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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pJV1 is an 11 kb, high-copy-number conjugative *Streptomyces phaeochromogenes* plasmid that replicates by the rolling circle mechanism (RCR). Sequencing combined with functional analysis of deletion, insertion and frameshift mutations was used to characterize the genes involved in plasmid transfer and chromosome mobilization (Cma), the single-strand origin for RCR and an associated strong incompatibility (Sti) determinant. pJV1 contains two essential transfer genes whose expression is regulated by an adjacent repressor gene with similarity to the GntR family of regulators. A consensus sequence specific for the helix–turn–helix motifs of repressor proteins of *Streptomyces* plasmids is proposed. Unregulated expression of the transfer genes by inactivation of the repressor is lethal. Three additional genes increase intramycelial plasmid spread resulting in pock formation but, unlike the essential transfer genes, are not required for Cma. The pJV1 transfer genes and their regulatory region, but not the minimal replication region encoding the double-strand replication origin and replication protein, are similar in their sequence and arrangement to those of the *Streptomyces nigrificans* plasmid pSN22, revealing a modular organization of *Streptomyces* RCR plasmids.

Keywords: *Streptomyces*, plasmid transfer, plasmid spread, modular organization, rolling-circle replication

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). Over-expression of the transfer functions of these plasmids is lethal to the host (Kil phenotype); negative regulation is exerted by 'Kil-override' (Kor) proteins (Kendall &

Abbreviations: Cma, chromosome-mobilizing ability; ds, double-stranded; Hyg, hygromycin B; HTH, helix–turn–helix; *ori*, replication origin; RBS, ribosome-binding site(s); RCR, rolling-circle replication; Rep, plasmid replication; Spc, spectinomycin; Spd, plasmid spread; ss, single-stranded; Th, thiostrepton; Tra, plasmid transfer.

The GenBank accession number for the sequence reported in this paper is U23762.

Cohen, 1987; Hagège *et al.*, 1993b; Kataoka *et al.*, 1991a, b).

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 (Servín-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 (*bis-2 leu-2 spc-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 (Servín-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 (Bolívar *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 pIJ2925 (Janssen & Bibb, 1993) digested with *EcoRI* and *HindIII*, and then deleting the *Apal* site in the polylinker with Klenow enzyme to restore blue-white colony selection; pB72, therefore has a modified pUCBM21 polylinker flanked by *Bgl*II sites, and was specifically used to insert pJV1 fragments into pB83 in both orientations (pB85, pB86, pB87, pB88; Fig. 1 and Table 1). pIJ486 (Ward *et al.*, 1986) was the source of the 1.1 kb *Bcl*I *tsr* fragment, pIJ702 (Katz *et al.*, 1983) was the source of the 1.5 kb *Bcl*I *mel* fragment carrying the tyrosinase gene, and pIJ963 was the source of the 1.7 kb *Kpn*I-*Bgl*II *hyg* fragment conferring resistance to hygromycin B; pIJ963 is a derivative of pIJ2922 (Janssen & Bibb, 1993) carrying the *Pst*I-*Bam*HI fragment containing *hyg* 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% Casamino 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 10⁷ 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⁺ Spc^R 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 10⁸ spores, was mixed with 10⁷ 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 [³⁵S]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 *Taq* DNA polymerase using a TAQuence 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 on-line 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/Recipon/blast_search.html) and the programs BLASTN, BLASTP and TBLASTN (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

Plasmid	Pock formation	Transfer*	Cma*	ssDNA accumulation	Comments†
None	NA	NA	$< 10^{-7}$	NA	
pJV1	+	1.0(24/24)‡	5.2×10^{-6}	—	Wild-type
pB2	—	1.1×10^{-5}	4.4×10^{-6}	—	pJV1 with <i>spdB</i> region substituted by <i>neo</i> and <i>tsr</i> genes from pIJ486 (Ward <i>et al.</i> , 1986): hybrid pJV1/pIJ101 ds <i>ori</i> (Servín-González, 1993)
pB45	—	NT	NT	+	<i>Bam</i> HI ₆ – <i>Kpn</i> I ₈ fragment of pJV1 ligated to <i>Bgl</i> II– <i>Kpn</i> I fragment of pIJ963. Minimal replicon, Hyg ^R
pB50	+	0.8	1.2×10^{-5}	—	<i>tsr</i> insertion in single <i>Bgl</i> II ₇ site of pJV1
pB53	+	0.5	2.2×10^{-5}	NT	<i>Eco</i> RI ₁ – <i>Bam</i> HI ₆ fragment from pJV1, containing <i>spdB</i> genes, cloned into <i>Eco</i> RI/ <i>Bgl</i> II-cut pB2; hybrid pJV1/pIJ101 ds <i>ori</i>
pB57	—	$< 10^{-7}$	$< 10^{-7}$	+	Large <i>Bam</i> HI _{6–11} fragment from pB50
pB58	—	$< 10^{-7}$	$< 10^{-7}$	NT	Deletion of <i>Nco</i> I _{12–18} fragment of pB50
pB59	—	$< 10^{-7}$	$< 10^{-7}$	NT	Deletion of <i>Bst</i> XI _{14–15} fragment of pB50
pB61	—	NT	NT	+	Minimal replicon, obtained by ligation of Klenow-treated <i>Bam</i> HI ₆ – <i>Kpn</i> I ₈ fragment of pB50; single <i>Bam</i> HI site for cloning is regenerated
pB62	—	NT	NT	—	Deletion of <i>Kpn</i> I ₈ – <i>Sca</i> I ₁₀ fragment of pB2; hybrid pJV1/pIJ101 ds <i>ori</i>
pB65	—	6.2×10^{-2}	2.0×10^{-6}	NT	Frameshift mutation by infilling the <i>Asp</i> 718I ₃ site in pB50
pB66	—	9.3×10^{-2}	2.0×10^{-6}	NT	Deletion of <i>Sst</i> I _{2–4} fragment of pB50
pB67	—	7.7×10^{-2}	1.6×10^{-5}	NT	Frameshift mutation by infilling the <i>Not</i> I ₅ site in pB50
pB68	+	0.6	4.0×10^{-5}	NT	pB2-derivative with <i>spdB</i> region from pB50 introduced as <i>Eco</i> RI– <i>Eco</i> RV fragment (<i>Eco</i> RV site is inside the <i>tsr</i> gene); hybrid pJV1/pIJ101 ds <i>ori</i>
pB69	—	0.8×10^{-5}	2.0×10^{-6}	NT	pJV1 with <i>spdB</i> region substituted by <i>neo</i> and <i>tsr</i> genes from pIJ486 (Ward <i>et al.</i> , 1986). Similar to pB2 with pJV1 ds <i>ori</i>
pB70	+	0.8	1.5×10^{-5}	NT	pB62-derivative with <i>spdB</i> region from pB50. Constructed in the same way as pB68
pB75	—	7.7×10^{-6}	4.2×10^{-7}	NT	<i>tsr</i> insertion in single <i>Asp</i> 700I ₁₈ site of pJV1
pB83	NT	NT	NT	+	1.5 kb <i>Bcl</i> I <i>mel</i> fragment from pIJ702 (Katz <i>et al.</i> , 1983) cloned into single <i>Bam</i> HI site of pB61. Minimal replicon with single <i>Bgl</i> II site inside <i>mel</i> fragment for cloning
pB85	NT	NT	NT	—	<i>Sca</i> I ₁₀ – <i>Nco</i> I ₁₂ fragment of pJV1 cloned into pB83 in the natural orientation
pB86	NT	NT	NT	+	<i>Sca</i> I ₁₀ – <i>Nco</i> I ₁₂ fragment of pJV1 cloned into pB83 in the opposite orientation
pB87	NT	NT	NT	+	<i>Nco</i> I _{12–18} fragment of pJV1 cloned into pB83 in the natural orientation
pB88	NT	NT	NT	+	<i>Nco</i> I _{12–18} fragment of pJV1 cloned into pB83 in the opposite orientation
pB89	—	5.9×10^{-2}	0.3×10^{-6}	NT	<i>tsr</i> insertion in single <i>Eco</i> RI ₁ site of pJV1

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^R colonies.

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RESULTS AND DISCUSSION

The restriction map of pJV1 (Bailey *et al.*, 1986) was refined by adding the sites for *Asp*700I, *Bpu*1102I, *Bst*XI, *Not*I, *Nco*I and *Sca*I (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 *Bcl*I fragment containing the thiostrepton-resistance gene (*tsr*) into the single *Bgl*II

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 *Bam*HI_{6–11} fragment), pB58 (2.4 kb deletion of *Nco*I₁₂–*Nco*I₁₈ fragment) and pB59 (deletion of 0.5 kb *Bst*XI₁₄–*Bst*XI₁₅ 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 *Bst*XI sites was inside a transfer gene. pB75, which has *tsr* in the single *Asp*700I₁₉ 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 *Sst*I_{2–4} 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 *Asp*718I₃ site (pB65) or the *Not*I₅ site (pB67), and also insertion of *tsr* in the single *Eco*RI₁ site (pB89) gave no pocks and a reduced transfer phenotype similar to that of pB66 (*Sst*I_{2–4} deletion). Deletion of the 2.5 kb *Eco*RI₁–*Bgl*II₇ fragment in pB69 reduced plasmid transfer 10⁵-fold and thus had a much more severe effect than the insertions in pB65, pB67, pB89 or the *Sst*I_{2–4} deletion in pB66 (plasmid transfer in the range 6–9 × 10^{–2}). 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 *Eco*RI₁–*Bam*HI₆ and *Eco*RI₁–*Bgl*II₇ fragments, respectively, have been added in their original place; both plasmids had about normal (wild-type) transfer and Cma, and produced pocks. The 1.7 kb *Kpn*I₈–*Sca*I₁₀ 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 *Bst*XI₁₅, *Nco*I₁₈ and *Asp*700I₁₉ in the transfer region, and *Eco*RI₁, *Kpn*I₃ and *Not*I₅ 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 *Bpu*1102I₁₃ 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 *Bpu*1102I₁₃ site and extending towards the *Eco*RI₁ site into the transfer region. When pB50 was cut with *Bpu*1102I and *Eco*RI, treated with Klenow enzyme and ligated, plasmids with the expected structure were readily obtained. Also, pB58 (see above), lacking the 2.4 kb *Nco*I_{12–18} fragment containing the *Bpu*1102I₁₃ site

and part of the transfer region, was viable. However, attempts to delete from pB50 the 1.3 kb *Sca*I₁₀–*Bpu*1102I₁₃ fragment again produced transformants with deletions extending into the transfer region. Therefore, it appeared that the *Bpu*1102I₁₃ 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 *Eco*RI 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 *traR*, 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 *Eco*RI site. The spread region contains three ORFs, named *spdB1–3* according to the partially homologous genes in pSN22 (*spdB3* 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 *spdA* (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 (Kataoka *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

[illegible]

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).

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 *tsr* insertion in pB75, which eliminates the C-terminal part of TraB (approximately 25 % of the sequence) shows a low but clearly detectable

The intergenic 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* intergenic 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 (TTGCCG instead of TTGCTT). 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 intergenic 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 (GAGGTGA; 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 *Sma*I site located between the GTG codon and an ATG codon further downstream had no phenotypic effect.

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-

Figure 1. The effect of the initial concentration of the monomer on the polymerization of α -methylstyrene initiated by BuLi in THF at -78°C . The concentration of the initiator was 1.0×10^{-2} mole/liter. The concentration of the monomer was 0.05 mole/liter (O), 0.1 mole/liter ($\square$), 0.2 mole/liter ($\triangle$), 0.3 mole/liter ($\diamond$), and 0.4 mole/liter ($\circ$). The polymerization was terminated by the addition of methanol.

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 10⁵-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 *clt* 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

Fig. 4. Alignment of Rep proteins of RCR *Streptomyces* plasmids. The sequences were aligned using the CLUSTAL program (PC/GENE release 6.8). Residues which are conserved in all five sequences are in upper-case in the consensus line, whereas residues conserved in four out of the five sequences are in lower-case. The three conserved motifs of RCR proteins described by Ilyina & Koonin (1992) are shown below the sequence (x, any amino acid; u, bulky hydrophobic residue).

protein resembled those of the Rep proteins of *Streptomyces* plasmids pIJ101 (36% identity; Kendall & Cohen, 1988), pSN22 (36% identity; Kataoka *et al.*, 1994c), pSLG33 (37% identity; Felsberg *et al.*, 1993), pSG5 (23% identity; Muth *et al.*, 1995) and that of pBL1 from *Brevibacterium lactofermentum* ATCC 13869 (22% identity; Filpula *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'.

Fig. 5. Palindromic repeats within ORFs of the putative *spdA* genes of pJV1, pSN22, and the homologous sequence in pIJ101. The numbers correspond to those of Kendall & Cohen (1988) for pIJ101 (complementary strand), Fig. 2 for pJV1 and Kataoka *et al.* (1994a) for pSN22; only the second and third repeats in the pSN22 sequence are shown. Asterisks indicate identical residues in pJV1 and pIJ101. Nucleotides which are conserved in all repeats are in bold.

pIJ101	CCGAGATACGTC ACGTCGAC GAATGCAGCAGCCTTCCATTCCGTC ACGTCGAC GAAGCTCGGGCCGCA	8126
pJV1	TTCCGTTTCGTC ACGTCGAC GATCGCCGACTCCTCCGATTCCGTC ACGTCGAC GAAGCGTGCCTC	7113
pSN22	CAGGAAAGTC ACGTCGAC TTTCCCGGTCGGCAGGCCCGCAGCAAGTC ACGTCGAC TTTCCAAAACCCCA	736

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 GTCACGTGAC 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*₈–*ScaI*₁₀ deletion 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

Fig. 6. Accumulation of ss plasmid DNA by different pJV1 derivatives. ssDNA was detected by Southern hybridization as described in Methods. See Table 2 for a description of the different plasmids. (a) Lanes: 1, pB61; 2, pB83; 3, pB85; 4, pB86; 5, pB50. (b) Lanes: 1, pB83; 2, pB87; 3, pB88; 4, pB50.

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 derivatives was tested (Table 1). The minimal replicon plasmids pB61 and pB83, as well as pB57 (4.7 kb *Bam*HI₆–₁₁ 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*₈–*ScaI*₁₀ fragment) did not. Orientation-specific ss *ori* activity was, however, detected in the 656 bp *ScaI*₁₀–*NcoI*₁₂ 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*₁₂–₁₈ fragment into pB83 in both orientations did not prevent ssDNA accumulation (pB87, pB88, Fig. 6b).

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

Plasmid	First sporulation round		Second sporulation round	
	Th*	Th + Hyg†	Th*	Th + Hyg†
pB83	100 % (5.4×10^8)	100 %	100 % (4.8×10^8)	96 %
pB50	100 % (5.9×10^8)	14 %‡	100 % (4.0×10^8)	7 %‡
pB85	100 % (6.9×10^8)	1 %‡	100 % (1.8×10^9)	0.0003 %‡
pB86	100 % (1.9×10^9)	90 %	100 % (8.9×10^8)	100 %

*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.

The pJV1 *ss ori* is associated with a strong incompatibility (Sti) determinant

Further experiments tested whether the *ss ori*-containing *ScaI*₁₀–*NcoI*₁₂ fragment was capable of conferring an Sti phenotype, as described by Deng *et al.* (1988) for pIJ101: different pB50 derivatives (Th^R) were introduced by transformation into *S. lividans* JT46 carrying plasmid pB45, a minimal replicon plasmid without *ss ori* (*Bam*HI₆–*Kpn*I₈ 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 (Th^R Mel⁺, no *ss ori*) was introduced into JT46(pB45), all the selected Th^R colonies were also Mel⁺ Hyg^R, indicating that the two plasmids were compatible. When, however, pB50 was introduced by transformation into JT46(pB45), not all of the Th^R 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 (inactive *ss ori*) was compatible. These results demonstrated that the 656 bp *ScaI*₁₀–*NcoI*₁₂ 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 Th^R 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 *ScaI*₁₀–*NcoI*₁₂ 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).

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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