stopped by adding chloroform/methanol (750 μL, 2:1, *v/v*). After addition of water (200 μL), the lipids were extracted, and the radioactivity of the polar upper phase, which contained [^{14}C]phosphorylcholine, was determined by scintillation counting.

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Keywords: biological activity · hydrolases · inhibitors · oxidation · sphingomyelinases

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Catalytically Active Tetramodular 6-Deoxyerythonolide B Synthase Fusion Proteins

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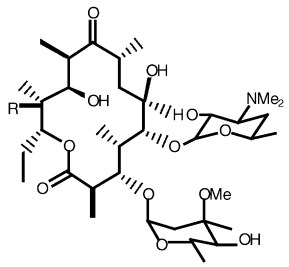
Large numbers of structurally diverse and medicinally important macrocyclic polyketides are produced through the action of the type I modular polyketide synthases (PKS) of actinomycete bacteria. In such systems, the individual catalytic domains required for each successive cycle of polyketide chain extension and subsequent reduction are ordered through covalent links into a multienzyme module. These modules are housed in the order in which they are used on three or more giant protein subunits. An individual giant PKS protein may house a single extension module, as in one subunit of the spiramycin-producing PKS,[1] or as many as six successive extension modules, as in one subunit of the rapamycin-producing PKS.[2] In general, the catalytic advantages of such multifunctional enzymes are thought to include more efficient channelling of enzyme-bound intermediates between successive active sites, and protection of the intermediates from side reactions.[3]

The erythromycin-producing PKS, 6-deoxyerythonolide B synthase (DEBS), which governs the biosynthesis of erythromycin A (**1**) and B (**2**) in *Saccharopolyspora erythraea*, is an



Erythromycin A (**1**): R = OH
Erythromycin B (**2**): R = H

established model system for investigations into the mechanism of polyketide biosynthesis. The three multienzyme polypeptides DEBS 1, DEBS 2 and DEBS 3 are readily separated during their purification from *S. erythraea* extracts, and this has prevented investigation of the overall architecture of the complex.[4–8] In this work we aimed to create tethered versions of DEBS by

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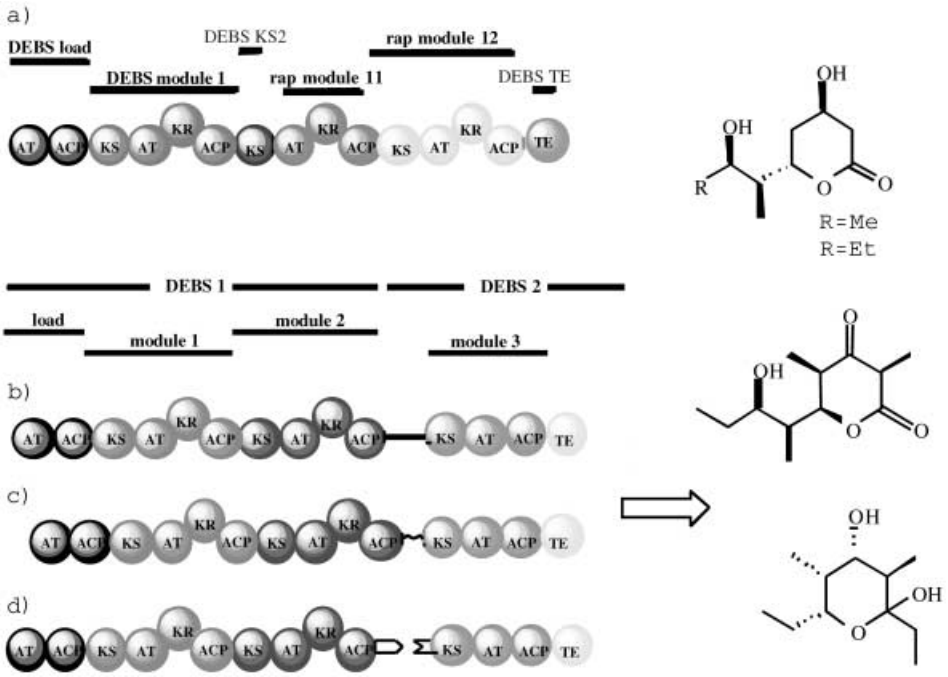
covalent fusion of two or even all three DEBS subunits, and to study the effect of this association on catalysis. Productive fusions have already been described for bi- and trimodular PKS systems (Scheme 1). For example, an active hybrid PKS has been engineered by tethering the loading module and ketosynthase of DEBS module 1 to modules 11 and 12 of the rapamycin PKS through a short interdomain linker peptide (Scheme 1 a).[9] Similarly, the C- and N-terminal regions of DEBS 1 and 2, which have been suggested to be involved in dimerisation[10] and/or interprotein 'docking' of DEBS 1 and DEBS 2[11] can be replaced by a 25 amino acid linker identical to the intermodular linker between modules 5 and 6 on DEBS 3 without loss of activity (Scheme 1 c).[11] More remarkably, when a translational fusion of DEBS 1 with half DEBS 2 was constructed by fusing the respective stop and start codons, the resulting trimodular synthase (Scheme 1 b) retained catalytic activity in vivo comparable to that of the control system, where module 3 resided on a separate protein (Scheme 1 d).[11] The respective C- and N-terminal residues of DEBS 1 and DEBS 2 must lie close enough together in the normal DEBS 1:DEBS 2 complex so that their fusion does not disrupt the overall structure.

In the inter-DEBS multienzyme fusions created in this work, we chose to retain the 'docking regions' at the termini of the DEBS proteins. The genes *eryAI* and *eryAII*, which encode the polypeptides DEBS 1 and DEBS 2, are separated in the chromosome by 1443 bp containing a nonessential transposon sequence. To construct the DEBS $1+2$ fusion this transposon sequence was removed. The TGA stop codon of *eryAI* was altered to a CTC codon for leucine and fused to the methionine codon at the start of *eryAII* that had been altered from GTG to ATG to allow for the incorporation of a unique *Bsp*HI restriction site for cloning and analysis (Figure 1 a). The flanking regions from *eryAI* and *eryAII* were amplified by PCR. Mutagenic PCR primers were used to introduce the *Bsp*HI restriction site at the point of fusion. The primers also included the *Sex*AI and *Bsm*I restriction sites, which are naturally present in *eryAI* and *eryAII*, respectively (Figure 1 b). The two PCR products were cloned separately into plasmid pUC18 then, following digestion of the resultant plasmids with *Bsp*HI and *Eco*RI, fused together at the *Bsp*HI restriction site in a triple ligation with *Eco*RI-digested pCJR24,[12] an intergrative expression vector for *S. erythraea*. The resultant plasmid, pCMS32 (Figure 1 c), was used to transform protoplasts of *S. erythraea* to introduce the mutated sequence into the chromosome in place of the wild-type counterpart by double homologous recombination.

The *eryAII* and *eryAIII* genes of *S. erythraea* encode the polypeptides DEBS 2 and DEBS 3 and lie adjacent to each other in the same reading frame. The DEBS $2+3$ fusion was therefore created by altering the TAG stop codon of *eryAII* to CTC, which codes for serine (Figure 2 a). This change was achieved by PCR amplification of the flanking regions of DNA with mutagenic oligonucleotide primers that introduce a *Bsp*HI site at the join and that incorporate the natural *Bam*HI site of *eryAII* whilst creating a new *Eco*RI site (Figure 2 b). The two PCR products were ligated by *Bsp*HI and cloned together into *Bam*HI/*Eco*RI-digested pUC18. The insert was excised (*Nde*I/*Eco*RI) and cloned into *Nde*I/*Eco*RI-digested pCJR24 to create pCMS51 (Figure 2 c). The DEBS mutation was introduced into the *S. erythraea* chromosome by double homologous recombination using pCMS51. The DEBS $1+2+3$ fusion strain was created by double homologous



Scheme 1. *a) TKS-AR7. DEBS loading module and module 1 are fused to rapamycin (rap) modules 11 and 12.[9] b) KAO318. DEBS 1 is fused to DEBS module 3, with the natural polypeptide linker retained. c) KAO330. DEBS 1 is fused to DEBS module 3 by a 25 amino acid linker, the intermodule segment originally connecting DEBS modules 5 and 6. d) CK13. Module 3 resides on a separate protein to modules 1 and 2. AT, acyl transferase; ACP, acyl carrier protein; KS, β-ketoacyl synthase; KR, β-ketoacyl reductase; ER, enoyl reductase; DH, dehydratase; TE, thioesterase.*

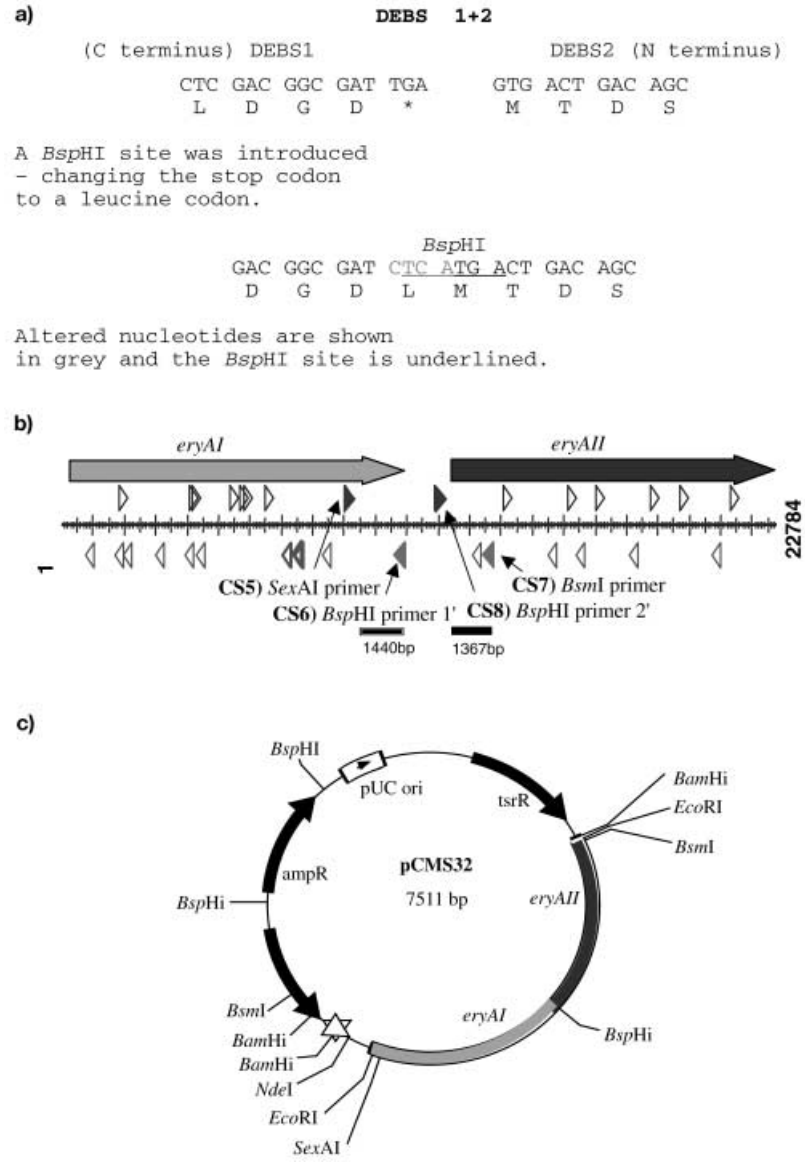


Figure 1. *a) Join region for splicing the first two polypeptides of DEBS. The two polypeptides are separated in the chromosome by 1443 bp containing a transposon sequence. This sequence is removed in joining the two polypeptides together. In this case, the methionine codon was also altered because GTG is an* Actinomycete *start codon and will only be recognised as a methionine when at the beginning of an open reading frame. Generally, this codon would be translated as valine. b) PCR of flanking regions from* eryAI *and* eryAII*, with mutagenic primers for the introduction of the* Bsp*HI site. The primers also include naturally present* Bsm*I and* Sex*AI sites for cloning and analysis. c) Regions flanking the* eryAI *3′ and* eryAII *5′ termini, joined through a* Bsp*HI fusion and cloned into a pCJR24 backbone.*

recombination between the DEBS 2 + 3 strain and pCMS32. The correct arrangement of the genes in each of the recombinants was checked by PCR and Southern blot analysis (data not shown).

The recombinant strains were initially assessed for erythromycin production by bioassay. Discs of agar cut from tap water medium (TWM)[13a] plates on which the *S. erythraea* strains had been grown for one week were placed on a TWM plate freshly inoculated with *Bacillus subtilis*. Discs of wild-type *S. erythraea* and of *S. erythraea* SGT2, a modified strain from which all three *eryA* genes have been deleted,[14] were used as controls. After overnight incubation of the plates at 37 °C, inhibition of bacterial growth by wild-type *S. erythraea*, DEBS 1 + 2 and DEBS 2 + 3 strains was noted; such results were not recorded for *S. erythraea* SGT2 or DEBS 1 + 2 + 3 (Figure 3). These results indicate that the tetramodular DEBS fusion proteins were catalytically active.

Cultures of the fusion strains were grown in sucrose succinate defined medium (SSDM, 500 mL)[13b] for analysis of erythromycin production. A culture of wild-type *S. erythraea* was grown for comparison. After five days growth in an orbital shaker at 27 °C, 200 rpm, cultures were harvested by centrifugation. The resultant mycelial pellets were resuspended in an equal volume of water and the pH value corrected to 9.00 before extraction into a double volume of ethyl acetate and methanol. Solvent was removed under reduced pressure and the extract resuspended in methanol and analysed by LC-MS. Results indicated that, though the strain in which all three of the DEBS multienzyme subunits are linked produced barely detectable levels of erythromycins A and B, production levels of erythromycin A and B by the DEBS 1 + 2 and DEBS 2 + 3 strains were equal to (or even better than) those produced by the wild-type sample.

In the current structural model for type I modular PKSs,[5, 15] the protein – protein interactions between successive modules in different PKS proteins are limited to the C terminus of the upstream module, and the N terminus of the downstream module. There is no functional distinction between this arrangement and that where the modules are tethered covalently by a short flexible linker within the same protein. The results in this paper provide additional evidence that switching the interaction from an inter- to an intramolecular one does not compromise the catalytic apparatus in vivo, at least for the four-module proteins. A number of alternative explanations can be suggested to account for the inefficiency of the hexamodular species, not least the problem of ensuring correct full-length synthesis and precise folding of a polypeptide of relative molecular mass 900 kDa. The synthesis of 6-deoxyerythronolide B in vitro by a mixture of purified DEBS 1, DEBS 2 and DEBS 3 has been reported but in disappointingly low yield.[15] The way is now open to test whether the fused DEBS enzymes might show a catalytic advantage over the native enzyme subunits in vitro. Finally, this work serves to confirm evidence that the amino acid residues at the extreme N and C termini of the proteins are not essential for the docking process between successive multienzymes, or for specific protein – protein interactions within the complex.[16]

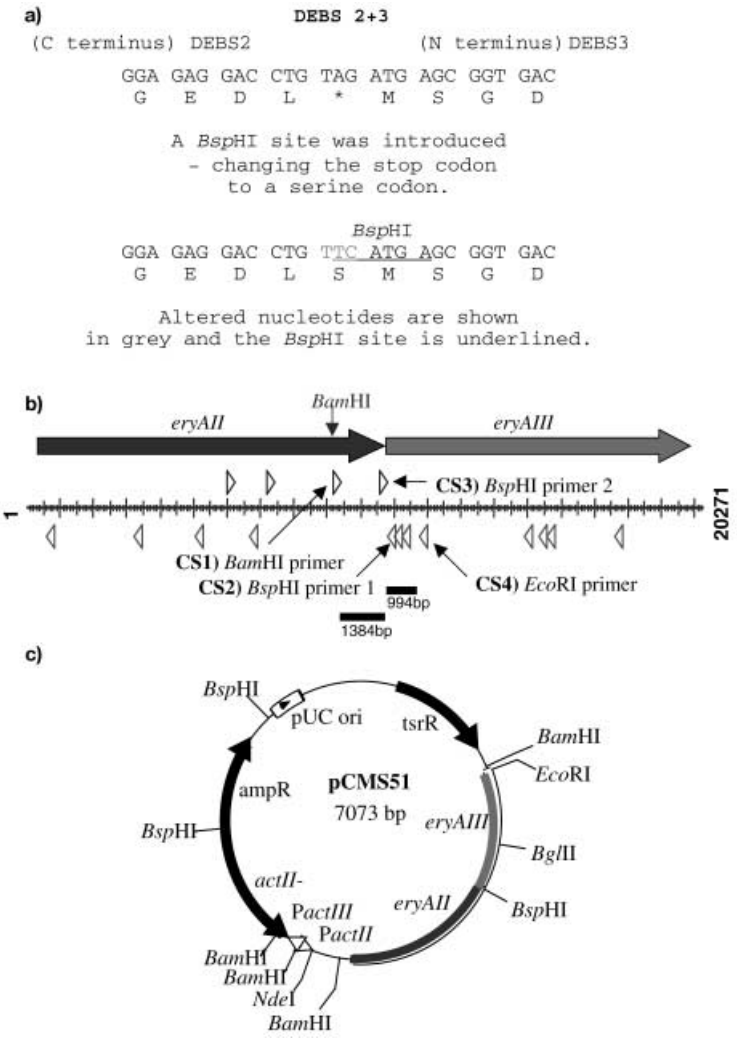


Figure 2. *a) Amino acid sequence in the region used for splicing the two polypeptides DEBS2 and DEBS3. The two polypeptides are adjacent to one another in the chromosome and are in the same reading frame. Therefore, the only change required was alteration of a stop codon to a serine codon. b) PCR of flanking regions from* eryAII *and* eryAIII*, with mutagenic primers for the introduction of a* Bsp*HI site. The primers also include* Bam*HI, naturally present in* eryAII*, and introduce a* Sex*AI into* eryAII *for cloning and analysis. c) Regions flanking* eryAII *3′ and* eryAIII *5′ termini, joined through a* Bsp*HI fusion and cloned into a pCJR24 plasmid vector.*

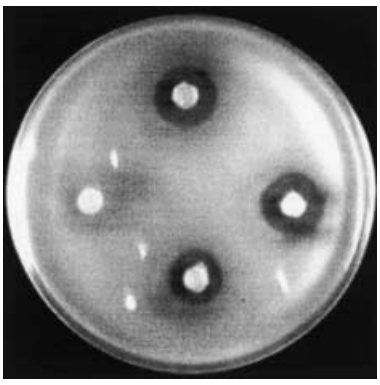


Figure 3. *Bioassay to assess the catalytic ability of fused DEBS proteins. Plugs of TWM agar on which* S. erythraea *had been grown were placed on TWM agar freshly inoculated with* B. subtilis *and incubated overnight. Clear areas indicate the production of erythromycins. Clockwise from top: DEBS 2 + 3 fused strain, wild type, DEBS 1 + 2, SGT2.*

Keywords: biosynthesis · enzyme catalysis · peptides · proteins

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