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Physical Mapping of the Linear Plasmid pSLA2-L and Localization of the *eryAI* and *actI* Homologs

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The 200-kb linear plasmid pSLA2-L was suggested to be involved in the production of lankamycin and lankacidin in *Streptomyces rochei* 7434AN4. In this study, we have constructed a physical map for 23 *Pst*I fragments of pSLA2-L, the sum of which was 206 kb. Detailed restriction maps for both ends of pSLA2-L revealed the presence of terminal inverted repeats, the size of which was found to be 2.1 kb by cloning and sequencing of the end-points. Hybridization experiments using two polyketide biosynthetic genes, *eryAI* and *actI*, located their homologous regions on *Pst*I fragments A and I, respectively.

Key words: *Streptomyces*; linear plasmid; physical mapping; pulsed-field gel electrophoresis; polyketide antibiotic

Introduction

Filamentous soil bacteria, *Streptomyces*, frequently have linear plasmids, which are a significant feature of this genus.[1] The plasmids vary greatly in size (12 kb to 1 Mb), contain terminal inverted repeats (TIRs) at both ends, and are blocked by a protein at the 5′-ends. SCP1, the most studied 350-kb linear plasmid[2] in *Streptomyces coelicolor* A3(2), carries the biosynthetic gene cluster for the antibiotic methylenomycin,[3] and after integration into the chromosome, it shows unique uni- or bi-directional gene transfer during conjugation.[4] However, the biological functions of other linear plasmids in *Streptomyces* have not been intensively studied.

Streptomyces rochei 7434AN4, a producer of two macrolide antibiotics, lankamycin[5] and lankacidin,[6,7] carries three linear plasmids, pSLA2-L (200 kb), M (100 kb), and S (17 kb).[8] The smallest plasmid, pSLA2-S, was the first linear plasmid isolated from bacteria.[9,10] pSLA2-S replicates bidirectionally from an origin near the center of the molecule, and at the telomeres, the ends of lagging-strand DNA were speculated to be completed by protein-priming DNA synthesis.[11] The largest plasmid, pSLA2-L, was suggested to be involved in the production of lankamycin and lankacidin on the basis of the plasmid profile of various mutants, transfer of the producing ability by protoplast fusion, and the structure of an antibiotic-deficient deletion plasmid, pSLA2-L1.[8] However, even the location of their biosynthetic genes on pSLA2-L has not been found. Although we constructed physical maps of pSLA2-L with several rare-cutting restriction endonucleases,[8] they were not fine enough for precise analysis.

As a first step toward biological studies of pSLA2-L and also to reveal its structural properties, we constructed a physical map for 23 *Pst*I fragments of pSLA2-L. The inside ends of the TIRs were analyzed in detail by cloning and nucleotide sequencing. Finally, regions homologous to the polyketide biosynthetic genes for erythromycin and actinorhodin were identified.

Materials and Methods

Bacterial strains and plasmids. *Streptomyces rochei* 7434AN4 and its mutant 51252, which carries only pSLA2-L, were described previously.[8] Two polyketide biosynthetic genes, *eryAI*[12] for erythromycin and *actI*[13] for actinorhodin, were used as probes for hybridization. For this purpose, pKS2/M5,[12] pUC19 carrying a 1.4-kb *Sma*I fragment of the KS/SU2 region of *eryAI* from *Saccharopolyspora erythraea*, and pIJ2345,[14] pBR329 carrying a 2.2-kb *Bam*HI fragment of *actI* from *S. coelicolor* A3(2), were kindly provided by L. Katz and D. A. Hopwood, respectively.

Pulsed-field gel electrophoresis (PFGE) and Southern hybridization. The DNA samples of *S. rochei* for PFGE analysis were prepared by the mycelium method as described.[15,16] We used a contour-clamped homogeneous electric fields (CHEF)[17] machine for PFGE. It was done at around 150 V in 0.5 × TBE buffer using 1.0% agarose gel at 15°C. The pSLA2-L DNA was separated with pulse-times of 20 sec, extracted from gels by electroelution and digested with restriction endonucleases. The digested fragments were separated by CHEF gel electrophoresis with pulse-times of 1.5–3.0 sec or by conventional agarose gel electrophoresis, and transferred to nylon membranes. DNA probes for hybridization were labeled with digoxigenin-labeled dUTP (Boehringer Mannheim, Mannheim, Germany).

DNA cloning and sequencing. pUC19 was used as a vector for construction of a *Bam*HI library and for cloning of *Hin*dIII fragments C and E, terminal *Eco*RI fragments E3 and E4, and their subfragments. Nucleotide

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sequences were determined by the dideoxy chain termination method using the Sequenase kit (Toyobo) and ^{32}P-dCTP.

Results and Discussion

Physical mapping of NdeI fragments

Although the restriction maps of pSLA2-L with rare-cutting restriction enzymes, *Dra*I, *Eco*RV, *Hin*dIII, and *Hpa*I, have been reported previously,[8] the maps were not fine enough, because no recognition site for these enzymes was present on the left 100-kb half of pSLA2-L. To construct a detailed physical map, a rare cutter that cleaves the left 100-kb half, was indispensable. Among enzymes tested, *Nde*I was found to cut this region; double digestion of pSLA2-L with *Eco*RV plus *Nde*I changed the size of *Eco*RV fragment A (Fig. 1(a)).

Digestion with *Nde*I of pSLA2-L gave five fragments, A (80 kb), B (45 kb), C1, 2 (doublet, 2 × 30 kb), and D (20 kb)(Fig. 1(a)). The order of five fragments was studied by hybridization of *Eco*RV fragments A and B, and *Hin*dIII fragment B, the locations of which had been identified[8] and are redrawn in Fig. 2. *Eco*RV fragment A hybridized to *Nde*I fragments A, B, and C1, *Eco*RV fragment B to *Nde*I fragment A, and *Hin*dIII fragment B to *Nde*I fragments C2 and D (data not shown). In addition, the left and right end-specific probes of pSLA2-L, pE4Ka and pE3Kc (see later), hybridized to *Nde*I fragments C1 and D, respectively. These results unequivocally determined the order of *Nde*I fragments to be C1-B-A-C2-D (Fig. 2). This order was finally confirmed by hybridization of *Nde*I linking plasmids. From the *Bam*HI library of pSLA2-L, three of the four *Nde*I linking clones were isolated and are indicated by an asterisk in Fig. 2; pND2 (carries a 17-kb insert and connects *Nde*I fragments B and A), pND3 (6.1-kb insert, *Nde*I-A and C2) and pND4 (4.4-kb insert, *Nde*I-C2 and D).

Physical mapping of PstI fragments

Digestion of pSLA2-L with *Pst*I and *Bam*HI gave 23 fragments (Fig. 1(b), 1(d)) and about 40 fragments, respectively. Since the number and sizes of *Pst*I fragments were suitable for fine mapping, this enzyme was used for further analysis. On the other hand, *Bam*HI fragments were too many for physical mapping and were used to construct the *Bam*HI plasmid library. The sizes of *Pst*I fragments are listed in Table, the sum (206 kb) of which agrees with the reported size (200 kb) of pSLA2-L itself.[8]

At first, all the *Pst*I fragments were divided into groups by hybridization with *Eco*RV and *Hin*dIII fragments. Hybridization of the *Eco*RV-A probe is shown in Fig. 1(c) as an example and all the hybridization results are summarized in Table.

The *Eco*RV-A group contains *Pst*I fragments A, B, C, E1, E2, and G (Fig. 1(c)). The positive hybridization of *Pst*I fragment K was due to the right terminal inverted repeat (TIR-R, see later). *Pst*I fragment E1 could be

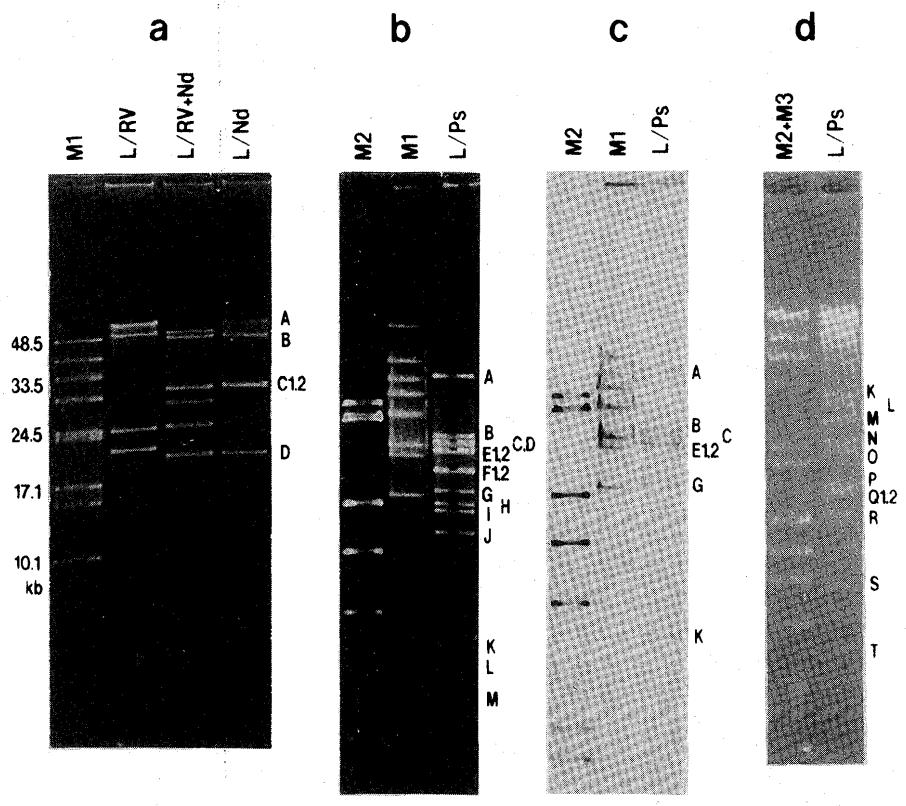

Fig. 1. Analysis of the pSLA2-L Digest by CHEF (a, b, c) and Conventional, Agarose Gel Electrophoresis (d).

(a) *Nde*I and *Eco*RV digest. (b, d) *Pst*I digest. (c) Southern hybridization analysis of the filter of (b). Digoxigenin-labeled *Eco*RV fragment A and λDNA were used as probes for hybridization. We checked in advance that λDNA did not hybridize to the pSLA2-L DNA. M1, λDNA-Mono Cut Mix (New England Biolab, Beverly, MA); M2, λDNA digested with *Hin*dIII; M3, φx174 DNA digested with *Hae*III. Nd, *Nde*I; Ps, *Pst*I; RV, *Eco*RV.

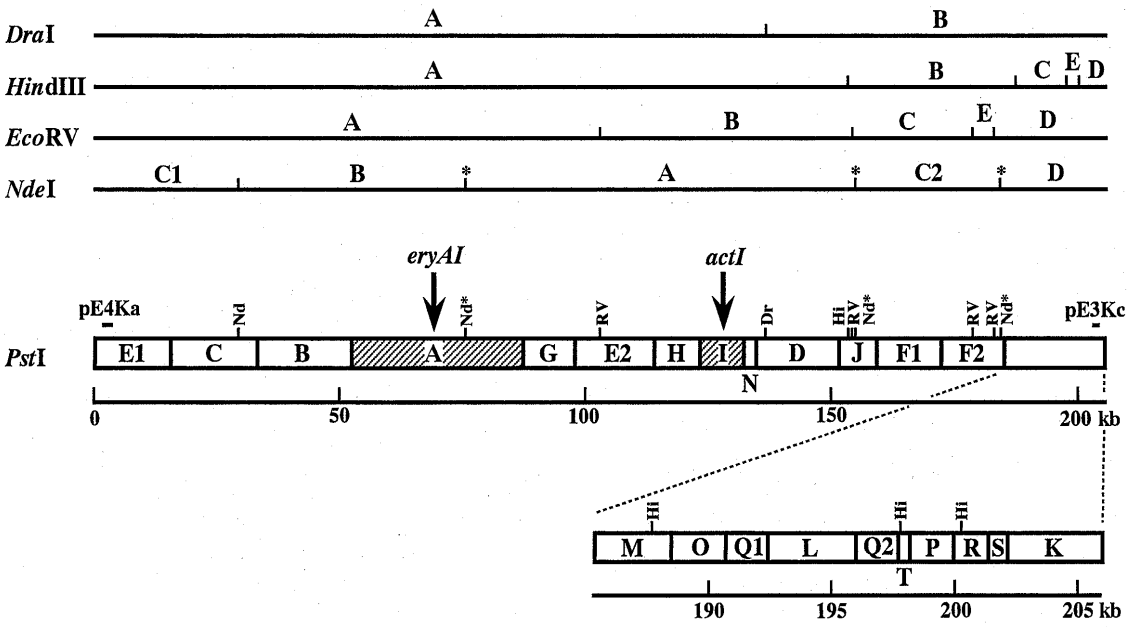

Fig. 2. Physical Map of the 23 *Pst*I Fragments of pSLA2-L.

The 22 *Pst*I sites and the sites for *Dra*I (Dr), *Eco*RV (RV), *Hin*dIII (Hi), and *Nde*I (Nd) are aligned on a linear map. The 20-kb right-end region is magnified 5 times below the main map. The restriction maps of *Dra*I, *Hin*dIII, *Eco*RV, and *Nde*I fragments are also shown above the main map to make the mapping process clear. *Nde*I linking clones have been isolated for three *Nde*I sites with an asterisk. The end-specific probes, pE4Ka and pE3Kc are shown as a bar. The *eryAI* and *actI* probes hybridized to *Pst*I fragments A and I, respectively.

Table. Size and Hybridization of *Pst*I Fragments of pSLA2-L

Fragment	Size (kb)	Hybridization								
		RV-A	RV-B	Nd-C	Nd-B	Nd-A	Hi-B	Hi-C	Hi-E	Hi-D
A	35	+			+	+				+*
B	19	+			+					
C	17.5	+		+	+					
D	17		+			+				
E1	16	(+)		(+)						
E2	16	(+)	(+)			(+)				
F1	13			(+)			(+)			
F2	13			(+)			(+)			
G	10.5	+				+				
H	9.3		+			+				
I	9.0		+			+				
J	7.7		+	+		+	+			
K	3.9	+*								+
L	3.6							+		
M	3.1						+	+		
N	2.4		+			+				
O	2.2							+		
P	1.8								+	
Q1	1.7							(+)		
Q2	1.7							(+)		
R	1.4								+	+
S	0.8									+
T	0.5								+	
	206.1									

RV, *Eco*RV fragment; Nd, *Nde*I fragment; Hi, *Hin*dIII fragment. +, positive hybridization. +*, positive hybridization due to TIR. (+), the hybridized fragment in the doublet band was not identified only by this experiment.

localized at the left end by hybridization with the left end probe pE4Ka (see later). Since *Pst*I fragment E2 changed its size by double digestion with *Eco*RV, this fragment was identified as the junction fragment between the *Eco*RV-A and B groups. The results of hybridization of *Nde*I fragments A, B, and C (Table),

and the location of the *Nde*I linking clone pND2 on *Pst*I fragment A unambiguously identified the order of *Pst*I fragments as E1-C-B-A-G-E2.

The *Eco*RV-B group contains *Pst*I fragments D, E2, H, I, J, and N. *Pst*I fragment J was identified as the right junction fragment by double digestion with *Eco*RV. Screening of the *Bam*HI library using *Pst*I fragment N as a probe enabled us to isolate the linking plasmid pIND (5.2-kb insert) which could connect three *Pst*I fragments, I, N, and D in this order. In addition, *Dra*I fragment B hybridized to *Pst*I fragments D and J. Thus, the order of the *Eco*RV-B group was E2-H-I-N-D-J.

*Hin*dIII fragments B, C, D, and E cover the remaining 50-kb right part of pSLA2-L. The *Hin*dIII-B group contains *Pst*I fragments F1, F2, J, and M. *Pst*I fragment M was located at the right junction by double digestion with *Eco*RV. Thus, the remained doublet fragments F1, 2 were found to be adjacent to each other and were named F1 and F2 in the right-handed direction. *Hin*dIII fragments C and E were cloned into pUC19 and were found to contain *Pst*I fragments L, M, O, Q1, Q2, and T, and *Pst*I fragments P, R, and T, respectively. Precise restriction mapping of two clones determined the order of *Pst*I fragments to be M-O-Q1-L-Q2-T-P-R. The *Pst*I map of *Hin*dIII fragment D at the right end of pSLA2-L was established to be R-S-K during the analysis of TIR-R as described below. Thus, the physical map of the 23 *Pst*I fragments of pSLA2-L has been completed as shown in Fig. 2.

Analysis of the TIRs

All the linear plasmids so far isolated from *Streptomyces* have TIRs at both ends, the size of which ranges from 44 bp of plasmid SLP2[18] to more than 95 kb of plasmid pPZG101.[19] In our previous study, *Hin*dIII fragment D was localized at the right end of pSLA2-L. The labeled *Hin*dIII fragment D hybridized to the opposite end fragment *Eco*RV-A, which suggested the presence of TIRs on pSLA2-L.[8]

Using *Hin*dIII fragment D as a probe, two *Eco*RI fragments located close to each end, were cloned and named pE3 (5.3 kb, right end) and pE4 (4.3 kb, left end), respectively. Both plasmids were found to carry an *Eco*RI fragment 0.5 kb from each end. Restriction analysis of pE3 and pE4 enabled us to construct the physical maps at both ends of pSLA2-L (Fig. 3). Thus, the order of *Pst*I fragments at the right end was K-S-R from the end.

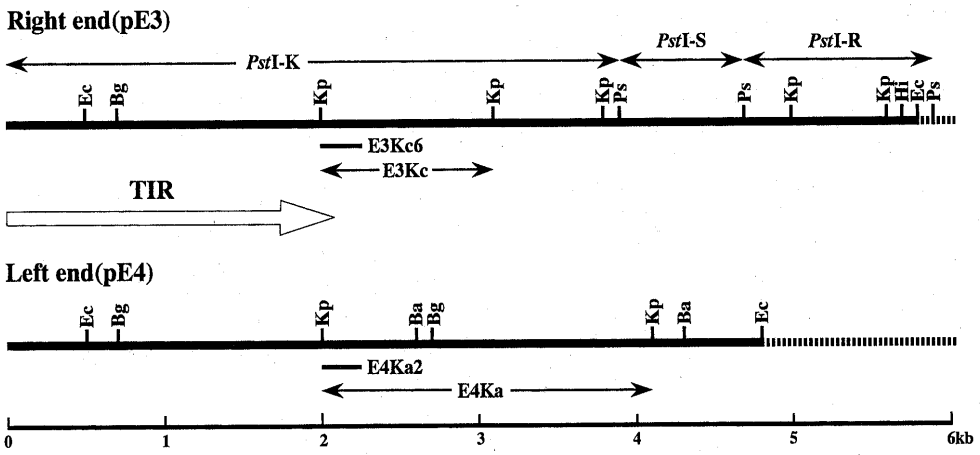

Fig. 3. The Restriction Maps of the Right and Left Ends of pSLA2-L.
The maps were constructed mainly by restriction analysis of two *Eco*RI clones, pE3 and pE4. Subclones pE3Kc and pE4Ka were used as end-specific probes for hybridization. Comparison of the nucleotide sequences of fragments E3Kc6 and E4Ka2 determined the end-points of the TIRs. The range of the TIR regions is indicated by a white arrow. Ec, *Eco*RI; Bg, *Bgl*II; Kp, *Kpn*I; Ps, *Pst*I.

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                                TIR-R                          ------ -  ->&lt;-  - ------
pE3Kc6  GGTACCGGTCCTGCTCGACCCCGGCGAGACCGCCGACAACATGCTCACCAGCCGGCCGTACAACGGGCTGGCcaagctgc  80
        **********************************************************************  ****
pE4Ka2  GGTACCGGTCCTGCTCGACCCCGGCGAGACCGCCGACAACATGCTCACCAGCCGGCCGTACAACGGGCTGGCgttgctgt
        KpnI                    TIR-L                  ------ -  ->&lt;-  - ------

        tggaggcgctcagggcgcacgatgcccgggtggtggagcagttggcgcacgagcaggctcccagcgcctacaagcccgtc 160
        *     *   *  **     * **  *  *  **     **   *   * ** *   **  *   * *   **
        tccgccaggcgccgctgctgaccggccacggtgaagaaggattccgggcggcgcggttcccagggttcgaggtgcgtccg

        cagaagggcgcgcagggtcaggagagccggggcgaggggaacgggtccggcggcccgtcggcgccggcgcggaagctgct 240
        ** *  *  **       ** ** ***     *     * *  ** *          ***** *   ** *
        gttctcggtgtacgcggcgcacagcgcggggcgtccgccggtccggcgagccggagtatcacgccgtccgcgatgtcttc

        gaagttctccgtgccgcgtgatc 263
        *   *   *          Sau3AI
        gctttcgatc 250
             Sau3AI
```

Fig. 4. The Nucleotide Sequences Around the Inside Ends of TIR-R and TIR-L of pSLA2-L.
Capital letters indicate the TIR regions. Identical nucleotide pairs in the right and left terminal strands are indicated by asterisks. The palindromic inverted repeat is shown by paired arrows.

Comparison of the restriction maps of fragments E3Kc and E4Ka showed that the end of TIR-L was present between the sites for *Kpn*I and *Bam*HI on the left side of E4Ka.

To identify the end-points of the TIRs, fragments E3Kc and E4Ka were digested with *Kpn*I and *Sau*3AI and then cross-hybridized with E4Ka and E3Kc, respectively. Only the outermost fragments E3Kc6 and E4Ka2 gave a positive signal and were different in size, which indicated that each end-point of the TIRs was located on these fragments. Both fragments were cloned into pUC19 and their nucleotides were sequenced (Fig. 4). Comparison of their sequences showed that the TIR regions extend 66 beyond the *Kpn*I site. Thus, the size of the TIRs was determined to be 2.1 kb.

In addition, the nucleotide sequences identified an imperfect palindromic inverted repeat at one base from each inside end of the TIRs. We previously found an insertion element IS*466*[20] at the inside end of TIR-R of the linear plasmid SCP1.[21] The functions of these structures around the inside ends of TIRs of linear plasmids deserve to be studied further.

Location of the eryAI and actI homologs

S. rochei 7434AN4 produces two structurally unrelated macrolide antibiotics, lankamycin and lankacidin. Lankamycin is a 14-membered macrolide antibiotic,[5] while lankacidin consists of a unique 17-membered macrolide ring.[6,7] Both antibiotics belong to the polyketides that are synthesized by type-I or type-II polyketide synthases (PKSs).[22] Lankamycin clearly belongs to the type-I polyketides, because it is quite similar to the typical type-I polyketide, erythromycin, whose macrolide ring is synthesized from seven propionate molecules.[12] On the other hand, the macrolide skeleton of lankacidin was synthesized from a starter glycine molecule followed by eight acetate molecules, and all C-methyl groups were derived from methionine.[23] Therefore, it is quite different from the usual *Streptomyces* type-I polyketides from a biosynthetic point of view. Consequently, the biosynthetic genes for erythromycin (*eryAI*) and actinorhodin (*actI*) were used as probes for the type-I and type-II systems, respectively.

The digoxigenin-labeled *eryAI* and *actI* were used to probe *Pst*I fragments of pSLA2-L. The former hybridized to *Pst*I fragment A (Fig. 5(a)) and the latter to *Pst*I fragment I (Fig. 5(b)). A weak positive signal on the top of *Pst*I fragment A in Fig. 5(a) seems to be a combined fragment of *Pst*I-A and B from its size. The *Pst*I site between these fragments might be a little bit resistant to digestion. It is specially noteworthy that both of the type-I and type-II PKS homologs are located in a small (80 kb) region of pSLA2-L.

We have previously revealed that the giant linear plasmid SCP1 in *S. coelicolor* A3(2) carries the methylenomycin biosynthetic gene cluster.[3] This is a unique example where the plasmid involvement in antibiotic production has been proved definitely. In this study, we found the *eryAI* and *actI* homologous regions on pSLA2-L and located them on *Pst*I fragments A and I, respectively. This has opened a way to precisely analyze another example, the biosynthetic genes for lankamycin and lankacidin on the linear plasmid pSLA2-L.

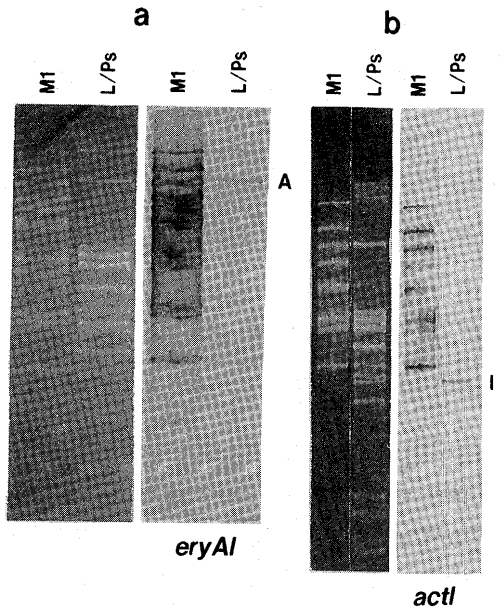

Fig. 5. Southern Hybridization of the *eryAI* and *actI* Probes to *Pst*I Fragments of pSLA2-L.

*Pst*I fragments of pSLA2-L were separated by CHEF gel electrophoresis, transferred to nylon membranes, and hybridized with λDNA and plasmids pKS2/M5 (a) or pIJ2345 (b), which carried the *eryAI* or *actI* genes, respectively.

Acknowledgments

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