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Biochemistry

An erythromycin analog produced by reprogramming of polyketide synthesis

(enoyl reductase/macrolide/metabolic engineering/*Streptomyces*)

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ABSTRACT **The polyketide-derived macrolactone of the antibiotic erythromycin is made through successive condensation and processing of seven three-carbon units. The fourth cycle involves complete processing of the newly formed β-keto group (β-keto reduction, dehydration, and enoyl reduction) to yield the methylene that will appear at C-7 of the lactone ring. Synthesis of this molecule in *Saccharopolyspora erythraea* is determined by the three large *eryA* genes, organized in six modules, each governing one condensation cycle. Two amino acid substitutions were introduced in the putative NAD(P)H binding motif in the proposed enoyl reductase domain encoded by *eryAII*. The metabolite produced by the resulting strain was identified as $\Delta^{6,7}$-anhydroerythromycin C resulting from failure of enoyl reduction during the fourth cycle of synthesis of the macrolactone. This result demonstrates the involvement of at least the enoyl reductase from the fourth module in the fourth cycle and indicates that a virtually complete macrolide can be produced through reprogramming of polyketide synthesis.**

A wide variety of natural compounds, exhibiting antibacterial, antihelminthic, antitumor, and immunosuppressive activities, contain a polyketide-derived skeleton. Biosynthesis of polyketides is mechanistically equivalent to formation of long-chain fatty acids (1), where the fatty acid synthase (FAS) condenses the extender unit malonate with the starter unit acetate and the resulting β-keto group undergoes three processing steps, β-keto reduction, dehydration, and enoyl reduction, to yield a fully saturated butyryl unit. The C_4 chain is elongated through repeated addition of two carbon atoms (derived from malonate) and fully processed at each cycle, until the proper length of a symmetrical chain has been reached. Many polyketides, in contrast, retain ketone, hydroxyl, or olefinic functions and contain methyl or ethyl side groups interspersed along an acyl chain of length comparable to that of common fatty acids. This asymmetry in structure implies that the polyketide synthase (PKS), the enzyme system responsible for formation of these molecules, although mechanistically equivalent to FAS, must somehow be programmed to produce the correct molecular structure.

The current model (2) for biosynthesis of complex polyketides (defined as compounds whose synthesis requires each FAS-like cycle to be usually different from the previous one) is exemplified, for the erythromycin aglycone DEB (Fig. 1), in Fig. 2. In *Saccharopolyspora erythraea*, three *eryA* genes govern the synthesis of this molecule and consist of six repeated units, termed modules, encoding six synthase units (SUs). We have proposed that each SU, which comprises a series of putative FAS-like activities, is responsible for one of the six elongation cycles required for DEB formation (2). A total of 28 distinct enzymatic activities, each unique for one catalytic event, reside in three large, multifunctional proteins—EryAI, EryAII, and EryAIII—encoded by *eryA* (Fig. 2). Thus, the noniterative processive synthesis of asymmetric acyl chains found in complex polyketides is accomplished through the use of a programmed protein template, where the nature of the chemical reactions occurring at each point is determined by the specificities of the domains contained in each SU.

The involvement of a distinct enzymatic activity in each synthesis step implies that a modification affecting a single activity should perturb only the corresponding step. Such modifications offer the potential for reprogramming the PKS, with the consequent production of novel polyketide structures. If each enzymatic activity can be assigned to a specific step in the biosynthetic pathway, the structure of the newly formed polyketide can be predicted. For DEB synthesis, the order in which the modules are arranged in the *Sac. erythraea* chromosome has been proposed to match the sequence in which the corresponding SUs are employed in the six elongation cycles (2). Previous work showed that deletion of the sequence in *eryAIII* corresponding to the KR domain of the fifth SU resulted in formation of a DEB derivative retaining the keto group introduced during the fifth cycle at the C-5 position, in accordance with the prediction from the model (2). Here we extend our previous finding that modified polyketides of predicted structure can be produced by genetic intervention, by demonstrating that polyketide synthesis can be reprogrammed by abolishing another activity involved in β-carbon processing, resulting in the production of a novel complete macrolide.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Plasmids. *Sac. erythraea* strains ER720 (3) and EER4S, described below, were cultivated as described (2, 4). Conditions for isolation of genomic DNA (5), integrative transformation (4, 6, 7), and gene replacement (8) have been reported. *Sac. erythraea* metabolites were isolated and analyzed by TLC (9). Plasmids were propagated and isolated from *Escherichia coli* DH5α (GIBCO/BRL) by following the supplier's conditions for transformation. Plasmids of the pUC series (10) were routinely employed for subcloning, and the vector pWHM3, which replicates poorly in *Sac. erythraea* (11), was used for integrative transformation. The *eryA* DNA has been described (2, 9).

Purification of $\Delta^{6,7}$-Anhydroerythromycin C. Spores of *Sac. erythraea* strain EER4S were grown as described (12).

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Abbreviations: ACP, acyl carrier protein; DEB, 6-deoxyerythronolide B; DH, dehydratase; ER, enoyl reductase; FAS, fatty acid synthase; KR, β-keto reductase; PKS, polyketide synthase; SU, synthase unit.

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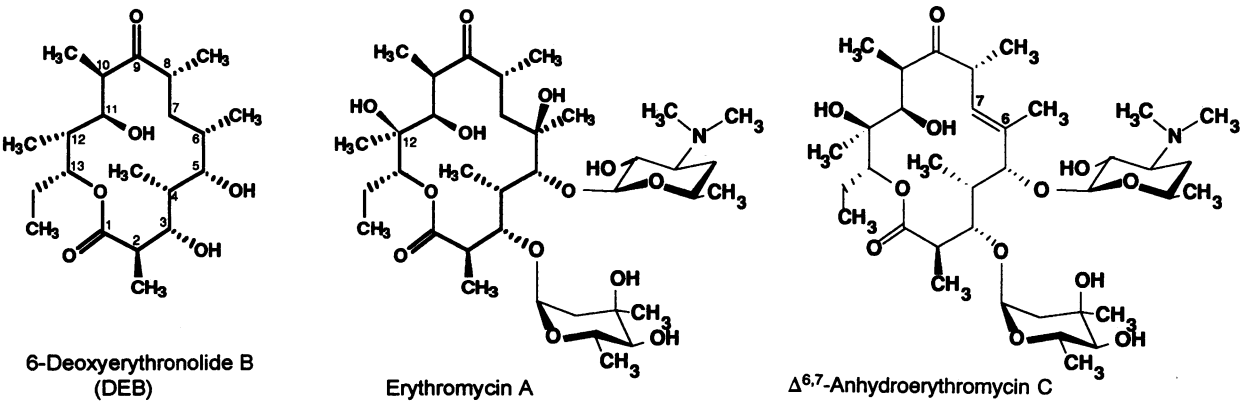

FIG. 1. Structures of 6-deoxyerythronolide B (DEB), erythromycin A, and $\Delta^{6,7}$-anhydroerythromycin C.

After 7 days, the pH was adjusted to 9 with NaOH and the beer was extracted with 2 half-volumes of methylene chloride. The combined concentrated extracts were digested in 1 liter of methylene chloride and extracted with 1 volume of 0.33 M citric acid. The aqueous layer was readjusted to pH 9 and extracted with 1 volume of methylene chloride. This second methylene chloride extract, after water washes and concentration to a residue (176 mg), was chromatographed on an Ito multilayered Planet Coil centrifuge in a system consisting of carbon tetrachloride, methanol, 0.01 M potassium phosphate (pH 7.2), 1:1:1 (vol/vol), with the lower phase stationary. The eluate was monitored by TLC and selected fractions were diluted with an equal volume of water, adjusted to pH 9 with NH_4OH, and extracted twice with equal volumes of methylene chloride, and the residue obtained after drying the combined organic layers was digested in C^2HCl_3 and subjected to ^1H NMR analysis. Fractions found to contain macrolides were combined and similarly extracted to yield 45 mg of a solid residue, which was chromatographed on an Ito multilayered Planet Coil centrifuge in a system consisting of heptane, benzene, 2-propanol, acetone, 0.01 M potassium phosphate (pH 7.0), 5:10:3:2:5, with the upper phase mobile. On the basis of TLC and ^1H NMR analysis, fractions were combined, concentrated, and partitioned between water (adjusted to pH 9) and methylene chloride. The organic phase was separated and concentrated to give a solid residue of $\Delta^{6,7}$-anhydroerythromycin C.

Spectral Determinations. NMR spectra were obtained from $^2HCl_3$ solutions with a General Electric GN500 spectrometer with data acquired at 500 MHz (^1H) and 125 MHz (^{13}C). Mass spectrometry was performed on a Kratos Analytical Instruments MS50 instrument operating in the fast-atom-bombardment positive-ion mode.

RESULTS

Rationale. According to the model for DEB formation (Fig. 2), module 4 governs the fourth elongation cycle, which requires full processing of the β-carbonyl, through the action of KR, DH, and ER, yielding the methylene that appears at the C-7 position of DEB (Fig. 3). The other functions in SU4, the β-ketoacyl-ACP synthase, the acyltransferase, and the ACP, are required for addition of the extender unit methylmalonate to the C_9 chain produced during steps 1–3. The extent of processing that the β-carbonyl will undergo during a particular cycle is predicted to result from the presence of functional domains within the SU governing that cycle. If the altered chain formed after inactivation of one of these domains can be a substrate for further elongation cycles, a modified polyketide with a less processed group at the corresponding position but otherwise identical to the native chain should be produced. Thus inactivation of the ER domain was predicted to result in formation of $\Delta^{6,7}$-anhydroerythronolide B. Since this macrolactone retains the C-3 and C-5 hydroxyls, the sites of deoxysugar attachment, the altered polyketide was expected to be a substrate for subsequent modification steps normally seen in the synthesis of erythromycin, leading to formation of a novel macrolide containing an unsaturation between C-6 and C-7.

Within the ER domain the putative NAD(P)H binding motif HAAAGGVGMA[2973] [the start codon for *eryAII*, as determined by Caffrey *et al.* (13), is set at 1] was identified by

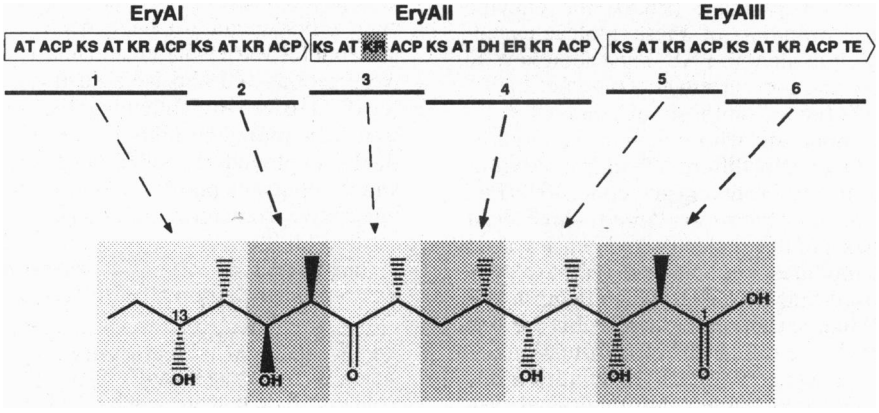

FIG. 2. Model for DEB synthesis. (*Upper*) The three *eryA*-encoded polypeptides and the FAS-like domains they contain. ACP, acyl carrier protein; AT, acyltransferase; DH, dehydratase; ER, enoyl reductase; KR, β-keto reductase; KS, β-ketoacyl-ACP synthase; TE, thioesterase. Note the shading of the KR in SU3 to denote a dysfunctional activity. Bars show the extent of each SU. (*Lower*) The completed polyketide chain. Arrows indicate the portions assembled by each SU.

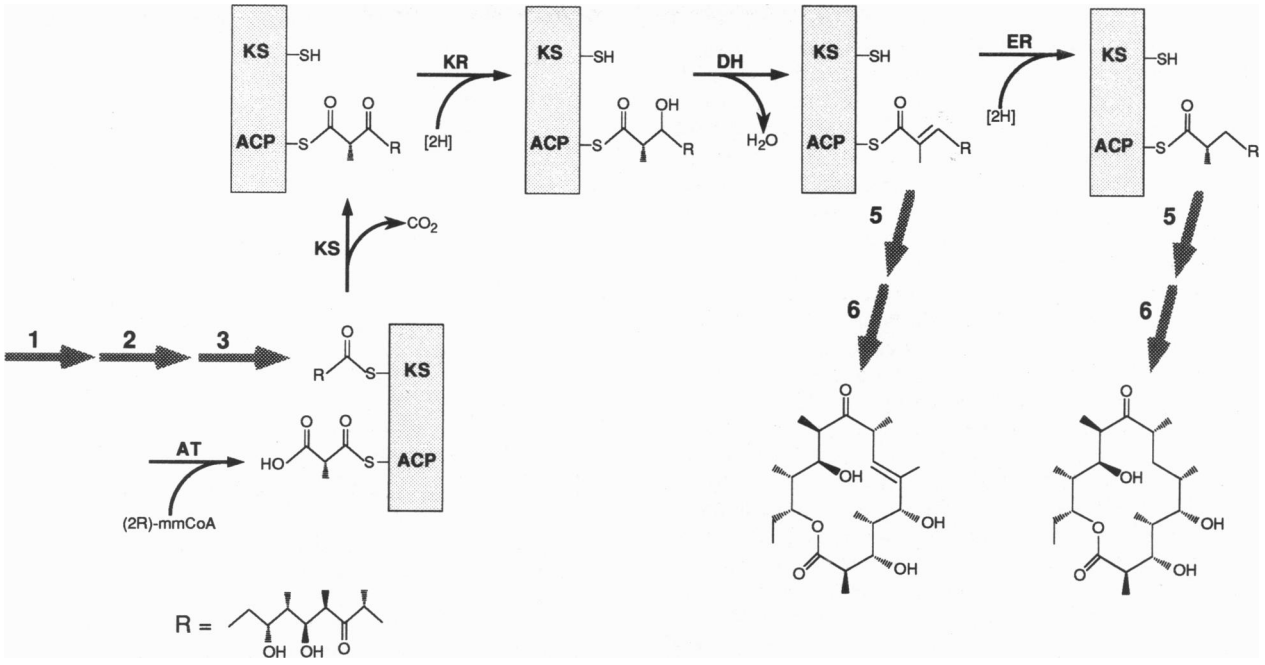

FIG. 3. Proposed role of SU4 in DEB synthesis. The reactions catalyzed by the individual domains in wild-type SU4 and in a strain where the enoyl reduction step is bypassed are illustrated. The proposed lactone ring formed in the latter case is also shown along with DEB. mmCoA, methylmalonyl-coenzyme A; for other abbreviations, refer to Fig. 2.

sequence comparison (14–16). This motif bears considerable similarity with the apparent consensus TGGtgglGxel (uppercase letters refer to invariant residues) found in five of the six *eryA*-encoded KR domains and proposed as the corresponding nucleotide binding site (2, 14, 16). The KR domain present in SU3, instead, was believed to be dysfunctional due to the presence of the sequence TGAASPVGDQL[1147] in place of the consensus, where two adjacent glycine residues are replaced by a serine and a proline (2). Visual inspection of the nucleotide-binding pocket in human glutathione reductase (17) suggested that introduction of both the hydroxyl side chain of serine and the turn imposed by proline would disrupt the binding cleft and impede accommodation of NAD(P)H. Thus, one possible strategy to inactivate the ER domain, in the absence of structural information, would be to change the sequence HAAAGGVGMA[2973] to HAAASPVGMA. Since the same two amino acid substitutions are encountered in the supposedly inactive KR domain within the same polypeptide, a reasonable prediction is that they would not perturb the ER domain structure in such a way as to affect the overall conformation of other domains.

Mutant Construction. The *eryAII* sequence GCAGGCGGTGTC[8910], encoding part of the ER domain, was changed to GCTAGCCCTGTC *in vitro* by replacing four bases to change the corresponding protein sequence and introduce an *Nhe* I site as a marker for the mutant allele (Fig. 4). The mutated segment was used to replace the chromosomal counterpart in *Sac. erythraea* by two plasmid-mediated, single reciprocal recombination events. Five independent isolates, when analyzed by Southern hybridization, lacked the 1.8-kb wild-type *Nhe* I band and exhibited two novel bands of 0.94 and 0.90 kb, indicating the presence of the mutant allele (data not shown). One of these isolates was selected for further studies and designated strain EER4S.

Metabolite Analysis. An ethyl acetate extract of the fermentation broth of EER4S contained very little bioactivity when compared with the parent strain grown under similar conditions. TLC analysis indicated the existence of two novel spots, with migration coefficients related to, but clearly distinct from, those of 3-α-mycarosylerythronolide B and erythromycin C, reference metabolites produced by the parent strain. The major alkaline compound produced by

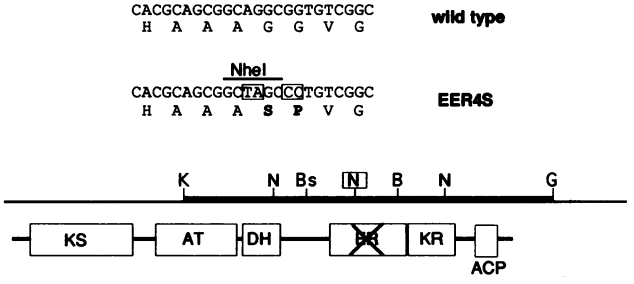

FIG. 4. Construction of strain EER4S. (*Upper*) Wild-type and mutant sequences. Changed nucleotides are boxed and corresponding changed amino acids are in bold type. (*Lower*) Mutant module 4 and the domains it encodes (see Fig. 2 for abbreviations), including the inactivated ER domain. Line represents the insert in plasmid pAER4S5 with relevant restriction sites. The newly introduced *Nhe* I site is boxed. B, *Bam*HI; Bs, *Bsp*EI; G, *Bgl* II; K, *Kpn* I; N, *Nhe* I. Two pairs of oligonucleotide primers (5′-CTGCGGTTCCGGACAACCC and 5′-ACACCGCTAGCCGCTGCGTGGATG; 5′-GCAGCGGCTAGCCCTGTCGGCATGGCGG and 5′-TTTTTGGATCCCAGCTCCCACGCC) were employed to amplify the *eryAII* segments between 8.37 and 8.91 kb and between 8.89 and 9.34 kb, respectively, and simultaneously introduce the desired changes. The resulting 0.54- and 0.45-kb fragments were digested with *Bsp*EI and *Nhe* I and with *Nhe* I and *Bam*HI, respectively, and ligated to *Bsp*EI/*Bam*HI-digested pAIK3, a pUC19 subclone carrying the 3.6-kb *Kpn* I fragment comprising the 3′ end of *eryAII* (only the 5′ *Kpn* I site is shown), to yield plasmid pAER4S. After verification of the predicted nucleotide sequence of the PCR-generated segment, the 2.5-kb *Eco*RI–*Bam*HI fragment from pAER4S (the *Eco*RI site comes from the pUC19 polylinker and is next to the *Kpn* I site shown) was ligated to a pWHM3 subclone carrying, at the *Bam*HI site and in the appropriate orientation, the 1.7-kb *Bam*HI–*Bgl* II fragment, to give plasmid pAER4S5. This latter construct was used to integratively transform *Sac. erythraea* ER720, selecting for resistance to thiostrepton. From one randomly picked colony, containing the plasmid integrated at the homologous site, 16 thiostrepton-sensitive segregants were obtained and examined for their genotype by Southern hybridization.

EER4S was identified as $\Delta^{6,7}$-anhydroerythromycin C (Fig. 1) from mass spectrometry and NMR analysis. The former gave an $(M+H)^+$ ion at M/Z 702 and an $(M+Na^+)$ ion at M/Z 724, 32 units lower than those obtained from erythromycin A. A complete assignment of the ^1H and ^{13}C NMR spectra was possible with the aid of correlated spectroscopy, heteronuclear multiple quantum correlation (18), and heteronuclear multiple bond correlation (19) experiments (data not shown). Most of the spectral values were very similar to those measured on 6-deoxyerythromycin C (J.B.M., unpublished data), an erythromycin derivative produced by genetic engineering (20). The spectral values showing significant difference between $\Delta^{6,7}$-anhydroerythromycin C and 6-deoxyerythromycin C are reported in Tables 1 and 2. The patterns of H-13 as a doublet of doublets and of the 12-methyl as a singlet, combined with the absence of an *O*-methyl singlet in the ^1H NMR spectrum, indicate that the product is an analog of erythromycin C. The 6,7-olefin is clearly evident by the deshielded H-7 signal at δ 5.78, which shows allylic coupling ($\approx$2 Hz) to the 6-methyl protons resonating at δ 1.88. Similarly, in the ^{13}C NMR spectrum the C-6 and C-7 are strongly deshielded and occur in the olefinic region at δ 142.1 and 125.2, respectively. Tables 1 and 2 show that significant differences between $\Delta^{6,7}$-anhydroerythromycin C and 6-deoxyerythromycin C are limited to carbons directly or indirectly linked to C-6 and C-7, and to hydrogens sterically close to these positions. One apparent exception may be represented by the 13 position, where both H and C values differ between the two compounds. The reason for this is not understood.

The configuration of the olefin was shown to be *E* by the strong cross peak between the H-8 and the 6-methyl protons in a rotating-frame Overhauser effect spectroscopy (ROESY) (21) experiment. For H-8, this was the only cross peak seen without antiphase coupling artifacts (e.g., to H-7 and the 8-methyl protons). The ROESY experiment also showed strong cross peaks of H-7 to H-10, H-4, and H-11 and weaker cross peaks to H-5 and H-1′ (data not shown). These interactions confirm the configuration of the olefin and indicate that the conformation of the macrolide ring is such as to direct H-7 toward the center of the ring. Hence, the structure shown in Fig. 1 represents a reasonable planar depiction of the geometry of the olefin.

The new macrolide isolated exhibited very weak bioactivity against a few Gram-positive bacteria and was generally 500 and 200 times less potent than erythromycins A and C, respectively (data not shown). This finding is not surprising, since all known 14- and 16-membered macrolides contain a methylene group at a position equivalent to C-7 in erythromycin (22). The amount of $\Delta^{6,7}$-anhydroerythromycin C produced by EER4S was estimated from the bioactivity

Table 1. Selected ^1H NMR spectral assignments for $\Delta^{6,7}$-anhydro- and 6-deoxyerythromycin C

Proton	$\Delta^{6,7}$-Anhydro	6-Deoxy
H-4	$\approx$1.75 m	1.60
H-5	3.84 d	3.42
H-6	—	1.33
H-7	5.78 dd	1.57, 1.68
H-8	3.39 dq	2.62
H-13	4.84 dd	4.94
6-CH_3	1.88 d	1.18
H-1′	4.12 d	5.03
H-1″	4.96 brd	4.17
H-5″	3.91 m	3.76

Values are reported as δ (ppm) from tetramethylsilane. Only values differing by $\geq$0.1 between the two compounds are shown. brd, Broad doublet; d, doublet; dd, doublet of doublets; dq, doublet of quartets; m, multiplet.

Table 2. Selected CMR spectral assignments for $\Delta^{6,7}$-anhydro- and 6-deoxyerythromycin C

Carbon no.	$\Delta^{6,7}$-Anhydro	6-Deoxy
2	45.7	45.2
4	46.9	44.0
5	83.5	84.2
6	142.1	35.2
7	125.2	34.2
8	47.6	45.8
9	215.5	220
10	41.4	38.7
13	78.7	76.4
4-CH_3	10.8 or 11.0*	9.7
5′	70.0	69.5
1″	100.0	99.2

Values are reported as δ (ppm) from tetramethylsilane. Only values differing by $\geq$0.5 between the two compounds are shown.
*Assignment of 4-CH_3 and 10-CH_3 may be interchanged.

detected in a concentrated extract and found to be approximately one-fifth the quantity of erythromycin A produced by the parent strain grown under similar conditions. That all the bioactivity resulted from $\Delta^{6,7}$-anhydroerythromycin C was confirmed by bioautoradiography, after resolution of macrolides by TLC (data not shown). This crude estimate suggests that the engineered PKS produced the altered polyketide at a level within an order of magnitude of that of the parent macrolide produced by the native enzyme.

DISCUSSION

We have shown here that a complete macrolide, differing from erythromycin C only by the presence of a 6,7 double bond, can be produced *in vivo* by an altered PKS obtained by disabling the ER domain from module 4. Consistent with the model for complex polyketide synthesis, only one step was affected by this mutation. To date, a few novel polyketides have been produced by genetic engineering, and most through intervention in the post-polyketide aspects of the pathways (20, 23–25). Only two altered polyketide skeletons have been produced through genetic intervention: 2-nor-DEB, produced by an as yet unclear mechanism in a strain lacking a functional acyltransferase from SU6 (12), and 5-deoxy-5-oxo-DEB, obtained by deletion of the KR domain from module 5 (2). In contrast, $\Delta^{6,7}$-anhydroerythromycin C represents a virtually complete macrolide of predicted structure produced by reprogramming of polyketide synthesis. This demonstrates not only that an altered polyketide chain can be produced and correctly lactonized but also that most of the post-polyketide modifications can occur on an altered lactone (see Fig. 1). The two previous examples dealt with the terminal (2-nor-DEB) or penultimate step (5-deoxy-5-oxo-DEB) in lactone synthesis; with the work described here, the altered polyketide chain has been further elongated by two distinct SUs. Thus it appears that the structure of the nascent chain may not play a critical role in polyketide synthesis and at least some altered polyketides can be substrates for further cycles. This implies that the correct transfer of the growing chain from one SU to the next, either intraprotein as in the case of 5-deoxy-5-oxo-DEB or interpolypeptide as in this example, may reside more in the specific juxtaposition of the various domains than in the specificity of a particular SU for the structure of the approaching acyl chain.

In previous work we provided direct evidence that keto reduction in synthesis step 5 is brought about by the KR domain of SU5 (2). This work has demonstrated that the methylene group present at C-7 of DEB is introduced via reduction of the double bond formed during step 4 and that the segment proposed as the ER domain in SU4 (14, 15) is

responsible for this reduction. Thus, one domain each from SU4 (ER), SU5 (KR), and SU6 (acyltransferase) has been directly demonstrated to be involved in the corresponding synthesis step, strengthening the argument of colinearity between genetic order of the modules and sequence of condensation cycles. Within the ER domain, the predicted NAD(P)H-binding motif must be essential for activity, since as few as two amino acid substitutions abolish this function. Because these same two amino acid changes are also encountered in the putative NAD(P)H-binding motif in the KR domain of SU3, the findings presented here provide additional support to the original notion that this KR is inactive (2), thus accounting for retention of a keto group during cycle 3 (Fig. 2), and that the lack of nucleotide binding is, at least in part, responsible for the inactivity.

Recent analysis of genes involved in avermectin formation has indicated that the corresponding PKS is organized similarly to that for the DEB PKS (26, 27). These two enzyme systems, recently referred to as modular PKSs (28) to distinguish them from those operating in processes that are mostly iterative, present a certain flexibility in the primary structure of SUs introducing a common degree of β-carbon processing. In avermectin synthesis, where five double bonds are retained, the DH-containing SUs completely lack ER domains (27). In the erythromycin PKS, a small change in the ER domain is sufficient to eliminate this activity, but a functional PKS deleted of the entire ER domain has not been successfully generated (S.D., unpublished work). Similarly, a keto group is retained in avermectin synthesis by a SU completely lacking a KR domain (27); for erythromycin, KR function can be lost either through the presence of a few point mutations, as in SU3, or after purposeful removal of this domain without noticeable effects on the other activities residing in the PKS (2).

Formation of $\Delta^{6,7}$-anhydroerythromycin C indicates that the lactone ring carrying the 6,7 unsaturation is a substrate for the post-polyketide modification enzymes: mycarosyl- and desosaminyltransferases and C-12 hydroxylase. These three reactions have thus far been observed with several ring structures in genetically modified *Sac. erythraea* (2, 12, 20), even when the alterations were very close to the site of modification. On the other hand, the isolation of a macrolide with an unmodified mycarose moiety in the present study indicates that the *O*-methyltransferase is unable to methylate the 3″ position of the unsaturated macrolide. This finding was not totally unexpected, as this enzyme is quite sensitive to small perturbations in substrate structure (29).

The erythromycin PKS represents a good model system for reprogramming polyketide synthesis. The entire sequence of the corresponding genes is known (2, 15, 30) and a role for each enzymatic domain has been proposed (2). In addition, methodologies for gene transfer in *Sac. erythraea* are available and the order of occurrence of the post-polyketide modifications is known. Thus, engineering the erythromycin pathway through PKS reprogramming offers the challenging opportunity of understanding the rules governing complex polyketide synthesis while producing, *in vivo*, novel macrolide structures.

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1. Hopwood, D. A. & Sherman, D. H. (1990) *Annu. Rev. Genet.* **24,** 37–66.
2. Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J. & Katz, L. (1991) *Science* **262,** 675–679.
3. DeWitt, J. P. (1985) *J. Bacteriol.* **164,** 969–971.
4. Donadio, S., Shafiee, A. & Hutchinson, C. R. (1990) *J. Bacteriol.* **171,** 350–360.
5. Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. & Schrempf, H. (1985) *Genetic Manipulation of Streptomyces: A Laboratory Manual* (John Innes Foundation, Norwich, U.K.).
6. Kieser, T. & Hopwood, D. A. (1991) *Methods Enzymol.* **204,** 430–458.
7. Weber, J. M. & Losick, R. (1988) *Gene* **68,** 173–180.
8. Donadio, S. & Hutchinson, C. R. (1991) *Gene* **100,** 231–235.
9. Tuan, J. S., Weber, J. M., Staver, M. J., Leung, J. O., Donadio, S. & Katz, L. (1990) *Gene* **90,** 21–29.
10. Vieira, J. & Messing, J. (1982) *Gene* **19,** 259–268.
11. Vara, J. A., Lewandoska-Skarbek, M., Wang, Y.-G., Donadio, S. & Hutchinson, C. R. (1989) *J. Bacteriol.* **170,** 5872–5881.
12. McAlpine, J. B., Tuan, J. S., Brown, D. P., Grebner, K. D., Whittern, D. N., Buko, A. & Katz, L. (1987) *J. Antibiotics* **40,** 1115–1122.
13. Caffrey, P., Bevitt, D. J., Staunton, J. & Leadlay, P. F. (1992) *FEBS Lett.* **304,** 225–228.
14. Donadio, S. & Katz, L. (1992) *Gene* **111,** 51–60.
15. Bevitt, D. J., Cortes, J., Haydock, S. F. & Leadlay, P. F. (1992) *Eur. J. Biochem.* **204,** 39–49.
16. Witkowski, A., Rangan, V. S., Randhawa, Z. I., Amy, C. M. & Smith, S. (1991) *Eur. J. Biochem.* **198,** 571–579.
17. Scrutton, N. S., Berry, A. & Perham, R. N. (1990) *Nature (London)* **343,** 38–44.
18. Muller, L. (1979) *J. Am. Chem. Soc.* **101,** 4481–4484.
19. Bax, A. & Summers, M. F. (1986) *J. Am. Chem. Soc.* **108,** 2093–2094.
20. Weber, J. M., Leung, J. O., Swanson, S. J., Idler, K. B. & McAlpine, J. B. (1991) *Science* **252,** 114–117.
21. Bothner-By, A. A., Stephens, R. L., Lee, J. M., Warren, C. D. & Jeanloz, R. W. (1984) *J. Am. Chem. Soc.* **106,** 811–813.
22. Omura, S. (1984) in *Macrolide Antibiotics: Chemistry, Biology and Practice*, ed. Omura, S. (Academic, Orlando, FL).
23. Epp, J. K., Huber, M. L. B., Turner, J. R., Goodson, T. & Schoner, B. E. (1989) *Gene* **85,** 293–301.
24. Hara, O. & Hutchinson, C. R. (1992) *J. Bacteriol.* **174,** 5141–5144.
25. Hopwood, D. A., Malpartida, F., Kieser, H. M., Ikeda, H., Duncan, J., Fujii, I., Rudd, B. A., Floss, H. G. & Omura, S. (1985) *Nature (London)* **314,** 642–644.
26. MacNeil, D. J., Occi, J. L., Gewain, K. M., MacNeil, T., Gibbons, P. H., Ruby, C. L. & Danis, S. J. (1992) *Gene* **115,** 119–125.
27. MacNeil, D. J., Occi, J. L., Gewain, K. M., MacNeil, T., Gibbons, P. H., Foor, F. & Morin, N. (1993) in *Genetics and Molecular Biology of Industrial Microorganisms*, eds. Baltz, R. H., Ingolia, T. & Hegeman, G. (Am. Soc. Microbiol., Washington, DC), in press.
28. Hopwood, D. A., Khosla, C., Sherman, D. H., Bibb, M. J., Ebert-Khosla, S., Kim, E.-S., McDaniel, R., Revill, W. P., Torres, R. & Yu, T.-W. (1993) in *Genetics and Molecular Biology of Industrial Microorganisms*, eds. Baltz, R. H., Ingolia, T. & Hegeman, G. (Am. Soc. Microbiol., Washington, DC), in press.
29. Paulus, T. J., Tuan, J. S., Luebke, V. E., Maine, G. T., Dewitt, J. P. & Katz, L. (1990) *J. Bacteriol.* **172,** 2541–2546.
30. Cortes, J., Haydock, S. F., Roberts, G. A., Bevitt, D. J. & Leadlay, P. F. (1991) *Nature (London)* **346,** 176–178.