The loading domain of the erythromycin polyketide synthase is not essential for erythromycin biosynthesis in *Saccharopolyspora erythraea*

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6-Deoxyerythronolide B synthase (DEBS) is a large multifunctional enzyme that catalyses the biosynthesis of the erythromycin polyketide aglycone. DEBS is organized into six modules, each containing the enzymic domains required for a single condensation of carboxylic acid residues which make up the growing polyketide chain. Module 1 is preceded by loading acyltransferase (AT-L) and acyl carrier protein (ACP-L) domains, hypothesized to initiate polyketide chain growth with a propionate-derived moiety. Using recombinant DNA technology several mutant strains of *Saccharopolyspora erythraea* were constructed that lack the initial AT-L domain or that lack both the AT-L and ACP-L domains. These strains were still able to produce erythromycin, although at much lower levels than that produced by the wild-type strain. In addition, the AT-L domain expressed as a monofunctional enzyme was able to complement the deletion of this domain from the PKS, resulting in increased levels of erythromycin production. These findings indicate that neither the initial AT-L nor the ACP-L domains are required to initiate erythromycin biosynthesis; however, without these domains the efficiency of erythromycin biosynthesis is decreased significantly. It is proposed that in these mutants the first step in erythromycin biosynthesis is the charging of KS1 with propionate directly from propionyl-CoA.

Keywords: *Streptomyces*, polyketide synthase, loading domain

INTRODUCTION

Polyketides are a large and diverse class of natural products that includes antibiotic, anticancer, anti-parasite, pigment and immunosuppressant compounds produced principally by *Streptomyces* and related filamentous bacteria. Biosynthesis of polyketides occurs by a mechanism that is similar to fatty acid biosynthesis but with some differences (Hopwood & Sherman, 1990; Katz & Donadio, 1993; Molnar *et al.*, 1993; Hutchinson & Fujii, 1995). Both polyketide synthases (PKSs) and

fatty acid synthases catalyse decarboxylative condensations between a thioester-linked nascent carbon chain and short-chain fatty acid extender units. PKSs, however, are considerably more diverse in the reactions that they can catalyse. This includes the use of different starters (acetate, propionate, butyrate) and chain extender units (malonate, methylmalonate, ethylmalonate) at various steps. Moreover, PKSs vary the extent of processing of the β -carbon after every condensation. Whereas the condensations of fatty acid biosynthesis are typically followed by a trio of reactions (β -ketoreduction, dehydration, enoylreduction) to give a fully saturated carbon chain, a PKS may employ some, all, or none of these reactions after each condensation, giving rise to a highly functionalized carbon chain (Hopwood & Sherman, 1990).

Erythromycin, a broad-spectrum macrolide antibiotic produced by *Saccharopolyspora erythraea*, is composed

Abbreviations: ACP, acyl carrier protein; ACP-L, ACP of loading domain; AT, acyltransferase; AT-L, AT of loading domain; DEB, 6-deoxyerythronolide B; DEBS, DEB synthase; DH, dehydratase; ER, enoyl reductase; *eryA*, genes encoding DEB synthase; KR, β -ketoreductase; KS, β -ketoacyl ACP synthase; PK, polyketide; PKS, polyketide synthase; RBS, ribosome-binding site.

Fig. 1. Organization of the erythromycin PKS: 6-deoxyerythronolide B (DEB) synthases 1, 2 and 3 (DEBS1, 2, 3). The domains are designated as follows: acyltransferase, AT; β -ketoacyl carrier protein synthase, KS; acyl carrier protein, ACP; β -ketoreductase, KR; dehydratase, DH; enoyl reductase, ER; thioesterase, TE. The loading domain of module 1, comprising AT-L and ACP-L, is boxed.

of a polyketide-derived macrocyclic lactone ring, 6-deoxyerythronolide B (DEB), onto which two sugars, L-mycarose and D-desosamine, are attached at C-3 and C-5, respectively. The DEB core of erythromycin is derived from six successive condensations between a propionyl-CoA starter and six (2S)-methylmalonyl-CoA extenders (Marsden *et al.*, 1994). The DEB synthase (DEBS) is a multifunctional, or type I, PKS, encoded by three large genes, *eryAI*, *eryAII* and *eryAIII* (Cortes *et al.*, 1990; Donadio *et al.*, 1991; Donadio & Katz, 1992; Caffrey *et al.*, 1992; Bevitt *et al.*, 1992). The three proteins encoded by the *eryA* genes are organized in modules (Donadio & Katz, 1992; Ruby & Danis, 1992; Swan *et al.*, 1994; Schwecke *et al.*, 1995). Each module contains the enzymic functions, as separate domains, for one of the six elongation cycles required for DEB formation. These include the β -ketoacyl-ACP synthase domain (KS), the acyltransferase domain (AT), and the acyl carrier protein domain (ACP). Also present may be β -ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains, responsible for the different degrees of reduction of the newly formed β -carbonyl group after each condensation step. In sum, DEBS comprises 28 enzymic activities, each dedicated to a single catalytic event (Fig. 1).

The PKS governs the selection of the starter molecule, the number and type of extender units added to the

growing chain, and the extent and stereochemistry of reduction at each cycle, contributing to the structural variation found in polyketides. In modular PKSs it appears that the order of functional domains largely reflects the sequence of biochemical reactions (Donadio *et al.*, 1991). Recently, the involvement of a distinct enzymic domain for each synthetic step has been exploited by genetically modifying specific domains, thereby reprogramming the PKS to produce novel polyketide structures (Donadio *et al.*, 1993; Olinynyk *et al.*, 1996; Bedford *et al.*, 1996; Kuhstoss *et al.*, 1996; Ruan *et al.*, 1997b).

The N-terminal end of module 1 of DEBS contains two additional enzymic domains, AT-L and ACP-L (Donadio *et al.*, 1991). The AT-L domain is thought to initiate polyketide synthesis by choosing and transferring propionate from propionyl-CoA to the pantotheine cofactor of ACP-L. From here the propionate moiety is delivered to the active-site cysteine of the ketosynthase domain of module 1 (KS1). This work describes efforts to further define the constraints on PKS reprogramming by examining the role of the initial domains in the erythromycin PKS. Here we report the construction of a DEBS that lacks the initial acyltransferase domain and another in which both AT-L and the first acyl carrier protein, ACP-L, have been deleted. Both DEBS mutants still produce erythromycin A, although

in lower yield than the wild-type. This suggests that the KS1 domain directly incorporates propionate from propionyl-CoA when the AT-L is absent in the mutant strains.

METHODS

Bacterial strains, plasmids and growth media. *Saccharopolyspora erythraea* ER720 (Dewitt, 1985) is the erythromycin-producing strain. Integrative transformation of *S. erythraea* protoplasts and routine growth and sporulation were carried out according to described procedures (Yamamoto *et al.*, 1986; Donadio *et al.*, 1991; Weber *et al.*, 1991). Competent *Escherichia coli* DH5 α and the vectors pUC19, M13mp18 and M13mp19 were obtained from Bethesda Research Laboratories. The plasmid pSL1180 was purchased from Pharmacia Biotech. Plasmid pWHM3 (Vara *et al.*, 1989) is a *Streptomyces-E. coli* shuttle vector that is poorly maintained episomally in *Saccharopolyspora erythraea*. Plasmid pWHM4 (Vara *et al.*, 1989) is also a *Streptomyces-E. coli* vector that can replicate in *Saccharopolyspora erythraea*. Plasmids constructed for this study are listed in Table 1. For growth of *S. erythraea* strains in liquid culture, either SGGP medium (Yamamoto *et al.*, 1986) or SCM medium (per litre of distilled H₂O: soytone, 20 g; soluble starch, 15 g; MOPS, 10.5 g; yeast extract, 1.5 g; CaCl₂, 0.1 g) was used. For the growth of *S. erythraea* strains on plates, R3M was used [1 litre contains sucrose, 103 g; K₂SO₄, 0.25 g; yeast extract, 4 g; Casamino acids, 4 g; tryptone, 4 g; agar, 22 g; H₂O, 830 ml; after sterilization the following solutions are added: 20 ml 2.5 M MgCl₂, 20 ml 50% glucose, 20 ml 2.5 M CaCl₂, 12.5 ml 2 M Tris/HCl pH 7.0, 0.2 ml of a 5000 $\times$ concentrated trace elements solution (Hopwood *et al.*, 1985), 2.5 ml 1 M NaOH, and 0.37 ml 0.5 M KH₂PO₄]. When selection of thiostrepton-resistant *S. erythraea* strains was required, 10 μ g thiostrepton

ml⁻¹ (Sigma) was used in liquid culture and 25 μ g thiostrepton ml⁻¹ for growth on plates. Plasmid-containing *E. coli* strains were grown in Luria Broth (LB) (Sambrook *et al.*, 1989) supplemented with 150 μ g ampicillin ml⁻¹ (Sigma). For bioassays, antibiotic medium 11 (Difco-Bacto) containing *Staphylococcus aureus* as an indicator strain was used.

DNA manipulation. Plasmid isolations from *E. coli*, restriction digestion, ligation, agarose gel electrophoresis, recovery of DNA from agarose gels, and Southern hybridization were done as described by Sambrook *et al.* (1989). Restriction enzymes and T4 DNA ligase were purchased from BRL/Life Technologies or New England Biolabs. Plasmid and chromosomal DNA from *S. erythraea* were isolated according to Hopwood *et al.* (1985). The Amersham Megaprime DNA labelling system was used for labelling of DNA fragments with [α -³²P]dCTP (Amersham). For PCR, oligonucleotide primers (described in Table 2) were used with *S. erythraea* chromosomal DNA or pATD600 (Table 1) as the template. A typical PCR reaction to amplify the characteristically high GC content actinomycete DNA was as follows. A 100 μ l reaction mixture contained 10 μ l 10 $\times$ PCR buffer (New England Biolabs), 16 μ l 1.25 mM dNTP mixture, 2 mM MgSO₄, 2% (v/v) glycerol, 10% formamide, 100 pmol of each primer and 100 ng template DNA. After 2 min incubation at 100 $^{\circ}$ C, 2 units Vent_r DNA Polymerase (New England Biolabs) was added, and then 30 cycles, each consisting of 20 s denaturation at 95 $^{\circ}$ C, 20 s annealing at 67 $^{\circ}$ C and a 2 min extension at 72 $^{\circ}$ C, were performed in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus).

Sequencing of the DNA fragments obtained by PCR was done with Sequenase 2.0 using 7-deaza-dGTP (United States Biochemical) according to the manufacturer's instructions and [α -³²P]dCTP. The Wisconsin Sequence Analysis Package program (Devereux *et al.*, 1984) was used for sequence analysis.

Table 1. Plasmids used in this study

Plasmid	Characteristics
pATD600	A pSL1180 derivative containing a 5.8 kb <i>EcoRI</i> – <i>EcoRV</i> fragment that includes the codons for the entire N-terminal region of DEBS1 (the 107 aa segment, AT-L, ACP-L, KS1 and a portion of AT1)
pATD607	A pWHM3 derivative containing a 4.45 kb fragment that includes a portion of the codons for the N-terminal region of DEBS1 (ACP-L, KS1 and the beginning of AT1, but not the 107 aa segment or AT-L); used to construct DEBS1-M1
pATD608	A pWHM3 derivative containing a 4.2 kb fragment that includes a portion of the codons for the N-terminal region of DEBS1 (KS1 and the beginning of AT1, but not the 107 aa segment, AT-L or ACP-L); used to construct DEBS1-M2
pATD615	A pWHM3 derivative containing a 4.1 kb fragment that includes a modified portion of the N-terminal region of DEBS1 (eryKS1 replaced by nidKS1) but lacks the codons for the 107 aa segment, AT-L and ACP-L; used to construct DEBS1-M7
pATD622*	A pWHM4 derivative containing the <i>ermE</i> * promoter
pATD623*	A pWHM4 derivative containing the <i>ermE</i> * promoter and the codons for the 107 aa fragment plus the AT-L domain
pATD624*	A pWHM4 derivative containing the <i>ermE</i> * promoter and the codons for the AT-L domain

* These plasmids were used in the complementation experiments.

Table 2. PCR primers designed for the construction of the plasmids used in this work

No.	Description	Oligonucleotide sequence
I	pATD607 N-terminal	5'-TTTTTTGAATTCACGCGTGGGCGTCGACTGGAACGC-3' <i>EcoRI MluI M</i>
II	pATD608 N-terminal	5'-TTTTTTGAATTCACGCGTGGCACAACGGGAAACCCGC-3' <i>EcoRI MluI M</i>
III	pATD607-8 C-terminal	5'-TTTTTTGGATCCGAGCTCGAGCATGAGCCGCTGCTG-3' <i>BamHI SstI</i>
VI	pATD614 N-terminal	5'-TTTTTTGAATTCACGCGTGTGTTCGTCTTCCCGGG-3' <i>EcoRI MluI M</i>
VII	pATD614 C-terminal	5'-TTTTTTGGATCCACGCGTCCATCGCGCCGCGAAAAAC-3' <i>BamHI MluI</i>
VIII	pATD622 permE* N-terminal	5'-TTTTTTAAGCTTGCGAGTGTCCGTTTCGAGT-3' <i>HindIII</i>
IX	pATD622 permE* C-terminal	5'-TTTTTTCTGCAGCGCTGGATCCTACCAACC-3') <i>PstI</i>
X	pATD623 Upstream N-terminal	5'-TTTTTTGAATTCCTGCAGAGGAGGGCCTGCTCATGGCGGACCTGTCAAAG-3' <i>EcoRI PstI RBS M</i>
XI	pATD623-24 Upstream C-terminal	5'-TTTTTTGGATCCACGCGTCCATCGCGGCGGCGA-3' <i>BamHI MluI</i>
XII	pATD624 Upstream N-terminal	5'-TTTTTTGAATTCCTGCAGAGGAGGGCCTGCTCATGGTGTTCGTCTTCCCGGGC-3' <i>EcoRI PstI RBS M</i>
XIII	pATD623-24 Downstream N-terminal	5'-TTTTTTGAATTCGCATGCGGCGGTTCTCTGCTGGCCG-3' <i>EcoRI SphI</i>
XIV	pATD623-24 Downstream C-terminal	5'-TTTTTTGGATCCTCTAGATTAGCCGGAATCGGCCGGCTC-3' <i>BamHI XbaI *</i>
XVII	pATD615 nidKS1 N-terminal	5'-TTTTTTGAATTCACGCGTCGTCAACGGCGAGACC-3' <i>EcoRI MluI</i>
XVIII	pATD615 nidKS1 C-terminal	5'-TTTTTTGGATCCACGCGTGT TCCCGTTGTGCGAC-3' <i>BamHI NsiI</i>
XIX	pATD615 eryAT1 N-terminal	5'-TTTTTTCTGCAGATGCATGCCATCATCGAGGAAGCT-3' <i>PstI NsiI</i>
XX	pATD615 eryAT1 C-terminal	5'-TTTTTTGGATCCGATATCCCTGCTCGGCGAGCGCAC-3' <i>BamHI EcoRV</i>

Construction of DEBS mutant strains. Plasmids pATD607, pATD608, pATD614 and pATD615 (Table 1) were constructed to produce specific deletions at the beginning of the *eryAI* gene. To prepare these plasmids, segments of DNA upstream and downstream of the region to be deleted in *eryAI* were amplified by PCR, and the amplified fragments were then ligated together (using restriction enzyme sites in the PCR primers that maintain the correct translational reading frame) in plasmid pWHM3. Several deletions were constructed taking advantage of our current understanding of the organization of the DEBS1 protein. Translation of DEBS1 initiates 107 amino acids upstream of the first recognizable enzymic domain, AT-L (Caffrey *et al.*, 1992). AT-L is presumed to determine the choice of the starter unit for DEB biosynthesis (Donadio *et al.*, 1991; Aparicio *et al.*, 1994). In plasmid pATD607, the codons for all of AT-L plus the 107 aa upstream segment have been deleted (346 codons in total). To construct pATD607, an *MluI*-*SstI* fragment (corresponding to nt 11540-12138: GenBank accession no. M63676) was PCR-amplified using

primers I and III (Table 2). The resulting fragment was then used to replace the native *MluI*-*SstI* fragment (nt 10174-12138: GenBank accession no. M63676) in the wild-type sequences of pATD600. Plasmid pATD608 is similar to pATD607, but also lacks the codons for ACP-L, thus a total of 421 codons have been deleted. To construct pATD608 another *MluI*-*SstI* fragment (corresponding to nt 11798-12138: GenBank accession no. M63676) was PCR-amplified using primers II and III (Table 2) and then used to replace the wild-type sequences, as before. The initiating GTG codon at the beginning of DEBS1 overlaps the *MluI* restriction site used for these constructions; consequently, in both pATD607 and pATD608 the initiation codon is retained as is the natural ribosome-binding site. Plasmid pATD615 lacks the 107 aa segment, AT-L, and ACP-L from DEBS1 plus KS1 is replaced by nidKS1 from the *Streptomyces caelestis* PKS involved in the biosynthesis of niddamycin (S. Kakavas and others, unpublished). To make pATD615, nidKS1-encoding sequences were amplified by PCR using oligonucleotides XVII and XVIII

(Table 2) as an *MluI*–*NsiI* fragment (corresponding to nt 2994–4285; S. Kakavas and others, unpublished). A segment downstream of *eryKS1* was also amplified by PCR as an *NsiI*–*EcoRV* fragment of 1121 bp (corresponding to nt 13107–14228; GenBank accession no. M63676) using oligonucleotides XIX and XX. These two fragments were then assembled and used to replace an *MluI*–*EcoRV* fragment in pATD607. In all cases fidelity of the PCR reactions was confirmed by sequence analysis, and plasmids were verified by restriction analysis.

S. erythraea mutant strains were constructed by two sequential, plasmid-mediated, single reciprocal recombination events. First, integrative transformation based on homologous recombination of the *S. erythraea* chromosome with the plasmids described above was carried out as detailed by Weber & Losick (1988) except that R3M plates were used for growth on solid media. Several of the resulting thiostrepton-resistant transformants were then subjected to at least two rounds of non-selective growth in SGGP medium followed by protoplasting and plating. Single colonies were then screened for sensitivity to thiostrepton, indicating loss of the integrated plasmid by a second recombination event. The chromosomal DNA of the resulting thiostrepton-sensitive colonies was examined by Southern analysis (Sambrook *et al.*, 1989). Between three and seven independent isolates in each case carried the appropriate mutation. Single isolates were then selected for further study and designated strains DEBS1-M1 (lacks AT-L and the upstream 107 aa segment), DEBS1-M2 (lacks the 107 aa segment, AT-L and ACP-L) and DEBS1-M7 (lacks the 107 aa segment, AT-L and ACP-L and *KS1* is replaced by *nidKS1*).

Another mutant strain was created that lacked the 107 aa segment and AT-L, and in which the methylmalonyl-specific AT1 was replaced by a malonyl-specific AT from *Streptomyces hygroscopicus* (Ruan *et al.*, 1997a). This mutant was constructed by transforming *Saccharopolyspora erythraea* EryAT1/‘Hyg’AT2 (Ruan *et al.*, 1997b) with plasmid pATD607 (Fig. 2), and has been designated DEBS1-M6 (lacks the 107 aa segment and AT-L, and AT1 is replaced by ‘Hyg’AT2).

Construction of the AT-L expression plasmids. Plasmids pATD622, pATD623 and pATD624 (Table 1) were constructed to complement the DEBS1 deletion strains described above. All three plasmids contain the strong, constitutive *ermE** promoter (Bibb *et al.*, 1994). The promoter was obtained by PCR amplification from pIJ4070 using primers VIII and IX (Table 2), which introduce *HindIII* and *PstI* sites that allow easy transfer into pWHM4. Plasmid pATD622 carries just the *ermE** promoter and was used as a control. Plasmid pATD623 resembles pATD622, but also includes a *PstI*–*XbaI* segment carrying the DEBS1 ribosome-binding site, the 107 aa segment and AT-L. To minimize the occurrence of PCR-induced errors, this *PstI*–*XbaI* insert was assembled from three pieces. The 5′ and 3′ ends of the fragment were generated by PCR using primers X–XI and XIII–XIV, respectively (Table 2), with pATD600 as the template. These short fragments (440 bp and 140 bp) were then joined to the central *MluI*–*SphI* fragment (810 bp) that was obtained from pATD600. Plasmid pATD624 is similar to pATD623 but it lacks the sequence encoding the 107 aa segment. In this case, PCR with primers XII–XI was used to generate a 110 bp fragment that replaces the 440 bp fragment described above in the construction of pATD623 (Table 2). Note that primers X and XII include the DEBS1 RBS and the GTG initiation codon while primer XIV introduces a TAA stop codon. In pATD623 and pATD624 this stop codon is presumed to terminate

translation of the AT-L domain. The position of this stop codon corresponds exactly to the extent of the deletion in strain DEBS-M1. Each of the plasmids is maintained in *S. erythraea* by thiostrepton selection. The fidelity of the PCR-amplified fragments was confirmed by sequencing.

Metabolite identification. Cultures of *S. erythraea* ER720 and the mutant strains, grown in SCM or SGGP for 3–4 d at 30 °C, were centrifuged for 5 min at 3500 r.p.m. The supernatants were adjusted to pH 9.0 with concentrated NH_4OH and were then extracted twice with 1 vol. ethyl acetate. The organic phases were combined, concentrated by rotary evaporation, and analysed on silica gel TLC plates (Sigma-Aldrich). The plates were developed using isopropyl ether/methanol/ NH_4OH (75:35:2, by vol.), and anisaldehyde/sulfuric acid/ethanol (1:1:9, by vol.) spray was used to visualize the compounds upon heating with a heat gun. For mass spectrometric analysis, the compounds were scraped from unstained TLC plates, and the portion of silica gel collected was extracted three times with ethyl acetate/methanol (2:1, v/v) and concentrated by evaporation. Bioassays were performed by spotting dilutions of extracts of mutant and wild-type cultures onto a paper disc. The discs were then air dried and placed on a plate containing 100 ml antibiotic medium 11 (Difco-Bacto) seeded with *Staphylococcus aureus* as an indicator strain. A curve generated from dilutions of an erythromycin A standard was used to compare and evaluate the amount of compound produced by the mutant strains. The inhibition zones were developed by overnight incubation of the plate at 37 °C.

RESULTS

The AT-L/ACP-L deletion mutants are able to produce low levels of erythromycin

The first polypeptide of the erythromycin PKS, DEBS1, has a 556 aa N-terminal extension, the loading domain, that includes an acyltransferase (AT-L) and an acyl carrier protein (ACP-L). It has been proposed that the AT-L and ACP-L domains are involved in choosing the propionate starter unit for erythronolide chain growth (Donadio *et al.*, 1991). Between the translation start site of DEBS1 and the beginning of the AT-L domain (defined by homology to type II soluble ATs) there is a stretch of 107 aa. This stretch precedes all AT domains in the DEBS1, DEBS2 and DEBS3 polypeptides, suggesting that the AT domains of a type I PKS may be larger than soluble type II AT enzymes. To examine the function of these elements in erythromycin biosynthesis, the N-terminus of DEBS1 was modified by introducing deletions into *eryA1*, the gene that encodes DEBS1. Plasmids which encode the N-terminal region of DEBS1 lacking the 107 aa segment and AT-L only (pATD607), or the 107 aa segment, AT-L and ACP-L (pATD608) (Table 1) were constructed. These mutated segments were then used to replace their chromosomal counterparts in *S. erythraea* (see Methods), creating two strains: *S. erythraea* DEBS1-M1, whose PKS lacks the AT-L domain and the 107 aa upstream segment, and *S. erythraea* DEBS1-M2, whose PKS additionally lacks the ACP-L domain (Fig. 2).

Extracts of fermentation broths from each strain were analysed by TLC, bioassay (Fig. 3) and mass spec-

Fig. 2. Schematic representation of the plasmids (a) and mutant strains (b) generated in this study. The hatched regions in (a) indicate deleted segments.

trometry. Surprisingly, both strains continued to produce a bioactive compound that appeared to be erythromycin based on its R_F on TLC and its mass spectrometric profile. The yield in each case was low, however: both strains produced approximately 0.5 % of the parental levels of erythromycin based on comparisons of inhibition zones produced by extracts of the mutant and parental strains (normalized for cell growth) (Table 3). Apparently, neither AT-L nor ACP-L is absolutely required for erythromycin biosynthesis, although they are important for efficient functioning of the PKS.

A soluble form of AT-L is able to partially restore erythromycin levels in the DEBS1-M1 mutant strain

To determine whether the mutant DEBS1 polypeptides that lack their N-terminal domains could be complemented *in vivo*, two plasmids were constructed that express either the AT-L domain and the 107 aa segment, pATD623, or the AT-L domain without the 107 aa segment, pATD624 (Fig. 2a). These plasmids and an isogenic control that lacks any *eryA*I sequences (pATD622) were then transformed into strains DEBS1-M1 and DEBS1-M2, and erythromycin A production of

the transformants was assessed following 3 or 4 d growth. As shown in Fig. 4, strain DEBS1-M1 produced approximately 20 times more erythromycin A (Table 3) when the AT-L domain plus the 107 aa segment were provided *in trans* (pATD623), indicating that the soluble N-terminal fragment of DEBS1 can still functionally associate with the truncated DEBS1 polypeptide and transfer propionate from propionyl-CoA to ACP-L. Interestingly, this association appears to require the 107 aa segment, since the AT-L domain lacking the 107 aa segment (pATD624) did not complement strain DEBS1-M1 (Fig. 4), although we cannot rule out the possibility that the 107 aa segment is required for proper folding or enzymic activity of the AT-L domain. As expected, neither pATD623 nor pATD624 complemented strain DEBS1-M2 (Fig. 4), which lacks ACP-L, indicating that the ACP-L domain must be present for the DEBS1 polypeptide to accept propionate from AT-L.

Initiation of erythromycin biosynthesis in the mutant strains occurs by direct loading of the KS1 domain

Production of erythromycin A in mutant strains DEBS1-M1 and DEBS1-M2 could occur in two ways. First, it is possible that the propionate starter for the initiation of

Fig. 3. TLC (a) and bioautography (b) of *S. erythraea* strains DEBS1-M1 and DEBS1-M2. (a) Lane 1, DEBS1-WT; lane 2, DEBS1-M1; lane 3, DEBS1-M2; each lane corresponds to 5 ml crude extract. (b) Lane A, DEBS1-WT; lane B, DEBS1-M1; lane C, DEBS1-M2; each lane corresponds to 100 µl crude extract.

erythromycin biosynthesis is loaded onto KS1 by the adjacent AT1 domain. Presumably, this would require decarboxylation of the methylmalonyl extender unit that is normally incorporated by AT1, followed by back-transfer to KS1. This hypothesis derives from the finding that the erythromycin PKS appears to catalyse decarboxylation of extender units and that the products of this reaction can then be used for polyketide biosynthesis in absence of the primer propionyl-CoA (Pieper *et al.*, 1996a, b). The second possibility is that the propionate starter (from propionyl-CoA) is loaded

Fig. 4. TLC of ethyl acetate extracts from 5 ml culture supernatants of *S. erythraea* strains DEBS1-M1 and DEBS1-M2 transformed with pATD622, pATD623 or pATD624. Lanes: 1, DEBS1-WT; 2, DEBS1-M1/no plasmid; 3, DEBS1-M1/pATD623; 4, DEBS1-M1/pATD624; 5, DEBS1-M1/pATD 622; 6, DEBS1-M2/no plasmid; 7, DEBS1-M2/pATD623; 8, DEBS1-M2/pATD624; 9, DEBS1-M2/pATD622.

directly onto the KS1 domain since the structure of the pantotheine thioester linkage of propionyl-ACP is identical to that of propionyl-CoA.

To distinguish between these alternatives, a new strain, DEBS1-M6 (Fig. 2), was constructed. DEBS1-M6 lacks both the 107 aa segment and AT-L domain (as in DEBS1-M1) but also has had the methylmalonyl-specific eryAT1 domain replaced by the malonyl-specific 'Hyg'AT2 domain (Fig. 2) from a type I PKS cluster of the rapamycin producer *Streptomyces hygroscopicus* ATCC 29253 (Ruan *et al.*, 1997a). Replacement of eryAT1 by 'Hyg'AT2 causes production of 12-

Table 3. Erythromycin production by the mutant strains alone and transformed with pATD622, pATD623 or pATD624

The values shown (μg erythromycin ml^{-1}) are means of five experiments on different isolates. Fresh transformants were used for each experiment.

Strain	No plasmid	pATD622	pATD623	pATD624
DEBS1-WT	25–35	25–35	–	–
DEBS1-M1	0.1–0.3	0.1–0.3	3–5	0.1–0.3
DEBS1-M2	0.1–0.4	0.1–0.4	0.1–0.4	0.1–0.4

Fig. 5. Schematic representation of DEBS1 in the wild-type and *S. erythraea* strains DEBS1-M1 and DEBS1-M6. In DEBS1-M1, the AT-L domain has been deleted. In DEBS1-M6, the AT-L domain has been deleted and the natural AT1 domain has been replaced by a malonyl-CoA specific domain from *Streptomyces hygroscopicus* ('Hyg'AT2).

desmethyl-12-deoxyerythromycin A (Ruan *et al.*, 1997b). If erythromycin synthesized by the AT-L-deleted DEBS1 strains is initiated by back-transfer (from AT1 to KS1) of a decarboxylated extender unit, then strain DEBS1-M6 should synthesize 12,14-didesmethyl-12-deoxyerythromycin A (Fig. 5), due to back-transfer of an acetate moiety (i.e. decarboxylated malonate) from 'Hyg'AT2 to KS1. Alternatively, if KS1 loads propionate directly from propionyl-CoA, then strain DEBS1-M6 will make the same compound, 12-desmethyl-12-deoxyerythromycin A, as the parent EryAT1/'Hyg'AT2 strain.

Mass spectrometric analysis of extracts obtained from fermentations of DEBS1-M6 revealed that this strain produces a compound of m/z 704, which corresponds to the molecular mass of 12-desmethyl-12-deoxyerythromycin A (Fig. 5). This indicates that back-transfer of

decarboxylated extender units from AT1 to KS1 does not occur and suggests that KS1 loads propionate directly from propionyl-CoA. As was the case for the other AT-L-deleted strains, the level of compound produced by DEBS1-M6 is noticeably reduced, resembling that of DEBS1-M1.

Because strain DEBS1-M6 appears to load KS1 directly with a propionate starter molecule (Fig. 5), we decided to analyse the substrate preference of KS1 domains by constructing strain DEBS1-M7 (Fig. 2), in which the entire loading domain (107 aa segment, AT-L and ACP-L) has been deleted and the eryKS1 domain is replaced by the nidKS1 domain from the niddamycin PKS cluster of *Streptomyces caelestis* (S. Kakavas and others, unpublished). Niddamycin is normally initiated with acetate from acetyl-CoA instead of propionate as in erythromycin. Consequently, the compounds produced

by strain DEBS1-M7 should reveal whether the KS1 domains possess specificity for their appropriate starter. Interestingly, DEBS1-M7 continued to produce erythromycin as shown by mass spectrometric analysis of extracts, albeit in the low amounts characteristic of the N-terminally deleted DEBS1 strains. There was no evidence of erythromycin derivatives initiated with acetate, although we cannot exclude the possibility that some 15-norerythromycin A is made together with erythromycin A, but below the detection limits of this experiment. This result seems to suggest either that *nidKS1* in strain DEBS1-M7 significantly prefers propionyl-CoA over acetyl-CoA or that propionyl-CoA levels in *S. erythraea* greatly exceed those of acetyl-CoA.

DISCUSSION

Erythromycin biosynthesis normally begins with the condensation of a coenzyme A thioester of a short-chain carboxylic acid 'starter unit', propionate, with a coenzyme A thioester of a dicarboxylic acid 'extender unit', methyl malonate. Partial proteolysis of DEBS has established that the N-terminal domain of DEBS1, corresponding to the loading domain, is specifically acylated by propionyl-CoA (Aparicio *et al.*, 1994), indicating that this region of the PKS is responsible for choosing the starter molecule for erythromycin biosynthesis. This idea is further supported by the finding that the 'starter unit' specificity of the spiramycin PKS can be changed from acetate to propionate when its loading domain is replaced with the corresponding domain from the tylosin PKS (Kuhstoss *et al.*, 1996). In this work, strains that lack the erythromycin PKS loading domain, in whole or in part, were constructed with the expectation that they would be incapable of producing erythromycin. Surprisingly, the deleted strains were still able to produce small amounts of erythromycin.

The KS domain in module 1 catalyses the condensation reaction between the starter and the extender incorporated by AT-L and AT1, respectively. One possible explanation for the continued synthesis of erythromycin in the deletion derivatives was that, in the absence of AT-L, AT1 can 'back-charge' KS1 with propionate (possibly formed from methylmalonate by decarboxylation). This does not appear to be the case, however, since strain DEBS1-M6 (which lacks AT-L and has had the methylmalonyl-specific AT1 replaced with a malonyl-specific AT) produces 12-desmethyl-12-deoxyerythromycin A. If back-charging had occurred, 12,14-didesmethyl-12-deoxyerythromycin would have been the expected product, reflecting the use of acetate (from the new AT1) as both starter and the first extender. Consequently, it seems most likely that the biosynthesis of erythromycin by the mutants DEBS1-M1, DEBS1-M2, DEBS1-M6 and DEBS1-M7 occurs by direct loading of propionate from propionyl-CoA onto the KS of module 1. (A similar, but more involved alternative would require direct loading onto KS1 of methylmalonate from methylmalonyl-CoA followed by de-

carboxylation, but this seems less likely.) The substrate normally accepted by KS1 is propionyl-ACP, whose propionyl-pantotheine group is structurally identical to that of propionyl-CoA. This similarity may be sufficient to enable propionyl-CoA to bind productively at the KS1 active site. Consistent with this idea, direct acylation of DEBS with chain elongation intermediates (synthesized as their *N*-acetylcysteamine derivatives, which also resemble the thioester portion of a phosphopantetheinylated molecule) has been demonstrated (Tsukamoto *et al.*, 1996).

Interestingly, no compounds produced by these mutants using acetyl-CoA as a starter were detected. Kao *et al.* (1994) developed an engineered host-vector system for the expression of recombinant PKSs in *Streptomyces coelicolor*, and when this system was used to express the complete DEBS structural genes production of DEB and 8,8a-deoxyoleandolide, which starts with acetate, was observed. It was suggested that the utilization of acetyl-CoA as a starter in this case could be due to the lower intracellular concentration of propionyl-CoA in the new host compared to the native erythromycin producer. 8,8a-Deoxyoleandolide has also been isolated as a minor component produced by a gene-disrupted *Saccharopolyspora erythraea* strain (Weber *et al.*, 1991). This demonstrates that DEBS can use acetate as starter in place of the normal propionate, although that does not seem to have taken place here even in the absence of AT-L. Recent *in vitro* work has demonstrated that DEBS1 has a 32-fold preference for a propionate starter over acetate and a 7.5-fold preference for propionate over butyrate (Pieper *et al.*, 1996). Moreover, *S. erythraea* appears to produce propionyl-CoA through the activity of a malonyl-CoA decarboxylase (Hsieh & Kolattukudy, 1994), a fact that may indicate that the pool size of propionyl-CoA in *S. erythraea* could be larger than the pool size of acetyl-CoA. Whatever the reason, in the absence of the loading domain the mutants generated in this work clearly utilize propionyl-CoA preferentially.

To determine if KS1 is involved in determining specificity for propionate in these mutants we constructed strain DEBS1-M7, in which the natural KS1 has been replaced by the niddamycin KS1 (this strain also lacks the loading domain). Niddamycin synthesis normally initiates with acetyl-CoA. Unexpectedly, the DEBS1-M7 mutant produced erythromycin A instead of 14-desmethylerythromycin, the compound expected if the *nidKS1* showed selectivity for acetyl-CoA. It seems that KS1 domains show some flexibility in the acyl chains that they will accept from a pantotheine donor, although in the context of *S. erythraea* the preference seems to be propionate over acetate. This may be because the concentration of propionyl-CoA greatly exceeds that of acetyl-CoA within the cell or because both the erythromycin and niddamycin KS1 domains bind propionyl-CoA more productively.

The levels of erythromycin synthesized by the AT-L deletion mutant DEBS1-M1 can be increased, although not to wild-type levels, by transforming the strain with

pATD623, a multicopy plasmid that expresses the 107 aa segment and AT-L driven by the *ermE** promoter. This suggests that it is possible to associate a soluble AT enzymic domain with the multienzyme PKS and it opens new possibilities for introducing different soluble AT domains to create new erythromycin derivatives. As expected, the levels of erythromycin in mutant DEBS1-M2, which lacks the 107 aa segment, AT-L and ACP-L, are not affected by the presence of plasmid pATD623. Presumably ACP-L is needed to accept propionate from AT-L to allow efficient synthesis of DEB.

A related experiment using pATD624, an equivalent plasmid that lacks the 107 aa segment, did not increase antibiotic production in the DEBS1-M1 strain. Indeed, deletion of just the 107 aa segment in the wild-type strain causes a loss of normal erythromycin biosynthesis (data not shown). This seems to indicate that the 107 aa segment is important for the activity of AT-L. We have detected a similar segment using BLAST searching (Altschul *et al.*, 1990) upstream of ATs from other type I PKSs, including the remaining ATs of the erythromycin PKS, the rapamycin PKS in *Streptomyces hygroscopicus* (Schwecke *et al.*, 1995), the oleandomycin PKS from *S. antibioticus* (Swan *et al.*, 1994), a PKS-like cluster of *S. ambofaciens* (Aigle *et al.*, 1996), mycocerosic acid synthase, a fatty acid elongating multifunctional enzyme from *Mycobacterium tuberculosis* (Mathur & Kolattukudy, 1992), and phenylalanine ammonia-lyases from plants (Osakabe *et al.*, 1995). Further work on the structure of this enzymic domain should reveal the relevance of the 107 aa segment to the activity of AT-L.

Up to now most erythromycin derivatives have been made by chemical modifications to the antibiotic, but these techniques can be limited. Recent efforts, however, show that erythromycin derivatives can be produced by genetically reprogramming erythromycin-producing strains (McAlpine *et al.*, 1987; Weber *et al.*, 1991; Donadio *et al.*, 1991, 1993; Jacoben *et al.*, 1997; Ruan *et al.*, 1997b). A better understanding of the structure and function of type I PKSs should enable the design of molecules with increased diversity and biological function.

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