Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 7305–7309, June 1998
Biochemistry

Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering

D. L. Stassi[†‡], S. J. Kakavas[†], K. A. Reynolds[§], G. Gunawardana[†], S. Swanson[†], D. Zeidner[†], M. Jackson[†], H. Liu[§], A. Buko[†], and L. Katz[†]

[†]Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, IL 60064; and [§]Institute of Structural Biology and Drug Discovery, Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, VA 23219

Edited by Arnold L. Demain, Massachusetts Institute of Technology, Cambridge, MA, and approved April 27, 1998 (received for review February 26, 1998)

ABSTRACT **A previously unknown chemical structure, 6-desmethyl-6-ethylerythromycin A (6-ethylErA), was produced through directed genetic manipulation of the erythromycin (Er)-producing organism *Saccharopolyspora erythraea*. In an attempt to replace the methyl side chain at the C-6 position of the Er polyketide backbone with an ethyl moiety, the methylmalonate-specific acyltransferase (AT) domain of the Er polyketide synthase was replaced with an ethylmalonate-specific AT domain from the polyketide synthase involved in the synthesis of the 16-member macrolide niddamycin. The genetically altered strain was found to produce ErA, however, and not the ethyl-substituted derivative. When the strain was provided with precursors of ethylmalonate, a small quantity of a macrolide with the mass of 6-ethylErA was produced in addition to ErA. Because substrate for the heterologous AT seemed to be limiting, crotonyl-CoA reductase, a primary metabolic enzyme involved in butyryl-CoA production in streptomycetes, was expressed in the strain. The primary macrolide produced by the reengineered strain was 6-ethylErA.**

Erythromycin (Er) is a broad-spectrum macrolide antibiotic produced by *Saccharopolyspora erythraea*. The backbone of the molecule is a 14-member macrocyclic ring (Fig. 1) that is produced through the sequential condensation of one molecule of propionyl-CoA and six molecules of methylmalonyl-CoA by a modular polyketide synthase (PKS). This enzyme complex comprises three large multifunctional polypeptides, DEBS1, DEBS2, and DEBS3, each of which contains two modules, and, in the case of DEBS1, a loading domain to initiate synthesis of the polyketide chain (1, 2). Each module contains the enzymatic activities necessary for one condensation and subsequent reduction of the extender to the growing chain. Selection of the appropriate extender unit is accomplished by the acyltransferase (AT) domain present in each module (3).

Recently, genetic engineering has joined synthetic chemistry in the production of novel macrolide structures. Hybrid PKSs have been constructed through the replacement of AT domains with those that specify different starter or extender units (3–6). These manipulations have resulted in the production of Er derivatives lacking methyl groups at C-10 and C-12 (5) and those incorporating the branched chain starter units of avermectin biosynthesis (6). To date, however, there have been no manipulations that allow the methyl side chains of Er to be replaced by ethyl groups. This type of modification would be useful in expanding the structural diversity of hybrid polyketides produced by combinatorial biosynthesis.

Niddamycin (Nd) is a 16-member macrolide with an ethyl side chain at C-6 (Fig. 1). The module 5 AT, which is believed to be responsible for incorporation of the ethyl side chain into the polyketide backbone, was identified by sequence analysis of the Nd PKS genes (7). In this paper we describe the construction of a hybrid PKS through the replacement of a methylmalonyl-specific AT of the Er PKS with that of the ethylmalonyl-specific AT of the Nd PKS. This substitution alone, however, was not sufficient to produce an Er derivative with an ethyl side chain; additional manipulations of the carboxylic acid precursor pools were necessary to generate the desired compound.

MATERIALS AND METHODS

Strains, Plasmids, and Media. The wild-type Er producer is *S. erythraea* ER720 (8). Plasmid pWHM3 (9) is an *Escherichia coli–Streptomyces* shuttle vector that is maintained in *S. erythraea* only when it contains heterologous DNA for chromosomal integration. Plasmid pDPE81 is a derivative of pKAS37 (10) in which a 1.7-kb *Bgl*II fragment containing the hygromycin resistance marker was inserted into the *Bgl*II site of pKAS37. *E. coli* DH5α (Life Technologies, Gaithersburg, MD) was the host used for plasmid construction and isolation. Liquid cultures of *S. erythraea* strains were grown in SGGP (11) for production of protoplasts and SCM (5) for metabolite or enzyme analysis. Plate cultures of *S. erythraea* strains were grown on R3M medium (5). Thiostrepton (Ts)-resistant strains of *S. erythraea* were grown in 25 μg/ml and 10 μg/ml Ts for plate and liquid cultures, respectively.

Plasmid pEAT4 (Fig. 2*A*) was constructed as follows. Cosmid pAIBX85, a pWHM3 derivative containing DNA from modules 3 and 4 of the Er PKS (corresponding to nucleotides 979-9349; GenBank accession no. M63677), was used to clone DNA flanking eryAT4. The 5′ flanking region was isolated by digesting pAIBX85 with *Msc*I and *Bst*EII (nucleotides 4247–6033), treating with the Klenow fragment of DNA polymerase, and ligating the fragment into the *Sma*I site of pUC19 to generate pUC/5′ flank. An *Avr*II site was engineered 13 bp downstream of the *Bst*EII site by PCR amplification of a 306-bp region of DNA from the *Pml*I site (nucleotide 5739) to 12 bp 3′ of the *Bst*EII site (nucleotide 6045). The engineered *Avr*II site does not change the Pro-Arg residues encoded by this region (Fig. 2*B*). A *Bam*HI site was also included on the PCR primer just downstream of the *Avr*II site. The resulting fragment was digested with *Pml*I and *Bam*HI and cloned into the *Pml*I/*Bam*HI site of pUC/5′ flank, replacing the native se-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/957305-5$2.00/0
PNAS is available online at http://www.pnas.org.

This paper was submitted directly (Track II) to the *Proceedings* office.

Abbreviations: 6-ethylErA, 6-desmethyl-6-ethylerythromycin A; AT, acyltransferase; Ccr, crotonyl-CoA reductase; Er, erythromycin; Nd, niddamycin; PKS, polyketide synthase; Ts, thiostrepton.

[‡]To whom reprint requests should be addressed at: Abbott Laboratories, CAPD, D-4FR F3-3, 1401 Sheridan Road, North Chicago, IL 60064. e-mail: diane.stassi@abbott.com.

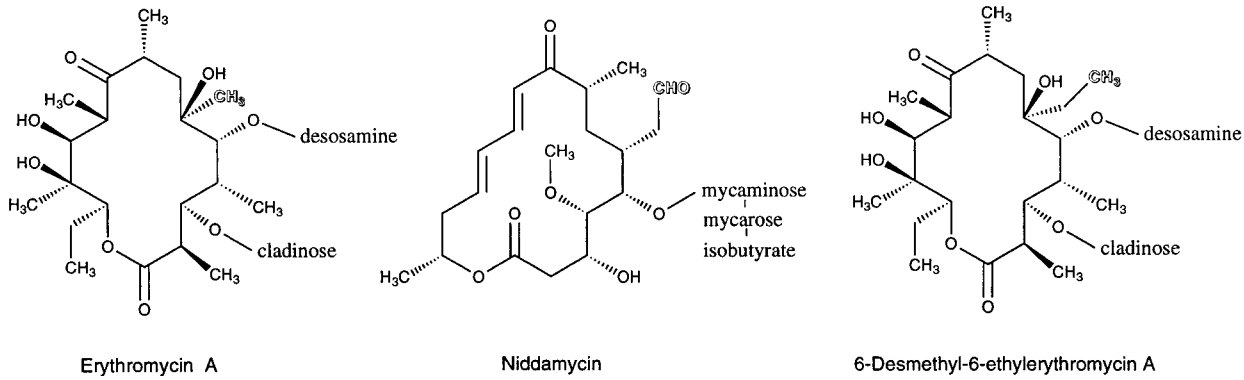

FIG. 1. Structures of Er, Nd, and 6-ethylErA. Shadowed letters represent relevant side groups.

quence and resulting in vector pUC/5′ flank/*Avr*II. The 3′ flanking DNA was isolated by digesting pAIXB85 with *Pml*I and *Msc*I (nucleotides 6999 and 8977), treating with the Klenow enzyme, and ligating the fragment into the *Sma*I site of pUC19, generating vector pUC/3′ flank. nidAT5 was isolated by PCR amplification of cosmid 13f5 (7), which contains DNA spanning module 5 of the Nd PKS cluster. The 5′ oligonucleotide was designed to create an *Avr*II site 12 nucleotides upstream of the beginning of the nidAT5 domain (nucleotide 25290; GenBank accession no. AF016585) and to place the amino acid sequence Pro-Arg-Lys-Pro in front of nidAT5 to correspond to the sequence that is found upstream of the eryAT4 domain. The 3′ oligonucleotide generated an *Fse*I site at the end of the nidAT5 domain (nucleotide 26284, GenBank accession no. AF016585), resulting in a conservative Val to Ala change. A *Bam*HI site was also incorporated after the *Fse*I site. The fragment was digested with *Avr*II and *Bam*HI and ligated into *Avr*II/*Bam*HI-digested pUC/5′ flank/*Avr*II, creating vector pUC/5′ flank/nidAT5. The 3′ flanking DNA was then cloned onto the 3′ end of nidAT5 by first digesting pUC/3′ flank with *Fse*I and *Bam*HI, gel purifying the 1920-bp fragment, and ligating it into *Fse*I/*Bam*HI-digested pUC/5′ flank/nidAT5, creating vector pUC/nidAT5/C6-flank. To generate pEAT4, the nidAT5/flanking DNA cassette was isolated from pUC/nidAT5/C6-flank by digestion with *Eco*RI and *Hin*dIII and then ligated to *Eco*RI/*Hin*dIII-digested pWHM3.

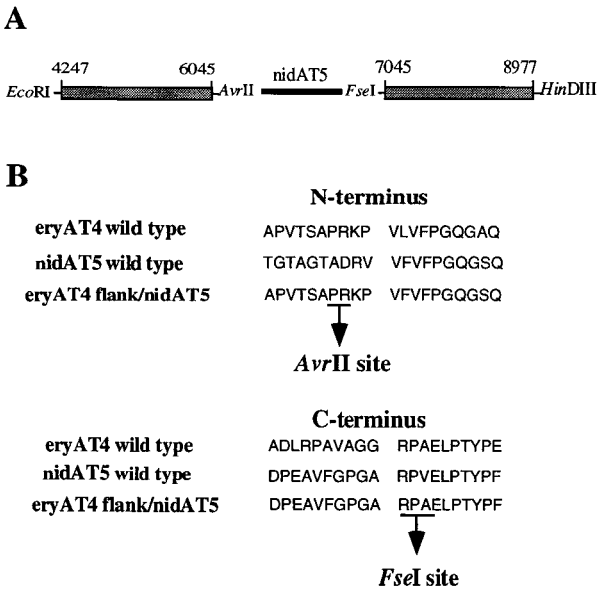

FIG. 2. (*A*) Diagrammatic representation of insert in vector pEAT4. DNA encoding the AT from module 5 of the Nd PKS cluster (nidAT5) was cloned between DNA flanking the 5′ and 3′ boundaries of the AT coding region in module 4 of the Er PKS cluster. The numbered boxes correspond to *eryA* sequence coordinates from GenBank accession no. M63676. (*B*) Amino acid comparisons in the junction regions of eryAT4, nidAT5, and the eryAT4 flank/nidAT5 construct. The amino acids encoding the *Avr*II and *Fse*I sites are indicated.

Plasmid pDPE-ccr (Fig. 3) was constructed as follows. The *Streptomyces collinus* crotonyl-CoA reductase (Ccr) gene (*ccr*) was subcloned from plasmid pZYB3 (12) by digestion with *Xba*I and *Bam*HI, which releases *ccr* and the upstream T7 ribosomal binding site. This fragment was treated with the Klenow enzyme and ligated into the polylinker of pDPE81 that had been digested with *Eco*RI and treated with the Klenow enzyme. The polylinker of pDPE81 is in the center of a 10-kb fragment of *S. erythraea* chromosomal DNA, which directs integration into the chromosome at a site that does not seem to affect Er production. Plasmid pDPE-ccr was designed so that the *ccr* gene expressed from the *ermE** promoter (13) and the Ts-resistance marker are left behind in the chromosome following a double-crossover event.

Genetic Manipulations. Standard molecular biology techniques were performed as described (14). Enzymes and reagents were purchased from Life Technologies. Protoplast transformation and marker replacement in *S. erythraea* were performed as described (5). One microgram of plasmid DNA was routinely used for protoplast transformations. Putative transformants were grown in SGGP medium containing 10 μg/ml Ts to confirm resistance. For chromosomal eviction of pEAT4, transformants were passaged twice in SGGP medium without Ts, cells were plated for spores, and individual colonies arising from spores were screened for Ts sensitivity.

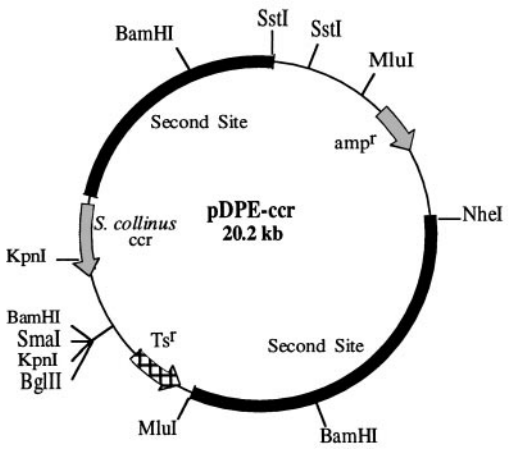

FIG. 3. Plasmid pDPE-ccr. The thick black lines represent *S. erythraea* DNA, which allows for integration of the plasmid by homologous recombination into the *S. erythraea* chromosome. Also indicated are genes for ampicillin (amp) and Ts resistance.

Electroporation was performed as described (15) by using 1 μg of pDPE-ccr DNA. Because the plasmid is unstable when integrated into the *S. erythraea* chromosome, two consecutive platings on Ts-containing R3M plates results in resolution and eviction of plasmid sequences. PCR and Southern hybridization were as described (7).

Ccr Assay. Seed cultures of *S. erythraea* grown in SCM and *S. collinus* grown in medium A (16) were diluted 5-fold into SCM and medium A, respectively, and grown for 48 h at 30°C. Cells were harvested, opened with a French pressure cell, centrifuged to obtain cell-free extracts, and assayed spectrophotometrically for Ccr activity as described (16). One unit of Ccr activity is defined as 1 μmol of NADPH oxidized in 1 min.

Isolation and Identification of Metabolites. Small-scale (milliliters) isolation of metabolites from *S. erythraea,* TLC, and MS analysis were performed as described (5). Large-scale isolation for compound identification was as follows. Fermentations were conducted in 42-liter LH fermentation series 2000 stainless steel vessels (LH Fermentation, Maidenhead, UK). Each fermentor was charged with 30 liters of a medium consisting of 1.5% soluble starch, 2.2% soybean flour, 0.2% $CaCO_3$, 0.15% brewer's yeast (Wind Gap Farms, Baconton, GA), 0.102% $MgSO_4 \cdot 7H_2O$, 0.0027% $FeSO_4 \cdot 7H_2O$, and 1% soybean oil. Sterilization was at 121°C and 15 psi (1 psi = 6.89 kPa). A two-step seed protocol was used. Vegetative growth from a frozen stock of *S. erythraea* strain EAT4-ccr was inoculated at 1.5% into a 500-ml Erlenmeyer flask containing 100 ml of the following medium: 1.5% glucose monohydrate/1% soluble starch/1% Soytone (Difco)/0.9% yeast extract (Difco)/0.21% Mops/0.001% Ts. For the second step, a 2-liter Erlenmeyer flask containing 600 ml of the same medium was inoculated at 5% with the first-step growth. Both steps were incubated at 28°C on a rotary shaker at 225 rpm for 48 h. The fermentor was inoculated at 5% with the second-step seed growth. Fermentor temperature was controlled at 32°C, agitation was 250 rpm, aeration was 1 vol$\cdot$vol$^{-1}\cdot$min^{-1}, and head pressure was 6 psi. Silicone antifoam was added at 0.01% initially and was available on demand. Harvest was at 108 h.

The fermentation broth (28 liters) was filtered, the pH was adjusted to 9 with NH_4OH, and the broth was extracted with CH_2Cl_2 (twice with 14 liters each). The combined extracts were concentrated, and the residue was partitioned between the two phases of the heptane/methanol/0.02 M K_2HPO_4 system (pH 6; 1:1:1). The aqueous phase was evaporated, and the residue was dissolved in 0.05 M aqueous potassium phosphate buffer (100 ml), adjusted to pH 9 with NH_4OH, and extracted twice with equal volumes of CH_2Cl_2. The CH_2Cl_2-soluble fraction (1.65 g) was separated by droplet countercurrent chromatography (100 vertical columns; 0.4 $\times$ 24 cm) by using the upper layer from the hexane/ethyl acetate/0.02 M K_2HPO_4 (pH 8; 1:1:1) system as the mobile phase. The fractions were analyzed by bioassay against *Staphylococcus aureus* and by ^{1}H NMR. The bioactive fractions that were shown to contain macrolides by NMR were pooled, dried (0.4 g), and chromatographed on a Sanki Engineering (Kyoto) HPLC centrifugal partition chromatograph by using the hexane/ethyl acetate/0.02 M K_2HPO_4 (pH 6; 1:1:1) system. The active fraction was further purified by HPLC on C_{18}-derivatized silica by using an acetonitrile/methanol/0.01 M $(CH_3)_4NOH$/0.05 M KH_2PO_4 (pH 6; 73:10:59:59) system. Two-dimensional NMR experiments, including double-quantum correlation, heteronuclear multiple quantum correlation, and heteronuclear multiple-bond correlation, performed in C^2HCl_3, were used for structure elucidation and the complete assignment of the ^{1}H and ^{13}C NMR signals.

***In Vitro* Antibacterial Activity.** Antibacterial activity was determined by the broth microdilution method (National Committee for Clinical Laboratory Standards (Villanova, PA) M7-A4, 1997), except that Brain Heart Infusion (Difco) broth was used as the test medium. Assays were incubated overnight at 35°C. Minimal inhibitory concentrations were defined as the lowest drug concentration (μg/ml) inhibiting bacterial growth.

RESULTS

Construction of *S. erythraea* EAT4. Plasmid pEAT4 was constructed to replace DNA encoding the AT domain of module 4 in the Er PKS (eryAT4) with DNA encoding the AT domain from module 5 of the Nd PKS (nidAT5) (7). *S. erythraea* ER720 protoplasts were transformed with pEAT4 DNA, and 10 transformants were obtained. Genomic DNA was extracted from one of the transformants for Southern analysis, in which probing with pWHM3 DNA confirmed the integration of pEAT4 at the appropriate location in the chromosome (data not shown). Nonselective growth to allow plasmid eviction through a double-crossover event yielded 96 colonies, of which 9 were found to be Ts sensitive. Southern analysis of the 9 clones showed that 3 had nidAT5 DNA sequences in place of eryAT4 chromosomal sequences, and the remaining 6 had segregated to wild type (data not shown).

Characterization of *S. erythraea* EAT4. To analyze the Er derivatives produced by *S. erythraea* EAT4, the three isolates and four of the wild-type segregants were grown in SCM medium for 4 d. The four wild-type segregants produced spots indistinguishable in color and R_f from the ErA standard. The three strains in which nidAT5 replaced the AT of module 4 of the Er PKS produced spots similar in R_f and color to the wild-type segregants, but the spots were much less intense (data not shown).

To determine the mass of the compound produced by *S. erythraea* EAT4, the supernatant of a 50-ml SCM culture of one of the isolates was extracted with ethyl acetate. The extract was subjected to TLC, but only the edges of the plate were sprayed with anisaldehyde to locate the region of interest. A 1-cm band of resin was scraped from the unsprayed portion of the plate at the R_f of ErA. The resin was extracted twice with 500 μl of an ethyl acetate/methanol (2:1) solution, and the organic phase was dried and then analyzed by electrospray ionization MS. Surprisingly, the mass of the protonated molecular ion of the compound was observed at m/z 734, which corresponds to the mass of ErA, not an ethyl-substituted ErA derivative.

Feeding of Ethylmalonyl-CoA Precursors to *S. erythraea* EAT4. One hypothesis for the failure of *S. erythraea* EAT4 to produce an ethyl-substituted derivative is that ethylmalonyl-CoA, the substrate for the nidAT5, is lacking. To test this hypothesis, cells were grown for 4 d in SCM medium containing precursor compounds, and ethyl acetate extracts of the cultures were analyzed. When either 50 mM butanol or 50 mM butyrate was added to the medium, a second spot running slightly faster than the R_f of ErA was seen. Butyrate-fed cultures (Fig. 4*A*) produced about a 1:1 ratio of the two spots, whereas the butanol-fed culture produced more of the ErA-like spot (data not shown). Addition of 10 mM ethylmalonate failed to produce significant amounts of either of the two spots described above. However, addition of 10 mM diethylethylmalonate was found to yield much more of the faster migrating compound than that seen with the other precursor compounds tested and very little compound migrating at the R_f of ErA (Fig. 4*A*). Cells grown in unsupplemented SCM medium produced only material migrating like ErA.

To characterize the newly synthesized compound, extracts of the butyrate- and the diethylethylmalonate-fed cultures were subjected to TLC, the region of interest was scraped from the plate and reextracted for electrospray ionization MS. The results showed that the butyrate-fed culture of *S. erythraea* EAT4 produced approximately equal amounts of compounds with protonated molecular ions at m/z 734 and 748. The 748 species is consistent with an additional methylene group on ErA, e.g., with an ethyl group replacing a methyl group on the

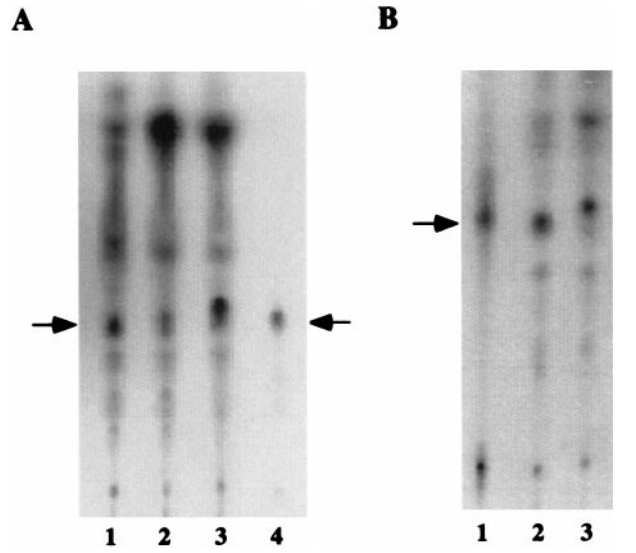

FIG. 4. TLC analysis of *S. erythraea* EAT4 and *S. erythraea* EAT4-ccr. (*A*) Ethyl acetate extracts of *S. erythraea* EAT4 cultures grown under various conditions. Lanes: 1, SCM medium; 2, SCM + 50 mM butyrate; 3, SCM + 10 mM diethylethylmalonate; 4, ErA standard (5 μg). The arrows indicate the position of ErA. (*B*) Ethyl acetate extracts of *S. erythraea* strains EAT4 (lane 2) and EAT4-ccr (lane 3) grown in SCM medium, along with 5.0 μg of ErA standard (lane 1). The arrow indicates the position of ErA.

macrolide ring. The diethylethylmalonate sample contained primarily a compound with a mass of 748, with only a trace of ErA (734) present.

Genetic Manipulation of Ethylmalonyl-CoA Levels in *S. erythraea* EAT4. Successful production of an Er derivative with a mass consistent with the addition of an ethyl side chain by butyrate- and diethylmalonate-fed *S. erythraea* EAT4 suggested that the levels of ethylmalonyl-CoA, the likely substrate of the nidAT5, must be limiting. In streptomycetes, Ccr catalyzes the last step in the reductive biosynthesis of butyryl-CoA from two molecules of acetyl-CoA (12). Because butyryl-CoA can then be carboxylated to form ethylmalonyl-CoA, the possibility that Ccr could be used to increase the levels of the ethyl-substituted Er derivative was investigated. The *ccr* gene of *S. collinus* was expressed from the strong *ermE** promoter at a site unlinked to the Er biosynthetic cluster in the *S. erythraea* EAT4 chromosome. Plasmid pDPE-ccr was electroporated into *S. erythraea* EAT4. About 40 Ts-resistant colonies were obtained, of which 7 were confirmed to be stable transformants. Genomic DNA was isolated from 2 of the transformants for Southern analysis. Probing with the *S. collinus ccr* gene confirmed that both isolates carried the gene at the expected location in the chromosome (data not shown). The strain was named *S. erythraea* EAT4-ccr.

To assess expression levels of the *ccr* gene, Ccr activity of *S. erythraea* EAT4-ccr was compared with that of *S. erythraea* EAT4 (parental strain) and wild-type *S. collinus*. We found (Table 1) that, although there was no detectable activity in the *S. erythraea* strain without the *ccr* gene, *S. erythraea* EAT4-ccr had about 20 times the relative activity of the wild-type *S. collinus* strain from which the gene originated.

Table 1. Ccr activity in *S. collinus* and engineered *S. erythraea* strains

Sample	Enzyme activity, milliunits/ml	Protein concentration, mg/ml	Specific activity, milliunits/mg
S. erythraea EAT4	<0.5 ± 0.0	3.0	<0.2 ± 0.0
S. erythraea EAT4-ccr	46.9 ± 2.0	3.0	15.6 ± 0.7
S. collinus wild type	5.5 ± 1.0	7.0	0.8 ± 0.1

To determine the effect of the *ccr* gene on production of an ethyl-substituted Er derivative, ethyl acetate extracts of supernatants of 4-d SCM-grown cultures of the EAT4 strains with and without the *ccr* gene were examined (Fig. 4*B*). TLC analysis of 10 ml of extracted cells demonstrated that EAT4 without the *ccr* gene produced a spot that migrated with ErA, whereas EAT4-ccr produced a spot that migrated faster than the ErA spot, with no ErA-like material visible.

Extracts were also analyzed by electrospray ionization MS. The EAT4 strain produced a compound with a protonated molecular ion at m/z 734, indicating ErA production. The EAT4-ccr strain produced a compound with a mass of 748, which is consistent with the production of the 6-ethylErA. No compound with a mass of 734 (ErA) was detected in extracts of this strain.

Structural Analysis and Biological Activity of Compound Produced by *S. erythraea* EAT4-ccr. The structure of 6-ethyl-ErA was confirmed by spectroscopic methods. The high resolution fast atom bombardment MS analysis of the sample gave an M + H^+ ion at m/z 748.4846 [calculated for $C_{38}H_{70}NO_{13}$ m/z = 748.4842 (Δ 0.5 ppm)], indicating that this molecule has an additional methylene function compared with ErA. The presence of an ethyl moiety in the molecule at the C-6 position and its relative stereochemistry were confirmed

Table 2. NMR data of 6-ethylErA

Carbon no.	^{13}C shift (δ), ppm	^{1}H shift (δ), ppm
1	175.5	
2	44.8	2.93
3	80.7	3.79
4	37.6	1.93
5	81.6	3.86
6	76.7	
7	38.4	1.72, 193
8	44.7	2.70
9	222.4	
10	37.7	3.01
11	68.9	3.82
12	74.4	
13	76.7	5.06
14	21.2	1.47, 1.91
15	10.7	0.84
2-CH_3	15.9	1.29
4-CH_3	9.7	1.14
6-$\underline{CH_2}$-CH_3	27.6	1.70, 2.02
6-CH_2-$\underline{CH_3}$	7.7	0.98
8-CH_3	18.6	1.17
10-CH_3	12.3	3.01
12-CH_3	68.9	3.82
1′	102.5	4.48
2′	72.5	3.21
3′	65.3	2.44
4′	28.3	1.22, 1.63
5′	68.7	3.52
6′	21.6	1.22
3′-$N(CH_3)_2$	40.7	2.29
1″	96.2	4.92
2″	35.3	2.34, 1.66
3″	72.4	
4″	77.7	3.02
5″	66.3	3.98
6″	21.6	1.29
3″-OCH_3	49.7	3.33
3″-CH_3	21.4	1.26

Numbering of carbon atoms of Er is as described (17).

by two-dimensional NMR. The stereospecificity of the ethyl moiety was found to be the same as that of the methyl group present at C-6 of ErA (Fig. 1). The ^{1}H and ^{13}C NMR assignments are shown in Table 2.

Antibacterial activity of 6-ethylErA was compared with that of ErA against a panel of *S. aureus, Staphylococcus epidermidis, Streptococcus pyogenes,* and *Enterococcus faecium* strains from the Abbott culture collection. Although 6-ethylErA does retain biological activity, it was found to be 15 to 60 times less potent than ErA against these strains. Representative minimal inhibitory concentrations (μg/ml) for 6-ethylErA and ErA, respectively, were as follows: *S. aureus* (4 and 0.06), *S. epidermidis* (4 and 0.12), *E. faecium* (4 and 0.12), and *S. pyogenes* (1 and 0.06).

DISCUSSION

In this work we have shown that an ethylmalonate AT can be substituted for a methylmalonate AT to produce a hybrid PKS that synthesizes the predicted macrolide product, 6-ethylErA. This suggests that Er analogs containing ethyl substitutions at positions other than C-6 or that ethyl analogs of other macrolides, such as rifamycin or rapamycin, can be prepared in a similar fashion by using the ethylmalonate-specific AT from the Nd PKS.

One key feature for the successful generation of 6-ethylErA was availability of ethylmalonyl-CoA as substrate for the ethylmalonate-specific AT of the Nd PKS in *S. erythraea*. This AT clearly demonstrates a relaxed specificity by reacting with both ethylmalonyl-CoA and methylmalonyl-CoA. In *S. erythraea* EAT4, the desired product could be obtained only by boosting the levels of ethylmalonyl-CoA by either precursor feeding or expression of Ccr, an enzyme involved in butyryl-CoA biosynthesis. These results suggest that ethylmalonyl-CoA derived from butyryl-CoA is not available at sufficient levels to compete with methylmalonyl-CoA for the ethylmalonate-specific AT. Ccr activity is not detectable in *S. erythraea* and attempts to detect a *ccr* gene in this organism by hybridization with the *S. collinus ccr* gene have failed (results not shown). In contrast, the *ccr* gene appears to be present in most streptomycetes (16), and in certain cases it appears to be clustered with antibiotic biosynthetic gene clusters. For example, a *ccr* homolog has been located in the biosynthetic cluster for tylosin (18), another 16-member macrolide with an ethyl side chain. Southern analysis of cosmid clones containing Nd biosynthetic genes also indicates that a *ccr* homolog may reside close to the Nd PKS (unpublished results).

It was demonstrated previously (5) that production of desmethyl Er derivatives by replacement of methylmalonate-specific ATs of the Er PKS with malonate-specific ATs from *Streptomyces hygroscopicus* and *Streptomyces venezuelae* was successful only in Er modules 1 and 2 (corresponding to C-12 and C-10 of Er, respectively) and did not give rise to a detectable polyketide when placed in module 4. In contrast, we successfully produced ethyl-substituted Er by replacement of the methylmalonate-specific AT of Er module 4 with the ethylmalonate-specific AT from the Nd cluster. In fact, in *S. erythraea* expressing the *S. collinus ccr* gene, replacements in modules 1 through 4 with the ethylmalonate-specific AT produced compounds with electrospray ionization mass spectra consistent with production of ethyl substitutions for methyl groups at C-12, C-10, C-8, and C-6, respectively. However, those in modules 5 and 6 did not produce compounds of mass 748 (data not shown). It is not known whether failure to produce the predicted compounds was caused by physical distortion of the PKS or its mRNA, inability of the PKS to process certain altered growing chains, or some structural instability of the macrolide itself because of the introduced change. Thus, even though genetic information may be present for the production of novel compounds, it is still not possible to predict which substitutions will yield detectable levels of product.

It has also been demonstrated that the malonyl-specific ATs can be distinguished from methylmalonyl-specific ATs through sequence alignments (3). By using this strategy, Ruan *et al.* (5) predicted that an AT from an unidentified PKS of *S. hygroscopicus* was a malonyl-specific AT, and they used it successfully to produce desmethyl Er derivatives. The Nd ethyl-specific AT was found to cluster with methylmalonyl-specific ATs (7) in similar AT alignments. This similarity may explain why methylmalonate was used as a substrate by nidAT5 in *S. erythraea* EAT4 to produce ErA when ethylmalonyl-CoA was not available.

Finally, structural determination by NMR not only confirmed the presence of the ethyl side chain on the Er derivative produced by *S. erythraea* EAT4 but also showed that the absolute configuration at this chiral center is the same as that in ErA. In Er biosynthesis, epimerization is required at C-6 because the PKS uses (2*S*)-methylmalonyl-CoA for chain extension (19). It is unclear whether the cognate epimerization occurs when ethylmalonate is used in the synthesis of 6-ethylErA because the stereochemistry of the ethylmalonate incorporated at C-6 is not known.

We thank Angela Nilius for performing minimal inhibitory concentration assays and Jim McAlpine, Rich Summers, and Thomas Vanden Boom for helpful discussions. This work was supported in part by Grant GM50542 (to K.A.R.) from the National Institutes of Health.

1. Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J. & Leadlay, P. F. (1990) *Nature* (*London*) **348,** 176–178.
2. Donadio, S. & Katz, L. (1992) *Gene* **111,** 51–60.
3. Oliynyk, M., Brown, M. J. B., Cortes, J., Staunton, J. & Leadlay, P. F. (1996) *Chem. Biol.* **3,** 833–839.
4. Kuhstoss, S., Huber, M., Turner, J. R., Paschal, J. W. & Rao, R. N. (1996) *Gene* **183,** 231–236.
5. Ruan, X., Pereda, A., Stassi, D., Zeidner, D., Summers, R., Jackson, M., Shivakumar, A, Kakavas, S. & Katz, L. (1997) *J. Bacteriol.* **179,** 6416–6425.
6. Marsden, A. F. A., Wilkinson, B., Cortes, J., Dunster, N. J., Staunton, J. & Leadlay, P. F. (1998) *Science* **279,** 199–202.
7. Kakavas, S. J., Katz, L. & Stassi, D. (1997) *J. Bacteriol.* **179,** 7515–7522.
8. DeWitt, J. P. (1985) *J. Bacteriol.* **164,** 969–971.
9. Vara J. A., Lewandowska-Skarbek, M., Wang, Y.-G., Donadio, S. & Hutchinson, C. R. (1989) *J. Bacteriol.* **171,** 5872–5881.
10. Maine, G., Post, D., Satter, M. & Stassi, D. (1997) International patent application WO 97/06266 (2/20/97).
11. Yamamoto, H., Maurer, K. H. & Hutchinson, C. R. (1986) *J. Antibiot.* **39,** 1304–1313.
12. Wallace, K. K., Han, L., Lobo, S. & Renolds, K. K. (1997) *J. Bacteriol.* **179,** 3884–3891.
13. Bibb, M. J., White, J., Ward, J. M. & Janssen, G. R. (1994) *Mol. Microbiol.* **14,** 533–545.
14. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
15. Fitzgerald, N. B., English, R. S., Lampel, J. S. & Vanden Boom, T. (1998) *Appl. Environ. Microbiol.*, **64,** 1580–1583.
16. Wallace, K. K., Bao, Z.-Y., Dai, H. Digate, R., Schuler, G., Speedie, M. & Reynolds, K. (1995) *Eur. J. Biochem.* **233,** 954–962.
17. Larty, P. A. & Faghih, R. (1993) in *Recent Progress in the Chemical Synthesis of Antibiotics,* eds. Lukacs, G & Ohno, M. (Springer, New York), p. 121.
18. Gandecha, A. R., Large, S. L. & Cundliffe, E. (1997) *Gene* **184,** 197–203.
19. Weissman, K. J., Timoney, M., Bycroft, M., Grice, P., Hanefeld, U., Staunton, J. & Leadlay, P. F. (1997) *Biochemistry* **36,** 13849–13855.