Sequence and functional analysis of the *Streptomyces phaeochromogenes* plasmid pJV1 reveals a modular organization of *Streptomyces* plasmids that replicate by rolling circle

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**INTRODUCTION**

Members of the genus *Streptomyces* are high G + C content Gram-positive mycelial soil bacteria with a complex life-cycle (Chater, 1989, 1993). Linear, circular and integrating plasmids, ranging in size from a few kilobases to several hundreds, are common in streptomycetes (reviewed by Hopwood *et al.*, 1986 and Hopwood & Kieser, 1993). Amongst these, the most studied are the 9–11 kb, circular high-copy-number plasmids pIJ101 (Kieser *et al.*, 1982), pSAM2 (Pernodet *et al.*, 1984) and pSN22 (Kataoka *et al.*, 1991a). They replicate by a rolling circle mechanism (RCR; Deng *et al.*, 1988; Hagège *et al.*, 1993a; Kataoka *et al.*, 1994c), like most small plasmids of low G + C content Gram-positive bacteria (reviewed by Gruss & Ehrlich, 1989). Despite their small size, they are highly self-transmissible by intermycelial conjugation, they spread efficiently in the recipient mycelium ('pock' formation), and they randomly mobilize the host chromosome (Cma) at low frequency. Plasmid transfer, spread and Cma require only a few plasmid-encoded genes (Kieser *et al.*, 1982; Kendall & Cohen, 1988; Smokvina *et al.*, 1991; Hagège *et al.*, 1993b; Kataoka *et al.*, 1991a, 1994a). Overexpression of the transfer functions of these plasmids is lethal to the host (Kil phenotype); negative regulation is exerted by 'Kil-override' (Kor) proteins (Kendall &
pJV1 is a high-copy-number, 11 kb circular plasmid from *Streptomyces phaeochromogenes* (Doull et al., 1983; Bailey et al., 1986). Several cloning vectors have been derived from pJV1 (Bailey et al., 1986; Miller, 1991; Denis & Brzezinski, 1992; Qin et al., 1994). The nucleotide sequence of the minimal pJV1 replicon was determined; the deduced sequence of the replication protein (Rep) is 35% identical to that of pIJ101, and the DNA sequences of the pIJ101 and pJV1 ds origins are similar around the nick site (Servin-González, 1993). In this paper, a detailed functional analysis and the complete DNA sequence of pJV1 is reported; it was found that the transfer functions of pJV1 are very different from those of pIJ101, but resemble those of the *Streptomyces* nigrifaciens plasmid pSN22.

## METHODS

### Bacterial strains and plasmids.

*Streptomyces lividans* JT46 (Tsai & Chen, 1987) and *Escherichia coli* JM101 (Yanisch-Perron et al., 1985) were used as host strains for the construction of different pJV1 derivatives. *S. lividans* TK64 (pro-2 str-6) and TK54 (his-2 leu-2 spo-1; Hopwood et al., 1983) were used in genetic crosses to assay plasmid transfer and Cma. *S. phaeochromogenes* (NRRL-B2559) was used as the source of pJV1.

All the *Streptomyces* plasmids used in this study (Table 1) were derived from pJV1, except for pB2 and its derivatives pB62, pB68 and pB70, which contain hybrid pJV1-pIJ101 ds origins of replication (Servin-González, 1993). Fig. 1 shows maps of all *Streptomyces* plasmids. pB65, pB66 and pB67 were constructed by cloning the 3 kb EcoRI-EcoRV fragment from pB50 (the single EcoRV site is located inside the *tsr* gene) into EcoRI/EcoRV-cut pBR322 (Bolivar et al., 1977) and, after modifying the fragment, re-introducing it into pB50. Some DNA manipulations were carried out in *E. coli* using pB72, which was constructed by inserting the *EcoRI*-HindIII polylinker fragment from pUCBM21 (Boehringer Mannheim) into pJl2925 (Janssen & Bibb, 1993) digested with *EcoRI* and *HindIII*, and then deleting the *ApaI* site in the polylinker with Klenow enzyme to restore blue-white colony selection; pB72, therefore, has a modified pUCBM21 polylinker flanked by *BglII* sites, and was specifically used to insert pJV1 fragments into pB83 in both orientations (pB85, pB86, pB87, pB88; Fig. 1 and Table 1). pJ1486 (Ward et al., 1986) was the source of the 1.1 kb *BeII* *tsr* fragment, pJ1702 (Katz et al., 1983) was the source of the 1.5 kb *BeII mel* fragment carrying the tyrosinase gene, and pJ1963 was the source of the 1.7 kb *KpnI-BglII* *bgg* fragment conferring resistance to hygromycin B; pJ1963 is a derivative of pIJ2922 (Janssen & Bibb, 1993) carrying the *pstl-BamHI* fragment containing *bgg* described by Lydiate et al. (1985).

### Media and growth conditions.

For plasmid isolation, *S. phaeochromogenes* and *S. lividans* were grown in liquid LB medium (Sambrook et al., 1989); for *S. lividans*, 34% sucrose was added to obtain dispersed growth. We found that LB-grown cultures gave much cleaner DNA preparations than those grown in media containing glucose, such as the widely used YEME medium (Hopwood et al., 1985). For protoplast preparation and transformation, *S. lividans* was grown in YEME medium. Hypertonic soft agar overlay medium was that of Baltz & Matsushima (1981). Protoplast regeneration was carried out on solid R2YE medium or R5 medium (Hopwood et al., 1985). R5 medium without sucrose was used for sporulation of *S. lividans*. The minimal medium used (MM) was that of Seno & Chater (1983), with 1.5% agar added; MMCY is MM supplemented with 0.2% Camasin acids and 0.1% yeast extract. Growth requirements and antibiotics were used at the concentrations described by Hopwood et al. (1985). Spores of *S. lividans* were stored as suspensions in 20% (v/v) glycerol at −20 °C and were thawed on ice prior to use.

### Genetic crosses.

Crosses were carried out by mixing approximately 10⁴ spores of *S. lividans* TK64, carrying a plasmid, with the same number of spores of plasmid-free strain TK54; the mixture was plated on R5 plates containing His, Leu, and Pro, and incubated at 30 °C until abundant sporulation occurred. Spores were then harvested, serially diluted in distilled water and plated on the different selective media. To quantify plasmid transfer the number of colonies growing on MMCY + Spc + Th was compared to the number of colonies growing on plates with MMCY + Spc. Cma was measured by comparing the number of *Pro*"His" Spe" recombinant colonies selected on MM + Spc + Leu to the number of colonies growing on MM + His + Leu + Pro.

### Pock formation.

A dilution of plasmid-carrying TK64, containing approximately 10⁴ spores, was mixed with 10⁴ spores of plasmid-free TK64, and plated on R5 plates using 3 ml hypertonic soft agar overlay. A plate containing only plasmid-free TK64 was used as a negative control. Plates were then incubated at 30 °C for up to 1 week and examined daily for the appearance of pocks.

### DNA manipulations.

Isolation of plasmid DNA from both *E. coli* and *Streptomyces* was done by the alkaline lysis procedure (Sambrook et al., 1989). Preparation and transformation of *S. lividans* protoplasts were essentially done as described by Hopwood et al. (1985), except that protoplasts were plated using a hypertonic soft agar overlay. Transformation of *E. coli* was done as described by Hanahan (1985), except that cells were grown in 2 × YT medium (Sambrook et al., 1989) supplemented with 10 mM MgSO₄ and 10 mM MgCl₂. Digestions with restriction enzymes, ligations and treatment with Klenow fragment of DNA polymerase I were done by standard protocols (Sambrook et al., 1989). All enzymes used were purchased from either GIBCO-BRL or Boehringer Mannheim.

### DNA sequencing and analysis.

Overlapping fragments of pJV1, no longer than 3 kb, were cloned into either M13mp18 or M13mp19 (Yanisch-Perron et al., 1985) and deletion series were obtained from these with exonuclease III, as described by Sambrook et al. (1989). Most sequencing was done with modified T7 DNA polymerase using Sequenase 2.0 kits (USB) and [³²P]dATP following the protocol supplied by the manufacturer; all templates were sequenced with dGTP and dITP mixes. Regions showing strong secondary structure were re-sequenced with *Tag* DNA polymerase using a TAlQuence 2.0 kit (USB) with dGTP and 7-deaza-dGTP. Both strands were sequenced.

Sequence analysis was carried out using the PC/GENE software package (release 6.8, IntelliGenetics) as well as the frame program (Bibb et al., 1984). Database searches were done online using the World Wide Web server for BLAST searches maintained at the National Center for Biotechnology Information (Bethesda, MD; URL = http://www.ncbi.nlm.nih.gov/BLAST, BLASTN and TABLASTN (Altschul et al., 1990).

### Detection of ss plasmid DNA.

Spores of *S. lividans* strain JT46 carrying the different plasmid derivatives were grown in 25 ml
Table 1. Phenotypes of the different pJV1 derivatives

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Pock formation</th>
<th>Transfer*</th>
<th>Cma*</th>
<th>ssDNA accumulation</th>
<th>Comments†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>&lt; 10⁻⁷</td>
<td>NA</td>
<td>Wild-type</td>
</tr>
<tr>
<td>pJV1</td>
<td>+</td>
<td>1·0(24/24)‡</td>
<td>5·2 × 10⁻⁴</td>
<td>—</td>
<td>pJV1 with spdB region substituted by neo and tsr genes from pIJ486 (Ward et al., 1986); hybrid pJV1/pIJ101 ds ori (Servín-González, 1993)</td>
</tr>
<tr>
<td>pB2</td>
<td>—</td>
<td>1·1 × 10⁻⁵</td>
<td>4·4 × 10⁻⁴</td>
<td>—</td>
<td>BglII–KpnI fragment of pJV1 ligated to BglII–Kpnl fragment of pIJ963. Minimal replicon, Hyg⁺</td>
</tr>
<tr>
<td>pB45</td>
<td>—</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>tsr insertion in single BglII site of pJV1</td>
</tr>
<tr>
<td>pB50</td>
<td>+</td>
<td>0·8</td>
<td>1·2 × 10⁻⁵</td>
<td>—</td>
<td>EcoRI1–BamHI fragment from pJV1, containing spdB genes, cloned into EcoRI1/BglII-cut pB2; hybrid pJV1/pIJ101 ds ori</td>
</tr>
<tr>
<td>pB53</td>
<td>+</td>
<td>0·5</td>
<td>2·2 × 10⁻⁶</td>
<td>NT</td>
<td>Large BamHI4–Asp71 fragment from pB50</td>
</tr>
<tr>
<td>pB57</td>
<td>—</td>
<td>&lt; 10⁻⁷</td>
<td>&lt; 10⁻⁷</td>
<td>+</td>
<td>Deletion of NeoI₁₂–₁₈ fragment of pB50</td>
</tr>
<tr>
<td>pB58</td>
<td>—</td>
<td>&lt; 10⁻⁷</td>
<td>&lt; 10⁻⁷</td>
<td>NT</td>
<td>Deletion of BsrXI₁₄–₁₈ fragment of pB50</td>
</tr>
<tr>
<td>pB59</td>
<td>—</td>
<td>&lt; 10⁻⁷</td>
<td>&lt; 10⁻⁷</td>
<td>NT</td>
<td>Minimal replicon, obtained by ligation of Klenow-treated BamHI₄–Kpnl fragment of pB50; single BamHI site for cloning is regenerated</td>
</tr>
<tr>
<td>pB60</td>
<td>—</td>
<td>NT</td>
<td>NT</td>
<td>—</td>
<td>Deletion of KpnI₄–ScaI₁₆ fragment of pB2; hybrid pJV1/pIJ101 ds ori</td>
</tr>
<tr>
<td>pB61</td>
<td>—</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>Frameshift mutation by infilling the Asp7181 site in pB50</td>
</tr>
<tr>
<td>pB62</td>
<td>—</td>
<td>NT</td>
<td>NT</td>
<td>—</td>
<td>Frameshift mutation by infilling the ScaI₁₆–₁₈ fragment of pB50</td>
</tr>
<tr>
<td>pB63</td>
<td>—</td>
<td>6·2 × 10⁻²</td>
<td>2·0 × 10⁻⁶</td>
<td>NT</td>
<td>Minimal replicon, obtained by ligation of Klenow-treated BamHI₄–Kpnl fragment of pB50; single BamHI site for cloning is regenerated</td>
</tr>
<tr>
<td>pB64</td>
<td>—</td>
<td>9·3 × 10⁻²</td>
<td>2·0 × 10⁻⁶</td>
<td>NT</td>
<td>Frameshift mutation by infilling the NeoI₁₆ site in pB50</td>
</tr>
<tr>
<td>pB65</td>
<td>—</td>
<td>7·7 × 10⁻²</td>
<td>1·6 × 10⁻⁵</td>
<td>NT</td>
<td>pB2-derivative with spdB region from pB50 introduced as EcoRI–EcoRV fragment (EcoRV site is inside the tsr gene); hybrid pJV1/pIJ101 ds ori</td>
</tr>
<tr>
<td>pB66</td>
<td>+</td>
<td>0·6</td>
<td>4·0 × 10⁻⁵</td>
<td>NT</td>
<td>pJV1 with spdB region substituted by neo and tsr genes from pIJ486 (Ward et al., 1986). Similar to pB2 with pJV1 ds ori</td>
</tr>
<tr>
<td>pB67</td>
<td>—</td>
<td>0·8 × 10⁻⁶</td>
<td>2·0 × 10⁻⁴</td>
<td>NT</td>
<td>pB62-derivative with spdB region from pB50. Constructed in the same way as pB68</td>
</tr>
<tr>
<td>pB68</td>
<td>+</td>
<td>0·8</td>
<td>1·5 × 10⁻⁵</td>
<td>NT</td>
<td>tsr insertion in single Asp7001₄ site of pJV1</td>
</tr>
<tr>
<td>pB69</td>
<td>—</td>
<td>7·7 × 10⁻⁶</td>
<td>4·2 × 10⁻⁷</td>
<td>NT</td>
<td>1·5 kb BglI mel fragment from pIJ702 (Katz et al., 1983) cloned into single BamHI site of pB61. Minimal replicon with single BglII site inside mel fragment for cloning</td>
</tr>
<tr>
<td>pB70</td>
<td>+</td>
<td>0·8</td>
<td>1·5 × 10⁻⁵</td>
<td>NT</td>
<td>ScaI₁₆–NeoI₁₄ fragment of pJV1 cloned into pB83 in the natural orientation</td>
</tr>
<tr>
<td>pB71</td>
<td>—</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>ScaI₁₆–NeoI₁₄ fragment of pJV1 cloned into pB83 in the opposite orientation</td>
</tr>
<tr>
<td>pB72</td>
<td>—</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>NeoI₁₂–₁₈ fragment of pJV1 cloned into pB83 in the natural orientation</td>
</tr>
<tr>
<td>pB73</td>
<td>—</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>NeoI₁₂–₁₈ fragment of pJV1 cloned into pB83 in the opposite orientation</td>
</tr>
<tr>
<td>pB74</td>
<td>—</td>
<td>5·9 × 10⁻²</td>
<td>0·3 × 10⁻⁸</td>
<td>NT</td>
<td>tsr insertion in single EcoRI₁ site of pJV1</td>
</tr>
</tbody>
</table>

NA, Not applicable.
NT, Not tested.

*Assayed as described in Methods. Frequencies for one representative experiment are shown. These varied slightly between repetitions of the same cross.
†See Methods for additional details.
‡Transfer for wild-type pJV1 was assayed by plasmid purification from individual Spc⁺ colonies.
of LB supplemented with 34% sucrose for 48 h at 30 °C with shaking. Mycelium was washed with 10% sucrose and exactly 50 mg (wet weight) of mycelium placed in Eppendorf tubes. The mycelium was then lysed and total DNA was extracted as described in procedure 4 of Hopwood et al. (1985); after step 3 of the procedure (phenol/chloroform extractions) 10 μl was loaded, without further treatment, on 1% (w/v) agarose gels, which were run overnight at 1 V cm⁻¹. The gels were then equilibrated in high-salt buffer (1.5 M NaCl, 1 M Tris/HCl, pH 7.5), and the DNA was transferred, without prior alkali denaturation, to PhotoGene Nylon membranes (GIBCO-BRL) and fixed in an oven at 80 °C for 1 h. The probe used in all cases was the BglII–Kpnl fragment from pJV1 that carries the rep gene and the ds replication ori (Servín-González, 1993), since this was the only fragment common to all derivatives used in this study; this probe was labelled with biotin-dCTP using a BioPrime kit (GIBCO-BRL). Hybridization and chemiluminescent detection of the hybridized probe were carried out using the PhotoGene system (GIBCO-BRL).

RESULTS AND DISCUSSION

Localization of genes involved in plasmid transfer and chromosome mobilization

The restriction map of pJV1 (Bailey et al., 1986) was refined by adding the sites for Asp7001, Bpu11021, BsrXI, NstI, NolI and Scal (Fig. 1). A series of pJV1 derivatives were constructed which carried insertions, deletions and frameshift mutations. These were tested in S. lividans for plasmid transfer, pock formation and Cma as described in Methods. Inserting the 1·1 kb BglII fragment containing the thiostrepton-resistance gene (tsr) into the single BglII...
site of pJV1, situated in a non-essential, probably non-coding region (Bailey et al., 1986; Servín-González, 1993) produced pB50 (Fig. 1). As expected, this plasmid was similar to pJV1 in its transfer, pock formation and Cma (Table 1). The Cma data agree with those of Bailey et al. (1986) but the observation of pocks (1–2 mm diameter) formed by pJV1 in lawns of S. lividans is new. The pB50 derivatives pB57 (4.7 kb large BamHI, fragment), pB58 (2.4 kb deletion of NeoI12–NeoI14 fragment) and pB59 (deletion of 0.5 kb BsrXI14–BsrXI15 fragment) have lost Cma, and the ability to transfer and form pocks (subscripted numbers after restriction enzyme names refer to the restriction site number in Fig. 1). This indicated that at least one of the BsrXI sites was inside a transfer gene. pB75, which has tsr in the single Asp70011g site gave 105-fold reduced plasmid transfer, no pocks and 10-fold reduced Cma; since these low transfer levels could also be attributed to spontaneous mutation, individual transformants were tested both for the presence of TK54 markers (His− Leu+) and pB75 plasmid DNA, which confirmed the low transfer levels in two independent crosses. pB66, lacking the 1.5 kb SsrI14–4 fragment formed no visible pocks, had approximately 10-fold reduced transfer and near normal Cma, which is indicative of disruption of spread functions. Frameshift mutations generated by infilling and re-ligation of the Asp7181g site of pJV1 (Fig. 1) and the single Asp70011g site (pB67) or the SacI14–4 deletion, also inserted into the single EcoRI site (pB98) gave no pocks and a reduced transfer phenotype similar to that of pB66 (SsrI14–4 deletion). Deletion of the 2.5 kb EcoRI1–BglII1 fragment in pB69 reduced plasmid transfer 106-fold and thus had a much more severe effect than the insertions in pB65, pB67, pB89 and the SsrI14–4 deletion in pB66 (plasmid transfer in the range 6–9 × 10−8). About the same low plasmid transfer was also observed with pB2, a homologue of pB69 with a hybrid pJV1-pIJ101 ds ori. pB53 and pB68 are derivatives of pB2 where the EcoRI1–BamHI1 and EcoRI1–BglII1, fragments, respectively, have been added in their original place; both plasmids had normal (wild-type) transfer and Cma, and produced pocks. The 1.7 kb KpnI1–SacI10 fragment opposite the spread region was deleted in pB68 without affecting plasmid transfer, pock formation and Cma (pB70). In summary, these results located the sites BsrXI15, NeoI10 and Asp70011g in the spread region, and EcoRI1, KpnI1 and NeoI14 in the spread region (required for pock formation) of pJV1.

**Location of a lethal function and its antidote**

When attempts were made to fill in the single Bpu1102118 site of pB50 these were always unsuccessful: the few transformants that were obtained either contained unmodified pB50 or, more often, plasmids that had suffered spontaneous deletions of different sizes, always removing the Bpu1102118 site and extending towards the EcoRI site into the transfer region. When pB50 was cut with Bpu11021 and EcoRI, treated with Klenow enzyme and ligated, plasmids with the expected structure were readily obtained. Also, pB58 (see above), lacking the 24 kb NeoI12–18 fragment containing the Bpu1102118 site and part of the transfer region, was viable. However, attempts to delete from pB50 the 1.3 kb SacI10–Bpu1102118 fragment again produced transformants with deletions extending into the transfer region. Therefore, it appeared that the Bpu1102118 site was located in a ‘Kil-override’ (Kor) function, which was necessary to repress a potentially lethal gene (Kil phenotype) in the transfer region of pJV1.

**DNA sequence of pJV1**

The entire pJV1 11 143 bp sequence, including that of the essential replication region which has been previously determined (Servín-González, 1993) is shown in Fig. 2; numbering starts at the single EcoRI site. The G+C content is 71.7 mol%, typical for *Streptomyces* DNA. Most of the deduced ORFs, indicated by the translation below the DNA sequence, read from left to right. The only exception is tsrR, which encodes the Kor function (see below). The ORFs are also indicated below the restriction map of pJV1 in Fig. 1. The transfer region contains two ORFs, traA and traB, the latter ending 45 bp before the EcoRI site. The spread region contains three ORFs, named *spaB*1–3 according to the partially homologous genes in *pSN22* (*spaB*3 was described previously; Servín-González, 1993). Downstream of the rep gene there is a 2 kb region which was non-essential for replication and transfer (see pB70). It contains only two small, possibly translated ORFs, named *orf84* and *spaD* (this last ORF is named after its *pSN22* homologue).

**Transfer genes**

The pJV1 traR gene would encode a 245 amino acid (26.5 kDa) protein. Similarity searches carried out on nucleotide and protein sequence databases revealed significant similarity with other regulatory proteins. The most similar proteins were TraR of *pSN22* (Katoaka et al., 1994a; 83% identity, 88% similarity), KorA of *pIJ101* (Kendall & Cohen, 1988; 28% identity, 42% similarity), KorSA of *pSAM2* (Hagège, et al., 1993b; 24% identity, 40% similarity), protein L of *pSCL1* (Wu & Roy, 1993; 27% identity, 38% similarity) and ImpA of *SLP1* (Shiffman & Cohen, 1993; 21% identity, 35% similarity). The putative regulator of the succinyl-CoA synthetase of *E. coli* (Buck & Guest, 1989), which belongs to the GntR family of bacterial regulators (Haydon & Guest, 1991), also showed significant similarity. The Kor proteins from RCR *Streptomyces* plasmids (excluding ImpA) could be aligned with 34% overall similarity (not shown). They all have near their N-terminus a putative helix–turn–helix (HTH) DNA-binding domain. Even though the *Streptomyces* proteins share significant overall similarity to other members of the GntR family of regulators (Hagège et al., 1993b), their HTH motifs conform poorly to the HTH consensus for this family (Bairoch, 1993; Fig. 3), and have low scores on prediction methods based on weight matrices (Fig 3; Dodd & Egan, 1990). There is substantial indirect evidence that these Kor proteins are transcriptional regulators; in addition, specific DNA-binding activity has been demonstrated for one member...
of this family of TraR homologues: the pSN22 TraR protein (Kataoka et al., 1994b). This implies that the unorthodox HTH motifs of these regulators do not reflect lack of DNA-binding activity, but merely divergence from those of other members of the GntR family, perhaps because of constraints placed on these proteins acting on G+C-rich DNA, as postulated by Molnar & Murooka (1993). Therefore, it seems appropriate to propose a specific HTH consensus sequence for TraR homologues (Fig. 3).

The pJV1 traA and traB genes would encode proteins of 184 amino acids (19.4 kDa) and 678 amino acids (71.7 kDa), respectively. The only proteins with significant similarity were the pSN22 TraA and TraB proteins (Kataoka et al., 1994a; 69 and 65% identity, respectively). pJV1 TraA has 29 amino acids at the N-terminus that are not present in the pSN22 equivalent. It is interesting to note that the ttr insertion in pB75, which eliminates the C-terminal part of TraB (approximately 25% of the sequence) shows a low but clearly detectable level of transfer, indicating that the C-terminal part of this protein is not absolutely necessary for its transfer function. pJV1 TraB has a P-loop ATP/GTP-binding motif (underlined in Fig. 2) in approximately the same position as in pSN22 TraB. The traB coding sequence of pSN22, but not that of pJV1, has a TTA leucine codon which is very rare in Streptomyces DNA (Chater, 1989). The TraB sequences of pJV1 and pSN22 reveal several hydrophobic regions which could be membrane-associated (data not shown); the longest hydrophobic stretch in pJV1 TraB, which could span a membrane (amino acids 318–338), is shown in Fig. 2.

The intercistronic region between the traR and traA genes has been studied in detail by Kataoka et al. (1991b, 1994b) in pSN22. These authors have shown the presence of divergent promoters in this region, one of them responsible for transcription of traR and the other for transcription of an operon comprising traA, traB and the spdB genes; transcription from both promoters is negatively regulated by the product of traR, which binds four 12 bp repeated sequences, called TRE boxes, overlapping the promoter sequences (Kataoka et al., 1994b). The traR–traA intercistronic region in pJV1 is very similar to that of pSN22. The −10 and the −35 regions of the traR promoter and the −10 region of the traA promoter are identical, but there are two mismatches in the traA promoter −35 region (TTGCGC rather than TTGTCC). The TRE boxes 1 and 2 are perfectly conserved, and there are two mismatches each in the TRE boxes 3 and 4 (Fig. 2). The pJV1 intercistronic region is 64 bp shorter than that of pSN22 (169 vs 233 bp), corresponding to a shorter leader in the pJV1 traA mRNA and a longer TraA protein (see above). The pJV1 TraA ORF is preceded by a likely RBS (GAGGTTA; Strohl, 1992); quite interestingly, this putative RBS is conserved in pSN22 just a few nucleotides upstream of a GTG codon in-frame with the traA ORF. Kataoka et al. (1994a) dismissed this GTG codon as the traA start codon since insertion of an 8 bp linker into the Smal site located between the GTG codon and an ATG codon further downstream had no phenotypic effect.

**Spread genes**

The organization and the DNA and deduced protein sequence of the pJV1 spread region also showed significant similarities to those of pSN22, although this was not as high as that observed for the tra genes. The pJV1 spd region consists of three ORFs showing translational coupling, since the spdB1 and spdB2 ter-

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**Fig. 2.** Nucleotide sequence of pJV1. The sequence has been numbered starting at the single EcoRI site. Restrictions sites for the enzymes shown above the sequence have been underlined, as have sequences which might function as RBSs. Translation of the different ORFs is shown below the nucleotide sequence. The coding strand is shown for all ORFs except for the traB ORF which is in the complementary strand; the translation of this ORF is shown in lower case letters. Potential membrane-spanning hydrophobic stretches in proteins are underlined and in bold. Asterisks above the sequence correspond to regions showing similarity to equivalent regions in pJV101 (Kendall & Cohen, 1988). Two black squares indicate the nick site inside the dso ori where replication starts. Black bars indicate regions corresponding to the TRE boxes of pSN22 (Kataoka et al., 1994a) in the traR–traA intercistronic region. The position of the nucleotide-binding motif (P-loop) in the TraB sequence is also shown. Inverted or direct repeats are indicated by arrows.

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**Fig. 3.** Alignment of HTH motifs of Kor proteins of RCR Streptomyces plasmids. The aligned HTH motifs were individually compared to the weight matrix of Dodd & Egan (1990); the scores for the HTH motifs and SD (in parentheses) were calculated as described by these authors. SD values below 2.5 are not considered significant unless there is evidence for the protein being a DNA-binding protein. The HTH motifs were also tested against the stringent stereochemical criteria of Shestopalov (1988); only the pSAM2 KorSA sequence did not conform to either of these rules. Positions perfectly conserved in all Kor proteins are marked by asterisks. The consensus HTH ‘signature’ for the GntR family of bacterial regulators is that of the PROSITE database (Bairoch, 1993).
mination codons overlap the start codon of the following gene. spdB1 is preceded by a likely RBS (underlined in Fig. 2; Strohl, 1992) and, in addition, sequences that could function as RBSs are found upstream of the initiation codons for the spdB2 and spdB3 (Fig. 2). This situation is not observed in pSN22, where none of the four ORFs described by Kataoka et al. (1994a) overlap.

The pJV1 spdB1 gene encodes a protein of 174 amino acids with three putative transmembrane helices (underlined in Fig. 2). It shows about 30% C-terminal identity to the 107 amino acid pSN22 SpdB1 protein.

The spdB2 gene of pJV1 encodes a 371 amino acid protein (putative transmembrane helices underlined in Fig. 2) 52% identical at the N-terminus to its 251 amino acid pSN22 homologue. The intercistronic region (nt 6094-6387) between the pSN22 spdB2 and spdB3 ORFs revealed an ORF showing Streptomyces codon usage and encoding an 89 amino acid protein with 63% identity to the C-terminal part of the pJV1 SpdB2 protein. This ORF was not considered in the analysis of Kataoka et al. (1994a).

pJV1 spdB3 encodes a very hydrophobic protein of 147 amino acids with three putative transmembrane helices (underlined in Fig. 2) and 63% identity to the 128 amino acid product of the pSN22 spdB4 gene.

No part of the pJV1 sequence showed similarity to the 70 amino acid product of pSN22 spdB3.

Analysis of mutations affecting the pJV1 spdB region revealed the existence of two distinguishable phenotypes. A series of frameshift mutations affecting the spdB1 and spdB3 ORFs, respectively, insertion into the single EcoRI site in the traB–spdB intercistronic region and partial deletion of ORFs spdB2 and spdB3 affected plasmid transfer, reducing its efficiency to levels around 5-10% of the wild-type (pB65, pB66, pB67, pB89; Fig. 1 and Table 1). On the other hand, deletion of the three spdB genes together with flanking regions reduced plasmid transfer about 100-fold but affected Cma only slightly (pB2, pB69; Fig. 1 and Table 1). One possibility to explain this difference is that the function of the SpdB proteins is independent and redundant, so that loss of one or two of the spdB genes can be partially overcome by the remaining one(s); in this case, a true mutant phenotype could only be obtained by deletion of the three ORFs. The most likely explanation, however, is that the deleted non-coding sequences flanking the spdB ORFs might be required in cis for plasmid transfer. A cis-acting function required for transfer has already been described for one RCR Streptomyces plasmid: pIJ101 (the ctt locus; Pettis & Cohen, 1994).

Replication region

The sequence of the pJV1 region containing the 528 amino acid Rep protein and the ds ori which are essential for plasmid replication has been previously reported (Servín-González, 1993). The sequence of the pJV1 Rep protein resembled those of the Rep proteins of Streptomyces plasmids pIJ101 (36% identity; Kendall & Cohen, 1988), pSN22 (36% identity; Kataoka et al., 1994c) and pSLG33 (37% identity; Felsberg et al., 1993), pSG5 (23% identity; Muth et al., 1995) and that of pBL1 from Brachybacterium lactofermentum ATCC 13869 (22% identity; Filipula et al., 1986; Fernández-González et al., 1994). All of these Rep proteins contained the three motifs characteristic of Rep proteins of RCR plasmids (Gruss & Ehrlich, 1989; Ilyina & Koonin, 1992), as shown in the alignment of the Rep sequences of the Streptomyces plasmids (Fig. 4). Note that there are several gaps introduced in the alignment and that some, not previously recognized, conserved regions exist in these Streptomyces Rep protein sequences.

Since the Tra and SpdB proteins of pJV1 were most similar in their sequence (61% average identity) and in their gene arrangement to those of pSN22, it was rather unexpected to find that the pJV1 Rep sequence exhibited only about 35% identity to pSN22 Rep, which itself is 95% identical to pIJ101 Rep; this near identity between pSN22 and pIJ101 is also seen in the nucleotide sequence and includes the non-coding region of the ds ori. The region of high similarity between pSN22 and pJV1, indicated in Fig. 1, excludes the whole pJV1 minimal replicon. Thus, pJV1 has a modular structure, as has been described for RCR plasmids of low G + C content Gram-positive bacteria (Projan & Novick, 1988; Novick, 1989). pJV1 appears to be a combination of a ‘pSN22-like’ transfer and spread module and a replication module different from that present in pSN22, which itself is ‘pIJ101-like’.
The pJV1 'spdA' region reveals conserved palindromic repeats

Kataoka et al. (1991a, b, 1994a) described in pSN22 the spdA locus approximately 500 bp downstream of traR and an ORF encoding a 154 amino acid putative SpdA protein. The sequence of pJV1 shows an ORF in the equivalent region with 35% identity (most of it restricted to the N-terminal portion) to the pSN22 equivalent. When the tblastn program was used to search for sequences similar to the product of pJV1 spdA, two sequences revealed significant similarity; one of them was, as expected, the product of pSN22 spdA, but a higher alignment score was obtained by the translation of sequences located just downstream of the pIJ101 rep gene; this is a region which has previously been considered as non-coding (Kendall & Cohen, 1988). Closer examination of this region in pIJ101 revealed the presence of an ORF (8377–7973, complementary strand) encoding a putative 135 amino acid protein. All three ORFs lack the bias in G+C content of the first, second and third codon position typical for coding regions in high G+C content Streptomyces DNA (Bibb et al., 1984). pJV1, pSN22 and pIJ101 have two or three (pSN22) palindromic repeats in their ‘spdA’ regions and all three plasmids have the sequence GTACGTCGAC in the central part of each palindrome (Fig. 5). Since the repeats are palindromic in all three plasmids, they could be viewed as direct repeats or as inverted repeats capable of forming stem-loop structures; in fact, closer examination shows that the pJV1 and pSN22 palindromic sequences would have the capability of forming quite stable stem-loop structures, while the sequences in pIJ101 would form a less stable structure (in this case the AAA sequence after both repeats could also be taken as evidence that these are better considered direct repeats; Fig. 5). The 1.7 kb KpnI–ScaI fragment of pB70 removed the N-terminal portion of the putative pJV1 spdA ORF, but not the palindromic sequences. pB70 was phenotypically normal (see above, Table 1, Fig. 1), suggesting that pJV1 spdA did not encode a protein involved in plasmid transfer. The conservation of the DNA sequence and secondary structure in the spdA region of pJV1, pSN22 and pIJ101 argues for a cis-acting function whose biological effects in pJV1 and pIJ101 are unknown. This, however, would not explain why potential frame-disrupting mutations in the pSN22 spdA ORF, that lie outside of the palindromic repeats, show an SpdA− phenotype (Kataoka et al., 1991a). Clearly, further experimentation is required to determine the function of the spdA region in pJV1 and its homologues.

pB70 also lacks orf84 (Fig. 1), which is the only likely coding sequence between the end of rep and the start of the putative spdA ORF; no proteins with significant similarity to the product of orf84 were found.

Identification of an ss ori

To locate the ss replication ori of pJV1, the accumulation of ss plasmid DNA by different pJV1 derivatives was tested (Table 1). The minimal replicon plasmids pB61 and pB83, as well as pB57 (4.7 kb BamHI, fragment of pB50) accumulated ss plasmid DNA, but pJV1, pB50 (complete replicons), pB2 (lacks spdB genes) and pB62 (lacks spdB genes and 1.7 kb KpnI–ScaI fragment) did not. Orientation-specific ss ori activity was, however, detected in the 656 bp ScaI–NcoI fragment: pB85, containing the fragment in its natural orientation, accumulated very little ss DNA, while pB86, with the fragment in the opposite orientation, accumulated about as much ss DNA as the vector plasmid pB83 (Fig. 6a). The ss ori of pSN22 has been mapped to a different, non-homologous region, upstream of traR (Kataoka et al., 1994b, c). The pJV1 regulatory region upstream of traR does not have ss ori activity: cloning the 2.4 kb NcoI–18 fragment into pB83 in both orientations did not prevent ssDNA accumulation (pB87, pB88, Fig. 6b).
**The pJV1 ss ori is associated with a strong incompatibility (Sti) determinant**

Further experiments tested whether the ss ori-containing *Sat110*-*Neo112* fragment was capable of conferring an Sti phenotype, as described by Deng et al. (1988) for pIJ101: different pB50 derivatives (ThR) were introduced by transformation into *S. lividans* JT46 carrying plasmid pB45, a minimal replicon plasmid without ss ori (*Bam*HI-*Kpn*I fragment of pJV1) containing the *hyg* gene for resistance to hygromycin B (Fig. 1). The results of these incompatibility tests are listed in Table 2. When pB83 (ThR Mel, no ss ori) was introduced into JT46(pB45), all the selected ThR colonies were also Mel, indicating that the two plasmids were compatible. When, however, pB50 was introduced by transformation into JT46(pB45), not all of the ThR colonies grew when replicated onto Hyg plates and, if they did, growth was often restricted to small sectors of the colonies, indicating that the plasmids were incompatible. Similar tests showed that pB85 (active ss ori) was incompatible with pB45 and pB86 (inverted, inactive ss ori) was compatible. These results demonstrated that the 656 bp *Sat110*-*Neo112* fragment of pJV1 contains in addition to the ss replication ori an orientation-specific Sti function, as is the case in pIJ101. Quantitative data confirming these results were obtained by re-plating spores from a pool of confluent ThR transformants on Th- and Th+Hyg-containing plates. Plating was repeated to monitor the stability of pB45 over a second spore generation. Surprisingly, pB85 seemed to express stronger incompatibility towards pB45 than pB50 (Table 2), indicating that incompatibility could be negatively regulated by some function present in pB50 and absent in pB85.

A database search revealed that a 200 bp stretch (nt 7250–7450) inside the 656 bp *Sat110*-*Neo112* fragment having ss ori and Sti activity was very similar to a stretch of similar size inside the smallest fragment of pIJ101 showing these same two functions (Zaman et al., 1993; Fig. 2). A similar sequence is also found downstream of *traR* in pSN22, but it lacks both ss ori and Sti activity (Kataoka et al., 1994c).

**Table 2. Demonstration of a pJV1 strong incompatibility (Sti) phenotype**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>First sporulation round</th>
<th>Second sporulation round</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Th*</td>
<td>Th + Hyg†</td>
</tr>
<tr>
<td></td>
<td>100% (5.4 × 10³)</td>
<td>100%</td>
</tr>
<tr>
<td>pB83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pB50</td>
<td>100% (5.9 × 10³)</td>
<td>14%†</td>
</tr>
<tr>
<td>pB85</td>
<td>100% (6.9 × 10³)</td>
<td>1%†</td>
</tr>
<tr>
<td>pB86</td>
<td>100% (1.9 × 10³)</td>
<td>90%</td>
</tr>
</tbody>
</table>

*Values in parentheses correspond to the actual number of colonies per ml in Th alone, which correspond to 100%.

†Numbers refer to the percentage of colonies growing in the presence of both antibiotics relative to the number growing on Th alone.

‡About 50% of these colonies remained small even after prolonged incubation, probably because of early loss of pB45 from the developing colonies.

**Conclusions**

Functional and sequence analysis of pJV1 revealed that it carries transfer and spread functions almost identical to those of pSN22. The replication functions of pJV1, however, are different from those of pSN22. The similarities and differences between pJV1, pSN22 and pIJ101 can be interpreted as evidence of a modular structure of RCR *Streptomyces* plasmids.

Despite the high similarity between pJV1 and pSN22 Tra and Spd proteins there are several differences: (i) the pJV1 TraA sequence has an N-terminal extension, and the *traR-traA* intergenic regulatory region is shorter in pJV1; (ii) the intergenic region of pSN22, but not that of pJV1 has ss ori activity; (iii) the spdB regions of pJV1 and pSN22 contain three and five ORFs, respectively, encoding very hydrophobic, potentially membrane-spanning putative proteins.

The TraR regulator of pJV1 is similar to the GntR family of regulators, but the putative HTH DNA-binding motif is poorly conserved. A consensus for *Streptomyces* plasmid regulators is proposed (Fig. 3). Like in other characterized conjugative *Streptomyces* plasmids, inactivation of the regulator with consequent overexpression of the transfer gene(s) is lethal.

The functions of the pJV1 *traA*, *traB* and *spdB* genes have been confirmed by deletion, insertion and frameshift mutations, but the precise functions of the corresponding putative proteins remain uncharacterized.

pJV1 does not seem to have a functional equivalent of the pSN22 *spdA* gene. It is also interesting that these two plasmids have completely different ss origins which are located in non-equivalent positions, despite high sequence similarity between them.

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