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Targeted gene inactivation for the elucidation of deoxysugar biosynthesis in the erythromycin producer *Saccharopolyspora erythraea*

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Abstract The production of erythromycin A by *Saccharopolyspora erythraea* requires the synthesis of dTDP-D-desosamine and dTDP-L-mycarose, which serve as substrates for the transfer of the two sugar residues onto the macrolactone ring. The enzymatic activities involved in this process are largely encoded within the *ery* gene cluster, by two sets of genes flanking the *eryA* locus that encodes the polyketide synthase. We report here the nucleotide sequence of three such ORFs located immediately downstream of *eryA*, ORFs 7, 8 and 9. Chromosomal mutants carrying a deletion either in ORF7 or in one of the previously sequenced ORFs 13 and 14 have been constructed and shown to accumulate erythronolide B, as expected for *eryB* mutants. Similarly, chromosomal mutants carrying a deletion in either ORF8, ORF9, or one of the previously sequenced ORFs 17 and 18 have been constructed and shown to accumulate 3-α-mycarosyl erythronolide B, as expected for *eryC* mutants. The ORF13 (*eryBIV*), ORF17 (*eryCIV*) and ORF7 (*eryBII*) mutants also synthesised small amounts of macrolide shunt metabolites, as shown by mass spectrometry. These results considerably strengthen previous tentative proposals for the pathways for the biosynthesis of dTDP-D-desosamine and dTDP-L-mycarose in *Sac. erythraea* and reveal that at least some of these enzymes can accommodate alternative substrates.

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Introduction

Erythromycin A is a clinically important macrolide antibiotic produced by the gram-positive bacterium *Saccharopolyspora erythraea*. As for many antibiotics, the cloning of the erythromycin biosynthetic genes has been facilitated by the fact that they are organised in a cluster which also includes the gene conferring self-resistance to erythromycin A. During the biosynthesis of erythromycin A, the aglycone 6-deoxyerythronolide B is first synthesised through the condensation of one propionate and six methyl malonate units, in a process very similar to fatty acid biosynthesis (for a review see Katz and Donadio 1993). The three polyketide synthase multienzymes involved in this process are encoded by three giant genes (*eryAI* to *III*) lying at the centre of the cluster. Further modifications of the macrolactone ring are introduced sequentially and require a number of additional enzymatic activities encoded by 18–20 genes located on both sides of the *eryA* locus (Figs. 1A and B). Of these, the *eryF* gene (ORF4) responsible for hydroxylation at position 6 of the macrolactone ring, the *eryG* gene (ORF6) responsible for O-methylation of L-mycarose to L-cladinose, and the *eryK* gene (ORF20) encoding the C-12 monooxygenase have been well characterised (Paulus et al. 1990; Haydock et al. 1991; Weber et al. 1991; Stassi et al. 1993; Lambalot et al. 1995). In contrast, the identity of the remaining ORFs and their roles in the biosynthesis and transfer of each of the two 6-deoxyhexoses activated as dTDP-D-desosamine and dTDP-L-mycarose, respectively, remain to be fully clarified. Generation of mutations in the region surrounding the *ermE* resistance gene has previously allowed the mapping of several *eryB* (dTDP-L-mycarose biosynthetic pathway) and *eryC* genes (dTDP-D-desosamine biosynthetic pathway) (Weber et al. 1985, 1990;

Dhillon et al. 1989). However, most of these mutants were generated prior to sequence analysis, leading in some cases to misinterpretation of phenotypes owing to polar effects on downstream genes.

Additional information on potential *eryB* and *eryC* genes has also been briefly summarised (without supporting evidence) in review articles (Donadio et al. 1993; Liu and Thorson 1994; Katz and Donadio 1995). We have recently reported (Gaisser et al. 1997) the complete nucleotide sequence of seven contiguous ORFs lying between *eryAI* and *eryK*, and have demonstrated that ORF15 is an *eryC* rather than the *eryB* gene previously proposed (Liu and Thorson 1994; Katz and Donadio 1995). ORF16 was also characterised as an *eryB* gene. In this paper, we present the nucleotide sequence of three additional ORFs (ORFs 7, 8, and 9), located immediately downstream of the *eryA* genes, and the results of targeted gene deletion of each of these three ORFs, as well as of four previously sequenced ORFs, ORFs 13, 14, 17 and 18. The properties of these mutants, together with details of the role of *eryBIII* (to be reported separately: S. Gaisser, G. A. Böhm, M. Doumith, N. Dhillon, M.-C. Raynal, J. Cortés and P. F. Leadlay, manuscript submitted) significantly strengthen our previous tentative proposals for the enzymology of deoxysugar formation in *Sac. erythraea* (Gaisser et al. 1997).

Materials and methods

Bacterial strains, plasmids and growth conditions

The *Streptomyces lividans* strain 1326 (Hopwood et al. 1983) was the kind gift of C. J. Thompson (University of Basel, Switzerland) and was maintained on R2YE (R5) plates (Hopwood et al. 1985). The *Sac. erythraea* NRRL 2338-red variant strain (Hessler et al. 1997) was obtained from J. M. Weber (Fermalogic, Chicago, Ill.) and was routinely grown on R2T2 (same as R2T without peptone; Weber et al. 1985) agar medium and in TSB medium (Oxoid) for liquid cultures, at 30° C. Preparation of *S. lividans* protoplasts, and subsequent transformation with plasmid DNA was performed according to Hopwood et al. (1985). Preparation of *Sac. erythraea* protoplasts and subsequent transformation with plasmid DNA was done as described (Gaisser et al. 1997). For optimal production of erythromycin A and its derivatives, cells were first grown at 28° C for 48 h in EP1 (erythromycin production) medium [5 g/l Solulys L corn steep liquor (Roquette frères), 10 g/l defatted soya flour (Cargill), 2 g/l $CaCO_3$, 5 g/l NaCl, pH 6.8, glucose was added from a 10× solution, to a final concentration of 15 g/l, after autoclaving] and subsequent subculture for 3 days in EP2 medium (10 g/l defatted soya flour, 0.2 g/l $CaCO_3$, 1 mg/l $CoCl_2 \cdot 6H_2O$, pH 6.8–7, final concentration of 20 g/l glucose, added from a 10× solution after autoclaving). *Escherichia coli* cells of the strains XL1-Blue (Stratagene), JM110 (Stratagene) and DH5α.MCR (Gibco-BRL) were used for transformation and plasmid preparation. These cells were routinely grown in LB liquid medium or on LB agar plates as described by Sambrook et al. (1989). Ampicillin (Sigma) was used at 100 μg/ml for plasmid selection. For erythromycin bioassays, agar plugs from *Sac. erythraea* culture lawns were placed on Antibiotic agar No. 1 (Merck) (AMerck) plates which had previously been overlaid with 4 ml of 0.5× AMerck inoculated with a spore suspension of *Bacillus pumilus* (ATCC 14884). Plates were incubated overnight at 37° C. The *E. coli-Streptomyces* shuttle vector pUWL218 (Wehmeier 1995), used for chromosomal integration in *Sac. erythraea*, was the kind gift of W. Piepersberg (University of Wuppertal, Germany). The pIJ486 plasmid (Ward et al. 1986) was obtained from C. J. Thompson. The pTZ18R phagemid was from Pharmacia Biotech. The pUC19 plasmid (New England Biolabs) was routinely used for subcloning experiments. The pORT1 shuttle vector was made by sub-cloning the 4-kb *Pst*I fragment isolated from pIJ486 (including both the thiostrepton resistance gene and the *Streptomyces* replicon) into the *Pst*I site of pUC19. The λSE5.5, pNCO28 and pNCO62 genomic clones of *Sac. erythraea* have already been described (Haydock et al. 1991; Gaisser et al. 1997). The pBK6-12 plasmid corresponds to a 4.5-kb *Kpn*I fragment isolated from the pBK25 plasmid clone described by Bevitt et al. (1992) and cloned into pTZ18R (Fig. 1B). Plasmids pK62 and pK23, respectively, correspond to the 5.8-kb and 10-kb *Kpn*I fragments isolated from λSE5.5 and cloned into pUC19 (Fig. 1A). The pEco2 plasmid corresponds to the 2.2-kb *Eco*RI fragment isolated from λSE5.5 and cloned into pUC19 (Fig. 1A). The plasmids pKB22 and pBK44 were derived from pK62 by sub-cloning each of the two 2.9-kb *Kpn*I-*Bgl*II fragments into the *Kpn*I/*Bam*HI sites of pUC19 (Fig. 1A). The pBIISB plasmid was derived by sub-cloning into pUC19 the 600-bp *Sal*I fragment isolated from pBK44 (Fig. 1A).

DNA manipulations

Digestion of DNA with restriction enzymes was performed as recommended by the enzyme suppliers (New England Biolabs and Boehringer Mannheim). Blunting of protruding ends was performed using the Klenow fragment of DNA polymerase I (Boehringer Mannheim) as described by Sambrook et al. (1989). Competent *E. coli* cells of commercial origin (Gibco BRL and Stratagene) were used for transformation with plasmid DNA following protocols recommended by the suppliers. Plasmid isolation from *E. coli* cells was performed using the Plasmid Midi kit (Qiagen) or the RPM kit (Bio101). Preparation of λ bacteriophage DNA was performed according to Ausubel et al. (1995). *Sac. erythraea* chromosomal DNA was isolated according to Hopwood et al. (1985) or with the G NOME DNA kit (Bio 101). For Southern blot analysis, DNA transfer onto GenescreenPlus membranes (Dupont NEN) was performed in 0.4 M NaOH as described (Ausubel et al. 1995). Oligonucleotides were 5′ end labelled using [γ^{32}P]ATP (Amersham) and polynucleotide kinase (Boehringer Mannheim) according to Sambrook et al. (1989). Hybridization was performed with the rapid hybridization buffer from Amersham, as recommended by the manufacturer. Polymerase chain reaction (PCR) analysis of DNA from *E. coli* cells were performed on 10 μl of an overnight culture in LB medium. For PCR analysis of *Sac. erythraea* DNA, 100 μl of a 3-day culture in TSB medium was centrifuged and 10 μl of the cell pellet (in TSB) was used for amplification in a geneAmp PCR system 9600 (Perkin Elmer Cetus). After heating for 3 min at 94° C, PCR reactions were carried out with AmpliTaq polymerase (Perkin Elmer) and appropriate oligonucleotide primers (25 pmol/μl) in the presence of 10% (v/v) dimethyl sulphoxide for 30 cycles of 1 min at 94° C, 1 min at 55° C and 3 min at 72° C, followed by an elongation period of 7 min at 72° C.

DNA sequencing and computer analysis

DNA sequencing was performed as described (Haydock et al. 1991). Additional sequencing analysis was performed on plasmid DNA samples (purified on Qiagen 100 columns) using an ABI Prism 377 automated sequencer (Applied Biosystems). In the latter case, sequencing reactions were performed using conventional M13 primers or synthetic primers with fluorescent dye-labelled dideoxynucleoside triphosphate terminators, and Taq FS polymerase (Perkin Elmer) in the presence of 5% (v/v) dimethyl sulphoxide. Sequence data were assembled as described (Haydock et al. 1991) and with the Autoassembler software package from Applied Biosystems. Sequence analysis was performed with the Genetic Computer Group software package (version 8.1) (Devereux et al. 1984).

Purification of erythronolide B and 3-α-mycarosyl erythronolide B

Erythronolide B and 3-α-mycarosyl erythronolide B were purified from the *eryCI* mutant WH2221 (Dhillon et al. 1989) by chromatography on aminopropyl silica gel and stepwise elution with n-butyl chloride/dichloromethane (100/0, 80/20, 50/50, 20/80, v/v), followed by gradient elution with n-butyl chloride/methanol (from 99/1 to 90/10, v/v). Fractions containing erythronolide B and 3-α-mycarosyl erythronolide B were identified by thin-layer chromatography, pooled and evaporated under reduced pressure. Erythronolide B was crystallised once from ethyl acetate/hexane and once from ethanol, while 3-α-mycarosyl erythronolide B was crystallised twice from ethyl acetate/hexane.

Generation of chromosomal mutants of *Sac. erythraea*

For the generation of chromosomal mutants of *Sac. erythraea* by homologous recombination, plasmids were constructed as follows.

1. ORF7 (*eryBII*). The pK62 plasmid was shortened on its left-hand side by removing a 598-bp *Bcl*I-*Bam*HI fragment (Fig. 1A). The resulting plasmid pBCK1 was further digested with *Mlu*I and *Bgl*II, to delete an 853-bp fragment internal to ORF7. After blunting with Klenow, the deleted plasmid was religated and transformed into *E. coli*. From the p19BIID plasmid thus generated, the 4.3-kb fragment harbouring the deletion was excised using *Kpn*I and *Hind*III (from the pUC19 polylinker) and sub-cloned into pUWL218 to generate plasmid pBIID.
2. ORF8 (*eryCIII*). As this ORF is translationally coupled to the downstream ORF7, an in-frame deletion was introduced to avoid any polar effect. A 663-bp *Sal*I deletion was created within ORF8 by sub-cloning into pUC19 the two *Sal*I fragments of 631 and 794 bp isolated from the pBK4 plasmid (Fig. 1A). The correct orientation of the two *Sal*I fragments was verified by the generation of a 600-bp fragment by PCR analysis of ampicillin-resistant clones, using as primers the C3-S and C3-R oligonucleotides described in Table 1. The resulting plasmid, pdel88, was then subjected to two additional sub-cloning steps in order to enlarge the flanking chromosomal regions available for homologous recombination. First, the 450-bp *Sac*I fragment in pdel88 was replaced by the 1.1-kb *Sac*I fragment from pEco2 (Fig. 1A) to generate plasmid pdel88A. In a second step, the pOBB plasmid was first derived by introducing the 4-kb *Pst*I fragment from pIJ486 (containing the thiostrepton resistance gene and the *Streptomyces* replicon) into the *Pst*I site of pBK44 (Fig. 1A). The 1.5-kb *Eco*RI fragment (carrying the deletion within ORF8) was then isolated from pdel88A and used to replace the 1.66-kb *Eco*RI fragment carrying the intact ORF8 in pOBB. The resulting plasmid, pCIIID, thus carries flanking chromosomal regions for homologous recombination of, respectively 1.27 kb of sequence upstream and 1.38 kb downstream of the deletion point.
3. ORF9 (*eryCII*). The pCIID plasmid carrying a 304-bp deletion in ORF9, which disrupts the reading frame, was constructed by sub-cloning the 1.1-kb *Sac*I-*Kpn*I fragment from plasmid pK23, together with the 1.7-kb *Eco*RI-*Kpn*I fragment from the pEco2 plasmid into the pORT1 vector previously digested with *Sac*I and *Eco*RI (Fig. 1A).
4. ORF13 (*eryBIV*). As this gene could be translationally coupled to the downstream ORF14, an in-frame deletion had to be introduced. First, the pPSP4 plasmid was constructed by sub-cloning the *Pvu*II-*Spe*I fragment from pBK6-12 and the *Spe*I-*Pst*I fragment from pNCO28 into pUC19 previously digested with *Sma*I and *Pst*I (Fig. 1B). A 510-bp *Bcl*I-*Nco*I fragment internal to ORF13 was then deleted and replaced by a 45-bp fragment derived from a

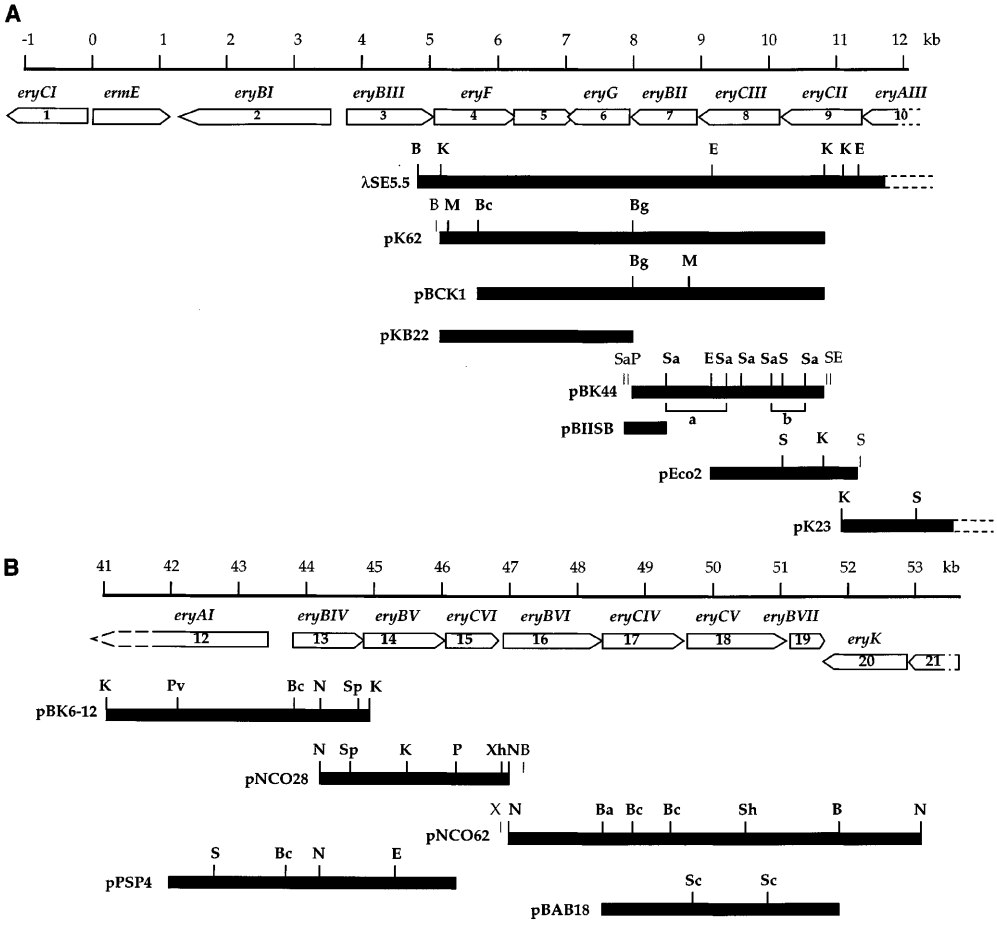

Fig. 1A, B Organisation of left (**A**) and right (**B**) regions of the erythromycin gene cluster in *Sac. erythraea*. Numbers refer to the distance (in kb) from the *Bam*HI site at the beginning of the *ermE* gene. ORFs are indicated by *open arrows* and named according to Liu and Thorson (1994) and Gaisser et al. (1997). Also shown are the locations of plasmids pNCO28 and pNCO62 (Gaisser et al. 1997) and of λSE5.5 (Haydock et al. 1991). Other clones are described in the text. **a** and **b** refer to the two *Sal*I fragments used to generate the pdel88 plasmid (see text). Restriction sites: B, *Bam*HI; Ba, *Bal*I; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; M, *Mlu*I; N, *Nco*I; P, *Pst*I; Pv, *Pvu*II; S, *Sac*I; Sa, *Sal*I; Sc, *Sca*I; Sh, *Sph*I; Sp, *Spe*I; X, *Xba*I; Xh, *Xho*I. Sites derived from polylinkers are *not* indicated in *bold face*

Table 1 Oligonucleotide primers used in the PCR analyses and Southern blot analysis of the different *ery* mutants of *Sac. erythraea*

Ery Mutant	Name of primer[a]	Oligonucleotide sequence
BII92	B2-S	5′-TTGGCGAAGTCGACCAGGTC-3′
	B2-R	5′-GCCGCTCGGCACGGTGAACTTCA-3′
CIII68	C3-S	5′-ATGCGCGTCGTCTTCTCCTCCATG-3′
	C3-R	5′-TCATCGTGGTTCTCTCCTTCC-3′
CII62	C2-S	5′-GGAATTCATGACCACGACCGATC-3′
	C2-R	5′-CGCTCCAGGTGCAATGCCGGGTGCAGGC-3′
BIV87	B4-S	5′-CAATATAGGAAGGATCAAGAGGTTGAC-3′
	B4-R	5′-AACTCGGTGGAGTCGATGTCGTCGCTGCGGAA-3′
BV88	B5-S	5′-AGGAGCACTAGTGCGGGTACTGCTGACGTCCTT-3′
	B5-R	5′-TCCGGAGGTGTGCTGTCGGACGGACTTGTCGGTCGGAAA-3′
CIV89	C4-S	5′-GGCCTATGTGGACTACGTGTTGAACGT-3′
	C4-R	5′-AGCGGCTTGATCGTGTTGGACCAGTAC-3′
CV90	C5-S	5′-TTCGCTCCCCGATGAACACAACTCGTA-3′
	C5-R	5′-AACGCCTCGTCCTGCAGCGGAGACACGAACA-3′

[a] Primers whose names have been *underlined* were used as probes for the Southern hybridization (Fig. 5B). In the case of the CII62 deletion mutant the C3-S primer was used as a probe

54-bp synthetic adapter. This adapter was generated by annealing together the complementary oligonucleotides: 5′-AATTGATCAAGGTGAACACGGTCATGCGCAGGATCCTCGAGCGGAACTCCATGGGG-3′ and 5′-CCCCATGGAGTTCCGCTCGAGGATCCTGCGCATGACCGTGTTCACCTTGATCAATT-3′, and digesting with *Bcl*I and *Nco*I (this adapter was also designed to introduce an *Xho*I site to assist in restriction analysis; see below). A *Sac*I-*Eco*RI fragment harbouring the modified ORF13 was further subcloned into the corresponding sites of pUWL218, thus generating pBIVD.

6. ORF14 (*eryBV*). The pBVD plasmid carrying a 712-bp deletion internal to ORF14 was generated by ligating the 1.1-kb *Bcl*I-*Kpn*I fragment from pBK6-12, together with the 1.1-kb *Kpn*I-*Bam*HI fragment from pNCO28, into the *Bam*HI site of pUWL218 (Fig. 1B).

7. ORF17 (*eryCIV*). The pNCO62 plasmid was digested with *Bal*I and *Bcl*I to generate a 949-bp deletion within ORF17 (Fig. 1B). Subsequent blunting of the ends and ligation generated pBCB17. A 2.68-kb fragment carrying the deletion was recovered from pBCB17 by digestion with *Xba*I and *Sph*I and sub-cloned into the corresponding sites of pUWL218 (Fig. 1B), thus generating pCIVD.

8. ORF18 (*eryCV*). A 3.5-kb *Bal*I-*Bam*HI fragment from pNCO62 was first sub-cloned into the *Sma*I-*Bam*HI sites of pUC19. From the resulting plasmid pBAB18, a 1-kb *Sca*I fragment internal to ORF18 was deleted (Fig. 1B). The fragment carrying the deletion was then recovered by digestion with *Hind*III and *Eco*RI (site in the pUC19 polylinker) and sub-cloned into the corresponding sites of pUWL218, resulting in the pCVD plasmid.

The plasmids pBIID, pCIIID, pCIID, pBIVD, pBVD, pCIVD and pCVD were individually used to transform protoplasts of *Sac. erythraea*. Selection of primary and secondary transformants was performed as described by Cortés et al. (1995), except that TSB (Oxoid) medium was used instead of M1-102 medium for the four rounds of subculture in liquid medium.

Metabolite identification

Cultures of *Sac. erythraea*, grown in EP2 medium for 72 h as described above, were centrifuged for 10 min at 5000 rpm. The supernatants were adjusted to pH 9.0–10.0 with sodium hydroxide and extracted with ethyl acetate. The organic phases were pooled, dried over anhydrous $MgSO_4$, concentrated by rotary evaporation, resuspended in dichloromethane and analysed on pre-coated silica gel 60 F_{254} thin-layer chromatography plates (Merck). Chromatography was performed using either dichloromethane-methanol (90/10, v/v) or isopropyl ether/methanol/ammonia (ca. 25% NH_3) (75/35/2, v/v). Alternatively, samples were chromatographed on pre-coated silica gel NH_2 F_{254} plates (Merck) in n-butyl chloride/methanol (90/10, v/v). The compounds were visualised by spraying the plates with p-anisaldehyde/sulphuric acid/ethanol (1/1/9, v/v) with subsequent heating for a few minutes at 80° C. Erythromycin A (Roussel Uclaf) was used as a standard. The antibiotic activity of metabolites was assessed in parallel by bioautography on agar seeded with *B. pumilus* (ATCC 14884). High-performance liquid chromatography (HPLC) was performed on a liquid chromatography system (Gilson) equipped with a variable wavelength detector at 215 nm, and a Kromasil C18 5µ stainless steel column (250 × 4.6 mm i.d.) fitted with a Kromasil C18 5µ pre-column (50 × 4.6 mm i.d.). The mobile phase was as follows: acetonitrile/methanol/0.065 M ammonium acetate pH 6.7 (35/15/50, v/v). The flow rate of the mobile phase was 1.0 ml/min and the column was operated at 30° C. HPLC coupled to mass spectrometry was carried out on a chromatography system (Waters) equipped with a Finnigan TSQ 7000 mass spectrometer.

Results

Cloning and sequencing of ORFs 7, 8, and 9

The strategy used for sequencing the genomic inserts in pRH3 and λSE5.5 has already been described (Dhillon et al. 1989; Haydock et al. 1991). Analysis of the nucleotide sequence of these clones allowed the identification of nine individual ORFs, in addition to the *ermE* resistance gene (Fig. 1A) (Haydock et al. 1991). Of these, five have already been published (Dhillon et al. 1989; Haydock et al. 1991). To complete the sequencing of ORFs 7, 8 and 9, the plasmids pKB22, pBK44, pBIISB and pEco2 (Fig. 1A) were subjected to automated sequencing as described in Materials and methods. The resulting nucleotide sequences of ORFs 7, 8 and 9 have been deposited in the EMBL database under the accession number Y14332. The sequence analysis of the remaining ORF, ORF2, will be reported elsewhere (Gaisser et al., manuscript submitted).

Sequence analysis of ORFs 7, 8 and 9

1. ORF7 (EryBII). The deduced product of ORF7 (a protein of 333 amino acid residues, M_r 35 867) shows

highest sequence similarity (51% identity, 61% similarity) to YrpG, the presumed product of an ORF uncovered by systematic sequencing of the *Bacillus subtilis* genome (accession number U93875). Although the function of YrpG is unknown, the ORF7 gene product also shows significant overall sequence similarity (40% identity) to MocA, a putative oxidoreductase from the pathway for catabolism of the crown-gall opine mannopine by *Agrobacterium tumefaciens* (Kim and Farrand 1996). It also shows moderate sequence similarity to other putative oxidoreductases, such as NorA (27% identity) in the aflatoxin biosynthetic gene cluster of *Aspergillus parasiticus* (accession number: U32377), to a rat liver gene implicated in the reduction of aflatoxin B1 aldehyde (accession number P38918), and to an authentic $NADP^+$-linked aryl alcohol dehydrogenase (26% identity) from the white-rot fungus *Phanerochaete chrysoporium* (accession number A55449) (Fig. 2).

2. ORF8 (EryCIII). The deduced protein product of ORF8 (a protein of 421 amino acid residues, M_r 45 929) shows convincing end-to-end sequence similarity to DauH (43% identity) and DnrS (47% identity), encoded by genes in the daunorubicin biosynthetic gene clusters of *Streptomyces peucetius* (Otten et al. 1995) and *Streptomyces* sp. C5 (Dickens et al. 1996), respectively. DauH and DnrS are believed to catalyse glycosyltransfer from dTDP-daunosamine to ε-rhodomycinone. The ORF8 gene product also shows convincing sequence identity (45%) to DnrH from *S. peucetius* (Scotti and Hutchinson 1996), which converts daunosamine to its polyglycosylated form, and to TylM2 (50% identity) from *S. fradiae*, which encodes the glycosyltransferase that adds mycaminose to the 5-hydroxy group of tylactone, the polyketide aglycone of tylosin (Gandecha et al. 1997). This family of related enzymes includes ORF14 (EryBV), also from the erythromycin biosynthetic gene cluster (Gaisser et al. 1997), which shows 44% sequence identity to ORF8 (Fig. 3).

3. ORF9 (EryCII). The deduced gene product of ORF9 (a protein of 361 amino acid residues, M_r 38 507) shows greatest sequence similarity to DnrQ, from the daunorubicin biosynthetic gene cluster of *S. peucetius* (38% identity) (Otten et al. 1995), a protein which is involved in daunosamine synthesis, and to ORF1* from the tylosin biosynthetic gene cluster of *S. fradiae* (40% identity) (Gandecha et al. 1997), which may be involved in mycaminose biosynthesis (Fig. 4). All these proteins also show end-to-end sequence similarity to cytochrome P450 enzymes from a variety of sources, but EryCII, DnrQ and ORF1* all lack the cysteine residue which provides the characteristic ligand to the heme iron in cytochrome P450.

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  ADH  .MVLPTAPEP PTLLGYHRIL SSSAGVRVSP LCLGTMSFGN GWKGV.MGEC
 AlcDH MNIWAPAPEP PTKLGRHRQL APGCGLHVSP IQLGAMSIGD KWHPYGMGTM
EryBII ..........  MTTDAATHVR LGRSALLTSR LWLGTVNFS. .......GRV
  YrpG ..........  .........  .....LRVSR LCLGTMNFG. .......VDT
  MocA ..........  .....MEYRL LGRSGLKVST LTVGTMTFGG VGWAKTVGDL
 AldRD ..........  ..........  .......... .......... ..........

  ADH  DQATSFNMLD TFYESGGNFI DVANFY.... QGGDTERWVG EWMAQRQNR.
 AlcDH DKEASFKLLD AFYNAGGNFI DTANVY.... QDETSEEFIG EWMEARGNR.
EryBII EDDDALRLMD HARDRGINCL DTADMYGWRL YKGHTEELVG RWLAQGGGRR
  YrpG DEKTAFRIMD EALDNGIQFF DTANIYGWGK NAGLTESIIG KWFAQGGQRR
  MocA GVTEAKRLVD LCLDAGINLI DTADVYS... .DGKSEEILG EIL..GGKRK
 AldRD MSQARPATVL GAMEMGRRMD VTSSSASVRA FLQRGHTEID TAFVYANGQS
                 *
  ADH  DEIVLSTKYT MGY...TMFG PQKIKSNY.Q GNHAKSLRLS VKASLQKLQT
 AlcDH DQMVVATKYS LVYKRGASFE EIPQKTQY.V GNSLKSMHIS VHDSLRKLRT
EryBII EDTVLATK.. ...VGGEMS. ..ERVND..S GLSARHIIAS CEGSLRRLGV
  YrpG EKVVLATK.. ...VYEPISD PNDGPNDM.R GLSLYKIRRH LEGSLKRLQT
  MocA GGALVATK.. ...ARFNMGP ...GPND..G GLSRQYLIAA CEASLKRLKT
 AldRD ETILGDLGLG LGRSGCKVKI ATKAAPMFGK TLKPADVRFQ LETSLKRLQC
                                                    **  *
  ADH  DYIDLLYVHM WDFTTSVEEV MRSLNHLVAN GKVLYLGVSD TPAWLVVKCN
 AlcDH SYIDIFYVHF WDYTCTIEEV MNGLHNLVAQ GKVLYLGVSD TPAWVVSKAN
EryBII DHIDVYQMHH IDRSAPWDEV WQAMDSLVAS GKVSYVGSSN FAGWHIAAAQ
  YrpG DHIELYQMHH IDRRTPWDEI WEAFETQVRS GKVDYIGSSN FAGWHLVKAQ
  MocA DVIDLYQLHE WDGQTPLEET MEALDTLVRQ GKVRYIGCSN FTGWQIMKAL
 AldRD PRVDLFYLHF PDHGTPIEET LQACHHVHQE GKFVELGLSN YVSWEVAEIC
           *   *      *                 **    * *     *
  ADH  AFARANGLTP FSVYQGHWSC AFRDFERDIL PMCESEGMGL APWGVLGRGQ
 AlcDH NYARMAGKTP FVIYEGEWNI TMRDMERDII PMCIHEGMAI APWNVLCAGK
EryBII ENAARRHSLG MVSHQCLYNL AVRHAELEVL PAAQAYGLGV FAWSPLHGGL
  YrpG AEAEKRRFMG LVTEQHKYSL LERTAEMEVL PAARDLGLGV VAWSPLAGGL
  MocA GISEKDKRQR FVSQQIHYTL EARDAEYELL PISVDQGLGV LIWSPLAGGL
 AldRD TLCKKNGWIM PTVYQGMYNA ITRQVETELF PCLRHFGLRF YAFNPLAGGL
                          *  *      *     *       *  *
  ADH  FRSAEEFSRE ......GRKM GPQD.....E KHRR...... ..LGEKLDQM
 AlcDH IRTDAEEERR LKSGEGGRTL LQFDGWLRNE TERK...... ..VSKALEKV
EryBII LSGALEKLAA ...GTAVKSA QGRAQVLLPS LRPA...... ..I.EAYEKF
  YrpG LGGKALKSNA ...GTRT... .AKRADLIEK HRLQ...... ..L.EKFSDL
  MocA LSGKHRRNQS APEGSRQFAG WTEPPVRDEE RLWN...... ..IVDTLLSV
 AldRD LTGRYKYQDK DGKNPESRFF GNPFSQLYMD RYWKEEHFNG IALVEKALKT

  ADH  AQQKNTKA.T SIAQAYVMHK APYV.....F PVIGGRKVEH LKENIEALGL
 AlcDH AEEIGAKSIT SVAIAYLMQK FPYV.....F PIVGGRKVEH LYANLEALDI
EryBII CRNLGEDP.A EVGLAWVLSR PGIA.....G AVIGPRTPEQ LDSALKASAM
  YrpG CKELGEKE.A NVALAWVLAN PVLT.....A PIIGPRTVEQ LRDTIKAVEI
  MocA ADGRGVSA.A QVALAWLIGR KAVT.....S IIIGGRTEAQ FKDNLAAADL
 AldRD TYGPTAPSMI SAAVRWMYHH SQLKGTQGDA VILGMSSLEQ LEQNLALVEE
                                        *
  ADH  .VLSEEEIRE IDDAEPFDVG FP.MNFSFET PTQSYRTNMT SKDIWQLSCN
 AlcDH .SLSPEQMQF LNDTVPFNKG FPYLLFGDGS DYNIVHKAAG HYDKWPAQQA
EryBII .TLDEQALSE LDEIFPAVAS GGAAPEAWLQ .......... ..........
  YrpG .SLDKEILRM LNDIFP..GP GGETPEAYAW .......... ..........
  MocA .QLSAEERKR LDDVSLLQLL YPYWHQRNNA SDRLSEADLE LLAPHLSKKG
 AldRD GPLEPAVVDA FDQAWNLVAH ECPNYFR... .......... ..........

  ADH  TRLETVPKQQ PIEPFQGAKY FGSASK
 AlcDH IRPQK..... .......... ......
EryBII .......... .......... ......
  YrpG .......... .......... ......
  MocA .......... .......... ......
 AldRD .......... .......... ......
```

Fig. 2 Comparison of the ORF7 gene product (EryBII) of *Sac. erythraea* with YrpG from *Bacillus subtilis* (U93875), MocA from *Agrobacterium tumefaciens* (U19620), NorA (ADH) from *A. parasiticum* (U32377), aflatoxin B1 reductase (aldRD) from rat liver (P38918) and aryl alcohol dehydrogenase (AlcDH) from *Penicillium chrysosporium* (A55449). Genbank accession numbers are given. Amino acid sequence identities are indicated by *asterisks*

Identification of chromosomal mutants of *Sac. erythraea*

To determine whether the products of ORFs 7, 8, 9, 13, 14, 17 and 18 are involved in the synthesis of either dTDP-L-mycarose or dTDP-D-desosamine, chromosomal mutants were constructed as described in Materials and methods. Gene replacement mutants were identified as follows. After screening for loss of resistance to thiostrepton, bioassays were performed to monitor the loss of erythromycin production. In all cases, *ery*$^-$ mutants could be identified (data not shown). The mutant strains BII92 (ORF7), CIII68 (ORF8),

```
   DauH  VRVLFATMAA RSHVYAQVTL ASALRTAGHE VLVASQPDVL DDIVRAGLTR
   DnrH  VRVLFATMAA RSHVYAQVTL ASALRTAGHE VLVASQPDVL DDIVRAGLTA
EryCIII  MRVVFSSMAS KSHLFGLVPL AWAFRAAGHE VRVVASPALT EDITAAGLTA
  TylM2  MRVLLTCIAH NTHYYNLVPV AWALRAAGHE VRVAAQPALT DTITASGLTA
   DnrS  MKVLVTAFAM DAHFNGVVPL AWALRAAGHD VRVASQPALT DSITRAGLTA
  EryBV  VRVLLTSFAH RTHFQGLVPL AWALRTAGHD VRVAAQPALT DAVIGAGLTA
           *     *    *    *   * * * ***  * *   *          ***

   DauH  VRIGEDLNIE EETREANASF EDDRNLGGLA MSNTRDDPLP WDHALGMFTA
   DnrH  VRIGEDLNIE EETREANASF EDDRNLGGLA MSNSRDDPFP WDHALGMFTA
EryCIII  VPVGTDVDLV DFMTHAGHDI IDYVR..SLD FSERDPATLT WEHLLGMQTV
  TylM2  VPVGGNESVL EFVTEIGGDP GPYQR..GMD FAETCGEPLS YEHALGQQTA
   DnrS  VPVGTDHQVQ AAMGAMAPGV FALHL..NPD YLENRPELLD LEFLEASTSM
  EryBV  VPVGSDHRLF DIVPEVAAQV HRYSF..YLD FYHREQELHS WEFLLGMQEA
         ** *

   DauH  MTAMVFQNVC PEPMVDDLVG LARDWRPDLV VWDPLTLAGP VAARLSGAAH
   DnrH  MTAMVFQNVC PEPMVDDLVG LARDWRPDLV VWDPLTLAGP VAARLSGAAH
EryCIII  LTPTFYALMS PDTLIEGMVS FCRKWRPDLV IWEPLTFAAP IAAAVTGTPH
  TylM2  MSALCFAPFN CDSTIDDMVA LARSWRPDLV LWEPFTYAGP IAAHACGAAH
   DnrS  LTAAFYAQIN NDSMIDEMVD FAAWWRPDLV VWEPFTFGGA VAAQVTGAAQ
  EryBV  TSRWVYPVVN NDSFVAELVD FARDWRPDLV LWEPFTFAGA VAARACGAAH
                        *     ******  * * *     **   *

   DauH  ARLLFGPDQM GRNRTAFRAL LDRQRPSCVT TRCAEWLTWT LERWRRQR.L
   DnrH  ARLLFGPDQM GRNRTAFRAL LDRQPPELRD DPLAEWLTWT LERCGGSA.G
EryCIII  ARLLWGPDIT TRARQNFLGL LPDQPEEHRE DPLAEWLTWT LEKYGGPA..
  TylM2  ARLLWGPDVI LNARAQFRRL APDSPEEPRE DPVAEWLGWT LERHGLTAER
   DnrS  ARLLWGPDLF LRVHDRFQQV LHEVPAERRD DALEEWLTWT LERHGA....
  EryBV  ARLLWGSDLT GYFRGRFQAQ RLRRPPEDRP DPLGTWLTEV AGRFGV....
         **** * *         *                      **

   DauH  DMSEELVLGQ WTIDPTPPSM RIPLDLPCVP VRYVPYNGP. .SLLPDWLRE
   DnrH  DMSEELVLGQ WTIDPTPPSM RIPLDLPCVP VRYVPYNGP. .SLLPGWLRE
EryCIII  .FDEEVVVGQ WTIDPAPAAI RLDTGLKTVG MRYVDYNGP. .SVVPEWLHD
  TylM2  ETVEELIGGQ WTLDPTAESL RCPRP.AVVP FRFVPYNGR. .SVLPDWLLR
   DnrS  AFGPEVISGH WTIDQMPPSV RFATARPTVP MRFVPYNGPV PAVVPPWLRA
  EryBV  EFGEDLAVGQ WSVDQLPPSF RLDTGMETVV ARTLPYNG.. ASVVPDWLKK
                  * *  *       *       *   ***       * **

   DauH  PPRHPRRLCL TLGVSLGEAT GAGTVAASDV LAAVDGLDVE VVATLSRNC.
   DnrH  PPRHPRRLCL TLGVSLGEAT GAGTVAASDV LAAVDGLDVE VVATLPRELR
EryCIII  EPERR.RVCL TLGISSREN. SIGQVSIEEL LGAVGDVDAE IIATFDAQQL
  TylM2  KPGRP.RVCF TLGVSARETY GRDAVPFHEL LAGLGDLDAE IVATLDPGQL
   DnrS  DPGRP.RVLL TQGITERSTG FTGLPRAGEL LASIAELDAE VVATVKAEER
  EryBV  GSATR.RICI TGGFSGLGLA .ADADQFART LAQLARFDGE IVVTGSGPDT
               *    * *                  *      * *    *

   DauH  QELGTLPANV RAVDFVRLNA LLPSCSGIIH HGGSGTFMTA LAHATPQLIV
   DnrH  EELGTLPANV RAVDFVPLNA LLPSCSGIIH HGGSGTFMTA LVHAIPQLIV
EryCIII  EGVANIPDNV RTVGFVPMHA LLPTCAATVH HGGPGSWHTA AIHGVPQVIL
  TylM2  SGAGEVPRNV RAVDFVPMDA LLPTCSAVVH HGGAGTCFTA TLNGLPQIVV
   DnrS  EGLPPLPGNV RVVDSLSLHV VLPSCAAVVH HGGAGTWATA ALHGVPQLAL
  EryBV  SA...VPDNI RLVDFVPMGV LLQNCAAIIH HGGAGTWATA LHHGIPQISV
               * *  *           *  *    * *** *   **       **

   DauH  PDMMWDAMEK AHGLARSGAG GYV..DAKDV SPDLLRERVL DLFDDPSYA.
   DnrH  PDMMWDAMEK AHGLARSGAG SYV..DARDV SPELLRERVL ALLDDPSYA.
EryCIII  PD.GWDTGVR AQRTQEFGAG IALPV..PEL TPDQLRESVK RVLDDPAH.R
  TylM2  AA.LWDAPLK GAQLAEAGAG VSIAP..EKL DAATLRAGVV RALEDEGHSR
   DnrS  AW.QWDDVFR AGQLEKLGAG IFLPPHGEGA SAGRVRDRLA QVLAEPSF.R
  EryBV  AH.EWDCMLR GQQTAELGAG IYLRP..DEV DADSLASALT QVVEDPTY.T
            **          ***

   DauH  AGARRVRAEI VGTPSPNDIV PVLERLTAEH QAGGPERSPA LKSPSTGGA
   DnrH  AGARRVRAEI VGTPSPNDIV PVLERLTAEH RAGGAEGGPA LKSPSTGGA
EryCIII  AGAARMRDDM LAEPSPAEVV GICEELAAGR REPR...... .........
  TylM2  RSAGLLRAEM LAEPTPAGLV PQLERLTALH RNGRSRSAPE R........
   DnrS  QGAARIRAEM LRTPAPGAVV PTLEQLTARH RAPAGQGVRH .........
  EryBV  ENAVKLREEA LSDPTPQEIV PRLEELTRRH AG........ .........
           *   *       * *   *    * *
```

Fig. 3 Comparison of the putative glycosyltransferases ORF8 (EryCIII) and ORF14 (EryBV) (Y11199) from *Sac. erythraea*, DauH from *Streptomyces* sp. C5 (U43704), DnrH (U77891) and DnrS (L47164) from *S. peucetius*, and TylM2 from *S. fradiae* (X81885). Accession numbers are given. Sequence identities are indicated by *asterisks*

```
   DnrQ  .......... MPTPTSAPPA APTDSELGRH LLTVRGFHFV FGALGDPYAR
TylORF1  MNTAAGPTGT AAGGTTAPAA AHDLSRAGRR LQLTRAAQWF AGNQGDPYGM
 EryCII  .......... ........MT TTDRAGLGRQ LQMIRGLHWG YGSNGDPYPM
                                    **  *   *        *  ****

   DnrQ  RLR.GEADHL SLGELVRDRG PL.HGSALGT WVTADGGISA RLLDDPLLGP
TylORF1  ILRAGTADPA PYEEEIRERG PLFHSELLGT WVTGSRHVAD AVTADDAFGA
 EryCII  LLCGHDDDPQ RRYRSMRESG ..VRRSRTET WVVADHATAR QVLDDPAF..
          *     *          *  *          * **              *

   DnrQ  RHPASEGPQE HVLENVWETW RTCHVTPLGE DLLTPAAADS DRLAALLGPV
TylORF1  LTADGARPGV RELPLSGSAL DAAHGNPGGP PL..PGG... .......WPH
 EryCII  TRATGRTP.. .......EWM RAAGAPP... ...AEWAQPF RDVHAASWEG
                *                  *

   DnrQ  LGPRTCTAWQ VDAGRAVHRV LDGLPPHFDV VSDLARPAIA GSLAAVLGLP
TylORF1  RPPDREERDD PDRHAADLLN AAGPGQVLDL V.PFARRLAA RTTGAWLGVP
 EryCII  EVPDVGELAE SFAG.....L LPGAGARLDL VGDFAWQVPV QGMTAVLG..
           *                      *    *   *   *          * **

   DnrQ  DEARAELPDL LAACGPVLDS ALCPPRLPVA RAMTQALRRV RELMAAAVAN
TylORF1  AERLPRFETA LTGCRRALDA LLCPQLLADA RA........ ..........
 EryCII  ..AAGVLRGA AWDARVSLDA QLSPQQLAVT EAAVAAL... ..........
                          **   *  *  *

   DnrQ  HLTAPADGAV SALLAVDPGG GRDPGDTVTA AVLSTVVGAE TAITTVANAV
TylORF1  ..GLAAEEAL RAVLGETPEA RGRPPGAVEA ARAHAVSAAE PIAVLLCNAV
 EryCII  ....PADPAL RALFA..... .......... .......GAE MTANTVVDAV
           *  *   *                                **          **

   DnrQ  MALLKHDEQW SLLRADPGRA ADAVEETLRW APPVTLRSLI TQGEVQIGGE
TylORF1  RELMERPAQW RALTADPGLA GAAITETLLW APPVRLESRV ARETAVLAGR
 EryCII  LAVSAEPGLA ERIADDPAAA QRTVAEVLRL HPALHLERRT ATAEVRLGEH
                        **  *       * *    *   *

   DnrQ  TLEADQHVVV LVDAAQRDPA LYEDPDRFRL D...RPRSPG FTHMALAGRD
TylORF1  TLPAGTHLVV LAAAANRDAC RNAGPAVTGF DVLRRASDGG PQPHGLPEDL
 EryCII  VIGEGEEVVV VVAAANRDPE VFAEPD..RL DVDRPDADRA LSAH....RG
                **    ** **       *      *

   DnrQ  HLGLVAPLVR VQCTAVLRAL AERLPGLRAE GEPLRRGRSP VVRAPLSLRL
TylORF1  HFRLSGPLVR RTAEAGLRAL AERFPGLRPA GPAVRVRRSP VLRGLGRLPV
 EryCII  HPGRLEELVT ALATAALRAA AKALPGLTPS GPVVRRRRSP VLRGTNRCPV
         *      **       * ***  *   ***    *   *  *** * *

   DnrQ  AQK...
TylORF1  APYVPE
 EryCII  EL....
```

Fig. 4 Comparison of the putative 3,4-keto isomerase ORF9 (EryCII) in *Sac. erythraea*, with DnrQ from *Streptomyces peucetius* (L47164) and the deduced protein product of ORF1* in *S. fradiae* (tylORF1) (X81885). Accession numbers are given. Sequence identities are indicated by *asterisks*

CII62 (ORF9), BIV87 (ORF13), BV88 (ORF14), CIV89 (ORF17) and CV90 (ORF18) thus generated were individually subjected to PCR analysis using the oligonucleotide pairs listed in Table 1. In each case, the size of the amplified fragment detected in the mutant strain analysed was identical to the one generated with the corresponding plasmid used to derive the mutant by homologous recombination, while the wild-type strain gave a PCR fragment of the expected higher mobility (data not shown). To confirm the presence of the deletion within the chromosome of each mutant, Southern blot analyses were performed as follows.

BII92. When *Kpn*I-digested chromosomal DNA prepared from the BII92 mutant strain was probed with the ^{32}P-end-labelled oligonucleotide B2-S (Table 1) a 4.9-kb band was detected, while the expected 5.8-kb band could be identified in the *Sac. erythraea* wild-type strain (data not shown).

CIII68. When *Eco*RI-digested chromosomal DNA prepared from the CIII68 mutant strain was probed with the C3-S oligonucleotide (Table 1) a 1.5-kb band was detected, as opposed to the 2.2-kb band identified in the wild-type strain (data not shown).

CII62. By Southern blot analysis using the C3-S oligonucleotide as a probe on *Eco*RI-digested chromosomal DNA a 1.8-kb band was detected in the CII62 mutant strain, while a 2.2-kb band was identified in the case of the wild-type strain (data not shown).

BIV87. Since an *Xho*I site had been introduced into the adaptor used to construct the pBIVD plasmid, this enzyme was used to monitor the presence of an additional *Xho*I site 2.7 kb upstream of the *Xho*I site located at the beginning of ORF16 (Fig. 1B). When genomic DNA from the BIV87 mutant was digested with *Xho*I and probed with the B4-R oligonucleotide (Table 1) a 2.7-kb band was detected, as opposed to the 5.4-kb signal detected in the wild-type strain (data not shown).
BV88. When *Nco*I-digested chromosomal DNA from the BV88 mutant strain was probed with the B5-R oligonucleotide (Table 1) a 2-kb band was detected, while a 2.7-kb band was identified in the wild-type strain (data not shown).
CIV89. By Southern blot analysis using the C5-R oligonucleotide as a probe (Table 1) on *Nco*I-digested chromosomal DNA, a 5.2-kb band was detected in the CIV89 mutant strain and a 6.2-kb band in the wild type (data not shown).
CV90. When *Nco*I-digested chromosomal DNA samples were probed with the C5-R oligonucleotide (Table 1), a 5.1-kb band and a 6.2-kb band were detected in the CV90 mutant and the wild-type strain, respectively (data not shown).

All these results confirmed the presence of the expected deletion within the chromosome of each mutant strain.

eryB and *eryC* phenotypes of chromosomal mutants of *Sac. erythraea*

In order to analyse the phenotype of each mutant, cells were grown in EP2 medium as described in Materials and methods, metabolites were extracted and analysed by thin-layer chromatography using purified erythronolide B, 3-α-mycarosyl erythronolide B and erythromycin A as standards. As shown in Fig. 5, mutants CIII68, CII62, CIV89 and CV90 all preferentially accumulate 3-α-mycarosyl erythronolide B, as well as varying amounts of erythronolide B, as expected for *eryC* mutants. Similarly, mutants BII92, BIV87 and BV88 all accumulate erythronolide B as the major compound, as expected for *eryB* mutants (Fig. 5). For all these ORFs, the nomenclature proposed by Katz and colleagues in several review articles (Donadio et al. 1993; Liu and Thorson 1994; Katz and Donadio 1995) therefore appears to be valid and the same designation is adopted here. In mutants BII92 and BIV87, minor metabolites of low mobility were detected in addition to erythronolide B (Fig. 5). In the case of BII92 and BIV87 the corresponding compounds display antibiotic activity, as assessed by bioautography performed on an agar plate seeded with the erythromycin-sensitive strain *B. pumilus* ATCC 14884 (data not shown). Similarly, minor compounds of low mobility were detected in mutant CIV89, in addition to erythronolide B and 3-α-mycarosyl erythronolide B (Fig. 5). In this case, bioautography of the products from a small-scale (50 ml) fermentation showed no antibiotic activity, but fermentation on a 2-l scale gave products which displayed clear antibiotic activity by this method (data not shown).

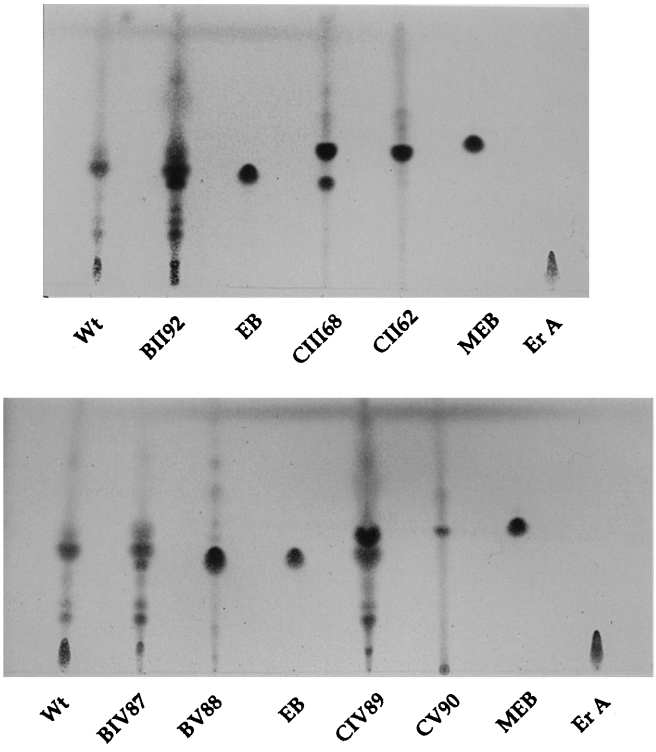


Fig. 5 Analysis by thin-layer chromatography of the metabolites produced by *ery*-mutants. Ten micrograms of the following standards were used: EB, erythronolide B; MEB, 3-α-mycarosyl erythronolide B; ErA, erythromycin A

Identification of hybrid compounds

In order to investigate further the structure of the minor compounds produced by mutants BII92, BIV87 and CIV89, extracts made from culture supernatants were analysed by HPLC coupled to mass spectrometry.

1. BII92. One metabolite, named M1, gave a parent peak at *m/z* 704 with a fragment peak at *m/z* 576, indicating the presence of desosaminyl erythronolide A. These results suggest that the *m/z* difference of 30 compared to erythromycin A (*m/z* 734) or 16 compared to erythromycin C (*m/z* 720) can be attributed to the neutral sugar residue. The M1 compound could therefore correspond to 3″-C desmethyl-2″, 3″-ene-erythomycin C. Apart from M1, traces of erythromycins A, B, C and D were also found (data not shown), as well as three other metabolites called M2, M3 and M4 which, according to their *m/z* values, could correspond to 3″-C desmethyl erythromycins A, C and D respectively (Table 2).
2. BIV87. The mass spectral data obtained with the BIV87 mutant clearly indicate that modified forms of erythromycins A, B, C and D are formed (indicated by the 4 K suffix in Table 3). The major component of the mixture appears to be a form of erythromycin D in

which two hydrogen atoms are missing (peaks at *m*/*z* 702 and 684 rather than 704 and 686). The presence of desosaminyl erythronolide B (*m*/*z* 560) as a side product of fragmentation clearly indicates that the difference in mass can be traced to the neutral sugar residue, even though a peak corresponding to the modified neutral sugar fragment itself has not been observed. The fragmentation patterns seen for the erythromycins A, B and C also show a difference of 2 in the *m*/*z* values, traceable to the neutral sugar moiety (Table 3).

Table 2 Mass spectrometry data for minor metabolites produced by the BII92 chromosomal mutant of *Sac. erythraea*

Proposed structure	Ion[a]	*m*/*z*	BII92
	$[M1]H^+$	704	+
M1	$[DesEA]H^+$	576	+
	Des	158	+
	$[M2]H^+$	706	+
M2	$[DesEA]H^+$	576	+
	Des	158	+
	$[M3]H^+$	690	+
M3	$[DesEB]H^+$	560	+
	Des	158	+
	$[M4]H^+$	720	+
M4	$[DesEA]H^+$	576	+
	Des	158	+

[a] Des = desosamine; DesEA = desosaminyl erythronolide A; DesEB = desosaminyl erythronolide B; M1 = 3″-C desmethyl-2″, 3″-ene-erythromycin C; M2 = 3″-C desmethyl erythromycin C; M3 = 3″-C desmethyl erythromycin D; M4 = 3″-C desmethyl erythromycin A

3. CIV89. The most significant minor metabolite produced by the CIV89 mutant showed a peak at *m*/*z* 720. Further fragmentation gave a specific ion at *m*/*z* 174, which is inconsistent with desosamine but would be compatible with hydroxydesosamine. A peak at 576 was seen which would be expected for hydroxydesosaminyl erythronolide B. These results suggest that the *m*/*z* difference of 16 compared to erythromycin D (*m*/*z* 704) can be attributed to the aminosugar residue. Smaller peaks at *m*/*z* 750, 736 and 734 were also seen, which would be consistent with the presence of hydroxydesosaminyl erythromycins A, B and C, but in these cases a peak for the hydroxydesosamine ion could not be detected above background (data not shown). Analysis of a larger-scale fermentation run with this mutant confirmed the presence of ions of the appropriate *m*/*z*, except that an ion of *m*/*z* corresponding to hydroxydesosaminosyl erythromycin B was not detected (data not shown).

4. BV88. MS analysis was also carried out in the case of the BV88 mutant strain, although for that mutant, thin-layer chromatography analysis did not show the presence of minor compounds in addition to erythronolide B (Fig. 5). Indeed, Gaisser et al. (1997) had reported the production of low amounts of desosaminyl erythronolide B, in addition to erythronolide B, by an *eryBVI*

Table 3 Mass spectrometry data for minor metabolites produced by BIV87, BV88 and CIV89 chromosomal mutants of *Sac. erythraea*

Proposed structure	Ion[a]	*m*/*z*	BIV87	BV88	CIV89
	$[4KErD]H^+$	702	+ +		
4KErD	$[4KErD\text{-}H_2O]H^+$	684	+ +		
	DesEB	560	+ +		
	Des	158	+		
	$[4KErC]H^+$	718	+		
4KErC	$[4KErC\text{-}H_2O]H^+$	700	+		
	DesEA	576	+		
	Des	158	+		
	$[4KErA]H^+$	732	+		
4KErA	$[4KErA\text{-}H_2O]H^+$	714	+		
	DesEA	576	+		
	Des	158	+		
	$[4KErB]H^+$	716	+		
4KErB	$[4KErB\text{-}H_2O]H^+$	698	+		
	DesEB	560	+		
	Des	158	+		
	$[DesEA]H^+$	576	+		
DesEA	$[DesEA\text{-}H_2O]H^+$	558	+		
	Des	158	+		
	$[DesEB]H^+$	560	+	+	
DesEB	$[DesEB\text{-}H_2O]H^+$	542	+	+	
	Des	158	+	+	
	$[EB\text{-}H_2O]$	385		+	
	$[4OHErD]\ H^+$	720			+
4OHErD	$[4OHErD\text{-}H_2O]H^+$	702			+
	4OHDesEB	576			+
	4OHDes	174			+

[a] Des = desosamine; DesEA = desosaminyl erythronolide A; DesEB = desosaminyl erythronolide B; EB = erythronolide B; 4KErA, B, C, D = 4″keto-erythromycin A, B, C, D; 4OHErD = 4′hydroxy erythromycin D; 4OHDesEB = 4′hydroxy-desosaminyl erythronolide B; 4OHDes = 4-hydroxy desosamine

(ORF16) mutant strain. Like the *eryBVI* mutant, the BV88 mutant was expected to be able to synthesise desosaminyl erythronolide B, since its desosamine biosynthetic and transfer enzymes had remained unaffected by the mutation introduced, even if the transfer of a neutral sugar residue had been abolished. The results obtained with HPLC coupled to mass spectrometry revealed a peak at *m*/*z* 560 which would indeed correspond to desosaminyl erythronolide B (Table 2). Further fragmentation of this ion gave a specific ion of *m*/*z* 158, corresponding to the loss of desosamine (Table 3).

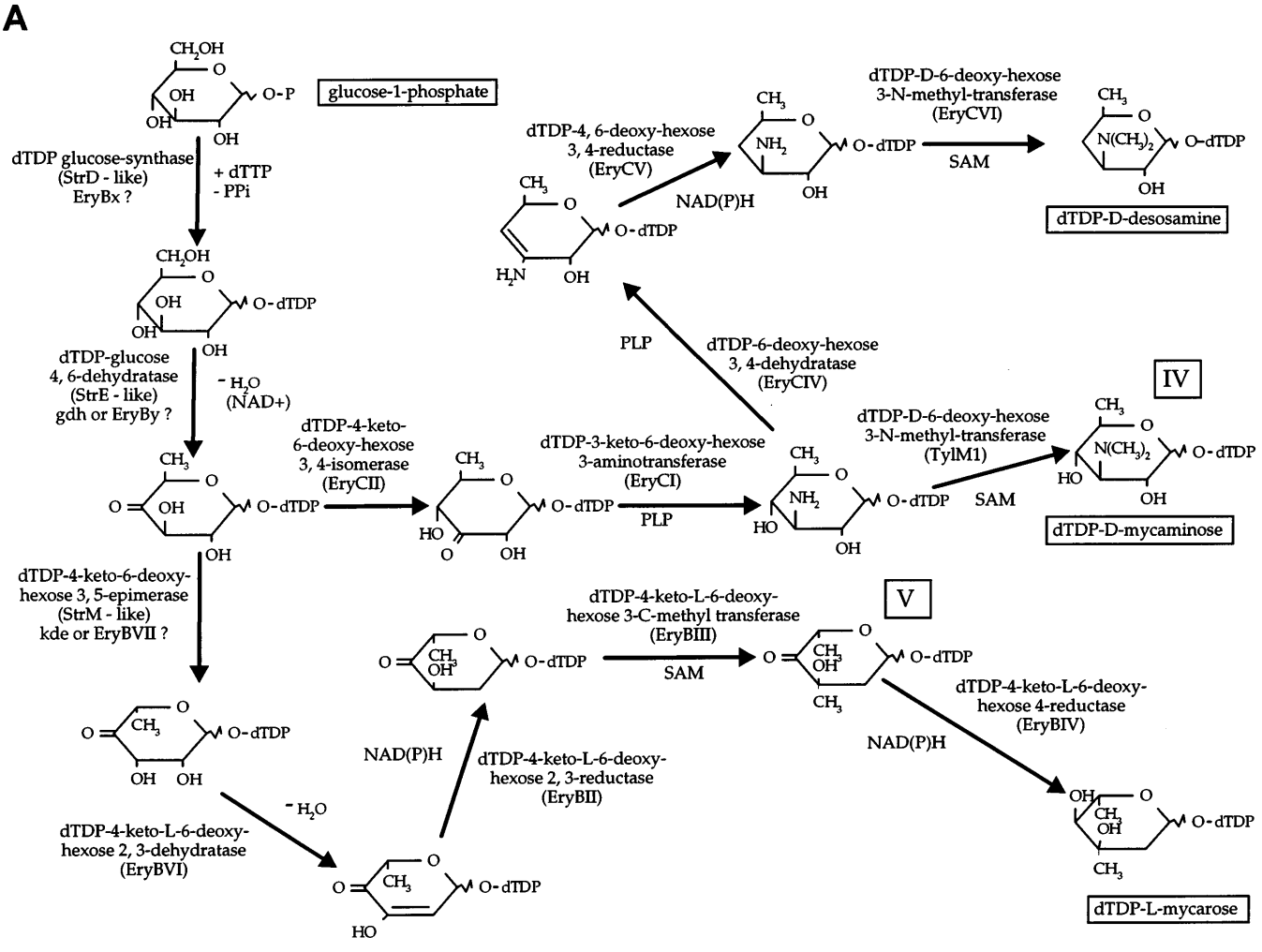
Discussion

The targeted deletion, where appropriate in-frame, of seven individual ORFs in the *ery* biosynthetic gene cluster has been accomplished and confirmed by Southern analysis. Thin-layer chromatography has provided unequivocal evidence that ORFs 7, 13 and 14 are *eryB* genes, because strains lacking such genes accumulated erythronolide B, as expected if they are specifically defective in the synthesis of dTDP-L-mycarose or in the transfer of the sugar to the macrolactone. Similarly, ORFs 8, 9, 17 and 18 have been shown to be *eryC* genes, because strains lacking any one of these genes accumulate 3-α-mycarosyl erythronolide B, as expected if they are defective in the synthesis of dTDP-D-desosamine or in subsequent transfer of the aminosugar to the macrolactone. This clarification of the role of these genes now permits us to make detailed proposals for the respective biosynthetic pathways that lead to these two sugars, which are summarised in Fig. 6A. Several deletion mutants also produced minor metabolites whose highly characteristic mass spectra have provided additional evidence for the role of the corresponding gene. The detection of a modified erythromycin in extracts from an *eryBIII* mutant alerted us to the potential of mass spectrometry in making these identifications, and for that mutant the mass spectrometric identification has been completely confirmed by NMR analysis of the purified metabolite (Gaisser et al., manuscript submitted).

The experimental confirmation that ORF8 is an *eryC* gene and that ORF14 is an *eryB* gene, and our finding that the sequence of ORF8 (*eryCIII*) reveals strong similarities both to ORF14 (*eryBV*) and to other glycosyltransferases (Fig. 3), together provide extremely strong evidence for the identification of these genes as governing the attachment of, respectively, mycarose (*eryBV*) and desosamine (*eryCIII*) to the aglycone. Mass spectrometric analysis of the BV88 mutant has provided evidence for species with the *m*/*z* values predicted for desosaminyl erythronolide B and its fragmentation products, which indicates that the normal pathway (in which desosamine is added only after prior attachment of mycarose) can be subverted in the absence of the *eryBV* gene product, and that the desosaminyltransferase can accept erythronolide B as an alternative (albeit poor) substrate, as previously observed with another *eryB* mutant (Gaisser et al. 1997). However the position in the aglycone to which the sugar residue becomes attached remains to be established.

The *eryBII* gene product displays significant sequence homologies with several oxidoreductases, such as MocA and NorA (Fig. 2). The evidence from mass spectrometry for the presence of 3″-C desmethyl-2″, 3″-ene-erythromycin C (see Fig. 6B for the corresponding neutral sugar residue incorporated) in the supernatant of the BII92 mutant strain strongly suggests that the *eryBII* gene encodes the enoyl reductase responsible for the reduction of the 2,3 double bond after the 2,3 dehydration by EryBVI, in the dTDP-L-mycarose biosynthetic pathway as depicted in Fig. 6A. Surprisingly, additional metabolites carrying a 2,3 reduced neutral sugar, such as erythromycins A, B, C, D and 3″-C desmethyl-erythromycins A, C, D, were also detected in the supernatant of the BII92 mutant. These results indicate the presence of a complementing enoyl reductase activity. As a tentative explanation for the presence of 3″-C desmethyl erythromycin derivatives, we propose that this complementing activity would generate the two alternative C3 epimers of the dTDP-6-deoxyhexose substrate and that one of them is not accepted as a substrate by the 3-C-methyltransferase (i.e. EryBIII; S Gaisser et al., manuscript submitted), although it can still be reduced by the 4-keto-reductase EryBIV, transferred to the macrolactone ring and further incorporated in the biosynthetic pathway of erythromycin A (see Fig. 6B for detailed pathways giving rise to the different neutral sugar residues incorporated in the macrolide structures synthesised by the BII92 mutant).

Fig. 6 **A** Proposed pathways for the biosynthesis of dTDP-D-desosamine and dTDP-L-mycarose in *Sac. erythraea*. **B** Proposed modified dTDP-L-mycarose pathways used in the case of the BII92 mutant strain to give rise to the low-mobility compounds with modified neutral sugar residues (see text). StrD, StrE and StrM, respectively, correspond to the dTDP-glucose synthase, dTDP-4,6-dehydratase and dTDP-6-deoxyhexose 3,5-epimerase characterised in *S. griseus* (Pissowotzki et al. 1991). Also shown is the connection between the biosynthetic pathways for dTDP-D-mycaminose and dTDP-D-desosamine. The TylM1 N-methyl transferase involved in the biosynthesis of dTDP-D-mycaminose in *S. fradiae* has been characterised by Gandecha et al. (1997). Compound I corresponds to the activated neutral sugar substrate used to generate the 3″-C desmethyl-2″,3″-ene-erythromycin C which would be produced by the BII92 mutant strain. Compound II corresponds to the activated neutral sugar substrate used to generate 3″-C desmethyl erythromycins A, C and D produced by the BII92 mutant strain. Compound III leads to the production of erythromycins A, B, C and D in the BII92 mutant strain. Compound IV corresponds to the aminosugar used to generate the 4′-hydroxy erythromycin D produced by the CIV89 mutant strain. Compound V corresponds to the activated 4-keto mycarose substrate used to generate the 4″-keto-mycarosyl erythromycins A, B, C and D produced by the BIV87 mutant strain (see text as well as Tables 2 and 3)


A
glucose-1-phosphate
dTDP glucose-synthase
(StrD - like)
EryBx ?
+ dTTP
- PPi
dTDP-glucose
4, 6-dehydratase
(StrE - like)
gdh or EryBy ?
- H2O
(NAD+)
dTDP-4-keto-
6-deoxy-hexose
3, 4-isomerase
(EryCII)
dTDP-3-keto-6-deoxy-hexose
3-aminotransferase
(EryCI)
PLP
dTDP-6-deoxy-hexose
3, 4-dehydratase
(EryCIV)
dTDP-4, 6-deoxy-hexose
3, 4-reductase
(EryCV)
NAD(P)H
dTDP-D-6-deoxy-hexose
3-N-methyl-transferase
(EryCVI)
SAM
dTDP-D-desosamine
dTDP-D-6-deoxy-hexose
3-N-methyl-transferase
(TylM1)
SAM
IV
dTDP-D-mycaminose
dTDP-4-keto-6-deoxy-
hexose 3, 5-epimerase
(StrM - like)
kde or EryBVII ?
dTDP-4-keto-L-6-deoxy-
hexose 2, 3-dehydratase
(EryBVI)
- H2O
dTDP-4-keto-L-6-deoxy-
hexose 2, 3-reductase
(EryBII)
NAD(P)H
dTDP-4-keto-L-6-deoxy-
hexose 3-C-methyl transferase
(EryBIII)
SAM
V
dTDP-4-keto-L-6-deoxy-
hexose 4-reductase
(EryBIV)
NAD(P)H
dTDP-L-mycarose
B
dTDP-4-keto-L-6-deoxy-
hexose 4-reductase
(EryBIV)
NAD(P)H
I
Reductase ?
NAD(P)H
dTDP-4-keto-L-6-deoxy-
hexose 4-reductase
(EryBIV)
NAD(P)H
II
Reductase ?
NAD(P)H
dTDP-4-keto-L-6-deoxy-
hexose 3-C-methyl transferase
(EryBIII)
SAM
dTDP-4-keto-L-6-deoxy-
hexose 4-reductase
(EryBIV)
NAD(P)H
III
dTDP-L-mycarose

The sequence analysis reported here clearly identifies the deduced protein product of ORF9 (*eryCII*) as being closely related to known cytochrome P450 hydroxylases, but the similarity is especially close to the DnrQ (DnmQ) protein involved in the biosynthesis of daunosamine in *S. peucetius* (Otten et al. 1995, 1997) and to ORF1* from *S. fradiae* (Gandecha et al. 1997) (Fig. 4). All three of these proteins lack the critical active-site cysteine residue that provides an axial ligand to the heme in authentic P450s, indicating that these enzymes represent a new family of proteins with unknown enzymatic activity. Comparison of the pathways postulated for the three aminosugars (dTDP-L-daunosamine, dTDP-D-desosamine and dTDP-D-mycaminose) show that they all involve a 3-keto-hexose as an intermediate. A plausible role for EryCII can therefore be suggested in catalysis of the isomerisation of dTDP-4-keto-6-deoxyglucose to dTDP-3-keto-6-deoxyglucose, with DnrQ (DnmQ) and ORF1* playing analogous roles in their respective gene clusters. However, there are no clues from this analysis as to the mechanism by which EryCII might accomplish this transformation.

The sequence of the gene product of ORF13 (*eryBIV*) contains a sequence motif characteristic of NAD^+-dependent ketoreductases (Gaisser et al. 1997) and we have tentatively proposed that EryBIV might be involved in the reduction of dTDP-4-keto-L-mycarose (Fig. 6A). Strong support for this is now provided by our finding that this gene is an *eryB* gene, and that the BIV87 deletion mutant accumulates, in addition to erythronolide B, small amounts of novel erythromycin-like species, for which the *m*/*z* values of each peak are consistently 2 mass units lower than those for the corresponding erythromycins A, B, C and D. These data (Table 3) are fully consistent with the absence, in the BIV87 mutant, of a specific 4-ketoreductase activity. It appears that the unreduced dTDP-4-keto sugar (i.e. compound V in Fig. 6A) can act as an alternative substrate for the mycarosyltransferase. The same conclusions have been independently reached by Katz and his collaborators (R. G. Summers, oral presentation, Bloomington, Ind., 1996).

As previously reported (Gaisser et al. 1997), the sequence of ORF17 (*eryCIV*) bears significant similarity to that of *eryCI* (Dhillon et al. 1989), and also to that of authentic pyridoxal phosphate-dependent enzymes. As previously demonstrated for *eryCI* (Dhillon et al. 1989), an ORF17 deletion mutant displays an *eryC* phenotype, as previously indicated by Katz and Donadio (1995). However, it has remained unclear why two such enzymes exist in a pathway requiring only one transamination. The deletion mutant CIV89 has been shown here to produce, in addition to 3-α-mycarosyl erythronolide B, small amounts of a novel erythromycin D-like molecule whose mass spectrum is fully consistent with the presence in this compound of an additional hydroxy group compared to erythromycin D. The mass spectrum contains peaks at the *m*/*z* values appropriate for the fragment ions hydroxydesosaminyl erythronolide B and for hydroxydesosamine, confirming that the alteration is localised to the deoxyaminosugar moiety (Fig. 6A).

These data on *eryCIV* provide several important clues to the organisation of the dTDP-desosamine biosynthetic pathway. First, the gene product of *eryCIV* cannot be the transaminase that introduces the amino group at C-3 in the course of the biosynthesis of dTDP-D-desosamine, and the transaminase must therefore be encoded by *eryCI*. The retention of a hydroxy group in minor products synthesized by the *eryCIV* mutant strongly implicates the *eryCIV* gene product as a pyridoxal phosphate-dependent dehydratase. Based on mechanistic considerations, transamination should precede dehydration, so this implies that *eryCI* acts before *eryCIV* (Fig. 6A). Finally, these data indicate that subsequent enzymes in the pathway, including the *eryCIII* glycosyltransferase, can to some extent accept an altered substrate. A subsequent reduction step would be required to complete the deoxygenation at C-4 in dTDP-desosamine biosynthesis. Our confirmation that ORF18 is an *eryC* gene (*eryCV*), together with the evidence that the inferred protein sequence contains a motif common to NAD^+-dependent oxidoreductases (Gaisser et al. 1997) make it very likely that the gene product of ORF18 (*eryCV*) performs this step. Both EryCIV and EryCV would therefore participate in the deoxygenation at C-4 of desosamine, rather than EryCII as recently postulated by Otten et al. (1997).

These results are summarised in the scheme shown in Fig. 6A. A number of important uncertainties have been removed concerning the identity and roles of the *eryB* and *eryC* genes. As the scheme shows, if further work confirms that the *eryCIV* mutant does lead to a 4-hydroxy substituent remaining on the hexose ring (i.e. mycaminose), a neat mechanism emerges for the evolution of separate but similar biosynthetic pathways to desosamine and mycaminose in different macrolide-producing actinomycetes (Fig. 6A). Even for those genes where a combination of sequencing information, gene inactivation, and accumulation of novel minor metabolites, has led to the most confident identifications, more needs to be done to confirm these findings. The role of ORF2/*ery*BI (Weber et al. 1990) and evidence to support the proposed role of ORF3 (*ery*BIII) as a methyltransferase (Fig. 6A), will be discussed separately (Gaisser et al., manuscript submitted). So far neither dTDP-glucose synthase nor dTDP-glucose-4,6-dehydratase has been identified within the *ery* gene cluster, and the involvement of the 4,6-dehydratase encoded by the *gdh* gene in the biosynthesis of erythromycin has not been demonstrated, since inactivation of that gene seems to be lethal (Linton et al. 1995). In addition, it will be important to determine whether the ORF19 gene product does indeed contribute to the biosynthesis of erythromycin A as a 3,5-epimerase (Fig. 6A), since another enzyme of this type has been cloned from *Sac. erythraea* (named *kde*; Linton et al. 1995).

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