample, the concentrated crude material was extracted twice with an equal volume of 0.05 M aqueous potassium phosphate (pH 4.25). The pooled aqueous phase was then adjusted to pH 9 with NH₄OH and reextracted twice as described above. The process was repeated, and the oily

<table>
<thead>
<tr>
<th>No.</th>
<th>Substitute AT domains</th>
<th>Oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>“Hyg” AT2 (N-terminal)</td>
<td>5'-CCGGAGATTCGTACGGTGGCGGTATGTTCA-3' EcoRI AvrII</td>
</tr>
<tr>
<td>2</td>
<td>“Hyg” AT2 (C-terminal)</td>
<td>5'-GGCCGATCCATGCATACGTCGCGAGGAGGTAAC-3' BamHI NsiI</td>
</tr>
<tr>
<td>3</td>
<td>“Ver” AT (N-terminal)</td>
<td>5'-TTTATTGTCAATGCTAGCTCGGGAAGGTCAGCAACCGG-3' EcoRI AvrII</td>
</tr>
<tr>
<td>4</td>
<td>“Ver” AT (C-terminal)</td>
<td>5'-TTTTTGATCATGCATACGTCGCGAGGAGGTAAC-3' BamHI NsiI</td>
</tr>
<tr>
<td>5</td>
<td>RAPS AT14 (N-terminal)</td>
<td>5'-TTTTGACATATTGCGTCGTCGCGAGGAGGTAAC-3' EcoRI AvrII</td>
</tr>
<tr>
<td>6</td>
<td>RAPS AT14 (C-terminal)</td>
<td>5'-TTTTGACATATTGCGTCGTCGCGAGGAGGTAAC-3' HindIII NsiI</td>
</tr>
<tr>
<td>7</td>
<td>DEBS AT1 flanking regions</td>
<td>5'-GCTGATATCCGCTACGGTGTCAGCAACCGG-3' EcoRI</td>
</tr>
<tr>
<td>8</td>
<td>Uptream (C-terminal)</td>
<td>5'-GACGGATCCGCGCTACGGTGTCAGCAACCGG-3' BamHI AvrII</td>
</tr>
<tr>
<td>9</td>
<td>Downstream (N-terminal)</td>
<td>5'-TTGGATATCCATGCTACGGTGTCAGCAACCGG-3' BamHI NsiI</td>
</tr>
<tr>
<td>10</td>
<td>Downstream (C-terminal)</td>
<td>5'-GGAAGGTCCGCGACCTGCCCAGCC-3' HindIII</td>
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<td>DEBS AT2 flanking regions</td>
<td>5'-TTTATTGACATATTGCGTCGTCGCGAGGAGGTAAC-3' HindIII</td>
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<td>Upstream (C-terminal)</td>
<td>5'-TTTTTTGTCAATGCTAGCTCGGGAAGGTCAGCAACCGG-3' PstI AvrII</td>
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<tr>
<td>13</td>
<td>Downstream (C-terminal)</td>
<td>5'-TTTTTTGTCAATGCTAGCTCGGGAAGGTCAGCAACCGG-3' PstI NsiI</td>
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<tr>
<td>14</td>
<td>Downstream (C-terminal)</td>
<td>5'-TTTTTTGTCAATGCTAGCTCGGGAAGGTCAGCAACCGG-3' EcoRI</td>
</tr>
</tbody>
</table>
Residue that remained after concentration of the pooled ethyl acetate extracts was then digested in 2.5 ml each of the upper and lower phases of a solvent system consisting of n-hexane, ethyl acetate, and 0.02 M aqueous potassium phosphate (pH 8), 1:1:1 (vol/vol/vol), and subjected to countercurrent chromatography in an Ito multilayer Planet Coil centrifuge, with the upper phase mobile. A second separation was then performed with a solvent system comprising n-hexane, ethyl acetate, and 0.02 M aqueous potassium phosphate (pH 6.4), 6:4:5 (vol/vol/vol), with the upper phase mobile.

Complete assignment of the 1H and 13C NMR spectra of both 10-desmethyl-erythromycin A, 6,9,12-spiroketal (10-desmethylanhydroerythromycin A) and 12-desmethyl-12-deoxyerythromycin A was possible with the aid of correlated spectroscopy, heteronuclear multiple quantum correlation, heteronuclear multiple bond correlation, and distortionless enhancement by polarization transfer spectroscopy, heteronuclear multiple quantum correlation, heteronuclear multiple bond correlation, and distortionless enhancement by polarization transfer experiments.

**RESULTS**

AT domains used to replace DEBS AT1 and DEBS AT2.

Three heterologous AT domains, “Hyg” AT2, “Ven” AT, and RAPS AT14, each thought to specify the use of malonyl-CoA for polyketide chain extension, in contrast to methylmalonyl-CoA, which is used for condensations in the biosynthesis of 6-deB, were chosen to replace the AT domains of modules 1 (DEBS AT1) and 2 (DEBS AT2) of DEBS1 (10). The “Hyg” AT2 domain was obtained from module 2 of the “Hyg” PKS gene cluster of the rapamycin producer *S. hygroscopicus* ATCC 29253 (28). RAPS AT14 was obtained from module 14 of the *S. hygroscopicus* ATCC 29253 and was proposed previously to encode a malonyl-CoA-specific AT domain based on correlation of the structure of rapamycin with the organization of its PKS (32). The “Ven” AT domain was obtained from an unknown PKS-like gene that is naturally found at this position in the original sequence of *S. venezuelae* (28). Thus, both “Hyg” AT2 and “Ven” AT also seemed likely to be specific for malonyl-CoA, based on their similarity to RAPS AT14 and other RAPS AT domains that were thought to specify the use of malonyl-CoA (Fig. 2).

**Construction of the AT replacement plasmids.** A cassette strategy was developed to facilitate construction of several plasmids, each harboring a different heterologous AT, to replace DEBS AT1 or DEBS AT2. Two unique restriction sites, *Avr*I and *Nsi*I, were introduced into the cassette by using PCR primers (Table 2 and Fig. 3). The boundaries of the erythromycin and heterologous AT domains were defined by amino acid sequence alignment of all known complex PKS AT domains, and these boundaries encompass the entire AT domain regions that were previously considered to be important for AT function (8). The boundaries were also chosen to tolerate the introduction of new restriction sites with a minimum of disruption to the original amino acid sequence of each AT domain. Within these constraints, the entire DEBS AT1 and DEBS AT2 domains could be replaced when *Avr*I and *Nsi*I sites were introduced at the N- and C-terminal boundaries, respectively. Likewise, the heterologous AT domains were also flanked by these restriction sites. As shown in Fig. 3A two residues near the N terminus (boxed) were changed to Pro (P) and Arg (R) by introducing the *Avr*I site. Notably, for the two residues changed at the N terminus, the P is conserved in similar positions in all of the RAPS AT domains (alignment not shown), and the R is already present in three of the five ATs shown in Fig. 3A. At the C terminus, P (boxed) is changed to Ala (A) upon introduction of the *Nsi*I site (Fig. 3A). Alanine is naturally found at this position in the original sequence of the “Ven” AT domain.

Cassettes carrying DNA flanking the resident DEBS AT1 (EryAT1) and DEBS AT2 (EryAT2) domains were constructed in several steps. For construction of the EryAT2 flanking cassette (Fig. 3B), the upstream flank was isolated from pUC19/EryAT1 upstream (Materials and Methods) and inserted into the *EcoRI/BamHI* sites of pUC19/EryAT1 downstream to generate construct pUC19/EryAT1-flanks (map not shown). The *EcoRI/HindIII* fragment that contained both flanking regions in their appropriate orientations was then isolated from pUC19/EryAT1-flanks and subcloned into the same sites of pHWM3 to generate the integrative cassette pHWM3/EryAT1-flank used for module 1 replacement (Fig. 3B). Similar steps were taken to prepare a second integrative cassette, pHWM3/EryAT2-flank, for module 2 (Fig. 3B).

Individual heterologous AT domains were inserted between the two flanking DNA regions of the DEBS AT1 or AT2 domains as shown in Fig. 3B. The PCR-generated DNA fragments carrying “Hyg” AT2, “Ven” AT, and RAPS AT14 were first isolated from the corresponding subclones (pUC18/“Hyg” AT2, pUC18/“Ven” AT, and pUC19/RapAT14, respectively) by digestion with *Avr*I and *Nsi*I and then subcloned in the integrative cassette pHWM3/EryAT1-flank to generate three plasmids, pHWM3/EryAT1/“Hyg” AT2, pHWM3/EryAT1/“Ven” AT, and pHWM3/EryAT1/RapAT14, for AT replacements at module 1 (Fig. 3B). Similar steps were taken to generate pHWM3/EryAT2/“Hyg” AT2 and pHWM3/EryAT2/RapAT14 for AT replacements at module 2 (Fig. 3B).

**Construction of the AT replacement strains of *S. erythraea* ER720.** The AT domains of modules 1 and 2 in DEBS1 of *S. erythraea* ER720 were replaced by using the above-described constructs containing the different heterologous ATs in a two-step procedure that involves homology-based integration of the recombinant plasmids into the chromosome of *S. erythraea* ER720, followed by homology-driven resolution of the inte-
grants to obtain the desired replacements. After protoplast
transformations with the AT replacement plasmids (3 mg plasmid DNA typically used for each transformation), one to
five Thio' integrative transformants were typically obtained.
Integration of the plasmid into the chromosome was verified
by Southern hybridization using either pWHM3 vector se-
quence or the heterologous AT sequence as a probe (data not
shown). One integrant from each transformation was then
used for resolution of the integration event. Two to six thio-
strepton-sensitive resolvants were commonly obtained from

FIG. 3. Scheme for the construction of the AT replacement plasmids. (A) Alignment of the amino acid sequences of the three heterologous ATs and two resident
AT domains to be replaced. The boxed residues shown at the N and C termini of the AT domains were defined as the boundaries for the AT replacements. Two unique
restriction sites, AvrII (CCTAGG) and NsiI (ATGCAT), were introduced in the boundary regions. The introduction of the AvrII site causes residue changes to P (Pro)
and R (Arg) in the N terminus (as arrows indicate), and the NsiI site causes a change to A (Ala) in the C terminus (as the arrow indicates). (B) The flanking region
cassettes for the AT replacements were constructed as pWHM3/EryAT1-flank for module 1 and pWHM3/EryAT2-flank for module 2. Numbers above each flanking
region refer to the relevant sequence positions in the eryA sequence deposited in GenBank (accession no. M63676). Three PCR-generated fragments carrying the
heterologous ATs were isolated from their corresponding subclones of pUC18/
"Hyg" AT2, pUC18/"Ven" AT, and pUC19/RapAT14 (see Materials and Methods) by
digestion with AvrII and NsiI and subcloned in pWHM3/EryAT1-flank or pWHM3/EryAT2-flank, respectively, to generate the AT replacement plasmids in module 1
the resolutions. These were then subjected to Southern analysis (data not shown) to determine whether resolution resulted in the desired genotype wherein the resident AT domain had been replaced by the heterologous AT. The five AT-replaced strains of \textit{S. erythraea} ER720 obtained were designated \textit{S. erythraea} EryAT1/“Hyg” AT2, \textit{S. erythraea} EryAT2/“Hyg” AT2, \textit{S. erythraea} EryAT1/“Ven” AT, \textit{S. erythraea} EryAT1/RapAT14, and \textit{S. erythraea} EryAT2/RapAT14 (Table 1).

\textbf{Novel erythromycin derivatives produced by the AT replacement strains.} Macrolide compounds produced by the AT replacement strains of \textit{S. erythraea} ER720 were isolated as described in Materials and Methods and characterized by TLC, TLC-bioautography, MS, and (for \textit{S. erythraea} EryAT1/“Hyg” AT2 and \textit{S. erythraea} EryAT2/“Hyg” AT2) NMR analysis.

The AT replacements in module 1 (\textit{S. erythraea} EryAT1/“Hyg” AT2, \textit{S. erythraea} EryAT1/“Ven” AT, and \textit{S. erythraea} EryAT1/RapAT14) all appear to produce the same erythromycin derivative, based on both TLC and MS analysis of culture broth extracts. As shown in Fig. 5, this new compound is visible as a blue-staining spot that runs slightly faster than erythromycin A. MS analysis of these samples after isolation from a TLC plate revealed a dominant M + H$^+$ peak at \textit{m/z} 704, which is 30 units lower than for erythromycin A (M + H$^+$ = \textit{m/z} 734). This result suggests that these compounds are erythromycin A derivatives lacking both a methyl group and a hydroxyl group. In agreement with the MS results, the major macrolide species produced by \textit{S. erythraea} EryAT1/“Hyg” AT2 was characterized by its \textit{1H} and \textit{13C} NMR spectra as 12-desmethyl-12-deoxyerythromycin A (Table 3 and Fig. 4B).

To determine whether 12-desmethyl-12-deoxyerythromycin A is biologically active, the culture broth extracts prepared from the three AT replacement strains in module 1 were subjected to TLC-bioautography. A unique inhibition zone corresponding to the position of the novel TLC spot (Fig. 5) was observed in all samples assayed (data not shown). This result indicates that the novel compound has significant bioactivity against \textit{S. aureus} and may be the only biologically active macrolide produced by these strains, since no other inhibition zone was observed even when a 10-fold-higher concentration of the crude samples was applied (data not shown). To compare the amounts of compound produced by these three strains, cells were grown in triplicate, extracts were normalized for cell amounts of compound produced by these three strains, and MS analysis of the culture broth extracts. As shown in Fig. 5, two novel spots, one running slightly faster than erythromycin A and another running slightly slower than erythromycin A, were identified by TLC in extracts of culture supernatants. MS analysis of the samples revealed two predominant species with the M + H$^+$ molecular masses of \textit{m/z} 720, corresponding to the slow-running TLC spot, and \textit{m/z} 704, corresponding to the fast-running TLC spot. This is in agreement with erythromycin A derivatives lacking a methyl group and lacking both a methyl and a hydroxyl group, respectively. The predicted structures of these two derivatives are shown in Fig. 4C. In addition, it was observed that the ratio of the two compounds (determined as the ratio of the peak height at \textit{m/z} 720 to that at \textit{m/z} 704) varied slightly with culture conditions and/or the extraction process.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Position} & \textbf{NMR chemical shift$^a$} \\
\hline
\textbf{1$^1$C} & \textbf{1$^1$H} \\
\hline
1 & 176.8 & 179.0 \\
2 & 45.1 & 45.6 \\
3 & 79.4 & 76.1 \\
4 & 41.7 & 42.1 \\
5 & 83.5 & 85.4 \\
6 & 75.1 & 81.6 \\
7 & 38.7 & 41.2 \\
8 & 43.7 & 39.8 \\
9 & 219.5 & 115.5 \\
10 & 46.1 & 42.5 \\
11 & 66.9 & 80.1 \\
12 & 40.3 & 83.9 \\
13 & 73.7 & 81.6 \\
14 & 28.0 & 24.2 \\
15$^a$ & 9.6 & 9.6 \\
2$^a$ & 14.4 & 13.0 \\
4$^a$ & 9.6 & 17.1 \\
6$^a$ & 26.3 & 27.7 \\
8$^a$ & 18.5 & 12.0 \\
10$^a$ & 10.6 & \\
12$^a$ & & 24.5 \\
1$^a$ & 103.2 & 102.4 \\
2$^a$ & 70.8 & 69.7 \\
3$^a$ & 65.4 & 65.8 \\
4$^a$ & 28.9 & 28.7 \\
5$^a$ & 69.2 & 69.4 \\
6$^a$ & 21.3 & 21.2 \\
8$^a$ & 40.3 & 40.4 \\
1$^a$ & 96.6 & 94.7 \\
2$^a$ & 34.9 & 34.6 \\
3$^a$ & 72.7 & 72.8 \\
4$^a$ & 77.9 & 78.3 \\
5$^a$ & 66.0 & 65.0 \\
6$^a$ & 18.4 & 17.8 \\
8$^a$ & 21.5 & 21.6 \\
OCH$_3$ & 49.5 & 49.3 \\
\hline
\end{tabular}
\caption{NMR data for 12-desmethyl-12-deoxyerythromycin A and 10-desmethylerythromycin A 6,9,9,12-spiroketal in CDCl$_3$}
\end{table}

$^a$ NMR chemical shift assignments for 12-desmethyl-12-deoxyerythromycin A (1) and 10-desmethylerythromycin A 6,9,9,12-spiroketal (2). The absence of \textit{13C} signals for the 12-CH$_3$ and 10-CH$_3$ indicates that these compounds are the expected desmethylerythromycin derivatives.

TLC-bioautography of the crude extract clearly indicates that both compounds are biologically active against \textit{S. aureus} (data not shown). In contrast to the production of 12-desmethyl-12-deoxyerythromycin A, the yield of the 10-desmethylerythromycin derivatives by \textit{S. erythraea} EryAT2/“Hyg” AT2 did not differ significantly from that produced by \textit{S. erythraea} EryAT2/RapAT14. In addition, as shown in Fig. 5, the production of 10-desmethylerythromycin derivatives by \textit{S. erythraea} EryAT2/“Hyg” AT2 and \textit{S. erythraea} EryAT2/RapAT14 seemed to be significantly lower than that of the 12-desmethylerythromycin derivative, based on the intensities of the TLC staining spots (Fig. 5).

For NMR structural studies, the erythromycin derivatives produced by \textit{S. erythraea} EryAT2/“Hyg” AT2 were isolated from a 27-liter fermentation as described in Materials and Methods. Unfortunately, the expected compound, 10-desmethylerythromycin A, was not recovered in sufficient yield for structure determination, but several milligrams of a presumed breakdown product, the 6,9,9,12-spiroketal derivative of 10-
desmethylerythromycin A, was isolated. From the $^1$H and $^{13}$C NMR spectra of this compound, it was clear that the methyl group normally attached to C-10 was absent and that the C-10 methine had been replaced by a methylene, as expected for a 10-desmethylerythromycin derivative (Table 3). The mass spectral data were also in agreement with the structural assignment, giving an $M^+$ ion at $m/z$ 702. It is known that erythromycin A can be converted to its 6,9:9,12-spiroketal derivative in the presence of aqueous acid (42). It appears that 10-desmethylerythromycin A is even more labile to such rearrangement than the parent compound.

**DISCUSSION**

Methylmalonyl-CoA is the only extender unit used by the erythromycin PKS (DEBS) to build the polyketide core of erythromycin. To alter the polyketide backbone structure, three heterologous AT domains, “Hyg” AT2, RAPS AT14, and “Ven” AT, which were predicted to use malonyl-CoA, based on sequence similarity to other putative malonyl-CoA-specific acyltransferases, were chosen to replace the AT1 and AT2 domains of the DEBS I subunit of the erythromycin PKS. The heterologous ATs were isolated from three different PKS clusters from two non-*Saccharopolyspora* actinomycetes. One of the three, RapAT14, was selected from the 14th module of the rapamycin PKS and, as shown in Fig. 2, appears to be the least conserved among the seven rapamycin malonylacyltransferase domains. The choice of RapAT14 was purely arbitrary. *S. erythraea* strains carrying these heterologous AT replacements produced novel erythromycin derivatives which lack a methyl group at the predicted position on the macrolactone ring. All three AT replacements in module 1 appeared to produce the same erythromycin derivative, 12-desmethyl-12-deoxyerythromycin A (12-desmethylerythromycin B). *S. erythraea* EryAT1/“Hyg” AT2 produced the highest level of the novel compound, comparable to the level of erythromycin A produced by the parental strain. The other two strains carrying the replacements at AT1 produced less 12-desmethyl-12-deoxyerythromycin A. Substantially lower yields, however, were observed when the AT in module 2 of DEBS1 was replaced: the level of the 10-desmethylerythromycins produced by both
Erythromycin A standard (510-desmethyl-12-deoxyerythromycin A producers S. erythraea EryAT1/RapAT14 was at least fivefold less than the level of erythromycin produced by S. erythraea EryAT2/"Hyg" AT2, S. erythraea EryAT1/RapAT14, and S. erythraea EryAT1/"Ven" AT, respectively; lane 5, erythromycin A standard (5 μg); lanes 6 and 7, 10-desmethylerythromycin A and 10-desmethyl-12-deoxyerythromycin A producers S. erythraea EryAT2/"Hyg" AT2 and S. erythraea EryAT2/RapAT14, respectively. Positions corresponding to erythromycin A are indicated by arrows.

S. erythraea EryAT2/"Hyg" AT2 and S. erythraea EryAT2/RapAT14 was at least fivefold less than the level of erythromycin produced by S. erythraea ERY720. Since wild-type levels of 12-desmethylerythromycin were made in at least one of the AT1 constructs, the basis for the large variations in rates of synthesis cannot be attributed to either the limitation of malonyl-CoA for incorporation or the efficiency of processing the nascent polyketide lacking a side chain in a single position. Rather, it is likely that the large variations in rates of synthesis are related to the effects of introducing heterologous domains on the structure and functions of the other domains in the multifunctional polypeptide.

The AT domains accept the extender unit and pass it to the ACP domain within the module that it occupies. Though AT domain swapping may be permitted, detrimental effects on the rates of extender unit transfer may occur when noncognate AT-ACP or putative AT-KS interactions take place. Thus, the large variations in the levels of polyketide production observed when different heterologous AT domains were used to replace a given resident AT domain in module 1 or 2 in DEBS 1 are not unexpected. Kuhstoss et al. (20) found that when the AT-ACP domains of the starter module (responsible for loading the starter unit in spiramycin synthesis) were replaced by a corresponding region of the starter module of the tylosin PKS, the rate of synthesis of the hybrid polyketide actually went up three- to fourfold. Until the structures of PKSs are understood, the rate of synthesis of the hybrid polyketide cannot be predicted.

These effects may also explain the results observed when replacements using the three heterologous malonyl AT domains were attempted in other modules of DEBS. In summary, strains which carried "Hyg" AT2 (the domain that yielded the highest level of hybrid polyketides in modules 1 and 2) in module 3, 4, 5, or 6 did not produce detectable levels of erythromycin analogs, nor did other strains carrying replacements using "Ven" AT or RapAT14 in selected modules (data not shown). It is likely, therefore, that more information on the structure of the PKS will be required before it can be predicted a priori whether a given modular replacement will yield productive levels of novel polyketides.

Productive heterologous AT-ACP interactions in aromatic PKSs have also been reported (19). Aromatic PKSs are composed of discrete monofunctional (or difunctional) polypeptides that assemble to form a complex. In these and later experiments (24a), one ACP was successfully substituted for another. Most aromatic PKS clusters do not contain a gene specifying an AT function, but since malonate is always used as the extender in aromatic polyketide synthesis, it is believed that these PKSs employ the AT component of the host fatty acid synthase complex (27a, 35). Thus, the noncognate interactions apparently took place between a fatty acid AT and a PKS ACP.

It was not surprising that hydroxylation at C-12 of the macro lactone ring, mediated by EryK, a P450 enzyme (34), did not take place on the 12-desmethylerythromycin derivative. P450-mediated hydroxylation involves a free radical mechanism that should be several hundred-fold less favored energetically than the secondary ([CH3]2 carbon rather than the tertiary [CH(CH3)]2 carbon normally found at C-12 of erythromycin. In the 10-desmethyl derivatives, approximately one-half of the compounds were C-12 hydroxylated. Since the overall level of production of the 10-desmethylerythromycins was much lower than the level of erythromycin produced by the parental strain, the finding of only 50% hydroxylation suggests very weak substrate activity of 10-desmethylerythromycin D for EryK. In the normal pathway to erythromycin A, erythromycin D is converted to erythromycin C by EryK; purified EryK exhibits a 1,900-fold preference for erythromycin D over erythromycin B as a substrate (21). Interestingly, no 12-desmethylerythromycin D or 10-desmethylerythromycin C or D was produced in any of the AT1 or AT2 domain replacement strains. These results indicate that EryG, the enzyme that O-methylates the C-3 hydroxyl on the L-mycarose moiety, can use either the 10- or 12-desmethyl derivative efficiently as a substrate. This could not have been predicted since it was previously discovered that some erythromycin derivatives that contained changes in the structure of the lactone ring, e.g., 6-deoxyerythromycin, 2-de methylerythromycin, and Δ^4'-anhydroerythromycin, C, had reduced activity as substrates for EryG (9, 24, 27, 40).

Replacement of a methylmalonyl with a malonyl extender in a polyketide through swapping of the AT1 domain of DEBS 1 with the AT domain from the second module of the rapamycin PKS was first reported by Oliynyk et al. (26). Although that work used a DEBS 1 model system that produced a triketide lactone, it showed unambiguously that the AT domains alone in DEBS 1 (and likely all complex PKSs) specify the structure of the extender unit incorporated during polyketide synthesis. In addition, replacement of either AT1 or AT2 domain with the AT domain from the second module of the rapamycin PKS was first reported by Oliynyk et al. (26). Although that work used a DEBS 1 model system that produced a triketide lactone, it showed unambiguously that the AT domains alone in DEBS 1 (and likely all complex PKSs) specify the structure of the extender unit incorporated during polyketide synthesis. In addition, a comparison of all AT domains in the rapamycin PKS with those that were previously described for DEBS allowed Schwecke et al. (32) to demonstrate that ATs could be grouped into two clusters on the basis of their sequences: those specifying malonate and those specifying methylmalonate. The findings reported here confirm and expand the previous work by demonstrating that two AT domains in DEBS 1 can be replaced by three different heterologous, malonyl-specifying ATs. However, the signature sequences proposed by Haydock et al. (12) for malonyl-incorporating ATs (ETGYAxxxxxxxQxxFGGL; 11 conserved amino acids) may apply only to the rapamycin PKS. The corresponding domain in “Hyg” AT2 has only four conserved residues (TdhxxxxxxxxxARxLw), and the “Ven” AT domain has only three conserved residues (TvtvxxxxxxxGRFl). Nonetheless, the AT groups proposed by Schwecke et al. (32) are very important. In two of the three cases, the AT used for replacement in either module 1 or module 2 of DEBS 1 was from a PKS-like gene cluster whose
characterized or partially characterized PKS components. It appears promising, therefore, that novel, rationally designed complex polyketides can be produced through the assembly of PKS components from a variety of organisms with only sequence information to guide the choice of AT domains.

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