ANALYSIS OF THE PROMOTER REGION OF THE CLONED KANAMYCIN RESISTANCE GENE (*kmr*) FROM *STREPTOMYCES KANAMYCETICUS*

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The appropriate location and orientation of the kanamycin resistance gene (*kmr*) cloned on multicopy plasmids were determined by subcloning experiments. The transcription start site was identified by high-resolution S1 mapping and the *kmr* mRNA was shown to have a long leader of about 400 bp. An additional transcript upstream of the *kmr* gene was also detected, which ran in the opposite direction and overlapped 2 to 3 nucleotides with the *kmr* transcript. The presumptive promoter region of this physiologically unidentified RNA was similar to the *Escherichia coli* promoter consensus sequence both in the −10 and −35 regions, whereas the putative promoter region of the *kmr* gene exhibited sequence similarities in the −10 region to the promoters of the endoglycosidase H (*endo*H) and the viomycin phosphotransferase (*vph*) genes from *Streptomyces*.

One of the goals of research on *Streptomyces* molecular genetics is to know how genes for secondary metabolism such as antibiotic biosynthesis are regulated so that they are expressed in the correct order at the appropriate time. It is possible that different sigma factors enable RNA polymerase to recognize unique classes of promoters and thereby activate selective sets of genes, as shown in *Bacillus subtilis*[1]. Two forms of RNA polymerase holoenzymes with different species of sigma factors have been identified from *Streptomyces coelicolor* and shown to recognize different promoter classes[2]. In addition, several types of *Streptomyces* promoters have been analyzed in the last few years; these include the aminoglycoside phosphotransferase (*aph*) gene from *Streptomyces fradiae*[3], the endoglycosidase H (*endo*H) gene from *Streptomyces plicatus*[2], the rRNA methylase genes from *Streptomyces azureus* (*tsr*)[3] and from *Streptomyces erythraeus* (*erm*)[4], the viomycin phosphotransferase (*vph*) gene from *Streptomyces vinaceus*[3], the genes of the glycerol operon (*gyl*) of *S. coelicolor*[3] and the hygromycin B phosphotransferase (*hyg*B) gene from *Streptomyces hygroscopicus*[5]. Comparative analyses of these revealed the diversity of *Streptomyces* promoters; thus the task remains to classify these promoters in relation to different species of RNA polymerase and the cellular differentiation process.

We have previously cloned and characterized the kanamycin resistance gene (*kmr*) from *Streptomyces kanamyceticus*[6]. In the present report we have determined the transcription start site and putative promoter of the gene cloned on multicopy plasmids in *Streptomyces lividans*.

Materials and Methods

Bacterial Strains and Plasmids

Streptomyces lividans 1326 (a derivative of *S. lividans* 66[7] provided by D. A. HOPWOOD) was used as a host in subcloning experiments. Transformation of *S. lividans* 1326 was done by the method of THOMPSON *et al.*[8]. The multicopy plasmids pIJ702[9] and pSL1[10,11] were used as cloning vectors

in *S. lividans*. pMCP5 was derived from pIJ702 and carried the *kmr* gene in the *Bgl* II site as previously described[6]. JM83 and pUC8[12] were used for the construction of recombinant plasmids in *Escherichia coli*. *Streptomyces* strains were grown at 28°C in Tryptic Soy Broth (Difco) with 1% glucose (TSBG medium) and 0.1% glycine. Thiostrepton and/or kanamycin were added in TSBG medium to 5 μg/ml and 50 μg/ml, respectively. *E. coli* strains were grown in LB medium[13] with 25 μg/ml of ampicillin.

DNA Preparation and Manipulation

Plasmid DNA from *Streptomyces* was isolated by alkaline lysis[14] followed by spermine treatment[15]. *E. coli* plasmid DNA was prepared according to the method of HOLMES and QUIGLEY[16]. Digestion with restriction enzymes and treatment with modifying enzymes were carried out as recommended by the commercial suppliers. DNA fragments were purified by the electroelution method of SMITH[17].

RNA Isolation

Total RNA was prepared from *S. lividans* 1326 carrying pMCP64 (CMA64) by a modification of the method of PENN *et al.*[18]. *S. lividans* CMA64 was grown in TSBG medium supplemented with kanamycin. Cells at mid-log phase were harvested and suspended in LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.8, 1% SDS) and then were added to an equal volume of phenol and a half volume of glass beads. The mixture was vortexed twice for 2 minutes and the aqueous phase was collected by centrifugation at 5,000 rpm for 10 minutes. The aqueous phase was re-extracted with phenol - chloroform and centrifuged at 10,000 rpm for 10 minutes. To the aqueous phase, 1/10 volume of 2 M LiCl and an equal volume of 2-propanol were added. The precipitate was collected and was treated with RNase-free DNase I (Worthington). After two phenol extractions, RNA was precipitated with 2-propanol.

Preparation of Hybridization Probes

Purified restriction fragments were treated with calf intestine alkaline phosphatase for 30 minutes at 37°C. The phosphatase was inactivated by heating for 10 minutes at 65°C and removed by phenol extraction. The dephosphorylated fragments were labeled at their 5′ ends with [γ-^{32}P]dATP by T4 polynucleotide kinase. The 3′ end of the *Eco*R I site was labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. The labeled fragments were used as hybridization probes directly or after separation as described by MAXAM and GILBERT[19].

S1 Nuclease Mapping

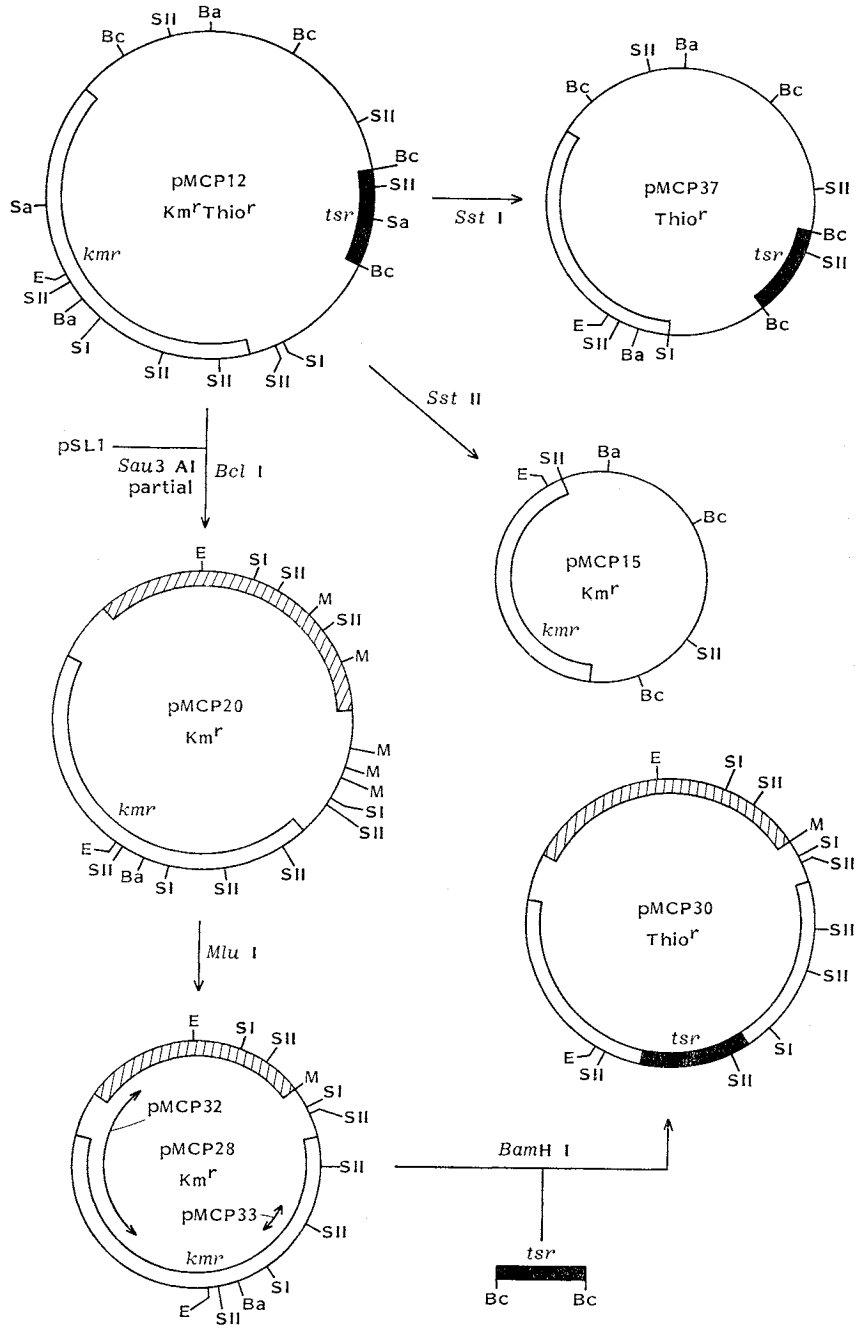
End labeled probes were hybridized with 50 μg total RNA or 50 μg carrier tRNA in 20 μl of hybridization solution (40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide). The hybridization mixture was heated for 10 minutes at 85°C and the temperature was decreased slowly to 61°C (Tm(DNA-DNA)+4°C) and the mixture incubated for 3 hours. Hybridization was terminated by the addition of 300 μl ice-cold S1 digestion buffer (280 mM NaCl, 30 mM NaOAc, 4.5 mM $Zn(OAc)_2$, 20 μg/ml carrier single-stranded DNA) containing 200 U of S1 nuclease. After incubation for 45 minutes at 37°C, protected hybrids were extracted with phenol, recovered by 2-propanol precipitation, rinsed and redissolved in formamide loading buffer. Samples were denatured for 2 minutes at 90°C, quickly chilled and analyzed by electrophoresis in polyacrylamide gels containing 7 M urea.

S1 nuclease mapping using single-stranded hybridization probes was performed as follows. End-labeled, single-stranded hybridization probes and RNA were hybridized in 20 μl of hybridization solution (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 2 mM EDTA, 0.1% SDS). The hybridization mixture was incubated under paraffin oil for 5 minutes at 90°C, the temperature was slowly decreased to 61°C and the mixture further incubated for 3 hours. S1 treatment and analysis by polyacrylamide gels were carried out as described above.

High-resolution S1 Mapping

S1 nuclease protected hybrids were electrophoresed on polyacrylamide-urea sequencing gels, next to sequencing ladders of the end-labeled test DNA.

Fig. 1. Subcloning of the kanamycin resistance gene (*kmr*).



Thin lines and hatched boxes are fragments derived from pIJ702 and pSL1, respectively. Double lines and dark segments represent kanamycin resistance and thiostrepton resistance fragments, respectively. The deleted regions in pMCP32 and pMCP33 are shown by arrows inside the pMCP28 map. Abbreviations for restriction enzymes used in this figure are: Ba: *Bam*H I, Bc: *Bcl* I, E: *Eco*R I, Sa: *Sal* I, SI: *Sst* I, SII: *Sst* II, M: *Mlu* I.

Results

Localization of the *kmr* Gene

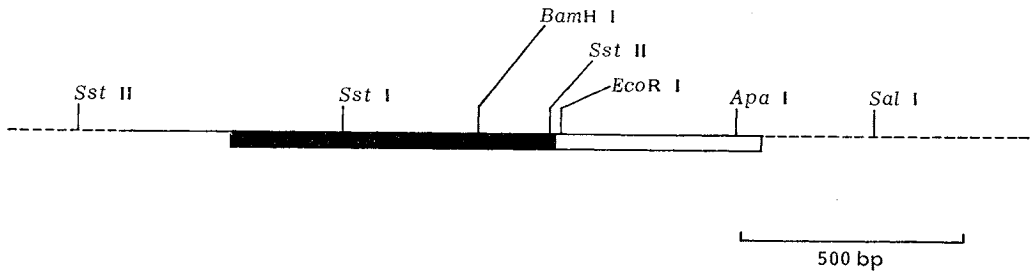
The *kmr* gene was located using a plasmid derivative (pMCP12) of pMCP5, the original recombinant plasmid carrying the *kmr* gene[6], as shown in Fig. 1. pMCP5 has three internal *Bcl* I sites within the insert[6]. We subcloned the largest *Bcl* I fragment (about 4 kb) into the *Bgl* II site of pIJ702. This plasmid, pMCP12, retained the ability to confer kanamycin resistance in *S. lividans*. Digestion of pMCP12 with *Sst* II followed by ligation generated the smaller plasmid pMCP15 that contained only two *Sst* II fragments of pMCP12 which carried the replication region and the *kmr* gene.

We also constructed the *kmr*-bearing plasmids pMCP20 and pMCP28, which are derivatives of the multicopy plasmid pSL1 isolated from *S. lavendulae*[10,11]. Considering that pMCP15 conferred the kanamycin resistance phenotype, the unique *Bam*H I site in pMCP28 was thought to be dispensable for expression of resistance. Consequently we cloned a *Bcl* I fragment harboring the *tsr* gene from pIJ702 into the *Bam*H I site. The resulting plasmid pMCP30 expressed thiostrepton but not kanamycin resistance in *S. lividans*. By comparing the restriction maps of pMCP15 and pMCP30, it was concluded that the *kmr* structural gene was located within the insert of pMCP15 and may be transcribed from the *Sst* II to the *Eco*R I site. These results also suggested that the *Bam*H I site was located within the regulatory region of the *kmr* gene, and that *kmr* expression in pMCP15 was due to transcription readthrough originating from the vector moiety of the plasmid. To locate the *kmr* regulatory region more precisely, we isolated pMCP37 which had undergone a deletion between the two *Sst* I sites of pMCP12. pMCP37 encoded thiostrepton resistance but failed to confer kanamycin resistance, suggesting that the regulatory region extended past the *Bam*H I site and included the region containing the *Sst* I site. Two spontaneous deletions from pMCP28, pMCP32 and pMCP33 (Fig. 1), made it possible to delimit the region required for *kmr* expression. From these results, we localized the *kmr* gene as shown in Fig. 2.

Mapping of Transcription Start Site with S1 Nuclease

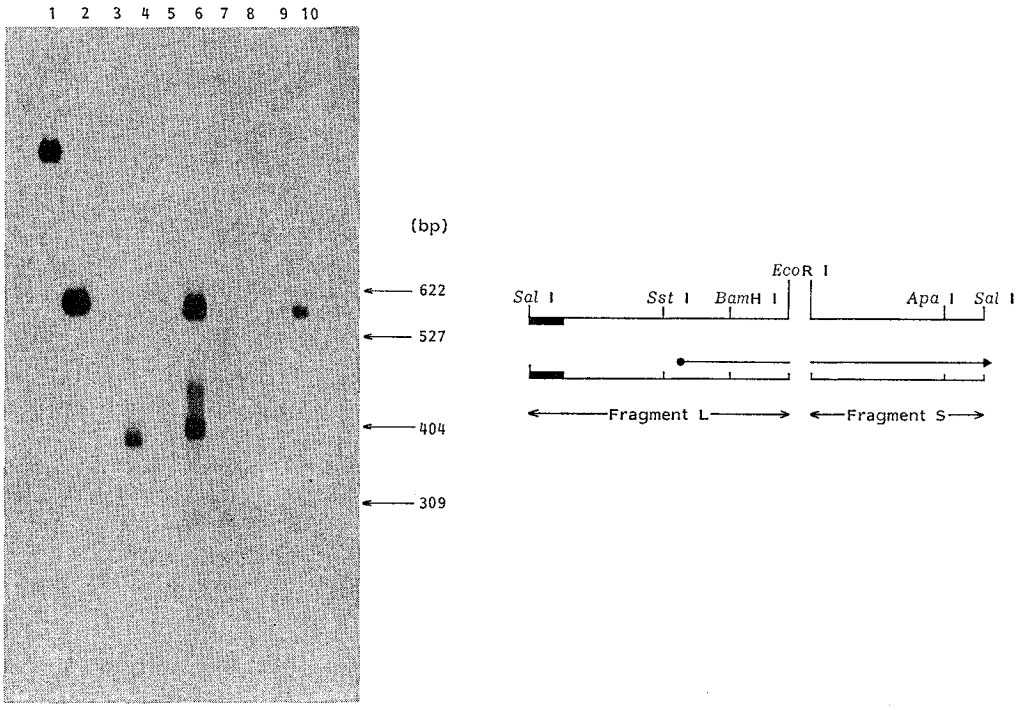
A spontaneous deletion in pMCP12 gave rise to plasmid pMCP64 which lacked the DNA upstream of the *kmr* gene and to the middle of the *tsr* gene (Fig. 1). A *Sal* I fragment of pMCP64 (Fig. 1), which contained the *kmr* gene as judged by the restriction analysis of pMCP32 and pMCP33, was introduced into the *Sal* I site of pUC8, giving rise to plasmid pMCP63. The *Sal* I insert of pMCP63 was digested with *Eco*R I which cleaved in the middle of the *kmr* gene (Fig. 3). The resulting two

Fig. 2. Restriction enzyme cleavage map of the kanamycin resistance gene.



The double line and dark segment represent the structural gene and regulatory region, respectively. Both ends of the structural gene are tentatively located. The thin line represents the limit of uncertainty of the regulatory region.

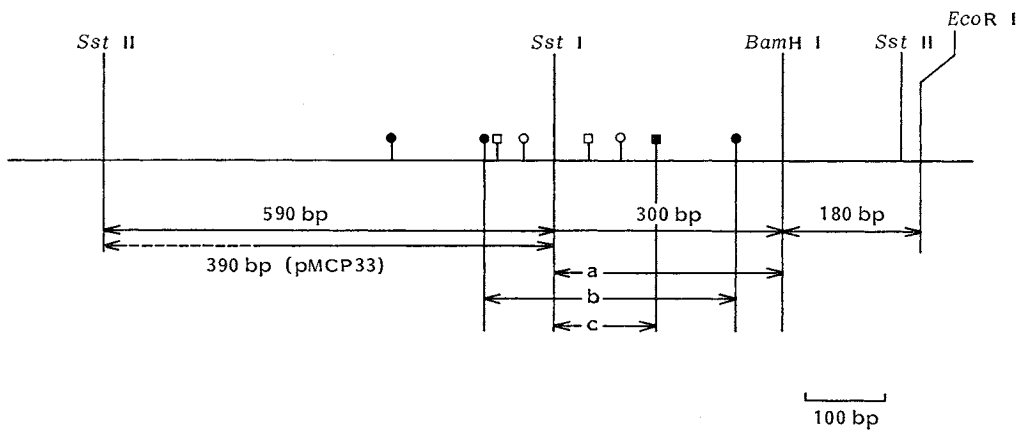
Fig. 3. S1 mapping analysis of the *kmr* gene.



The restriction map shows the *kmr*-carrying *Sal* I fragment of pMCP63. Lane 1: Fragment L in the absence of RNA and S1 nuclease, lane 2: fragment S in the absence of RNA and S1 nuclease, lane 3: fragment L labeled at 5′ ends, hybridized with tRNA, lane 4: same as lane 3 except hybridization with CMA64 RNA, lane 5: fragment S labeled at 5′ ends, hybridized with tRNA, lane 6: same as lane 5 except hybridization with CMA64 RNA, lane 7: fragment L labeled at 3′ end of *Eco*R I site, hybridized with tRNA, lane 8: same as lane 7 except hybridization with CMA64 RNA, lane 9: fragment S labeled at 3′ end of *Eco*R I site, hybridized with tRNA, lane 10: same as lane 9 except hybridization with CMA64 RNA. The molecular weight markers are pBR322 digested with *Hpa* I. The circle and the arrow in the restriction map represent the presumptive transcription start site and the direction of transcription, respectively. The thick lines show the thiostrepton resistance gene in pMCP64.

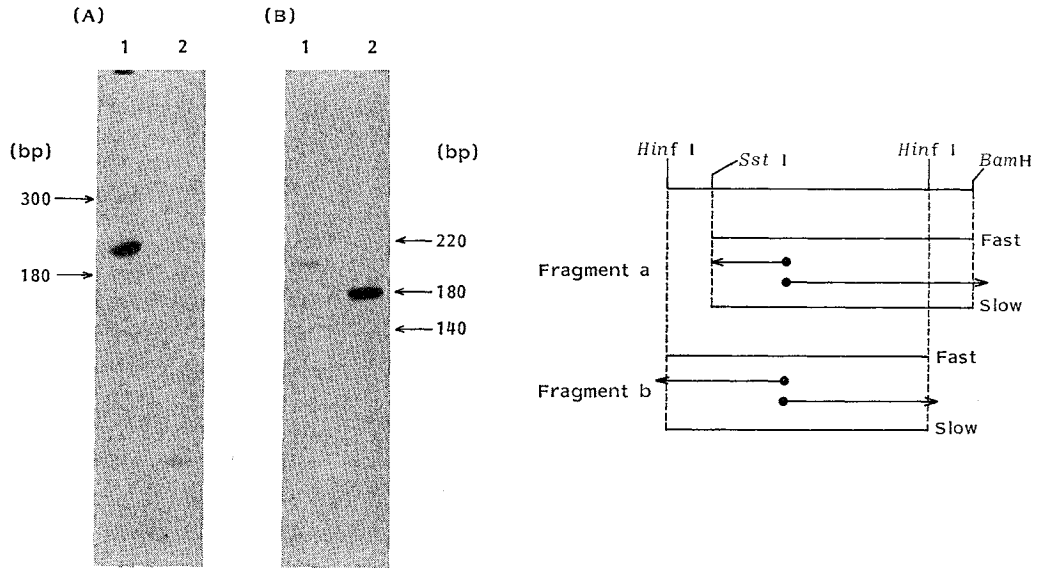
fragments were separated by agarose gel electrophoresis and recovered by electroelution. Each fragment was labeled at the 5′ ends of the *Sal* I and *Eco*R I sites and at the 3′ end of the *Eco*R I site as described in Materials and Methods. Each radiolabeled fragment was combined under hybridization conditions with RNA isolated from *S. lividans* CMA64 and the mixture was treated with S1 nuclease. Fig. 3 shows the S1 nuclease-resistant fragments which were resolved by electrophoresis. When the larger, 5′ end labeled fragment (1,040 bp) was used as the probe, a 380 bp fragment was protected by CMA64 RNA (lane 4); however, no DNA was protected using the same fragment labeled at the 3′ end (lane 8). As described below, there is another transcript that is identified when the opposite strand is used as a hybridization probe. However, only the 30 bp fragment protected by the *kmr* transcript is visible here because the transcript that is synthesized from the opposite strand would not protect the DNA labeled at the *Sal* I site, which lies within the plasmid-encoded thiostrepton gene. The smaller fragments (600 bp), both labeled at the 5′ end and 3′ end, were protected intact by CMA64 RNA (lanes 6 and 10). There are several other protected fragments in lane 6 of Fig. 3. We cannot conclude if they are derived from internal transcription start sites or due to secondary structure of

Fig. 4. Promoter region of the *kmr* gene.



DNA fragments used as probes for S1 mapping in Fig. 5 and Fig. 6 are shown. a: *Bam*H I - *Sst* I fragment, b: *Hinf* I fragment, c: *Hae* III - *Sst* I fragment. Symbols for restriction enzymes are: ● *Hinf* I, ○ *Rsa* I, □ *Nar* I, ■ *Hae* III. Other *Hae* III sites except the one noted in this figure were not checked.

Fig. 5. S1 mapping analysis of the *kmr* gene using single-stranded probes.



(A) Fragment a in Fig. 4 was labeled with ^{32}P at the 5′ ends followed by strand separation. CMA64 RNA was hybridized with the slow-migrating fragment a (lane 1) or the fast-migrating fragment a (lane 2). (B) Fragment b in Fig. 4 was labeled at 5′ ends followed by strand separation. CMA64 RNA was hybridized with the slow-migrating fragment b (lane 1) or fast-migrating fragment b (lane 2).

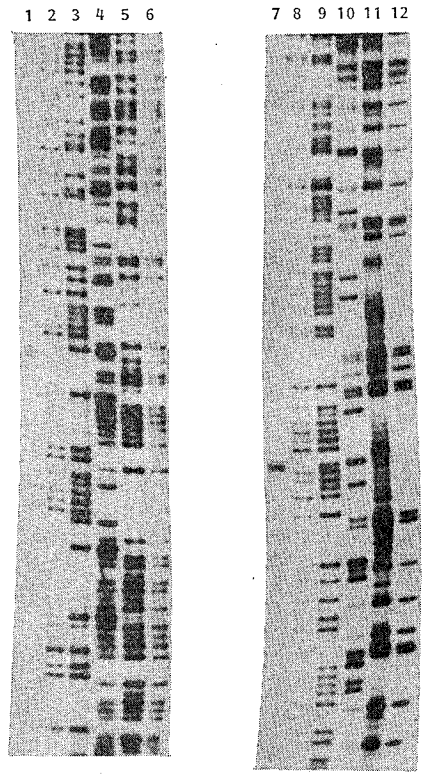
the hybridization probe. These results, which are summarized at the right side of Fig. 3, indicate that the transcription data are consistent with the results of the subcloning experiment described above.

A more detailed restriction enzyme analysis of the regulatory region was performed (Fig. 4). Two fragments (a and b in Fig. 4) were isolated which were believed to contain the transcription start site based on the restriction analysis. Single-stranded DNA of fragments a and b which were labeled

at the 5′ ends were used as probes for S1 nuclease protection experiments. Fig. 5 shows that the transcription start point of the *kmr* gene was located 210 bp upstream from the *Bam*H I site as expected, because a S1-resistant fragment of this size was generated with the *Bam*H I - *Sst* I fragment a that was ^{32}P-labeled at the *Bam*H I end (lane 1 of Fig. 5A). Another transcript was detected which ran in the opposite direction to the *kmr* transcript as shown by the smaller S1-resistant fragment from the complementary strand of the *Bam*H I - *Sst* I fragment labeled at the *Sst* I end (lane 2 of Fig. 5A). These results were confirmed by the results using fragment b as a probe (Fig. 5B). The precise start sites of these transcripts were identified by high-resolution S1 mapping using single stranded fragment c (Fig. 4). Fig. 6, lane 1 shows the S1-resistant DNA corresponding to the transcript that is produced by transcription originating upstream of *kmr* gene but proceeding in the opposite direction. Lane 7 shows the S1-resistant DNA fragment corresponding to the putative *kmr* transcript. The multiple S1-generated bands present in Fig. 6 are not uncommon as shown by BROSIUS *et al.*[20], who suggested that the 5′-terminal triphosphate group of the prokaryotic mRNA sterically inhibits S1 nuclease from cleaving the probe exactly at the junction of the RNA/DNA hybrid. The nucleotide sequences around the start sites and promoters are shown in Fig. 7. The RNA (RNA-R) transcribed opposite to the *kmr* transcript was initiated at the sites 2 and 3 nucleotides downstream from the start point of the *kmr* transcript.

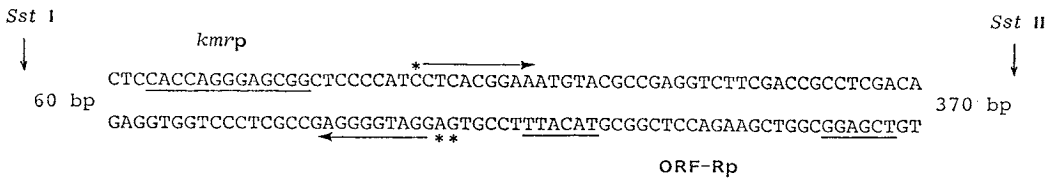
Fig. 8 shows the promoter sequences of the two genes. The nucleotide sequence upstream of the open-reading frame R (ORF-Rp) showed significant similarity to the consensus −10 and −35

Fig. 6. High-resolution S1 nuclease mapping of the kanamycin resistance fragment.



Fragment c in Fig. 4 was used as a probe after strand separation. Lanes 1~6: Slow-migrating strand, lanes 7~12: fast-migrating strand. Lanes 1 and 7: S1 protected DNA, lanes 2 and 8: G sequencing ladder, lanes 3 and 9: G+A sequencing ladder, lanes 4 and 10: A>C sequencing ladder, lanes 5 and 11: T+C sequencing ladder, lanes 6 and 12: C sequencing ladder.

Fig. 7. Nucleotide sequencing of the *kmr* promoter region.



Asterisks indicate the transcription start points and the direction of transcription is shown by arrows. Possible −35 and −10 regions are underlined.

Fig. 8. Nucleotide homologies between putative *kmr* and ORF-Rp (*kmr*) promoters and the known *Streptomyces* promoters.

(A)

```
CTCGGCGCCCACTCCTCCACCAGGGAGCGGCTCCCCATC*     kmr
                  |    |||||||
                  CCGGCGGGAGCGA              vphP1
                  |    |||   ||
CTGATTGACGCGC     CCGGCGGGCAGGG              endoH
                       |||    |
AGGTTTAAATCCTTATCGTTATGGGTATTG              ctc
```

(B)

```
TCGAGGCGGTCGAAGACCTCGGCGTACATTTCCGTGA**     ORF-Rp (kmr)
||||                    ||  |  |
TCGAAC      -17 bp-     TAGAGT              gylP2
|  |  |                 ||   ||
TTGGCG      -19 bp-     TAGGTT              gylRp
|  ||                   ||  |  |
TTGACA      -17 bp-     TATAAT              Escherichia coli
|  ||  |                 |  |  |
TTGACG      -19 bp-     GAGACT              gylP1
|  ||                   ||    |
TGGACA      -14 bp-     TAGGAT              ermEP1
|  ||  |                 |    |
TTGACG      -18 bp-     GAGGAT              ermEP2
  |  |                  ||    |
CTGCGA      -18 bp-     TAGCAT              ORFP1 (ermE)
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(A) Nucleotides of putative *kmr* promoter. (B) Nucleotides of the putative ORF-Rp (*kmr*) promoter. Asterisks represent transcription start points determined by high-resolution S1 mapping.

promoter sequences of *E. coli*[21] and the putative *S. coelicolor gyl* and *erm*E promoters[3] (Fig. 8B). The *kmr* promoter showed no similarity to the consensus *E. coli* promoter and only the sequence around the −10 region had significant similarity to those of *vph*P1[3] and *endo*H[2] (Fig. 8A).

Discussion

We have shown that there is a second transcript upstream from the *kmr* gene and oriented in the opposite direction. Deletion of the DNA from which this RNA was transcribed led to the inactivation of expression of the *kmr* gene as shown in pMCP37. Therefore, it might be possible that the unknown RNA or protein is involved in the expression of the *kmr* gene. This point was analyzed with a plasmid compatible with pMCP37[22] without definite result (data not shown).

The two promoters identified here are classified into different types based on their sequences. One belongs to a class of promoters represented by the *Streptomyces* genes *vph*P1 and *endo*H which have been shown to be transcribed by purified *ctc*-utilizing RNA polymerase[2]. Among these four promoters of the *kmr*, *vph*P1, *endo*H genes from *Streptomyces* and the *ctc* gene from *B. subtilis*, perfect homology could be only found in the three successive G's (Fig. 8A) which were shown to be functionally important in the *ctc* promoter[2,23,24].

The other gene, which may encode a DNA binding protein as speculated from the nucleotide sequence (data not shown), has a promoter sequence similar to the consensus *E. coli* promoter and the *erm*E and *gyl* promoters from *Streptomyces*. The spacing (18 bp) between the −10 and −35 regions of the gene is also close to the optimal distance (17 bp) observed in *E. coli*[21]. In the −10 region, the most highly conserved nucleotides (T and A at position 6 and 2, respectively) and the next most highly conserved nucleotide (T at position 1) shown by BIBB *et al.*[4] are also found in the ORF-Rp (*kmr*)

(Fig. 8B). In the −35 region, G at position 3 is conserved in all of the promoters listed in Fig. 8B. The next most highly conserved nucleotides in the −35 region are T at position 1 (7 out of 8 cases) and A at position 4 (6 out of 8 cases). The existence of this class of promoters is in keeping with the finding that the *Streptomyces* RNA polymerase can recognize *E. coli* transcriptional signals[25]. However, very few promoters from *Streptomyces* are utilized by *E. coli* RNA polymerase[26,27]. A collection of *Streptomyces* promoters which are functional in *E. coli* have nucleotide sequences which exhibit close homology to the *E. coli* consensus sequence in the −35 region (5 out of 6 bases in all 5 cases)[28,29]. The *endo*H gene of *S. plicatus* was shown to be expressed in *E. coli*[30]. Reexamination of the *endo*H promoter reveals that the −35 region (TTGACG) is closely homologous (5 out of 6 bases) to the *E. coli* consensus sequence (Fig. 8A). Therefore, it might be possible that the minimum requirement for the utilization of *Streptomyces* promoters in *E. coli* is the 5 nucleotide match in the −35 region to the *E. coli* consensus sequence.

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