JOURNAL OF BACTERIOLOGY, Mar. 2000, p. 1243–1250 Vol. 182, No. 5
0021-9193/00/$04.00+0

KorSA from the *Streptomyces* Integrative Element pSAM2 Is a Central Transcriptional Repressor: Target Genes and Binding Sites

GUENNADI SEZONOV,† CHRISTOPHE POSSOZ, ANNICK FRIEDMANN, JEAN-LUC PERNODET, AND MICHEL GUÉRINEAU*

Laboratoire de Biologie et Génétique Moléculaire, Institut de Génétique et Microbiologie, UMR CNRS 8621, Université Paris-Sud, 91405 Orsay, France

Received 15 July 1999/Accepted 29 November 1999

pSAM2, a 10.9-kb mobile integrative genetic element from *Streptomyces ambofaciens*, possesses, as do a majority of *Streptomyces* conjugative plasmids, a *kil-kor* system associated with its transfer. The *kor* function of pSAM2 was attributed to the *korSA* gene, but its direct role remained unclear. The present study was focused on the determination of the KorSA targets. It was shown that KorSA acts as a transcriptional repressor by binding to a conserved 17-nucleotide sequence found upstream of only two genes: its own gene, *korSA*, and *pra*, a gene positively controlling pSAM2 replication, integration, and excision. A unique feature of KorSA, compared to Kor proteins from other *Streptomyces* conjugative plasmids, is that it does not directly regulate pSAM2 transfer. KorSA does not bind to the pSAM2 genes coding for transfer and intramycelial spreading. Through the repression of *pra*, KorSA is able to negatively regulate pSAM2 functions activated by Pra and, consequently, to maintain pSAM2 integrated in the chromosome.

A large number of mobile genetic elements, including plasmids and integrative elements capable of site-specific integration into the chromosome, have been identified in *Streptomyces*, *Saccharopolyspora*, *Amycolatopsis*, and other gram-positive bacteria belonging to the order *Actinomycetales* (12, 18). Most of the *Streptomyces* elements are self-transmissible and able to mobilize chromosomal markers. Transfer in *Streptomyces* is usually associated with pock formation characterized by a local inhibition of growth and development of the recipient strain in contact with the donor strain (1, 12). Bacterial plasmids often contain conditionally lethal genes (reviewed in reference 9). Certain genes (*kil* genes) specify functions lethal to either the host or the plasmid, while others (*kor* genes) encode products that control expression of the *kil* phenotype. It is impossible to obtain transformants with derivatives in which the *kor* gene is inactivated. The presence of a *kil-kor* system associated with transfer has been identified for all *Streptomyces* plasmids able to form pocks during conjugative transfer. The *kil* function was attributed to the *tra* genes (transfer genes), whose expression was lethal in the absence of the *kor* gene. Most of the identified *kor* genes code for proteins that belong to the GntR family of transcriptional regulators (8) and contain in their sequences a DNA binding motif. For all known cases, Kor directly regulates the expression of *tra* and also the transcription of its own gene. The best-studied *Streptomyces* plasmid, pIJ101, carries two *kil-kor* systems, *kilA-korA* and *kilB-korB* (16), and both KorA and KorB directly regulate the expression of the genes involved in transfer as well as their own synthesis (16, 26, 27). The different affinities of the processed form of KorB for its operators situated in the promoters of *kilB* and *korB* (34) allow it to maintain the repression established by KorB (29). For pSN22, the TraR (Kor) binding sites were located in the overlapping promoters of the *traR* gene and the *traA-traB-spdB* operon (14, 15). Conjugative plasmid pSG5 from *Streptomyces ghanaensis*, which is unable to form pocks during its transfer, is the only known example in which the *tra* gene (major transfer gene) does not represent a *kil* function. A gene similar to *kor* was found in its sequence, but its direct role remains unknown (17).

The organization of the *kil-kor* system for integrative elements follows the same pattern, with the Kor protein being indispensable for the transcriptional repression of the *tra* gene. The *kil-kor* systems of the integrative element pMEA300 from *Amycolatopsis methanolica* (31) and of SLP1 from *Streptomyces coelicolor* (4) are examples. ImpA (Kor) of SLP1, which is thought to be a regulator of SLP1 transfer, can bind to a 16-bp operator situated in the promoter of its own gene (24).

pSAM2 is present as one integrated copy in the chromosome of *Streptomyces ambofaciens* strain B2 ($pSAM2_{B2}$) and is found simultaneously as one integrated copy and 5 to 10 replicating copies per genome in the independently obtained strains B3 and B4 ($pSAM2_{B3}$ and $pSAM2_{B4}$) (19). A *kil-kor* system associated with transfer seems to be present in pSAM2. The major transfer gene, *traSA*, as well as the genes *spdA*, *-B*, *-C*, and *-D*, which are possibly involved in intramycelial spreading, have been identified (7). The hypothesis about the direct role of *korSA* in the regulation of *kil*, based on the inability of the deletion derivatives lacking *korSA* and also *pra* to transform was reconsidered after the discovery that *pra* is indispensable for the establishment of pSAM2 in the new host, as it activates pSAM2 replication and integration (22).

In this study we demonstrate that the KorSA protein encoded by pSAM2 is a transcriptional repressor of its own gene, *korSA*, and of the *pra* gene, which was shown to code for an activator of pSAM2 replication, integration, and excision (22, 23). While the expression of *traSA* in pSAM2 seems to be under the control of KorSA, no binding sites could be detected in that region, contrary to the widely adopted model of the organization of the *Streptomyces kil-kor* systems. *korSA* seems to code for the pSAM2 major repressor controlling, via the *pra* gene, the main functions of this genetic element.

* Corresponding author. Mailing address: Laboratoire de Biologie et Génétique Moléculaire, Institut de Génétique et Microbiologie, UMR CNRS 8621, Bâtiment 400, Université Paris-Sud, 91405 Orsay, France. Phone: 33 (0) 1 69 15 69 17. Fax: 33 (0) 1 69 15 45 44. E-mail: michel.guerineau@igmors.u-psud.fr.

† Present address: Laboratoire de Génétique Microbienne, Institut Jacques Monod, UMR CNRS 7592, Université Paris VI, 75251, Paris, Cedex 05, France.

TABLE 1. Plasmids[a]

Plasmid	Construction
pOS530	*pra* gene promoter (a fragment from nt 1766 to 1904 in AJ005260) from $pSAM2_{B2}$ cloned after PCR amplification as the *Bam*HI-*Eco*RI fragment in the promoter probe vector pIJ487/Hygro
pOS556	*Bam*HI(154)-*Bam*HI(1746) fragment of $pSAM2_{B3}$ containing the *korSA* gene cloned in the integrative vector pTO1* (22); *orf131* present in the fragment was inactivated by deletion of the *Bgl*II(297)-*Bsr*GI(955) fragment; contains the *tsr* gene conferring resistance to thiostrepton
pOS699	$pSAM2_{B3}$ derivative with the *korSA* gene inactivated by deletion of the *Nco*I-*Nco*I fragment; the *Nco*I site was modified by Klenow polymerase, introducing a frameshift
pOS702	*korSA* gene promoter (a fragment from nt 884 to 995 in AJ005260) from $pSAM2_{B3}$ cloned after PCR amplification as the *Bam*HI-*Hin*dIII fragment in the promoter probe vector pIJ487/Hygro
pIJ487/Hygro	pIJ487 (32) derivative in which the *hyg* gene (33) was cloned in the *Eco*RV site; Hyg^r Tsr^s

[a] All plasmids were constructed in this work.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Streptomyces lividans* TK24 is the commonly used host strain for cloning experiments (13). *S. ambofaciens* strains have been described elsewhere (19). The plasmids used are listed in Table 1.

Culture and transformation conditions. General culture and genetic techniques for *Streptomyces* spp. were as described by Hopwood et al. (11), and those for *Escherichia coli* were as described by Sambrook et al. (21). *Streptomyces* transformants carrying the thiostrepton resistance (*tsr*) gene (30) were selected using 50 μg of nosiheptide per ml.

Recombinant clones of *S. lividans* TK24 containing derivatives of pIJ487/Hygro carrying the *aph* resistance gene transcribed from a heterologous promoter (pOS530 and pOS702) were selected on R2YE medium containing 80 μg of hygromycin B per ml. To determine the level of kanamycin resistance, spore suspensions of the recombinant clones were spread on minimal medium (11) containing increasing concentrations of kanamycin monosulfate (Sigma). For strains *S. lividans*/pOS699/pOS702 and *S. lividans*/pOS556/pOS530, the initial transformants were obtained after double selection on medium containing nosiheptide and hygromycin B.

DNA isolation. Plasmid DNA was isolated from *E. coli* and from *Streptomyces* spp. by alkaline lysis (11). Total DNA was isolated as described by Hopwood et al. (11).

Status of pSAM2 derivatives in *S. lividans*. Total DNAs of recombinant strains digested with *Eco*RI were checked for the presence of free and/or integrated pSAM2 sequences by Southern hybridization with the [α-^{32}P]dATP-labeled *Eco*RI-*Eco*RI fragment of pSAM2 containing the *attP* site.

RNA isolation, Northern hybridization, and high-resolution S1 mapping. Total RNA from *Streptomyces* was isolated as described by Hopwood et al. (11). For Northern hybridization, total RNA (40 to 50 μg) was denatured with glyoxal and dimethyl sulfoxide and, after electrophoresis, was transferred to a Hybond-N filter (Amersham). The filter was hybridized with a radiolabeled probe corresponding to the *korSA* gene (the 0.8-kb PCR fragment synthesized with the oligonucleotides GS1 [5′ GCGACGTGGCTTCCTGTGGTTGACT] and GS13 [5′ CCCGGCCGTGGTGCGGGCTTCCCGG]).

Low- and high-resolution S1 mappings were performed as described by Hopwood et al. (11). For low-resolution S1 mapping, the 1.6-kb *Bam*HI(154)/*Bam*HI(1746) fragment was used as a probe. For high-resolution S1 mapping, the probe was prepared as described by Raibaud et al. (20), using the oligonucleotide GS9 (5′ CACTTGGCACCATGTCGCCGGGCTT), which is situated 115 bp downstream of the presumed start codon of the *korSA* gene. The same oligonucleotide was used for sequencing.

pSAM2 sequence. All restriction sites and positions of the cloned fragments are numbered according to the sequence submitted previously to the EMBL data bank under accession number AJ005260.

Purification of the KorSA protein. The KorSA protein was produced in *E. coli* and purified as a maltose-binding protein (MBP)–KorSA fusion protein, using the cloning and purification kit of New England Biolabs. The *korSA* gene coding part was amplified by PCR and cloned in the expression vector pMAL-c2, where it is expressed under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *tac* promoter. The MBP-KorSA protein was purified by affinity chromatography using a column with amylose resin, as recommended by the kit producer. This method of purification allowed us to obtain MBP-KorSA protein that was electrophoretically pure.

Gel retardation assays. DNA fragments were incubated with the MBP-KorSA protein for 10 min at 30°C in binding buffer (5× binding buffer contains 50 mM Tris-HCl [pH 7.5], 50 mM $MgCl_2$, 500 mM $NaCl_2$, 1 mM dithiothreitol, and 50% glycerol). Protein-DNA complexes were separated by electrophoresis at 4°C on polyacrylamide (PAA) gels. The acrylamide concentration is indicated in the figure legends. The nondenaturating gel contained acrylamide-bis-acrylamide, 10% glycerol, and 0.25× Tris-borate-EDTA.

To determine the total number of KorSA binding sites in pSAM2, the entire set of unlabeled DNA fragments obtained after digestion of 1 μg of the circular form of $pSAM2_{B3}$ was used in the gel shift assay. After migration, the PAA gel was colored with ethidium bromide. The DNA fragments bound with MBP-KorSA disappeared from their normal position in the gel, indicating the presence of KorSA binding sites in these regions of pSAM2, which were identified using the complete pSAM2 sequence.

Radiolabeled DNA fragments corresponding to these regions were used in the second round of the gel shift analysis. [γ-^{32}P]dATP-labeled fragments (0.05 pmol) of 125 bp and 174 bp were bound to MBP-KorSA and analyzed in a PAA gel.

DNase I footprinting. DNase I footprinting was performed as described by Holmes et al. (10). The DNA fragment containing the KorSA binding region was synthesized by PCR using the oligonucleotides Bam-kor-pr (5′ ACGGGATCC TTCGCGCACCACCACTCCAGC) and Hind-Kor-pr (5′ TTCAAGCTTGGTT CCCATAGTCCTTCTCTG). This 111-bp DNA fragment containing the *korSA* gene promoter (0.4 pmol) was radiolabeled with Klenow polymerase (*Bam*HI site) and then incubated with the MBP-KorSA protein at 30°C for 10 min in binding buffer. Samples were diluted twofold with DNase I buffer (10 mM HEPES-HCl [pH 7.8], 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 15 mM NaCl) and treated with 5 ng of DNase I for 1 min at room temperature. Samples were precipitated with ethanol in the presence of 0.3 M sodium acetate, resuspended in 95% formamide containing 20 mM EDTA, and loaded onto a 6% PAA sequencing gel. The gel was fixed in 10% acetic acid–10% methanol and dried, and radioactive bands were visualized by autoradiography.

RESULTS

Transcriptional analysis of *korSA*. To obtain data concerning transcription of the *korSA* gene in different pSAM2 derivatives, Northern hybridization and S1 low-resolution mapping were carried out with total RNA isolated from the strains *S. lividans*/$pSAM2_{B2}$ and *S. lividans*/$pSAM2_{B3}$. These experiments gave the same result. The *korSA* gene is constantly transcribed in both variants of pSAM2, either integrated only ($pSAM2_{B2}$) or integrated and replicating ($pSAM2_{B3}$). The presence in both strains of a 1-kb mRNA corresponding to the size of *korSA* (Fig. 1) indicates that it is a monocistronic transcript (data not shown).

The start point of *korSA* transcription was localized by high-resolution S1 mapping. Transcription is initiated from the adjacent A or T nucleotide situated 18 and 19 nucleotides (nt) upstream of the putative initiation codon ATG (data not shown) (see Fig. 5). These results allowed us to locate the *korSA* promoter. Its sequence does not show similarity to the consensus sequence proposed for *Streptomyces* promoters similar to those recognized by *E. coli* RNA polymerase containing the σ^{70} subunit (28).

The KorSA protein binds to two regions in pSAM2. The deduced protein KorSA belongs to the GntR family of transcriptional regulators (8) and contains a helix-turn-helix DNA binding motif (7). Determination of the positions of the KorSA binding sites in the pSAM2 sequence could give direct indications of the targets of this regulator. The protein KorSA was expressed in *E. coli* as a fusion with the N-terminal part of MBP (MBP-KorSA) and purified as described in Materials and Methods. The MBP-KorSA fusion protein was used for further analysis.

To determine the number and positions of all of the KorSA binding sites present in the sequence of pSAM2, we took advantage of the relatively small size of pSAM2 (10.9 kb) and of the knowledge of its whole nucleotide sequence (references 2, 3, 5–7, and 23 and our unpublished results; the published part of the pSAM2 sequence has been deposited in the EMBL

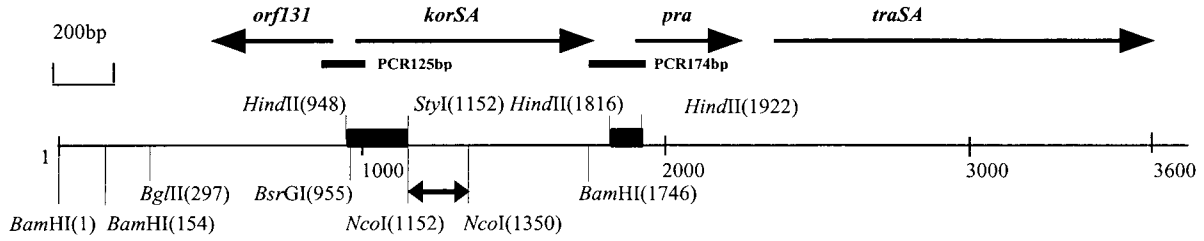


FIG. 1. Physical and genetic map of the pSAM2 region surrounding the *korSA* gene. The two rectangular blocks correspond to the positions of the KorSA binding regions. The two black bars correspond to the positions of the PCR fragments used in gel shift experiments (PCR 125bp and PCR 174bp). The double arrow represents the extent of the deletion inactivating *korSA* in pOS699.

database under accession number AJ005260). The total number of KorSA binding sites in pSAM2 was determined directly in gel shift experiments using the entire set of unlabeled DNA fragments obtained after digestion of the circular form of $pSAM2_{B3}$ with restriction enzymes and separation of these DNA fragments on a PAA gel. If these fragments were mixed with the MBP-KorSA protein prior to gel electrophoresis, any fragment whose migration is retarded could be expected to bind to KorSA, and thus the binding region could be localized. The restriction enzymes (*Aat*II, *Acc*I, *Ava*II, *Dde*I, *Hin*dII, *Rsa*I, *Sty*I, and *Xho*II) were chosen because each cuts pSAM2 into 15 to 25 fragments that could be identified from the pSAM2 sequence. In addition, every region of pSAM2 is represented at least by a fragment that is of optimal size for gel shift experiments (100 to 1,000 bp) and well separated from the neighboring fragments. Typical results obtained after cutting with *Dde*I and *Sty*I are shown in Fig. 2.

For every restriction enzyme used, no more than two fragments of pSAM2 were retarded (Fig. 2). Comparison of the results obtained using different endonucleases allowed us to identify the minimal fragments containing the binding regions. They were relatively short and located in the promoters of the *korSA* [the 204-bp *Hin*dII((948)/*Sty*I(1152) fragment] and *pra* [the 106-bp *Hin*dII(1816)/*Hin*dII(1922) fragment] genes. Binding of the MBP-KorSA protein was not observed with the fragments containing the *traSA* gene or its promoter. This was unexpected, since in *Streptomyces* plasmids such as pIJ101 (26, 27) or pSN22 (15), Kor was shown to repress directly the expression of the intermycelial transfer genes by binding in their promoter regions.

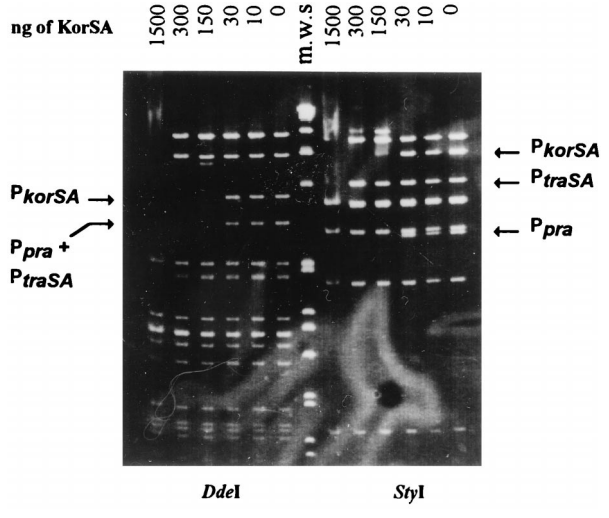


FIG. 2. Gel shift experiments performed with the KorSA protein, using unlabeled fragments of $pSAM2_{B3}$ obtained after restriction with *Dde*I and *Sty*I. The fragments containing *korSA*, *pra*, and *traSA* promoters are indicated (P_{korSA}, P_{pra}, and P_{traSA}, respectively). The fragments were separated in a 3.5% nondenaturing PAA gel. The DNA fragments binding KorSA protein did not migrate compared to the control. Binding was specific when 30 to 300 ng of KorSA was added. m.w.s., molecular weight standards.

The fragments corresponding to the regions localized after the preliminary gel shift experiments with the whole DNA of pSAM2 were synthesized by PCR, radioactively labeled, and tested in a new round of gel shift experiments with the MBP-KorSA protein. The first region, upstream of *korSA*, corresponds to the 125-bp fragment (positions 880 to 999 in Fig. 1 and in the AJ005260 sequence and additional nucleotides creating the *Bam*HI and *Hin*dIII sites at the ends of the fragment). The second region, upstream of *pra*, corresponds to the 174-bp fragment (positions 1760 to 1922 in the same sequence and additional nucleotides creating the *Asp*718I and *Bam*HI sites at the ends of the fragment). The results obtained confirmed and refined the first suggestion about the position of the KorSA protein binding sites. As shown in Fig. 3A, MBP-KorSA was able to bind efficiently to the 174-bp fragment corresponding to the *pra* gene promoter from the variants $pSAM2_{B2}$, $pSAM2_{B3}$, and $pSAM2_{B4}$. This means that the mutations found in the *pra* promoter in $pSAM2_{B3}$ and $pSAM2_{B4}$, while strengthening *pra* expression, did not abolish the binding of KorSA.

To refine the analysis, the 174-bp fragment from $pSAM2_{B2}$ corresponding to the *pra* gene promoter was cut with *Apa*LI (giving fragments of 88 and 86 bp) and with *Hin*dII (giving fragments of 69 and 105 bp). Both *Apa*LI subfragments were able to bind MBP-KorSA, but only the 105-bp *Hin*dII fragment was retarded (Fig. 3B). It was concluded that each *Apa*LI fragment contains at least one site for KorSA and that no functional site is situated on the 69-bp *Hin*dII fragment. It is interesting that the point substitution present in $pSAM2_{B4}$ and located in the core sequence of binding site 2 (see Fig. 5) significantly decreases but does not abolish the binding of the 88-bp *Apa*LI DNA fragment to KorSA (data not shown). This indicates that this nucleotide (G) is important for KorSA binding, as predicted by the footprint experiment (see below). In addition it confirms the validity of the KorSA targets determined by gel retardation experiments.

Binding of KorSA to its own promoter was confirmed with the 125-bp fragment corresponding to the *korSA* gene promoter (Fig. 3C). The protein KorSA has 10 to 20 times more affinity for the *pra* gene promoter than for its own promoter, since, using equal amounts of DNA, 10 to 20 times more KorSA was required to obtain the same pattern of retardation with the *korSA* promoter as with the *pra* promoter (Fig. 3C).

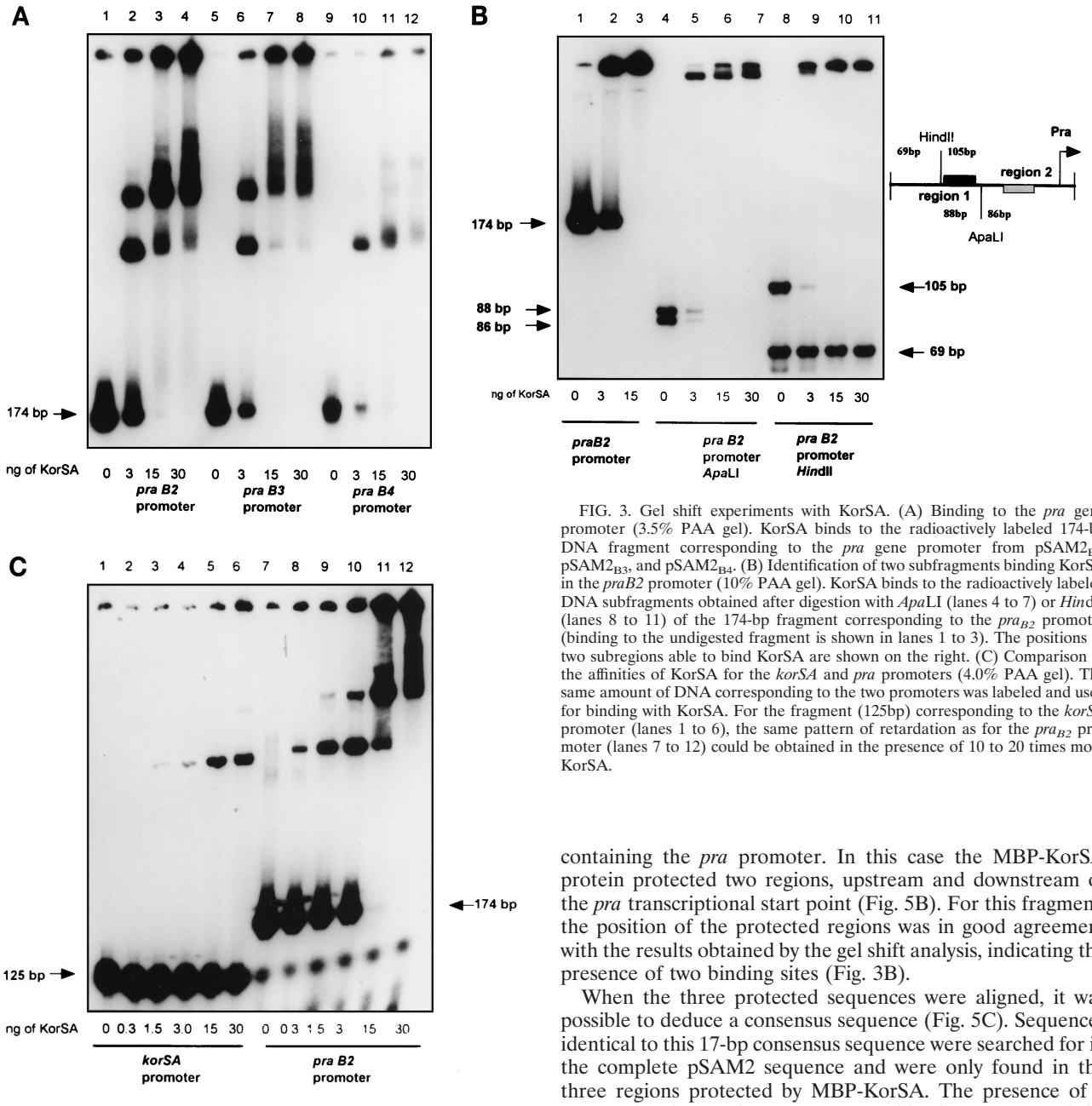


FIG. 3. Gel shift experiments with KorSA. (A) Binding to the *pra* gene promoter (3.5% PAA gel). KorSA binds to the radioactively labeled 174-bp DNA fragment corresponding to the *pra* gene promoter from $pSAM2_{B2}$, $pSAM2_{B3}$, and $pSAM2_{B4}$. (B) Identification of two subfragments binding KorSA in the *praB2* promoter (10% PAA gel). KorSA binds to the radioactively labeled DNA subfragments obtained after digestion with *Apa*LI (lanes 4 to 7) or *Hin*dII (lanes 8 to 11) of the 174-bp fragment corresponding to the pra_{B2} promoter (binding to the undigested fragment is shown in lanes 1 to 3). The positions of two subregions able to bind KorSA are shown on the right. (C) Comparison of the affinities of KorSA for the *korSA* and *pra* promoters (4.0% PAA gel). The same amount of DNA corresponding to the two promoters was labeled and used for binding with KorSA. For the fragment (125bp) corresponding to the *korSA* promoter (lanes 1 to 6), the same pattern of retardation as for the pra_{B2} promoter (lanes 7 to 12) could be obtained in the presence of 10 to 20 times more KorSA.

To confirm the absence of binding sites for KorSA in the *traSA* gene sequence, three fragments corresponding to the promoter and 5′ coding part, to the central part, and to the 3′ coding part of the *traSA* gene were synthesized by PCR, radioactively labeled, and used in gel shift assays. None of these fragments were retarded in the presence of MBP-KorSA (data not shown). These results could also be considered to be a control of the specificity of the KorSA binding.

Determination of the KorSA binding site by footprint analysis. To determine the sequence recognized by KorSA, footprint analysis was performed. In the fragment containing the *korSA* promoter, the MBP-KorSA protein was able to protect against DNase I the 25- to 27-bp region situated immediately upstream of the *korSA* transcriptional start point (Fig. 4). The same type of experiment was performed with the fragment containing the *pra* promoter. In this case the MBP-KorSA protein protected two regions, upstream and downstream of the *pra* transcriptional start point (Fig. 5B). For this fragment, the position of the protected regions was in good agreement with the results obtained by the gel shift analysis, indicating the presence of two binding sites (Fig. 3B).

When the three protected sequences were aligned, it was possible to deduce a consensus sequence (Fig. 5C). Sequences identical to this 17-bp consensus sequence were searched for in the complete pSAM2 sequence and were only found in the three regions protected by MBP-KorSA. The presence of a 9-nt perfect inverted repeat was noted in the sequence of the KorSA binding site 3. Due to these repeats, the 17-bp consensus is present in both strands for site 3.

KorSA is a transcriptional repressor of the two genes *pra* and *korSA*. The KorSA protein, like other known *Streptomyces* Kor proteins, belongs to the GntR family of transcriptional repressors (8). To determine whether KorSA plays a similar role in pSAM2, the strengths of the *pra* and *korSA* promoters, in the presence or absence of KorSA, were compared using the promoter probe vector pIJ487 (32). In this vector the kanamycin resistance gene is transcribed from the inserted promoter. The level of transcription from a tested promoter was estimated by the level of resistance to kanamycin. It was observed that when the *korSA* gene is present in *trans*, the pra_{B2} promoter ($Ppra_{B2}$) is repressed, demonstrating that KorSA acted as a transcriptional repressor of the *pra* gene. For example, at a kanamycin concentration of 3 μg/ml, the presence of *korSA*

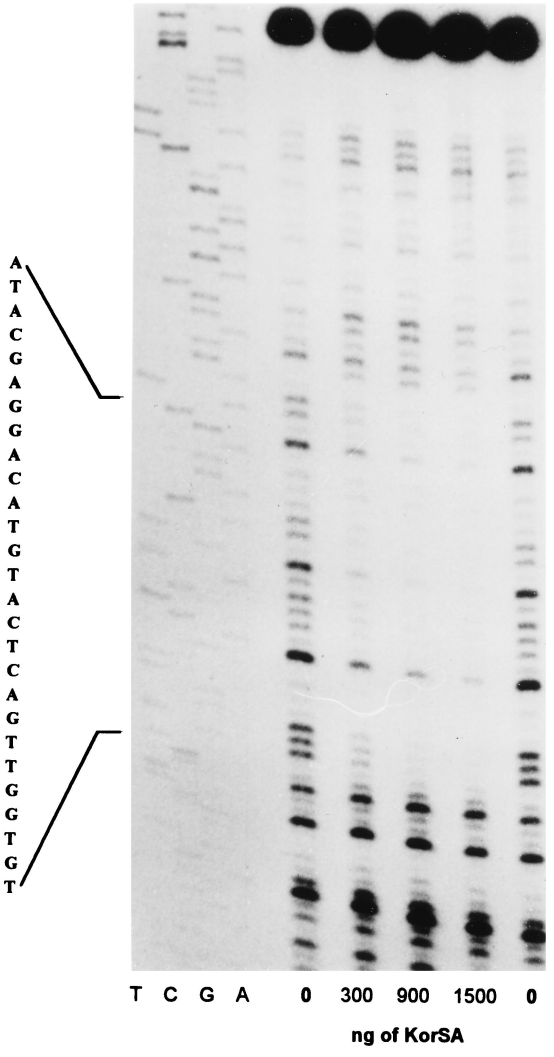


FIG. 4. Footprint analysis of KorSA binding to the *korSA* promoter. Different amounts of KorSA protein were added to the 111-bp PCR-synthesised DNA fragment corresponding to the *korSA* promoter. The DNA sequence corresponds to the MBP-KorSA protected region. The sequence was determined using the same DNA fragment with the oligonucleotide Bam-kor-pr (described in Materials and Methods).

reduces survival about 10-fold (Fig. 6A). Surprisingly, in the presence of pOS699 with *korSA* disrupted, $Ppra_{B2}$ was found to be activated, indicating that pSAM2 codes for another, positive, regulator of *pra* expression. The $Ppra_{B3}$ promoter is much stronger than $Ppra_{B2}$. For instance, with $Ppra_{B3}$ cloned in pIJ487, survival was at least 50% with kanamycin at 100 μg/ml (23), while survival was about 5% with kanamycin at 5 μg/ml with $Ppra_{B2}$ (Fig. 6A). The repression of $Ppra_{B3}$ in the presence of *korSA* in *trans* was detectable only at kanamycin concentrations higher than 100 μg/ml (data not shown).

Using the same approach, it was demonstrated that KorSA is also a transcriptional repressor of its own gene (Fig. 6B). The *korSA* gene promoter is repressed only by KorSA, since in the presence of pOS699 containing the whole pSAM2 sequence, but with the *korSA* gene disrupted, no repression of the *korSA* promoter was observed.

Inactivation of the *korSA* gene. A variant, pOS699 (Fig. 1; Table 1), containing an internal deletion in the *korSA* gene was constructed from a derivative of $pSAM2_{B3}$, pTS39, which has been used for the functional analysis of pSAM2 (25). pOS699 was able to transform *S. lividans* with the same efficiency as pTS39. For pTS39 the integrated and replicative forms coexist in *S. lividans*. However, pOS699 was present only as a replicative form (Fig. 7). The introduction of a copy of the *korSA* gene in *trans* in the strain *S. lividans*/pOS699 restored the presence of the pOS699 integrated form (Fig. 7) but, for unknown reasons, did not restore the usual proportion between the free and integrated forms for $pSAM2_{B3}$. It should be emphasized that strong activation of pSAM2 excision was previously observed for the derivative pOS548, in which the *pra* gene was overexpressed under the control of an inducible and very strong heterologous promoter, *ptipA* (22). These results, together with the binding of KorSA to the pra_{B3} promoter, allow us to conclude that KorSA is still able to repress the *pra* gene promoter in the mutant $pSAM2_{B3}$.

The status of pOS699 could be explained by the absence of repression of *pra*. Nevertheless, it should be stressed that the role of KorSA cannot be limited only to the repression of *pra*. Inactivation of *korSA* had other consequences. *S. lividans* was transformed by pOS699 with the same efficiency as pTS39, but transformants appeared after more than 1 week, compared to 3 days for pTS39 transformants. Moreover, after 2 to 3 weeks, the clones obtained by transformation did not give progeny when picked on new plates. This phenotype was considered to be an attenuated Kil phenotype and was suppressed by complementation in *trans* with a single copy of the *korSA* gene in the strain *S. lividans*/pOS699/pOS556.

DISCUSSION

The conjugative *S. ambofaciens* integrative element pSAM2 possesses a *kil-kor* system. The *korSA* gene has been identified as a key element of this system, and the *kil* locus has been located in the region of *traSA*, the main transfer gene (7, 25), but the direct role of KorSA in the regulation of the *kil* locus remained unknown. In this study we characterized the targets of the KorSA protein in the pSAM2 sequence.

Considering the relatively small size of the pSAM2 genome and the availability of its complete sequence, gel shift experiments were performed with the totality of the DNA fragments obtained after pSAM2 digestion with restriction endonucleases. Using this new approach, it was possible to demonstrate directly that KorSA binds only to the promoter regions of two pSAM2 genes, *pra* and *korSA*, with no other binding sites detected. Unlike other known actinomycete mobile elements, KorSA did not bind either to *traSA*, the main pSAM2 transfer gene, or to the *spdA*, *-B*, *-C*, and *-D* genes involved in pSAM2 spreading. These conclusions were confirmed after determination by footprint analysis of the sequence recognized by KorSA. This 17-nt consensus sequence was found only upstream of the *korSA* and *pra* genes and not elsewhere in pSAM2. The promoters of these genes, cloned from the wild-type $pSAM2_{B2}$ and studied in a *Streptomyces* promoter probe vector, were shown to be repressed in vivo in the presence of *korSA* in *trans*, thus confirming the in vitro studies.

These data allowed us to conclude that KorSA negatively regulates the expression of another regulator gene of pSAM2, *pra*, characterized to be essential for the expression of genes involved in integration-excision and replication of pSAM2. The presence of genes similar to *pra* has not been shown in any other exhaustively studied actinomycete mobile elements (a *pra*-like gene is present in the sequence of pSA1 of *Streptomyces azureus* ATCC 1421 [EMBL accession number AB010724]).

Although the presence of functional pSAM2 has never been observed in the *S. coelicolor* genome, several open reading

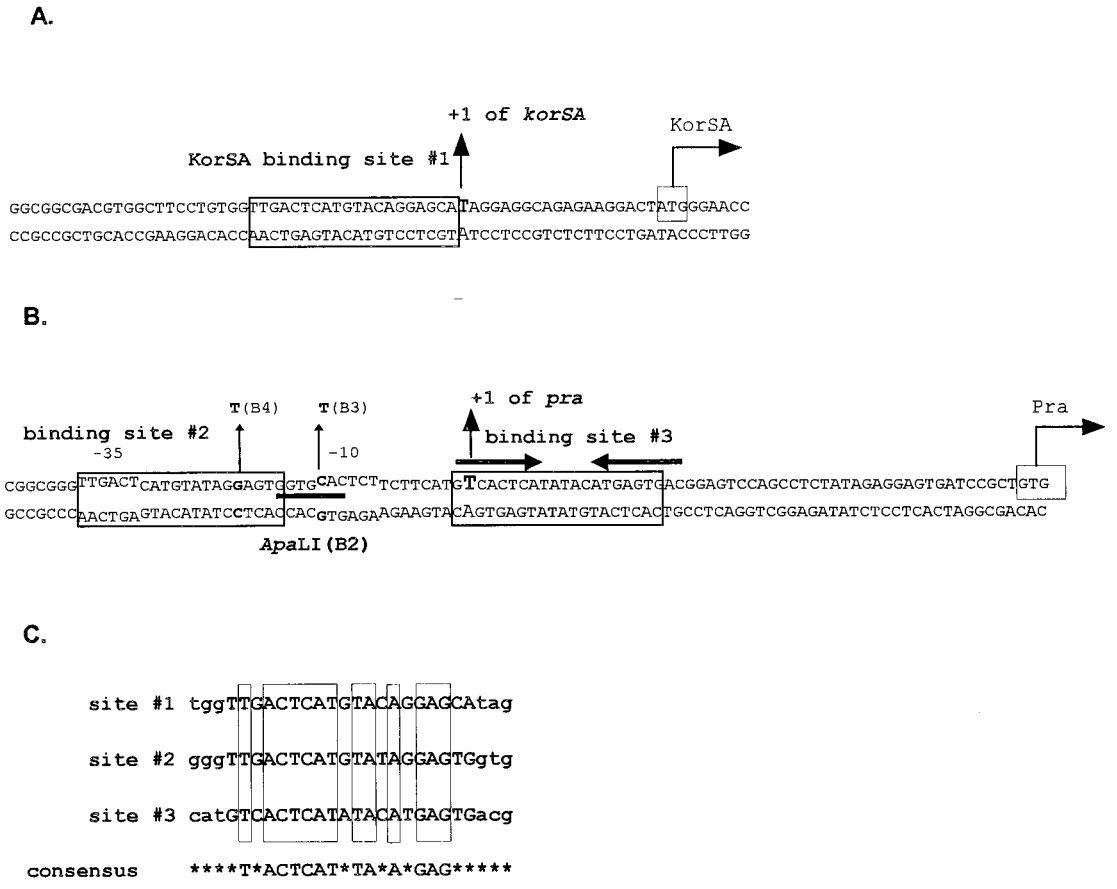


FIG. 5. Positions of the KorSA binding sites upstream of the *korSA* and *pra* genes. (A) DNA sequence upstream of the *korSA* gene translation initiation codon. (B) DNA sequence upstream of the pra_{B2} gene translation initiation codon. (C) Alignment of the KorSA binding sites found in the pSAM2 sequence. The positions of the sequences corresponding to the KorSA binding sites are boxed. The positions of the point mutations found upstream of the *pra* gene in $pSAM2_{B3}$ and $pSAM2_{B4}$ are indicated as T (B3) and T (B4).

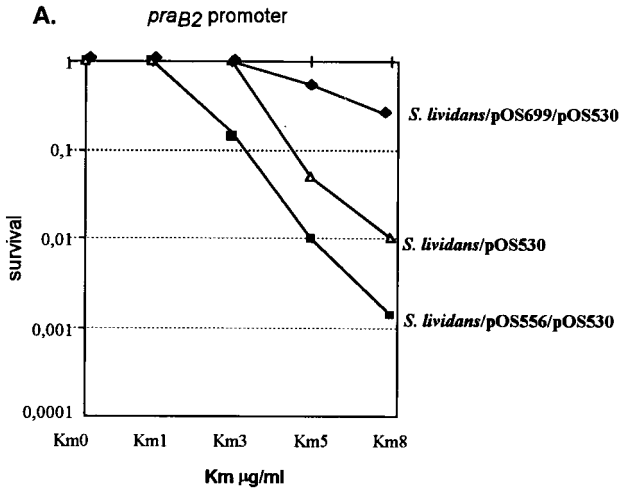
frames exhibiting very high similarity with pSAM2 genes were recently found during the sequencing of the complete *S. coelicolor* chromosome. These sequences could be detected by hybridization of *S. coelicolor* total DNA with a pSAM2 probe. Nothing is known about the possible expression and role of these open reading frames in *S. coelicolor*. However, when hybridization of a pSAM2 probe was performed under the same conditions with total DNA of *S. lividans*, no hybridizing fragment could be detected (data not shown). Therefore, these open reading frames, which are highly similar to pSAM2 genes, are absent from *S. lividans* and could not interfere with the functional study of pSAM2 performed in this strain.

Functional analysis confirms the role of KorSA as the *pra* repressor. In the derivative pOS699, in which *korSA* is inactivated, strong activation of pSAM2 excision was observed, a phenomenon very similar to that obtained by overexpression in *cis* of the *pra* gene (22).

Another role discovered for KorSA, i.e., as a negative regulator of its own synthesis, is consistent with the data published for the *kor* genes of other actinomycetes conjugative elements (see the introduction). The affinity of KorSA for the *korSA* promoter, which is weaker than that for *pra*, probably allows this protein to maintain its concentration at a level sufficient to keep the *pra* gene constantly repressed. It should be noted that the affinity of the fused protein MBP-KorSA could be slightly different from that of KorSA, but unfortunately, the cleavage of the fused protein was extremely inefficient.

It has been demonstrated that $pSAM2_{B3}$ is different from the wild-type form $pSAM2_{B2}$ by the coexistence of replicative and integrated forms, while $pSAM2_{B2}$ is present in the cells only as an integrated sequence. This difference is a consequence of the point substitution in the *pra* gene promoter (23). This mutation causes a more efficient transcription of pra_{B3}, whereas mRNA of pra_{B2} was not detected. A plausible explanation of this phenomenon might have been the different affinities of KorSA for the mutated and nonmutated sequences. However, the mutation in the *pra* gene promoter is not located in the KorSA binding sites, and KorSA binds to the *praB3* promoter with roughly the same affinity as it does to the *praB2* promoter. These results indicate that the mutated *praB3* promoter is still regulated by KorSA.

The absence of a direct connection between KorSA and the expression of the main transfer gene, *traSA*, presents a significant difference between the genetic organizations of transfer control in pSAM2 and in other well-studied conjugative elements of actinomycetes. The derivative of $pSAM2_{B3}$ lacking functional *korSA* expressed an attenuated Kil phenotype. This raises the question of the mechanism by which KorSA regulates this determinant. Generally, the *kil* function is attributed to a transfer gene(s). KorSA did not bind to any region in the



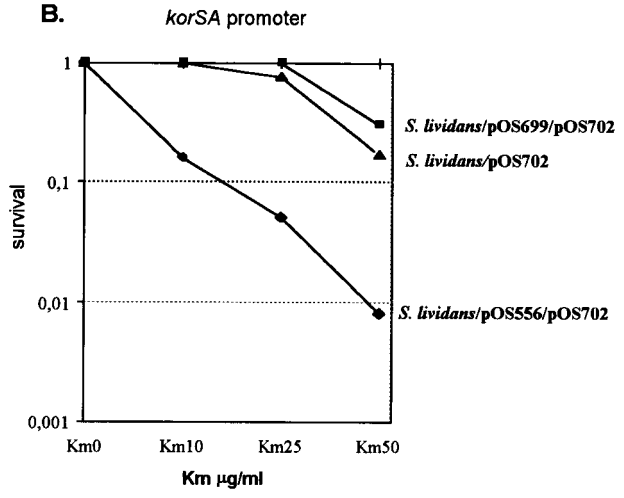


FIG. 6. KorSA is a repressor of the *korSA* gene and *pra* gene promoters. The sensitivity to kanamycin (Km) of *S. lividans* TK24 harboring the plasmids pOS530 (A) and pOS702 (B) increases in the presence of the functional *korSA* gene (pOS556).

traSA gene. *traSA* is not cotranscribed with *pra* (23), and overexpression of *pra* from the *tipA* promoter, not controlled by KorSA, but in the presence of intact *korSA* (pOS548 [22]), does not cause the Kil phenotype. It could be supposed that either Pra or KorSA needs an unidentified partner to regulate *traSA* expression or that another regulatory gene is involved.

In pSAM2, KorSA exhibits unique properties: it regulates integration, excision, and replication through the control of *pra* but does not directly regulate the main pSAM2 transfer gene *traSA*. KorSA could be considered the main negative transcriptional regulator of pSAM2. This suggested that KorSA alone may be responsible for the silent status of the pSAM2 integrated form, and it should be noted that *korSA* was always expressed, even in $pSAM2_{B2}$ which was found only integrated. Activation of the excision and replication of pSAM2, which are indispensable for transfer, should include a mechanism for relieving KorSA repression under conditions favorable for transfer.

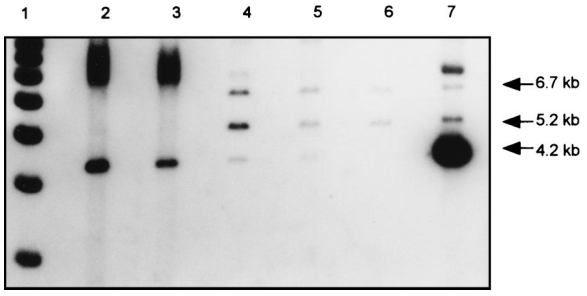


FIG. 7. Effect of *korSA* gene disruption on the status of pSAM2. Total DNA, restricted with *Eco*RI, was analyzed by Southern hybridization with the ^{32}P-labeled 4.2-kb *Eco*RI-*Eco*RI fragment of pSAM2 containing the *attP* integration site. The two DNA bands of 6.7 and 5.2 kb correspond to integrated pSAM2, while the 4.2-kb band corresponds to the free form of pSAM2. DNA was extracted from *S. lividans* TK24 containing pOS699 clones 1 and 2 (lanes 2 and 3), pOS699 plus pOS556 clones 1 and 2 (lanes 4 and 5), $pSAM2_{B2}$ (lane 6), and pTS39 ($pSAM2_{B3}$ derivative) (lane 7). Lane 1, 1-kb ladder DNA.

ACKNOWLEDGMENTS

We are grateful to M. J. Bibb and K. Flärdh for the information concerning the pSAM2-like sequence in the *S. coelicolor* genome. We thank R. d'Ari for helpful comments on the manuscript. We thank R. Morosoli for helpful and pleasant discussions during this work.

This work has been carried out as part of the "Bioavenir" program supported by Rhône-Poulenc with the participation of the French Ministères de la Recherche, et de l'Enseignement superieur, de l'Industrie et du Commerce Extérieur.

REFERENCES

1. **Bibb, M. J., R. F. Freeman, and D. A. Hopwood.** 1977. Physical and genetical characterization of a second sex factor, SCP2, for *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. **154:**155–166.
2. **Boccard, F., T. Smokvina, J.-L. Pernodet, A. Friedmann, and M. Guérineau.** 1989. The integrated conjugative plasmid pSAM2 of *Streptomyces ambofaciens* is related to temperate bacteriophages. EMBO J. **8:**973–980.
3. **Boccard, F., T. Smokvina, J.-L. Pernodet, A. Friedmann, and M. Guérineau.** 1989. Structural analysis of loci involved in pSAM2 site-specific integration in *Streptomyces*. Plasmid **21:**59–70.
4. **Grant, S. R., S. C. Lee, K. Kendall, and S. N. Cohen.** 1989. Identification and characterization of a locus inhibiting extrachromosomal maintenance of the *Streptomyces* plasmid SLP1. Mol. Gen. Genet. **217:**324–331.
5. **Hagège, J., F. Boccard, T. Smokvina, J.-L. Pernodet, A. Friedmann, and M. Guérineau.** 1994. Identification of a gene encoding the replication initiator protein of the *Streptomyces* integrating element, pSAM2. Plasmid **31:**166–183.
6. **Hagège, J., J.-L. Pernodet, A. Friedmann, and M. Guérineau.** 1993. Mode and origin of replication of pSAM2, a conjugative integrating element of *Streptomyces ambofaciens*. Mol. Microbiol. **10:**799–812.
7. **Hagège, J., J.-L. Pernodet, G. Sezonov, C. Gerbaud, A. Friedmann, and M. Guérineau.** 1993. Transfer function of the conjugative integrating element pSAM2 from *Streptomyces ambofaciens*: characterization of a *kil-kor* system associated with transfer. J. Bacteriol. **175:**5529–5538.
8. **Haydon, D. J., and J. R. Guest.** 1991. A new family of bacterial regulatory proteins. FEMS Microbiol. Lett. **79:**291–296.
9. **Holcík, M., and V. N. Iyer.** 1997. Conditionally lethal genes associated with bacterial plasmids. Microbiology **143:**3403–3416.
10. **Holmes, D. J., J. L. Caso, and C. J. Thompson.** 1993. Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*. EMBO J. **12:**3183–3191.
11. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
12. **Hopwood, D. A., T. Kieser, D. J. Lydiate, and M. J. Bibb.** 1986. *Streptomyces* plasmids: their biology and use as cloning vectors, p. 159–220. *In* S. W. Queener and L. E. Day (ed.), The bacteria: a treatise on structure and function, vol. IX. Academic Press, Orlando, Fla.
13. **Hopwood, D. A., T. Kieser, H. M. Wright, and M. J. Bibb.** 1983. Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Microbiol. **129:**2257–2269.
14. **Kataoka, M., Y. M. Kiyose, Y. Michisuji, T. Horiguchi, T. Seki, and T. Yoshida.** 1994. Complete nucleotide sequence of the *Streptomyces nigrifaciens* plasmid, pSN22: genetic organization and correlation with genetic properties. Plasmid **32:**55–69.

15. **Kataoka, M., S. Kosono, T. Seki, and T. Yoshida.** 1994. Regulation of the transfer genes of *Streptomyces* plasmid pSN22: in vivo and in vitro study of the interaction of TraR with promoter region. J. Bacteriol. **176:**7291–7298.
16. **Kendall, K. J., and S. N. Cohen.** 1987. Plasmid transfer in *Streptomyces lividans*: identification of a *kil-kor* system associated with the transfer region of pIJ101. J. Bacteriol. **169:**4177–4183.
17. **Maas, R.-M., J. Götz, W. Wohlleben, and G. Muth.** 1998. The conjugative plasmid pSG5 from *Streptomyces ghanaensis* DSM 2932 differs in its transfer functions from other *Streptomyces* rolling-circle-type plasmids. Microbiology **144:**2809–2817.
18. **Omer, C. A., and S. N. Cohen.** 1989. SLP1: a paradigm for plasmids that site-specifically integrate in the Actinomycetes, p. 289–296. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
19. **Pernodet, J.-L., J.-M. Simonet, and M. Guérineau.** 1984. Plasmids in different strains of *Streptomyces ambofaciens*: free and integrated forms of plasmid pSAM2. Mol. Gen. Genet. **198:**35–41.
20. **Raibaud, A., M. Zalacain, T. G. Holt, R. Tizard, and C. J. Thompson.** 1991. Nucleotide sequence analysis reveals linked *N*-acetyl hydrolase, thioesterase, transport, and regulatory genes encoded by the bialaphos biosynthetic gene cluster of *Streptomyces hygroscopicus*. J. Bacteriol. **173:**4454–4463.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Sezonov, G., A.-M. Duchêne, A. Friedmann, M. Guérineau, and J.-L. Pernodet.** 1998. Replicase, excisionase and intergase genes of the *Streptomyces* element pSAM2 constitute an operon positively regulated by the *pra* gene. J. Bacteriol. **180:**3056–3061.
23. **Sezonov, G., J. Hagège, J.-L. Pernodet, A. Friedmann, and M. Guérineau.** 1995. Characterization of *pra*, a gene for replication control in pSAM2, the integrating element of *Streptomyces ambofaciens*. Mol. Microbiol. **17:**533–544.
24. **Shiffman, D., and S. N. Cohen.** 1993. Role of the *imp* operon of the *Streptomyces coelicolor* genetic element SLP1: two *imp*-encoded proteins interact to autoregulate *imp* expression and control plasmid maintenance. J. Bacteriol. **175:**6767–6774.
25. **Smokvina, T., F. Boccard, J.-L. Pernodet, A. Friedmann, and M. Guérineau.** 1991. Functional analysis of the *Streptomyces ambofaciens* element pSAM2. Plasmid **25:**40–52.
26. **Stein, D. S., and S. N. Cohen.** 1990. Mutational and functional analysis of the *korA* and *korB* gene products of *Streptomyces* plasmid pIJ101. Mol. Gen. Genet. **222:**337–344.
27. **Stein, D. S., K. J. Kendall, and S. N. Cohen.** 1989. Identification and analysis of transcriptional regulatory signals for the *kil* and *kor* loci of *Streptomyces* plasmid pIJ101. J. Bacteriol. **171:**5768–5775.
28. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. **20:**961–974.
29. **Tai, J. T.-N., and S. N. Cohen.** 1993. The active form of the KorB protein encoded by the *Streptomyces* plasmid pIJ101 is a processed product that binds differentially to the two promoters it regulates. J. Bacteriol. **175:**6996–7005.
30. **Thompson, C. J., J. M. Ward, and D. A. Hopwood.** 1980. DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. Nature **286:**525–527.
31. **Vrijbloed, J. W., N. M. Put, G. I. Hessels, and L. Dijkhuizen.** 1995. Identification and functional analysis of the transfer region of the plasmid pMEA300 of the methylotrophic actinomycete *Amycolatopsis methanolica*. J. Bacteriol. **177:**6499–6505.
32. **Ward, J. M., G. R. Janssen, T. Kieser, M. J. Bibb, M. J. Buttner, and M. J. Bibb.** 1986. Construction and characterisation of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn*5* as indicator. Mol. Gen. Genet. **203:**468–475.
33. **Zalacain, M., A. Gonzalez, M. C. Guerrero, R. J. Mattaliano, F. Malpartida, and A. Jiménez.** 1986. Nucleotide sequence of the hygromycin B phosphotransferase gene from *Streptomyces hygroscopicus*. Nucleic Acids Res. **14:**1565–1581.
34. **Zaman, S., H. Richards, and J. M. Ward.** 1992. Expression and characterisation of the *korB* gene product from *Streptomyces lividans* plasmid pIJ101 in *Escherichia coli* and determination of its binding site on the *korB* and *kilB* promoters. Nucleic Acids Res. **20:**3693–3700.