Identification of two polyketide synthase gene clusters on the linear plasmid pSLA2-L in *Streptomyces rochei*

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Abstract

The 200 kb linear plasmid pSLA2-L was suggested to be involved in the production of two macrolide antibiotics, lankamycin (Lm) and lankacidin (Lc), in *Streptomyces rochei* 7434AN4. Hybridization experiments with the polyketide synthase (PKS) genes for erythromycin and actinorhodin identified two *eryAI*-homologous regions and an *actI*-homologous region on pSLA2-L. The nucleotide sequence of a 3.6 kb *SacI* fragment carrying one of the *eryAI*-homologs revealed that it codes for part of a large protein with four domains for ketoreductase, acyl carrier protein, ketosynthase, and acyltransferase. Gene disruption confirmed that the two *eryAI*-homologs are parts of a large type-I PKS gene cluster for Lm. A 4.8 kb DNA carrying the *actI*-homologous region contains four open reading frames (ORF1–ORF4) as well as an additional ORF, i.e. ORF5, which might code for a thioesterase. Deletion of the ORF2–ORF4 region showed that it is not involved in the synthesis of Lm or Lc. Thus, it was confirmed that pSLA2-L contains two PKS gene clusters for Lm and an unknown type-II polyketide. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Antibiotic; Biosynthesis; Lankacidin; Lankamycin; Macrolide

1. Introduction

The soil bacteria *Streptomyces* produce a large number of secondary metabolites, including antibiotics, immunosuppressants, herbicides, enzyme inhibitors and other physiologically active substances. The biosynthetic genes for antibiotics in *Streptomyces* form a large gene cluster and are usually located on the chromosome. However, the biosynthetic gene cluster for methylencillin is located on the 350 kb giant linear plasmid SCP1 in *Streptomyces coelicolor A3(2)* (Chater and Bruton, 1985; Kinashi et al., 1987).

Abbreviations: ACP, acyl carrier protein; act, actinorhodin biosynthetic genes; Ar, actinorhodin; AT, acyltransferase; CHEF, contourclamped homogenous electric fields; CLF, chain length factor; CYC, cyclohex; DEBS, 6-deoxyerythronolide B synthase; Em, erythromycin; *eryAI*, erythromycin synthase genes; Km, kanamycin; KR, ketoreductase; KS, ketosynthase; Lc, lankacidin; Lm, lankamycin; ORF, open reading frame; PFGE, pulsed-field gel electrophoresis; PKS, polyketide synthase; TE, thioesterase; Ts, thiostrepton.

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*Streptomyces rochei* 7434AN4 carries three linear plasmids, pSLA2-L (200 kb), -M (100 kb) and -S (17 kb) (Kinashi et al., 1994). This strain produces two structurally unrelated polyketide antibiotics, lankamycin (Lm) and lankacidin (Lc) (Fig. 1). Lm is a 14-membered macrolide antibiotic structurally similar to erythromycin (Em). On the other hand, Lc contains a unique 17-membered macrolide ring, which distinguishes it from the usual even-membered macrolide antibiotics. The largest plasmid pSLA2-L was suggested to be involved in the production of both antibiotics, on the basis of the plasmid profile of various mutants, transfer of the producing ability by protoplast fusion, and the structure of the deletion plasmid pSLA2-L1 in the antibiotic-deficient mutant KE32 (Kinashi et al., 1994). Hybridization experiments of pSLA2-L using the typical polyketide gene probes, *eryAI* for Em and *actI* for actinorhodin (Ar), revealed that homologous regions to these probes were located on *PstI* fragments A and I respectively (Kinashi et al., 1998).

In this study, we analyzed 35 kb *PstI* fragment A, which hybridized with *eryAI*. We found two *eryAI*-
Fig. 1. Chemical structures of the polyketide antibiotics, lankamycin, erythromycin, lankacidin C, and actinorhodin. R1, 4-acetyl-\(\text{l}\)-arcanose; R2, \(\text{d}\)-chalcose; R3, \(\text{l}\)-cladinose; R4, \(\text{d}\)-desosamine.

homologous regions (\(\text{eryAI}\)-homologs A and C), cloned and sequenced a 3.6 kb \(\text{SacI}\) fragment carrying \(\text{eryAI}\)-homolog A, and finally confirmed by gene disruption that both homologs are parts of a large Lm synthase gene cluster. We also cloned and sequenced a 4.8 kb DNA extending over \(\text{PstI}\) fragments H and I, which carried the \(\text{actI}\)-homologous region, and showed that this region is not involved in the production of Lm nor Lc.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The properties of \(S.\) rochei strains used in this study are listed in Table 1. Two PKS genes, \(\text{eryAI}\) and \(\text{actI}\), were used as probes for hybridization. For this purpose, pKS2/M5 (Donadio and Katz, 1992), pUC19 carrying a 1.4 kb \(\text{SmaI}\) fragment of the KS/SU2 region of \(\text{eryAI}\) from \textit{Saccharopolyspora erythraea}, and pIJ2345 (Malpartida et al., 1987), pBR329 carrying a 2.2 kb \(\text{BamHI}\) fragment of \(\text{actI}\) from \textit{Streptomyces coelicolor} A3(2), were provided by L. Katz and D.A. Hopwood respectively. The shuttle vector pRES18 (Ishikawa et al., 1996), which was used for gene disruption, was given by J. Ishikawa. The kanamycin resistance (\(\text{Km}\)) gene cartridge plasmid pUC4-KIXX (Barany, 1985) was purchased from Pharmacia. YM medium used for antibiotic production contained 4 g of yeast extract, 10 g of malt extract, and 4 g of glucose per liter (pH 7.3).

Table 1

\(S.\) rochei strains used in this experiment

<table>
<thead>
<tr>
<th>Strains</th>
<th>Properties</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>7434AN4</td>
<td>Wild-type strain which carries pSLA2-L, -M and -S</td>
<td>Kinashi et al. (1994)</td>
</tr>
<tr>
<td>51232</td>
<td>Derivative of 7434AN4 which carries only pSLA2-L. All the disruptants were prepared from this strain</td>
<td>Kinashi et al. (1994)</td>
</tr>
<tr>
<td>LM-BA1</td>
<td>Disruptant of (\text{eryAI})-homolog A</td>
<td>This work</td>
</tr>
<tr>
<td>LM-BC1</td>
<td>Disruptant of (\text{eryAI})-homolog C</td>
<td>This work</td>
</tr>
<tr>
<td>AC47-L</td>
<td>Disruptant of (\text{actI})-homolog</td>
<td>This work</td>
</tr>
</tbody>
</table>

2.2. DNA preparation, PFGE and Southern hybridization

\(S.\) rochei strains were shake cultured at 28°C in a 500 ml Sakaguchi flask, which contained 100 ml of YEME medium (Hopwood et al., 1985) or YM medium plus 0.5% glycine. The mycelium was harvested by centrifugation and washed twice with 10.3% sucrose. The DNA samples for PFGE analysis were prepared by the mycelium method as described (Kinashi, 1994; Lezhava et al., 1995). We used contour-clamped homogeneous electric fields (CHEF) (Chu et al., 1986) for PFGE analysis. This was carried out in 0.5 x TBE buffer using 1% agarose gel at 15°C. The pSLA2-L band was excised from gels and extracted by electrodissolution. Total DNA was prepared by a neutral method according to Tanaka et al. (1977) with slight modifications. The mycelium from a 50 ml culture was suspended in 3 ml of TSE–sucrose (30 mM Tris, 50 mM NaCl, 5 mM EDTA, pH 8.0, 10.3% sucrose), to which 1 ml of 0.5 M EDTA (pH 8.0) and 4 ml of lysozyme (5 mg/ml in TSE, Kaken Seiyaku, Tokyo) was added and incubated at 37°C for 30 min, 2 ml of Actinase E (5 mg/ml in TSE, Kaken Seiyaku, Tokyo) was added and incubated at 37°C for 30 min, and then 2 ml of 10% sodium dodecyl sulfate was added and shaken for an additional 30 min. 2 ml of 5 M NaCl was added and mixed for 30 min and left at 4°C overnight. The mixture was centrifuged at 12,000 rpm for 20 min, and the supernatant fluid was ethanol-precipitated. The precipitate was dissolved in TE (10 mM Tris, 1 mM EDTA, pH 8.0), extracted with phenol, incubated with RNase (100 μg/ml) at 37°C for 1 h, and extracted again with phenol-chloroform.
The pSLA2-L DNA and total DNA were digested with restriction endonucleases, separated by agarose gel electrophoresis, and transferred to nylon membranes. Hybridization was carried out using the DIG system (Boehringer Mannheim) overnight at 70°C in standard buffer according to the supplier’s protocol. After hybridization, washing was done twice for 5 min each in 2 x wash solution at room temperature, and then twice for 15 min each in 0.1 x wash solution at 70°C.

2.3. DNA sequencing

The nucleotide sequences of eryAI-homology A and the aclI-homolog were determined by the dideoxy termination method using Sequenase kit (Toyobo) and P-dCTP, or the dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) and an automatic DNA sequencer (Prism-373, PE Applied Biosystems).

2.4. Construction of the targeting vectors

The targeting vectors were constructed by using a shuttle vector pRES18 (Ishikawa et al., 1996) and a Km gene cassette from pUC4-KIXX (Barany, 1985).

The targeting vector for disruption of eryAI-homolog A, was constructed as follows (see Fig. 4-1). The SacI-SphI and SphI-SacI fragments of pLBA101, which carries the 3.6 kb SacI fragment of BamHI fragment A, were subcloned into pUC19 to generate pLBA102 and pLBA103 respectively. The EcoRI-HindIII fragment of pLBA102, the HindIII-SacI fragment of pLBA103, and pRES18 digested with EcoRI plus SacI, were ligated to give pLBA104. Cleavage of the central HindIII site of pLBA104 by partial digestion followed by ligation with the 1.6 kb HindIII fragment of pUC4-KIXX generated pLBA105. All manipulations hitherto described were carried out in *Escherichia coli* HB101.

pLBC105, the targeting vector for eryAI-homolog C, was constructed as follows (Fig. 4-2). The BamHI-SphI and SphI-KpnI fragments of pLBC101, which carries the 4.4 kb BamHI-KpnI fragment of BamHI fragment C, were subcloned into pUC19 to generate pLBC102 and pLBC103 respectively. The BamHI-HindIII fragment of pLBC102, the HindIII-EcoRI fragment of pLBC103, and pRES18 digested with BamHI plus EcoRI, were ligated to give pLBC104. Insertion of the Km gene cassette into the central HindIII site of pLBC104 generated pLBC105.

pLAC105, the targeting vector for the aclI-homolog, was constructed more easily (Fig. 4-3). The 5.4 kb BamHI fragment extending over *PstI* fragments H and I was cloned into pUC19 to generate pLAC101. The 1.2 kb Svals fragment of pUC4-KIXX was introduced into the Eco47III-digested pLAC101 to give pLAC104, the insert of which was re-ligated to BamHI-digested pRES18 to afford pLAC105.

2.5. Gene disruption

The targeting vectors were propagated once in *E. coli* ET12567 (dam dem hsdM, MacNeil et al., 1992) to overcome the restriction and modification barrier in Streptomyces. Strain 51252 was cultured in YEME medium and subjected to protoplast formation, transformation, and regeneration as described by Hopwood et al. (1985). One randomly selected transformant, which was resistant to both Km and thiostrepton (Ts), was cultured in liquid YEME medium containing 10 µg/ml Km to full growth, and then 2% of the broth was transferred to the same fresh medium once a day and cultured for 4 days. Protoplasts were prepared and regenerated to obtain single colonies. The antibiotic resistance of 50 colonies was tested using three different YM plates, which contained either no antibiotic, 10 µg/ml of Km, or 50 µg/ml of Ts.

3. Results

3.1. Location of eryAI-homologs

The physical map of 23 *PstI* fragments of pSLA2-L was constructed and the *eryAI* probe hybridized to 35 kb *PstI* fragment A (Kinashi et al., 1998). To locate the *eryAI*-homologous region(s) more precisely, restriction and hybridization analysis of pSLA2-L was performed. Two discrete hybridizing bands were observed in the digest with BamHI and KpnI; the BamHI fragments A (16.5 kb) and C (13.5 kb), and two KpnI fragments (10 and 7.1 kb). Therefore, BamHI fragments A and C were cloned into pUC19 to give plasmids pLBA100 and pLBC100. As shown in Fig. 2-1, BamHI fragments A and C were found to be located adjacent to each other inside of *PstI* fragment A. The 7.1 kb KpnI fragment and the 4.4 kb BamHI-KpnI fragment were located on BamHI fragments A and C respectively. These *eryAI*-homologous regions were named *eryAI*-homologs A and C.

3.2. Nucleotide sequence of *eryAI*-homolog A

To determine the nucleotide sequence of *eryAI*-homolog A, a 3.6 kb SacI fragment (Fig. 2-1) was cloned to give plasmid pLBA103. The insert of this plasmid was digested with several restriction endonucleases, sub-cloned into pUC19, and subjected to sequencing. The sequence of 3573 bp (data are available in the DDBJ/EMBL/GenBank databases under accession number AB016763) has a G+C content of 76.1%. As shown in Fig. 3, frame analysis revealed that this frag-
ment does not contain initiation or stop codons and, therefore, encodes for part of a large multifunctional protein with four domains: complete domains for ketoreductase (KR, 184 aa), acyl carrier protein (ACP, 84 aa), ketosynthase (KS, 418 aa), and an N-terminal half of a domain for acyltransferase (AT, 235 aa) are present.

The nucleotide sequence was compared with three 6-deoxyerythronolide B synthase (DEBS) genes of *Sarcina ventriculi* (*eryAI, eryAII, and eryAIII* (Donadio and Katz, 1992)). The identities are 71.3%, 65.7%, and 64.3% respectively on the nucleotide level, and 64.2%, 55.6%, and 56.8% respectively on the amino acid level. The amino acid sequences of individual domains encoded by *eryAI-homolog A* were compared with those of the most similar protein, DEBS1 coded by *eryAI* (Fig. 3), which revealed the following identities: 77.2% for KR; 67.9% for ACP; 70.1% for KS; 62.0% for AT. The structural similarity of Em and Lm and the high homology of *eryAI* and *eryAII-homolog A* on both the nucleotide and amino acid levels strongly suggest that this region is part of a Lm synthase gene cluster.

### 3.3. Disruption of *eryAI-homolog A*

To prove the involvement of the 3.6 kb *SucI region* in Lm synthesis, gene disruption experiments were performed. For this purpose, we used a *Streptomyces-E. coli* shuttle vector, pRES18 and a Km<sup>+</sup> cassette from plasmid pUC4-KIXX. The targeting vector pLBA105 carried the 1.6 kb HindIII fragment of pUC4-KIXX in place of the 0.1 kb *SphI* fragment located in the KS domain (Fig. 4-I). To overcome the restriction-modification barrier in *Streptomyces*, which frequently makes transformation in this genus difficult, pLBA105 was first introduced into modification-minus *E.coli* ET12567 and then used to transform *Streptomyces* 51252, which carries only pSLA2-L. To facilitate the integration of pLBA105 into pSLA2-L, one Km- and Ts-resistant transformant was cultured in liquid medium containing only Km with serial transfers. Then, protoplasts were prepared and regenerated to obtain single colonies. Among 50 regenerated colonies, 19 were resistant to both Km and Ts, and 31 were resistant to only Km. Five colonies were picked up from the latter group and their DNAs were analyzed by CHEF and hybridization. The Km<sup>+</sup> gene (probe 1) hybridized to the pSLA2-L band of all of the five strains (Fig. 5-I, only the result of one disruptant LM-BA1 is shown here and below), but not to that of strains 7434AN4 and 51252, which indicated that the vector had been integrated into pSLA2-L.

To learn the structure of the integrated vector in the disruptants, total DNAs were extracted, digested with *SacI*, and analyzed by hybridization. Probe 1 hybridized to a 5.1 kb band in the disruptant LM-BA1, whereas no hybridizing signal was observed in strain 51252 (Fig. 5-2). When the original 3.6 kb *SucI fragment* (probe 2) was used, the same 3.6 kb band was observed in strain 51252, whereas the enlarged 5.1 kb band was detected in the disruptant. The large fragment observed in both strains 51252 and LM-BA1 might be due to *eryAI-homolog C*. These results indicate that the 0.1 kb *SphI* fragment in the KS domain of *eryAI-homolog A* was replaced by the 1.6 kb Km<sup>+</sup> cassette by a double crossover (Fig. 4-I).

The antibiotic production of the five disruptants was tested by bioautography as described previously (Kinashi et al., 1994). None of the disruptants showed an inhibition zone corresponding to Lm, but all of the strains still showed a zone for Le (Fig. 6; only the result of disruptant LM-BA1 was shown). These results confirmed that the *eryAI-homolog A* is part of the Lm synthase gene cluster.

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**Fig. 2.** The restriction maps of pSLA2-L around *eryAI-homologs A and C*. (1) and the *actI-homologous region* (2). See Kinashi et al. (1998) for the *PolI* fragment map of pSLA2-L. (1) The restriction map of *PolI* fragment A. All sites for *PolI* (Pi), *BamHI* (Ba), *KpnI* (Kp), and *NdeI* (Nd) are shown. For *SacI* (Sc), only two sites flanking the 3.6 kb fragment, which was used for sequencing, are indicated. The shaded regions carry either *eryAI-homolog A* or C. The gene direction of *eryAI-homolog A* is shown by a broken arrow. (2) The restriction map of *PolI* fragment I and the right part of *PolI* fragment H. For *BglII* (Bgl) and *SalI* (Sal), only the sites described in the text are indicated. The shaded region hybridized to the *actI* probe. The positions, lengths and directions of five ORFs are shown by arrows.
Fig. 3. Homology of the amino acid sequences coded by eryAI-homolog A and eryAI. The amino acid sequence encoded by eryAI-homolog A (upper sequence, accession number AB016763) is shown in the one-letter code and compared with that of DEBS1 of Sac. erythraea encoded by eryAI (lower sequence). The amino acid numbering of DEBS1 follows that of the database (accession number M63676). Identical and homologous amino acids between two sequences are indicated by asterisks and dots respectively. The four active domains are shaded according to Donadio and Katz (1992).

3.4. Disruption of eryAI-homolog C

One question remained unsolved after the above work. Is another eryAI-homolog on BamHI fragment C also part of the Lm synthase gene cluster or is it involved in Lc biosynthesis? To solve this problem, gene disruption experiments were also performed on eryAI-homolog C. The targeting vector pLBC105 carried the Km\(^R\) cassette in place of the 1.8 kb SphI fragment in the center of eryAI-homolog C (Fig. 4-2). pLBC105 was treated as the case for eryAI-homolog A. Among 50 regenerated S. rochei colonies, 13 were resistant to both Km and Ts, while 37 were resistant to only Km. Two colonies from the latter were analyzed further. Probe 1 hybridized only to the mutant DNA; the pSLA2-L band in CHEF (data not shown) and the 4.2 kb BamHI–KpnI fragment in agarose gel electrophoresis (Fig. 5-3, only the result of disruptant LM-BC1 is shown here and below). On the other hand, the original 4.4 kb BamHI–KpnI fragment (probe 3) hybridized to the same fragment in strain 51252, and to the reduced 4.2 kb fragment also part of the Lm synthase gene cluster or is it involved in Lc biosynthesis? To solve this problem, gene disruption experiments were also performed on eryAI-homolog C. The targeting vector pLBC105 carried the Km\(^R\) cassette in place of the 1.8 kb SphI fragment in the center of eryAI-homolog C (Fig. 4-2). pLBC105 was treated as the case for eryAI-homolog A. Among 50 regenerated S. rochei colonies, 13 were resistant to both Km and Ts, while 37 were resistant to only Km. Two colonies from the latter were analyzed further. Probe 1 hybridized only to the mutant DNA; the pSLA2-L band in CHEF (data not shown) and the 4.2 kb BamHI–KpnI fragment in agarose gel electrophoresis (Fig. 5-3, only the result of disruptant LM-BC1 is shown here and below). On the other hand, the original 4.4 kb BamHI–KpnI fragment (probe 3) hybridized to the same fragment in strain 51252, and to the reduced 4.2 kb fragment also part of the Lm synthase gene cluster or is it involved in Lc biosynthesis? To solve this problem, gene disruption experiments were also performed on eryAI-homolog C. The targeting vector pLBC105 carried the Km\(^R\) cassette in place of the 1.8 kb SphI fragment in the center of eryAI-homolog C (Fig. 4-2).
3.5. Nucleotide sequence of actI-homolog

As recently reported (Kinashi et al., 1998), the actI probe hybridized to PstI fragment I of pSLA2-L. To locate the actI-homologous region more precisely, the PstI fragment I was cloned (pLAC100) and analyzed. The actI probe hybridized to the leftmost 1.3 kb PstI-BamHI fragment of PstI fragment I (see Fig. 2.2). Therefore, this fragment was subcloned and sequenced, the result of which showed the presence of two incomplete ORFs similar to actI-ORF1 and -ORF2. To reveal the gross gene organization of the actI-homologous region, the upstream 1.0 kb BamHI fragment and the downstream 2.5 kb SalI-PstI fragment were also subcloned and sequenced.

The nucleotide sequence of a total of 4767 bp (DDBJ/EMBL/GenBank databases, accession number AB021222) revealed four complete ORFs, ORF1 (419 aa), ORF2 (415 aa), ORF3 (88 aa) and ORF4 (318 aa), and a C-terminal part of ORF5 (198 aa) oriented in the same direction in Fig. 2.2. The GC content of this region is 76.2%. The 3′ end of ORF1 has a 4 bp overlap with the predicted start site of ORF2, which begins at nt 1850. This situation is the same as the relation between actI-ORF1 and -ORF2 (Fernandez-Moreno et al., 1992). In addition, a 4 bp overlap is also seen between ORF5 and ORF1. Therefore, three ORFs (ORF5, ORF1 and ORF2) are likely to be translationally coupled as discussed by Zalkin and Ebbole (1988). ORF2 and ORF3 are separated by 55 bp of non-coding DNA, and ORF3 and ORF4 by 40 bp of non-coding DNA.

ORF1, ORF2 and ORF3 are similar to the minimal PKS genes of the Ar biosynthetic gene cluster (actI-ORF1, -ORF2 and -ORF3), and ORF4 to actIV-ORF (Fernandez-Moreno et al., 1992). The identities of ORF1–ORF4 to the corresponding act-ORFs are 72.1, 69.5, 63.3 and 66.5% on the nucleotide level, and 63.4, 56.7, 38.8 and 54.5% on the amino acid level. From these data, ORF1–ORF4 are deduced to code for KS, chain length factor (CLF), ACP and cyclase (CYC) respectively for the synthesis of a type-II polyketide metabolite. As expected, ORF1 (KS) contains a cysteine residue at the putative catalytic site for condensation (Cys–X–Ser–X–Leu, aa 165–169), whereas ORF2 (CLF) does not.

The most interesting feature of this type-II PKS gene cluster is the presence of ORF5, which was deduced to code for TE. It contains the amino acid motif for TE (Gly–X–Ser–X–Gly) positioned near the N-terminus (Cho and Cronan, 1993). No TE gene has been found carries the 1.2 kb KmR cassette in place of the 1.8 kb EcoRV fragment of pLAC104. Gene disruption was done by the replacement of the 4.4 kb BamHI-KpnI fragment in pSLA2-L by the 4.8 kb BamHI-KpnI fragment in pLAC105. (2) pLBA105, the targeting vector for eryAI-homolog A, carries the 1.6 kb KmR cassette in place of the 0.1 kb SphI fragment in the KS region of eryAI-homolog A. pLBA105 was introduced into S. rochei 51252 to give the disruptant LM-BA1, where the 3.6 kb SphI fragment in pSLA2-L was replaced by the 5.1 kb SphI fragment in pLBA105 by a double crossover. (2) pLBC105, the targeting vector for eryAI-homolog C, carries the 1.2 kb KmR cassette in place of the 1.8 kb SphI fragment in the center of eryAI-homolog C. Gene disruption was done by the replacement of the 4.4 kb BamHI-KpnI fragment in pSLA2-L by the 4.2 kb BamHI-KpnI fragment in pLBC105. (3) pLAC105, the targeting vector for the actI-homolog, carries the 1.2 kb KmR cassette in place of the 1.8 kb EcoRV fragment of pLAC104. Gene disruption was done by the replacement of the 5.4 kb BamHI fragment in pSLA2-L by the 4.8 kb BamHI fragment in pLAC105. Ba, BamHI; Bg, BglII; Kp, KpnI; Ps, PstI; Sa, SalI; Sm, Smal; Sp, SphI; 47, Eco47III.
Fig. 5. Southern hybridization analysis of the disruptants of eryAI-homologs A (1, 2) and C (3), and the actI-homolog (4), using CHEF (1) and conventional agarose (2–4) gel electrophoresis. (1) The DNA samples prepared in gels were separated by CHEF gel electrophoresis and hybridized with the KmR cassette (probe 1). CHEF was conducted at 130 V with 3 min pulses for 36 h. The wild-type strain 7434AN4 contains three linear plasmids, pSLA2-L, M, and S. (2–4) Total DNAs were digested with SacI (2), BamHI plus KpnI (3), or BamHI (4), separated by conventional agarose gel electrophoresis, and hybridized with the following probes; probe 1, the KmR cassette; probe 2, the 3.6 kb SacI fragment which carries eryAI-homolog A; probe 3, the 4.4 kb BamHI–KpnI fragment which carries eryAI-homolog C; probe 4, the 5.4 kb BamHI fragment which carries the actI-homolog.

3.6. Disruption of actI-homolog

The actI-homologous region on PstI fragments H and I seems unlikely to code for the Lc biosynthetic genes, because the chemical structures of Lc and Ar are totally different (Fig. 1). To answer this question, gene disruption of this region was carried out. The targeting vector pLAC105 was constructed by replacing the 1.8 kb Eco47III fragment on the 5.4 kb BamHI fragment by the 1.2 kb SmaI fragment of pUC4-KIXX (Fig. 4-3). By this manipulation, the C-terminal region of ORF2 and the complete regions of ORF3 and ORF4 were removed. pLAC105 was treated as described for disruption of the eryAI-homologs. Among 50 regenerants obtained, 18 were resistant to both Km and Ts, and 32 were resistant to only Km.

Two colonies from the latter group were analyzed further. Probe 1 hybridized to the pSLA2-L band in CHEF gel electrophoresis (data not shown) and to the 4.8 kb BamHI fragment in agarose gel electrophoresis (Fig. 5-4, only the result of disruptant AC47-1 is shown here and below). On the other hand, the original 5.4 kb BamHI fragment (probe 4) hybridized to the same
fragment in strain 51252, and to the 4.8 kb fragment in the disruptant. This indicates that gene replacement occurred in the actI-homologous region of pSLA2-L. However, bioautography showed that the disruptant AC47-1 still produced both Lm and Lc (Fig. 6). These results revealed that the type-II PKS gene cluster in pSLA2-L is not involved in the production of Lc or Lm. This situation is similar to that in *Streptomyces cinnamoneus*, where type-II PKS genes were shown by gene targeting not to be involved in the synthesis of the polyether antibiotic monensin (Arrowsmith et al., 1992).

4. Discussion

Polyketide synthases (PKSs) are divided into two types, large multifunctional proteins (type-I system) and individual monofunctional proteins that associate as a multienzyme complex (type-II system) (Hopwood, 1997). The type-I PKSs are further subdivided into iterative PKSs and modular PKSs. The latter include actinomycete PKSs such as the one for erythromycin synthesis where six condensation reactions are carried out by six different sets of domains located on three multifunctional enzymes. Lm clearly belongs to the modular-type polyketides because its chemical structure is quite similar to that of Em. As expected, we identified two *eryAI*-homologs on *pPl* fragment A of pSLA2-L, and confirmed that both homologs were parts of the modular-type PKS gene cluster for Lm, therefore, they are abbreviated as the *lkm* genes. The 3573 nucleotide sequence of *eryAI*-homolog A revealed the presence of four domains for KR, ACP, KS and AT in this order. All of the four domains, especially the KR domain, are quite similar to those coded by *eryAI*.

Biosynthesis of Lc is different from the usual modular-type polyketides. Because, its macrolide skeleton is synthesized from a starter glycine molecule followed by eight acetate molecules (Uramoto et al., 1978). All the methyl groups at C-2, C-4, C-10 and C-16 are derived from methionine by C-methylation but not from propionate. Consequently, we tested the type-II PKS probe identified an *actI*-homologous region (Kinashi et al., 1998). The 3573 nucleotide sequence of the 4.8 kb *actI*-homologous region revealed that it codes for KS, CLF, ACP and CYC similar to actI-ORFs. In addition, this region contains a TE gene (ORF1) upstream of ORF1. However, gene disruption of ORF2-ORF4 did not cause any effect on the production of Lm or Lc. Thus, we have not yet identified the location of the Lc synthase genes or the chemical structure of the type-II polyketide metabolite, the synthesis of which is coded by the *actI*-homologous genes. The structure of the latter metabolite may be quite interesting, because a TE gene has been found for the first time in the type-II PKS gene cluster.

Gene replacement in *Streptomyces* sp. is frequently difficult. We had tested several plasmids to disrupt the *lkm* genes in *S. rochei* and finally succeeded in this by using pRES18. pRES18/19 are shuttle vectors constructed by fusion of plasmids pUC18/19, the T3 resistance gene, and plasmid pRES1 isolated from *Streptomyces griseus* (Ishikawa et al., 1996). It is noteworthy that gene replacement occurred at high frequencies (62–74%) in the protoplast regenerants of *S. rochei*. Most of the vectors that have hitherto been used for gene disruption in *Streptomyces* were non-replicating in the hosts or could be easily eliminated by culturing at a high temperature. On the other hand, gene replacement in *Sac. erythraea* was succeeded by using the plasmid pWHM3 (Vara et al., 1989), which is basically a fusion of pUC18 and pIJ702. Katz (1997) suggested that pIJ702 does not stably replicate in *Sac. erythraea* but carries out abortive replication to generate single-stranded intermediates which are the DNA elements that undergo genetic exchange. Similarly, it is likely that pRES18/19 could not stably replicate in *S. rochei* and, therefore, frequently induced recombinations.

Genetic and cloning studies of Hopwood and his colleagues showed that the methylzymycin biosynthetic (*mzy*) gene cluster are located on an unisolated plasmid SCP1 (Kirby and Hopwood, 1977; Chater and Bruton, 1985). We revealed that SCP1 is a 350 kb giant linear plasmid (Kinashi et al., 1987; Kinashi and Shimajima-Murayama, 1991) and located the *mzy* gene cluster on it (Redenbach et al., 1998). Pandza et al. (1998) showed that the oxytetracycline biosynthetic (*otc*) gene cluster can be transferred by recombination from the end of the linear chromosome of *Streptomyces rimosus* to the linear plasmid pZUG101. The present study has confirmed that two PKS gene clusters for Lm and an unknown type-II polyketide are located on pSLA2-L. Therefore, linear plasmids may sometimes carry an antibiotic biosynthetic gene cluster and be involved in its horizontal transfer in *Streptomyces*.

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References


