DNA homology between *Saccharopolyspora* strains and other erythromycin-producing actinomycetes

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The macrolide antibiotic erythromycin is produced by different genera of the *Actinomycetales*, including strains of *Saccharopolyspora*, *Arthrobacter*, *Micromonospora* and possibly *Streptomyces*. Erythromycin is also produced by several unclassified strains, four of which could be assigned to the genus *Saccharopolyspora* on the basis of bacteriophage host specificity. We used cloned erythromycin biosynthetic and resistance genes from *Saccharopolyspora erythraea* as hybridization probes to determine if the DNA sequences encoding these genes are conserved in different genera. Our results indicated strong hybridization to genomic restriction fragments from strains that were assigned to the genus *Saccharopolyspora*, and weak hybridization to DNA from strains which produce erythromycin or other macrolides but belong to other genera.

Introduction

Erythromycin A is a clinically important macrolide antibiotic composed of a 14-membered lactone ring (erythronolide) and two sugars, desosamine and cladinose. Erythromycin A was first isolated by McGuire *et al.* (1952) from cultures of *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*; Labeda, 1987), a strain that was subsequently used in many laboratories for studies on the genetics and biochemistry of erythromycin production (Seno & Hutchinson, 1986). Erythromycin biosynthetic genes from *Sap. erythraea* have been cloned and expressed in *Streptomyces lividans* (Stanzak *et al.*, 1986); they are located together with the erythromycin resistance gene (*ermE*) on a 35 kb DNA fragment. Specific genes have been mapped in this region by Donadio *et al.* (1989), Weber & Losick (1988) and Weber *et al.* (1989).

The erythromycin resistance gene *ermE* encodes a ribosomal RNA methylase that also gives resistance to other macrolide-lincosamide-streptogramin-B-type (MLS-type) antibiotics (Thompson *et al.*, 1982; Skinner *et al.*, 1983). A related MLS resistance gene from *Arthrobacter* (*ermA*) has been cloned (Roberts *et al.*, 1985), based on DNA sequence similarity between *ermE*

and *ermA*. Although other MLS-resistance genes isolated from bacilli, streptococci and staphylococci do not possess comparable DNA sequence similarities (Gryczan *et al.*, 1984), many of these resistance genes (including *ermE* and *ermA*) exhibit substantial similarity at the amino acid level, an observation consistent with the proposal that antibiotic resistance genes may have evolved in antibiotic-producing actinomycetes and were subsequently transferred to other micro-organisms (Benveniste & Davies, 1973).

The micro-organisms that produce erythromycin (and hence are also resistant to erythromycin) include members of several taxonomically distinct genera of the *Actinomycetales* (e.g. *Saccharopolyspora*, *Arthrobacter*, *Micromonospora* and possibly *Streptomyces*; see Bowen *et al.*, 1989; Labeda, 1987; and Stackebrandt *et al.*, 1983, for taxonomic relationships), which typically contain a G+C content of around 70 mol%. We have been interested in determining the extent to which erythromycin biosynthetic and resistance genes are conserved in these diverse micro-organisms, and whether they show homology to genes from other macrolide producers, as indicated by Southern hybridization with cloned erythromycin biosynthetic and resistance genes. Our results show that erythromycin biosynthetic and resistance gene sequences are not highly conserved in taxonomically distinct strains of actinomycetes, and do not show extensive homology to DNA from other macrolide producers.

Abbreviations: MLS, macrolide-lincosamide-streptogramin B; TS broth, trypticase soy broth.

Methods

Strains and bacteriophages. Bacterial strains used in this study are listed in Table 1. Of the bacteriophages, ϕ SE6 and ϕ SE69 were isolated from *Sap. erythraea* (Grund & Hutchinson, 1987); FP4, FP22, FP46, FP50 and FP62 are broad-host-range streptomycete bacteriophages (Cox & Baltz, 1984; Matsushima & Baltz, 1989); and OP1, OP2, OP3, OP4, OP5, OP6, OP15 and OP16 were isolated on *Amycolatopsis orientalis* (K. Cox, M. McHenney & R. H. Baltz, unpublished). ϕ SE6 and ϕ SE69 were propagated on *Sap. erythraea* and dilutions of the phage were plated on mycelial fragments generated by ultrasound (Baltz, 1978) from strains listed in Table 2 as described by Cox & Baltz (1984). The plating efficiencies of ϕ SE6 and ϕ SE69 were calculated relative to plating efficiencies on *Saccharopolyspora hirsuta* ATCC 27875. The FP phages and the OP phages were propagated and plated on *S. griseofuscus* and *A. orientalis*, respectively, as described by Cox & Baltz (1984).

Media and culture conditions. The bacterial cultures were grown in TS broth (Baltz, 1978). The media and procedures for preparing phage stocks and phage platings were those described by Cox & Baltz (1984). Erythromycin resistance was determined by plating mycelial fragments after ultrasonic treatment (Baltz, 1978) on TS agar plates containing 1 mg erythromycin ml⁻¹. Erythromycin production was verified by growing the cultures at 30 °C in modified S medium (Stanzak *et al.*, 1986), except for strain A37609, which was grown in PNS medium. PNS medium contained (per litre): 25 g glucose; 10 g corn starch; 5 g Bacto-peptone (Difco); 4 g NZ amine A (Sheffield Products); 5 g black strap molasses; 0.5 g MgSO₄·7H₂O; 2 g CaCO₃ and 2 ml Czapek's mineral stock. The mineral stock contained (per litre): 100 g KCl; 100 g MgSO₄·7H₂O and 2 g FeSO₄·7H₂O. After 3 d of growth, the broths were adjusted to pH 8.5 and extracted twice with ethyl acetate; the extracts were concentrated approximately fivefold by evaporation. Samples (about 25 µl) of the concentrated extracts were spotted onto aluminium-backed cellulose plates (EM Reagents) and the chromatograms developed in K₂HPO₄/ethanol/H₂O (1.74:3.0:95, w/v/v). The TLC plates were air-dried, and placed onto TS agar plates seeded with *Micrococcus luteus* ATCC 9341. The chromatograms were removed and the TS agar plates were incubated at 34 °C for 16–24 h and examined for antibiotic zones.

Southern hybridization analysis. Total DNA was extracted according to the procedure of Chater *et al.* (1984) from 10 ml cultures grown in TS broth for 3 d at 30 °C. The DNA was digested with restriction enzymes in buffers recommended by the manufacturers (New England Biolabs or Boehringer Mannheim). Agarose gel electrophoresis and Southern hybridization analysis were performed in 50% (v/v) formamide and 5 × SSPE (1 × SSPE = 9 mM-NaCl, 0.5 mM-NaH₂PO₄, 0.05 mM-EDTA, pH 7.7) at 42 °C. The filters were washed in 0.1 × SSPE at 60 °C as described by McHenney & Baltz (1988). Under these conditions, hybridization can be detected with DNA fragments of approximately 80% similarity or greater.

Results and Discussion

Bacteriophage host range analysis

Several different bacteriophages, which form plaques on species of *Streptomyces*, *Amycolatopsis* or *Saccharopolyspora*, were used to characterize the strains listed in Table 1. ϕ SE6 and ϕ SE69 were reported to form plaques on *Sap. erythraea* (formerly *S. erythraeus*) but not on *Streptomyces* spp. (Grund & Hutchinson, 1987). Both

ϕ SE6 and ϕ SE69 plated very efficiently on *Sap. erythraea* (Table 2), and on *Saccharopolyspora hirsuta* NRRL B-5792. Phage ϕ SE69 also plated on *Sap. hirsuta* NRRL 12045, but at a low efficiency. Neither ϕ SE6 nor ϕ SE69 plated on the strains of *Streptomyces*, *Amycolatopsis*, *Actinoplanes*, *Arthrobacter* and *Micromonospora* listed in Table 1. These results indicate that the host range of ϕ SE6 and ϕ SE69 is limited to the genus *Saccharopolyspora*. Four of the seven unclassified erythromycin-producing strains (A57583, A51740, A51741 and A51742) also supported plaque formation by ϕ SE6 and ϕ SE69 (Table 2). We then tested these strains for plaque formation by five broad-host-range *Streptomyces* phages (FP4, FP22, FP46, FP50 and FP62). As expected from previous studies (Cox & Baltz, 1984), plaque formation was observed on *Streptomyces ambofaciens*, *Streptomyces griseofuscus* and *Streptomyces lividans*, but not on *Sap. erythraea* (not shown). None of the unclassified strains supported plaque formation by any of these five phages. The unclassified strains were also tested for plaque formation by eight bacteriophages isolated on *Amycolatopsis orientalis*. These phages formed plaques on both *Amycolatopsis* species tested, but not on any of the other strains listed in Table 1, including the unclassified erythromycin producers. The bacteriophage host range data are therefore consistent with the reassignment of *S. erythraeus* to the genus *Saccharopolyspora* (Labeda, 1987); further, four of the unclassified erythromycin-producing strains (A57583, A51740, A51741 and A51742) can be tentatively assigned to the genus *Saccharopolyspora*.

Southern hybridization analysis of erythromycin-producing strains

In *Sap. erythraea*, erythromycin biosynthetic genes are clustered on the chromosome and linked to the erythromycin resistance gene *ermE* (Stanzak *et al.*, 1986; Weber *et al.*, 1985, 1989; Weber & Losick, 1988; Donadio *et al.*, 1989). To determine the extent of sequence relatedness among the erythromycin biosynthetic genes in different strains producing erythromycin, we used cloned erythromycin biosynthetic genes from *Sap. erythraea* as a hybridization probe.

The strains we chose for the analysis included classified strains (e.g. *Sap. erythraea*) and several unclassified strains isolated from soils collected at various locations around the world (see Table 1). Genomic DNA was isolated and digested with *Bam*HI and the resulting restriction fragments were separated by agarose gel electrophoresis and analysed by Southern hybridization using plasmid pKC488 as a probe. This plasmid was isolated from a genomic library of *Sap. erythraea* (Stanzak *et al.*, 1986) and carries erythromycin

Table 1. *Actinomycete strains used*

Strain	Source or geographical origin	Antibiotic produced	Reference(s)
<i>Saccharopolyspora erythraea</i>	NRRL 2338*	Erythromycin†	Labeda (1987); Seno & Hutchinson (1986)
<i>Saccharopolyspora hirsuta</i>	NRRL B-5792‡	Aminoglycoside KA-5685	Labeda (1987)
<i>Saccharopolyspora hirsuta</i>	NRRL 12045	Nargenicin A1	Whaley <i>et al.</i> (1980)
<i>Streptomyces ambofaciens</i>	ATCC 15154	Spiramycin	Matsushima & Baltz (1985)
<i>Streptomyces griseofuscus</i>	ATCC 23916	Bundlin	Cox & Baltz (1984)
<i>Streptomyces lividans</i> TK23	NRRL 15826	Actinorhodin	Hopwood <i>et al.</i> (1984)
<i>Amycolatopsis orientalis</i>	ATCC 19795	Vancomycin	Barna & Williams (1984); Lechevalier <i>et al.</i> (1986)
<i>Amycolatopsis</i> sp.	NRRL 8156	Glycopeptide A35512	Barna & Williams (1984); Lechevalier <i>et al.</i> (1986)
<i>Actinoplanes teichomyceticus</i>	ATCC 31121	Teichoplanin	Hunt <i>et al.</i> (1984)
<i>Arthrobacter</i> sp.	NRRL 3381	Erythromycin†	Roberts <i>et al.</i> (1985)
<i>Micromonospora rosaria</i>	ATCC 29337	Rosaramicin	Ganguly <i>et al.</i> (1982)
<i>Micromonospora</i> sp. 1225	Schering	Erythromycin†	Seno & Hutchinson (1986)
A37609§	Palestine	Erythromycin†	This report
A57583§	Barbados	Erythromycin†	This report
A51735§	Kenya	Erythromycin†	This report
A51740§	Kenya	Erythromycin†	This report
A51741§	Kenya	Erythromycin†	This report
A51742§	Kenya	Erythromycin†	This report
A51744§	India	Erythromycin†	This report

* Formerly *Streptomyces erythraeus*.

† Erythromycin production and resistance to erythromycin were verified as described in Methods.

‡ Type strain.

§ Not formally identified.

Table 2. *Efficiency of plating of Sap. erythraea phages ϕ SE6 and ϕ SE69 on erythromycin-producing and other actinomycetes*

Strain	Relative efficiency of plating	
	ϕ SE6	ϕ SE69
<i>Saccharopolyspora erythraea</i>	5×10^{-1}	10^{-1}
<i>Saccharopolyspora hirsuta</i> (NRRL B-5792)	1.0	1.0
<i>Saccharopolyspora hirsuta</i> (NRRL 12045)	$< 10^{-9}$	10^{-7}
<i>Streptomyces ambofaciens</i>	$< 10^{-9}$	$< 10^{-9}$
<i>Streptomyces griseofuscus</i>	$< 10^{-9}$	$< 10^{-9}$
<i>Streptomyces lividans</i> TK23	$< 10^{-9}$	$< 10^{-9}$
<i>Amycolatopsis orientalis</i>	$< 10^{-9}$	$< 10^{-9}$
<i>Amycolatopsis</i> sp.	$< 10^{-9}$	$< 10^{-9}$
<i>Actinoplanes teichomyceticus</i>	$< 10^{-9}$	$< 10^{-9}$
<i>Arthrobacter</i> sp.	$< 10^{-9}$	$< 10^{-9}$
<i>Micromonospora rosaria</i>	$< 10^{-9}$	$< 10^{-9}$
<i>Micromonospora</i> sp. 71-1225	$< 10^{-9}$	$< 10^{-9}$
A37609	$< 10^{-9}$	$< 10^{-9}$
A57583	2×10^{-5}	2×10^{-3}
A51735	$< 10^{-9}$	$< 10^{-9}$
A51740	4×10^{-3}	10^{-2}
A51741	3×10^{-3}	10^{-1}
A51742	3×10^{-8}	10^{-6}
A51744	$< 10^{-9}$	$< 10^{-9}$

biosynthetic and resistance genes on a 35 kb DNA insert (see Fig. 1). The erythromycin resistance gene (*ermE*) is located on a 3 kb *Bam*HI restriction fragment contained within the 35 kb insert in pKC488. As shown in Fig. 2(a), intense hybridization was observed between pKC488 and several genomic *Bam*HI restriction fragments from six of the erythromycin-producing unclassified strains (lanes 2–5, 7 and 8). Weaker hybridization to fewer genomic restriction fragments was observed between pKC488 and DNA from strain A37609 (lane 6), *Arthrobacter* sp. (lane 9) and *Micromonospora* sp. (Fig. 2b, lane 2), and very weak hybridization to one or two restriction fragments was observed with DNA from the *Sap. hirsuta* strains. No hybridization was observed to *S. lividans* DNA (Fig. 2a, lane 11). To investigate whether DNA sequences similar to the resistance gene *ermE* exist in the erythromycin-producing strains, we treated the hybridization filters to remove the radiolabelled probe (pKC488) and rehybridized the filter-bound DNA with pKC407, a plasmid containing the coding sequence for *ermE* on a 1.8 kb *Bam*HI–*Pst*I restriction fragment. As expected, pKC407 hybridized to a 3 kb *Bam*HI restriction fragment from the *Sap. erythraea* chromosome (Fig. 3a, lane 1). The plasmid also hybridized to a 3 kb *Bam*HI

Fig. 1. Restriction map of the 35 kb insert in pKC488.

Fig. 2. Southern hybridization analysis of genomic restriction fragments generated with *Bam*HI and hybridized with plasmid pKC488. (a) Genomic DNA from: *Sap. erythraea* (lane 1); A51583 (lane 2); A51741 (lane 3); A51735 (lane 4); A51744 (lane 5); A37609 (lane 6); A51742 (lane 7); A51740 (lane 8); *Arthrobacter* sp. (lane 9); *S. griseofuscus* (lane 10); *S. lividans* TK23 (lane 11). (b) Genomic DNA from: *Sap. erythraea* (lane 1); *Micromonospora* sp. (lane 2); *Sap. hirsuta* ATCC 27875 (lane 3); *Sap. hirsuta* NRRL 12045 (lane 4). (c) is a longer exposure of the film shown in (b). A *Hind*III digest of λ DNA was used as a standard.

restriction fragment from genomic DNA of the five unclassified strains that hybridized strongly with pKC488 (Fig. 3a, lanes 2–5, 7, 8); however pKC407 did not hybridize to DNA from strains that hybridized weakly with pKC488, including A37609, *Arthrobacter* sp., *S. griseofuscus*, *Micromonospora* sp., and the two *Sap. hirsuta* strains, and did not hybridize to DNA from *S. lividans*.

To determine whether the strong hybridization between the probes (pKC488 and pKC407) and the genomic restriction fragments from the six unclassified

strains is confined to the region containing the erythromycin biosynthetic and resistance genes, we repeated the Southern hybridization analysis with pKC491, a cosmid from the *Sap. erythraea* DNA library containing a 35 kb DNA insert that does not cross-hybridize with the DNA insert in pKC488. Using pKC491 as a probe, we observed strong hybridization with DNA from five of the unclassified strains (A51583, A51741, A51735, A51744 and A51740), weak hybridization with DNA from A51742 and no hybridization with DNA from A37609, *S. griseofuscus*, *S. lividans*, *Micromonospora* sp.

Fig. 3. Southern hybridization analysis of genomic restriction fragments hybridized with the erythromycin resistance gene *ermE*. The blots shown in Fig. 2 were treated to remove the hybridization probe and rehybridized with pKC408. (a) Genomic DNA from: *Sap. erythraea* (lane 1); A51583 (lane 2); A51741 (lane 3); A51735 (lane 4); A51744 (lane 5); A37609 (lane 6); A51742 (lane 7); A51740 (lane 8); *Arthrobacter* sp. (lane 9); *S. griseofuscus* (lane 10). (b) Genomic DNA from: *Sap. erythraea* (lane 1); *Micromonospora* sp. (lane 2); *Sap. hirsuta* ATCC 27875 (lane 3); *Sap. hirsuta* NRRL 12045 (lane 4). A *Hind*III digest of λ DNA was used as a standard.

or the two *Sap. hirsuta* strains (data not shown). These results suggest that the five unclassified strains are more closely related to *Sap. erythraea* than to the two other members of the genus *Saccharopolyspora*. However, these five strains are not identical since the lengths of the hybridizing restriction fragments generated with *Bam*HI and *Xho*I varied with each strain.

Based on these results, we could place the erythromycin-producing strains into two groups. Members of one group possess sequences that hybridize strongly with the erythromycin biosynthetic gene probe, and with a random probe (pKC491) from the *Sap. erythraea* library, indicating that these strains are closely related to *Sap. erythraea*. The bacteriophage typing also suggested that at least four of these strains are members of the genus *Saccharopolyspora*. Members of the other group possess sequences that hybridize weakly with the erythromycin biosynthetic gene probe, and not at all with the random probe, pKC491. The regions that hybridize with the erythromycin biosynthetic gene probe appear to be confined to a small number of restriction fragments which do not include those encoding the erythromycin resistance genes. The lack of extensive DNA sequence homology among diverse erythromycin-producing

strains suggests several evolutionary possibilities: (1) that erythromycin biosynthetic genes evolved before the divergence of actinomycete genera; (2) that these genes were transferred horizontally between certain genera early on the evolutionary time scale; or (3) that all or parts of the erythromycin pathway genes evolved more than once. In the case of β -lactam antibiotics that are produced by several species of *Streptomyces* and by several genera of fungi, there is evidence to suggest that the genes evolved in a streptomycete and were transferred around 370 million years ago to a fungal progenitor of *Aspergillus*, *Cephalosporium* and *Penicillium* (Weigel *et al.*, 1988). More detailed studies on the structural organization of erythromycin biosynthetic genes in different actinomycetes, and DNA sequence analysis of several specific genes cloned from taxonomically distinct micro-organisms should provide additional insights into the evolution of erythromycin biosynthetic genes.

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