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Identification of DEBS 1, DEBS 2 and DEBS 3, the multienzyme polypeptides of the erythromycin-producing polyketide synthase from *Saccharopolyspora erythraea*

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The *ery A* region of the erythromycin biosynthetic gene cluster of *Saccharopolyspora erythraea* has previously been shown to contain three large open reading frames (ORFs) that encode the components of 6-deoxyerythronolide B synthase (DEBS). Polyclonal antibodies were raised against recombinant proteins obtained by overexpression of 3′ regions of the ORF2 and ORF3 genes. In Western blotting experiments, each antiserum reacted strongly with a different high molecular weight protein in extracts of erythromycin-producing *S. erythraea* cells. These putative DEBS 2 and DEBS 3 proteins were purified and subjected to N-terminal sequence analysis. The protein sequences were entirely consistent with the translation start sites predicted from the DNA sequences of ORFs 2 and 3. A third high molecular weight protein co-purified with DEBS 2 and DEBS 3 and had an N-terminal sequence that matched a protein sequence translated from the DNA sequence some 155 base pairs upstream from the previously proposed start codon of ORF1.

Erythromycin; Polyketide synthase; *Saccharopolyspora erythraea*

1. INTRODUCTION

The first stage in the biosynthetic pathway to the macrolide antibiotic erythromycin A in *Saccharopolyspora erythraea* involves the stepwise construction of a polyketide chain, which is then cyclised to form the 14-membered lactone ring of 6-deoxyerythronolide B, the earliest intermediate so far isolated [1,2]. The nature of the polyketide synthase that catalyses this multistep process, 6-deoxyerythronolide B synthase (DEBS), has been extensively investigated by genetic analysis [3–5] and by nucleotide sequencing of the structural genes encoding the synthase [6–8]. These *eryA* genes are found clustered near *ermE*, the erythromycin resistance gene [9], and extend over approximately 45 kilobase-pairs (kbp) of DNA.

Partial nucleotide sequencing of this entire region by Katz and his colleagues at Abbott Laboratories (summarised in [10]) revealed multiple copies of each potential enzymic component of the synthase, as judged by sequence similarity to active-site regions in authentic fatty acid synthases [10] and other simpler polyketide synthases [11,12]. These authors proposed that different enzymes operated in each cycle of polyketide chain extension, some of these enzymes possibly being bifunctional. Meanwhile, complete DNA sequencing of 11 kbp from the *ermE*-proximal region of the *eryA* locus [6] confirmed the existence of multiple versions of each enzymic component and showed that nine distinct active sites were housed in a single polypeptide chain of predicted M_r 332, 104. Further, the enzymes were grouped in two sets along the polypeptide chain, each set having the activities required to accomplish one of the six required cycles of chain extension. The order of activities in each set is exactly that observed in vertebrate fatty acid synthases (see e.g. [13,14]) and in the polyketide synthase from *Penicillium patulum* that synthesises 6-methylsalicylic acid [15]. It was tentatively proposed that the product of this gene catalysed the fifth and sixth cycles of polyketide chain synthesis.

Further sequencing of *eryA* genes [7,8] has amply supported both the idea of 'an enyme for every move' (results quoted in [10]) and the likely domain organisation of the synthase [6], with three unusually large multienzyme polypeptides (DEBS 1, DEBS 2 and DEBS 3) housing a total of at least 28 distinct active sites. Targetted deletion in vivo of the ketoreductase domain in the N-terminal half of DEBS 3 [7] led to the accumulation of the expected metabolite, both confirming the identification of this domain and strengthening the prospects for rational modification of polyketide antibiotics.

The only enzymological study on DEBS so far has

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involved the high-level heterologous expression in *Escherichia coli* of the C-terminal portion of DEBS 3, predicted to house an acyl carrier protein (ACP) domain and a thioesterase domain that might act as a cyclase to produce 6-deoxyerythronolide B directly [16]. However, it has not so far been possible to demonstrate erythronolide biosynthesis in extracts of *S. erythraea*, and no specific assay for these enzymes has yet been developed. We report here the direct immunological detection of DEBS polypeptides in *S. erythraea* extracts, confirming their unusual size and opening the way to their purification and study.

2. MATERIALS AND METHODS

2.1. *Bacterial strain and growth conditions*

S. erythraea CA340 was the kind gift of Drs. L. Katz and J.P. DeWitt, Abbott Laboratories, Chicago, USA. Cells were grown from spore suspensions on a minimal medium (0.2 M sucrose, 20 mM succinic acid, 20 mM potassium phosphate (pH 6.6), 5 mM magnesium sulphate, 100 mM potassium nitrate and 2 ml trace elements solution [17] per litre. After about 5 days of growth, cells were harvested by filtration and the paste was stored at −80°C.

2.2. *Preparation of protein samples*

About 50 g of cell paste was dispersed in 100 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 50% (v/v) glycerol, 2 mM DTT, 0.4 mM PMSF, 1 mM phenanthroline, 100 μg/ml lysozyme, 50 μg/ml DNAse and 20 μg/ml RNAse. The suspension was sonicated eight times for 30 s at 0°C then centrifuged at 40,000×g for 2 h at 4°C. The supernatant fraction was used as a source of polyketide synthase components.

To purify the DEBS proteins, 70 ml of crude extract (usually about 2,000 mg protein) was applied to a DE52 DEAE-cellulose column (2.6×40 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 20% (v/v) glycerol and 2 mM DTT (buffer A). The column was washed with 200 ml buffer A and then bound proteins were eluted with a linear 600 ml gradient of 0–400 mM NaCl in buffer A . Alternate 15 ml fractions were analysed by SDS-polyacrylamide gel electrophoresis and those containing the DEBS proteins were pooled. This pool (475 mg protein in 45 ml) was applied to a Sephacryl S400 HR gel filtration column (90×2.6 cm) equilibrated with buffer A and proteins were eluted at a flow rate of 1.5 ml/min. Fractions containing the DEBS proteins were combined. The sample (80 mg protein in 80 ml) was mixed with 20 ml of buffer A containing 1 M ammonium sulphate, filtered and applied to a Pharmacia phenyl-Sepharose 16/10 Hiload column equilibrated with 200 mM ammonium sulphate in buffer A. Bound proteins were eluted with a 40 ml linear gradient of 200–0 mM ammonium sulphate in buffer A. Elution of proteins was monitored by recording the absorbance of the eluent at 280 nm. Peak fractions were analysed by SDS-polyacrylamide gel electrophoresis and stored at −80°C. At this stage the sample had a volume of 16 ml and contained 1.4 mg protein.

2.3. *Production of antisera*

The C-terminal ACP-thioesterase region of DEBS 3 was purified as described previously [16]. The C-terminal half of DEBS 2 was purified by preparative SDS-gel electrophoresis from lysates of an *E. coli* strain carrying a pT7-7 expression plasmid for this domain. The construction of this expression plasmid and characterization of the recombinant protein will be described elsewhere (D.J. Bevitt, S.A. Morris and P.F. Leadlay, manuscript in preparation). Antibodies against each recombinant protein were raised in New Zealand White female rabbits as described in [18].

2.4. *Protein chemical methods*

Protein concentrations were determined by the method of Lowry *et al.* [19] or by the dye-binding method of Bradford [20]. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [21] using 4–15% gradient gels. Western blotting was carried out by the method of Burnette [22]. N-terminal amino acid sequences were determined from protein bands which had been transferred to ProBlott poly(vinylidene difluoride) membranes by the method of Matsudaira [23]. Sequences were determined using an Applied Biosystems model 477A pulsed-liquid protein sequencer, fitted with an on-line model 120A analyser for detection of phenylthiohydantoin-amino acids.

3. RESULTS AND DISCUSSION

3.1. *Detection of 6-deoxyerythronolide synthase B components by Western blotting*

In Western blotting experiments, the antiserum raised against the C-terminal ACP-thioesterase of DEBS 3 reacted with a minor protein band of apparent M_r 270,000 present in crude lysates of erythromycin-producing *S. erythraea*. Antibodies against the C-terminal half of DEBS 2 reacted with a different, slightly larger protein band. The two immunoreactive bands were therefore tentatively identified as the DEBS 2 and DEBS 3 proteins. Examination of Coomassie blue-stained gels revealed that the cell extracts contained five polypeptides with apparent M_r greater than that of myosin. These proteins were more readily visualised in gels after their partial purification and concentration by anion-exchange chromatography using a DE52 ion-exchange column (Fig. 1). In all extracts examined, an additional band was observed which migrated between the DEBS

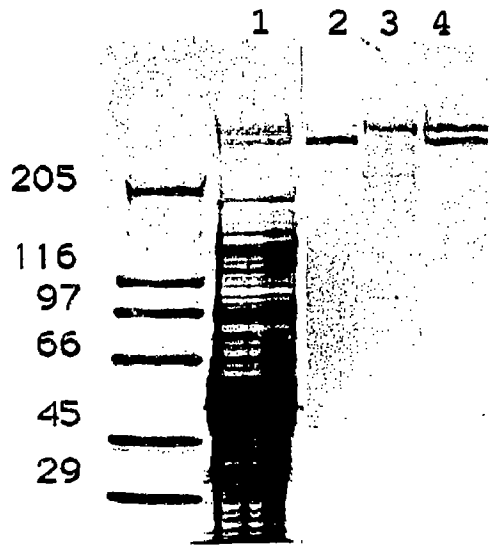

Fig. 1. Western blot analysis of *S. erythraea* proteins using antisera against C-terminal domains of DEBS 2 and DEBS 3. All lanes contain 30 μg of protein from the pooled fractions obtained after DEAE-cellulose chromatography. Lane 1 is from a Coomassie blue-stained gel. Lanes 2, 3, and 4 are from Western blots probed with anti-DEBS 3, anti-DEBS 2 and anti-DEBS 3 plus anti-DEBS 2 antisera, respectively. The molecular weights (kDa) of the standard proteins are also shown.

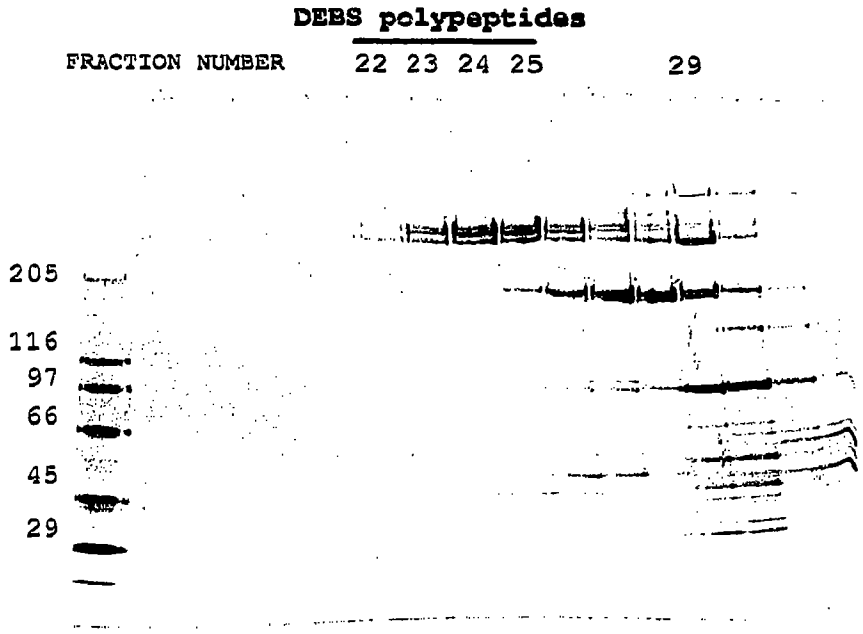

Fig. 2. Analysis of proteins eluted from the hydrophobic interaction column. A 25 μl sample of each 4 ml fraction was analysed by SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue. Fractions indicated by a bar contained all three DEBS proteins and were pooled. The DEBS 3 protein also appeared in a later peak (fraction 29) along with several contaminating proteins.

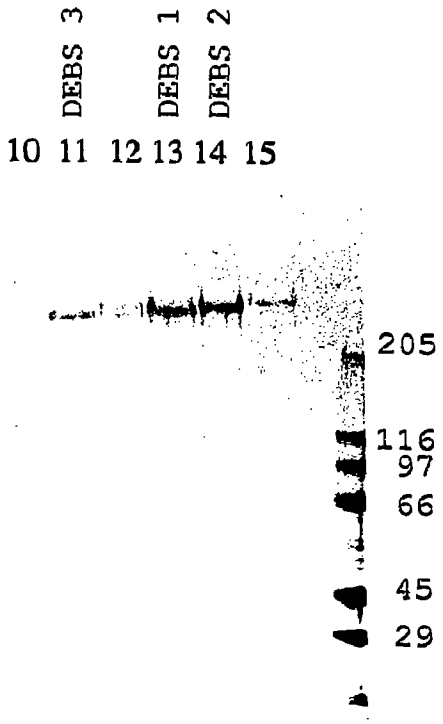

Fig. 3. Separation of the three DEBS polypeptides by anion-exchange chromatography on a MonoQ HR 5/5 column. Consecutive protein-containing fractions (1.5 ml) were analysed by SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue. The peak fraction for each DEBS polypeptide is labelled.

2 and DEBS 3 proteins. This band was considered the most likely to be the DEBS 1 protein since the M_r of DEBS 1 predicted from the DNA sequence [7] is intermediate between the calculated M_r values of DEBS 2 and DEBS 3.

3.2. *N-terminal amino acid sequence analysis*

In preliminary attempts to sequence these large proteins, and thereby confirm their identity, DEAE-cellulose-purified proteins were separated by SDS-gel electrophoresis and electroblotted onto a ProBlott membrane. However, there were insufficient amounts of the target proteins at this stage to yield satisfactory sequence data. The immunoreactive proteins were therefore further purified by gel filtration on a Sephacryl S-400 HR column and by hydrophobic interaction chromatography on a phenyl-Sepharose column. The DEBS-containing fractions from the latter chromatographic step are shown in Fig. 2. Interestingly, fractions were obtained which contained both the two immunoreactive proteins and also the putative DEBS 1 protein. However, the three proteins apparently did not form a stable complex: they could be separated completely by subsequent chromatography on a MonoQ HR 5/5 column using a shallow gradient (25 ml, 250–400 mM NaCl in buffer A) (see Fig. 3). For N-terminal sequencing, 15–30 μg of each purified polypeptide was subjected to electrophoresis on a 5% SDS/polyacrylamide gel and electroblotted onto a ProBlott membrane. With these loadings each polypeptide formed an

DEBS 1	found	A D L(S)K L(S)D X R X X(Q)
	predicted	S G P R S R T T S R R T S
	upstream sequence	A D L S K L S D S R T A Q
DEBS 2	found	T D S E K V A E Y L(R)X X X
	predicted	T D S E K V A E Y L R R A T
DEBS 3	found	S G D N G M(T)E E K X X X(Y)
	predicted	S G D N G M T E E K L R R Y

Fig. 4. N-terminal amino acid sequences of the DEBS proteins. The N-terminal sequences determined by protein sequencing are aligned with those predicted from the DNA sequence of *eryA*. X signifies an unidentified amino acid. For residues in parentheses, the identification was tentative.

intense band approximately 2 cm × 0,5 cm, when stained with Coomassie Blue. Initial yields of 20 pmol, 20 pmol and 50 pmol were obtained for the putative DEBS 2, DEBS 1 and DEBS 3, respectively. After five cycles these yields had decreased by about 50%. For all three polypeptides, the first ten or so residues could be identified before the increasing background noise prevented further sequencing. The N-terminal sequences obtained are presented in Fig. 4. For DEBS 2 and DEBS 3, the protein sequences were consistent with the translation start sites predicted from the nucleotide sequences of the corresponding genes. In the case of the putative DEBS 1, the protein sequence exactly matched an animo acid sequence translated from the DNA sequence some 155 base pairs upstream from the previously-proposed start codon of the gene encoding DEBS 1, and in a different reading frame to that proposed [7] (see Fig. 4). The coding sequence that agrees with our protein sequence is immediately preceded by a GTG codon, presumably encoding N-formylmethionine, and a plausible ribosome binding site is located 6–10 nucleotides further upstream [7]. These results suggest that the protein is indeed DEBS 1, but that the true start codon for ORF 1 is upstream from the one originally proposed on the basis of DNA sequence analysis alone. This region of *eryA* is being resequenced to determine the reason for this discrepancy.

3.3. *Conclusions*

Until now, the only evidence for the existence of three unusually large polypeptides as components of the erythromycin-producing polyketide synthase (DEBS) has been provided by DNA sequence analysis [6,7,8]. Here we report the detection of all three of these proteins in cell-free extracts of *S. erythraea*. The N-terminal sequence data and the reactions of the polypeptides with antisera directed against their C-terminal domains confirm that the products of the three large *eryA* genes are very likely to function as intact multienzyme polypeptides rather than undergoing proteolytic processing to form smaller functional domains. Furthermore, conditions have been found under which DEBS 1, 2 and 3 co-purify to near homogeneity. Although we have so far been unable to demonstrate overall synthesis of 6-deoxyerythronolide B using any of our preparations (P.C. and P.F.L., unpublished data), the ability to detect and isolate DEBS components from *S. erythraea* will greatly assist current attempts to overcome these difficulties.

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