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pIJ101, a Multi-Copy Broad Host-Range *Streptomyces* Plasmid: Functional Analysis and Development of DNA Cloning Vectors

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Summary. *Streptomyces lividans* ISP 5434 contains four small high copy number plasmids: pIJ101 (8.9 kb), pIJ102 (4.0 kb), pIJ103 (3.9 kb) and pIJ104 (4.9 kb). The three smaller species appear to be naturally occurring deletion variants of pIJ101. pIJ101 and its in vivo and in vitro derivatives were studied after transformation into *S. lividans* 66.

pIJ101 was found to be self-transmissible by conjugation, to elicit "lethal zygosis" and to promote chromosomal recombination at high frequency in both *S. lividans* 66 and *S. coelicolor* A3(2). A restriction endonuclease cleavage map of pIJ101 was constructed for 11 endonucleases; sites for five others were lacking. Many variants of pIJ101 were constructed in vitro by inserting DNA fragments determining resistance to neomycin, thiostrepton or viomycin, and having *Bam*HI termini, into *Mbo*I or *Bcl*I sites on the plasmid, sometimes with deletion of segments of plasmid DNA. The physical maps of these plasmids were related to their phenotypes in respect of lethal zygosis and transfer properties. In vivo recombination tests between pairs of variant plasmids were also done. These physical and genetic studies indicated that determinants of conjugal transfer occupy less than 2.1 kb of the plasmid. A second segment is required for spread of the plasmid within a plasmid-free culture to produce the normal lethal zygosis phenotype: insertion of foreign DNA in this region caused a marked reduction in the diameter of lethal zygosis zones. The minimum replicon was deduced to be 2.1 kb or less in size; adjacent to this region is a 0.5 kb segment which may be required for stable inheritance of the plasmid. The copy number of several derivatives of pIJ101 in *S. lividans* 66 was between 40 and 300 per chromosome and appeared to vary with the age or physiological state of the culture. pIJ101 derivatives have a wide host range within the genus *Streptomyces:* 13 out of 18 strains, of diverse species, were successfully transformed.

Knowledge of dispensable DNA segments and the availability of restriction sites for the insertion of DNA, deduced from the properties of plasmids carrying the *E. coli* plasmid pACYC184 introduced at various sites, was used in the construction of several derivatives of pIJ101 suitable as DNA cloning vectors. These were mostly designed to be non-conjugative and to carry pairs of resistance genes for selection. They include a bifunctional shuttle vector for *E. coli* and *Streptomyces;* a *Streptomyces* viomycin resistance gene of this plasmid is expressed in both hosts.

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Introduction

Since the first physical characterisation of a *Streptomyces* plasmid (Schrempf et al. 1975), several others have been described in a variety of species (e.g. Hayakawa et al. 1979; Malik and Reusser 1979; Okanishi et al. 1980; Nakano et al. 1980). Two plasmids, SCP2* and SLP1.2, have been used as cloning vectors in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans* 66 (Bibb et al. 1980; Thompson et al. 1980). Both have a low copy number (1–5 per chromosome) and a rather narrow host range. These features do not detract from the utility of SCP2* or SLP1.2 derivatives as vectors for many cloning purposes (Chater et al. 1982). However, for other applications of cloning, for example in gene amplification, or the transfer of DNA between a variety of different streptomycete hosts, plasmids of higher copy number and/or broader host range would be useful. We therefore set out to try to discover such plasmids by surveying a series of streptomycetes, by agarose gel electrophoresis, for the presence of CCC DNA. Since we wished to be able to compare the properties of any new plasmids in the well-characterised genetic backgrounds of *S. coelicolor* A3(2) and *S. lividans* 66, we used for the survey a set of strains in the "*S. violaceoruber*" group, to which these two strains belong, in order to maximise the chance that new plasmids could subsequently be transferred to them. Out of 24 strains studied, 11 appeared to contain plasmid DNA and one of them, *Streptomyces lividans* ISP5434, had four small plasmids (pIJ101-104) of high copy number and broad host range (Hopwood et al. 1981). The physical and biological properties of pIJ101-104 are reported here. They, and the several genetically marked derivatives described, have applications both for genetic manipulation in *Streptomyces,* by in vivo and in vitro recombination, and for the understanding of such properties as the nature of conjugal transfer of *Streptomyces* plasmids.

Materials and Methods

Bacterial Strains and Plasmids

Streptomyces lividans ISP5434 was obtained from two sources: Dr L.J. Nisbet of Glaxo Group Research (John Innes Stock No. 2896); and the German Culture Collection (DSM 40434; John Innes Stock No. 2908). Various derivatives of *S. coelicolor* A3(2) and *S. lividans* 66 (Lomovskaya et al. 1972; John Innes Stock No. 1326) are referred to in the text. Apart from strain 1326 itself, which contains two genetically defined plasmids, SLP2 and SLP3 (Kieser et al., to be published), derivatives TK19,

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TK21, TK24, TK126 and 3106 were used as hosts for routine transformations. TK19 (SLP2$^-$ SLP3$^+$) was derived from strain 1326 by loss of SLP2 after protoplasting and regeneration. TK21 (SLP2$^-$ SLP3$^-$) was derived from strain TK19 by loss of SLP3 (Kieser et al., to be published). TK24 (*str-6* SLP2$^-$ SLP3$^-$) is a streptomycin resistant mutant of strain TK21. 3106 (*his-2 leu-2 ura-6 ath-8 ilv-3 str-6* SLP2$^-$ SLP3$^-$) was constructed by repeated rounds of protoplast fusion between derivatives of strain TK21 carrying various auxotrophic mutations. TK126 (Cmls SLP2$^-$ SLP3$^+$), picked as a colony grown from a regenerated 1326 protoplast, was sensitive to 8 μg/ml chloramphenicol and had lost SLP2. Other *Streptomyces* strains, used in studies of the host range of pIJ101, are listed in Table 4. *E. coli* Rec$^+$ strain W5445 (Chi et al. 1978) was used as host for chimaeric plasmids containing *E. coli* plasmid pACYC184 (Chang and Cohen 1978) or pIJ36 (Thompson et al. 1982b).

Culture Conditions and Transformation Procedures

E. coli strain W5445 was grown in Luria broth or on Luria agar (Miller 1972) and transformed by the method of Dagert and Ehrlich (1979). Antibiotic resistant *E. coli* transformants were selected on Luria agar containing 50 μg/ml chloramphenicol, 15 μg/ml tetracycline, 50 μg/ml ampicillin or 1,000 μg/ml viomycin. Protoplasting and transformation of *S. lividans* were as described (Thompson et al. 1982a) except that after the addition of 25% polyethyleneglycol 1,000 the protoplast suspension was spread directly on regeneration medium (R2YE: Thompson et al. 1980) without further washes. For direct selection of antibiotic resistant transformants the regeneration plates (containing 25 ml medium) were incubated for 8–20 h at 32° C to allow for expression of the resistance genes and then overlayed with 2.5 ml soft Nutrient Agar (Difco) containing 100 μg/ml neomycin, 500 μg/ml thiostrepton or 300 μg/ml viomycin.

General cultural and genetic techniques for *Streptomyces* were those of Hopwood et al. (1973). Tests of lethal zygosis were made on plates of R2YE spread with dense suspensions of various indicator strains. Crosses were made on slants of R2YE supplemented with required growth factors. Progeny from crosses were selected by plating on suitably supplemented minimal medium (MM: Hopwood 1967). Antibiotic concentrations in MM were 1 μg/ml neomycin, 4 μg/ml streptomycin, 50 μg/ml spectinomycin, 50 μg/ml thiostrepton or 30 μg/ml viomycin.

DNA Isolation and Manipulation

Plasmids were isolated from *E. coli* by the procedure of Birnboim and Doly (1979). For the isolation of plasmid pIJ101 and its derivatives from *S. lividans* several methods were used successfully. The procedure finally adopted is based on the methods of Birnboim and Doly (1979) and McMaster et al. (1980). It gave plasmid DNA suitable for digestion with restriction enzymes, ligation and transformation. 50 ml TNB (Tryptone Soya Broth, Oxoid CM129) were inoculated with ca. 10^7 spores and heated to 50° C for 10 min. Incubation in shaken flasks containing stainless steel springs for dispersion was at 30–33° C for 16–20 h. Mycelium was harvested by filtration or centrifugation, washed with 10.3% sucrose and incubated for ca. 30 min in 2 ml lysozyme solution (10.3% sucrose, 100 ml; 2.5% K_2SO_4, 1 ml; 0.5% KH_2PO_4, 1 ml; 2.5 M $MgCl_2$, 0.1 ml; 0.25 M $CaCl_2$, 1 ml; 1 M TRIS-HCl (pH 8), 2.5 ml; 50% glucose, 4 ml; lysozyme, 100 mg). Lysis was achieved by adding 2 ml of 50° C lytic mix (5 M NaOH, 10 ml; 10% SDS, 20 ml; 0.25 M CDTA (cyclohexane diamine tetraacetate, Sigma), 25 ml; water, 45 ml). Thorough mixing was achieved by vigorous pipetting. After ca. 15 min incubation at room temperature, with further mixing at intervals, 4 ml phenol/chloroform (AnalaR phenol, 500 g; hydroxyquinoline, 0.5 g; 0.5 M NaCl, 200 ml; chloroform, 500 ml) were added and the mixture was agitated on a vortex mixer for 30 s. The samples were then centrifuged for 10 min at 10,000 rpm in a Sorvall SS 34 rotor to separate the phases. The upper aqueous phase could be loaded directly on to agarose gels without additions. For transformation and enzyme reactions phenol was removed by ether extraction and the samples were twice precipitated with ethanol.

DNA ligase was prepared by C.M. Bruton by the method of Murray et al. (1979) and used as described by Hepburn and Hindley (1979). *Sal*GI endonuclease was prepared by C.M. Bruton; all others were from commercial sources. DNA polymerase buffer (10 mM TRIS-HCl pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol) was used for all restriction enzyme digestions except for *Bam*HI, *Eco*RI, *Mbo*I, *Sma*I and *Xho*I which were used under the conditions recommended by the manufacturer. Reactions were terminated by heating to 65° C for 5 min, except for *Bcl*I where ethanol precipitation was used to stop the reaction. Agarose gel electrophoresis was performed with horizontal slab gels in either 90 mM TRIS-borate-2 mM EDTA or in 40 mM TRIS-5 mM sodium acetate-1 mM EDTA, adjusted to pH 7.8 with glacial acetic acid. Agarose concentrations of 0.7, 1.0 and 1.4% were used, depending on the fragment sizes to be resolved. For the separation of linear fragments, ethidium bromide (0.5 μg/ml) was generally included in the electrophoresis buffer. Polyacrylamide gel electrophoresis was performed with vertical slab gels in 90 mM TRIS-borate-2 mM EDTA by the method of Maniatis et al. (1975). Fragment sizes were determined by the method of Southern (1979), using a Huston digitizer connected to an Apple II computer for measuring the migration distances. Phage lambda DNA digested with *Hin*dIII (Daniels et al. 1980) and pBR322 DNA digested with *Hae*III (Sutcliffe 1978) were used as size standards.

Plasmid Copy Number Determination

For the determination of plasmid copy number mycelium was grown and protoplasted in the same way as for plasmid preparation. Lysis was achieved by the addition of 1 volume 1% SDS, 25 mM CDTA. Protoplasts and lytic mix were mixed by pipetting and then heated to 70° for 10 min. After cooling, the samples were vortexed for 1 min with 1 volume phenol:chloroform (1:1) and spun for 15 min in an Eppendorf centrifuge. The aqueous supernatant was then serially diluted in 14% sucrose, 10 mM EDTA, 100 μg/ml preheated RNase and 0.1% bromphenol blue. After incubation at 37° C for 1 h samples for each dilution were loaded on to agarose gels for electrophoresis. The gels were stained for ca. 2 h in ethidium bromide (0.5 μg/ml) and irradiated with short wavelength UV in order to nick the CCC plasmids. The gels were destained in several changes of water and photographed. The negatives were scanned with a Joyce-Loebel densitometer to determine the peak areas of chromosomal and plasmid DNA. The dilution factor necessary to equalise these peak areas then gave the ratio of plasmid to chromosomal DNA.

Results

Characterisation of Small Plasmids in S. lividans ISP5434

S. lividans ISP5434 was obtained from two sources (see Materials and Methods). Both cultures, 2896 from Glaxo and 2908 from the German Culture Collection, contained small-sized plasmid

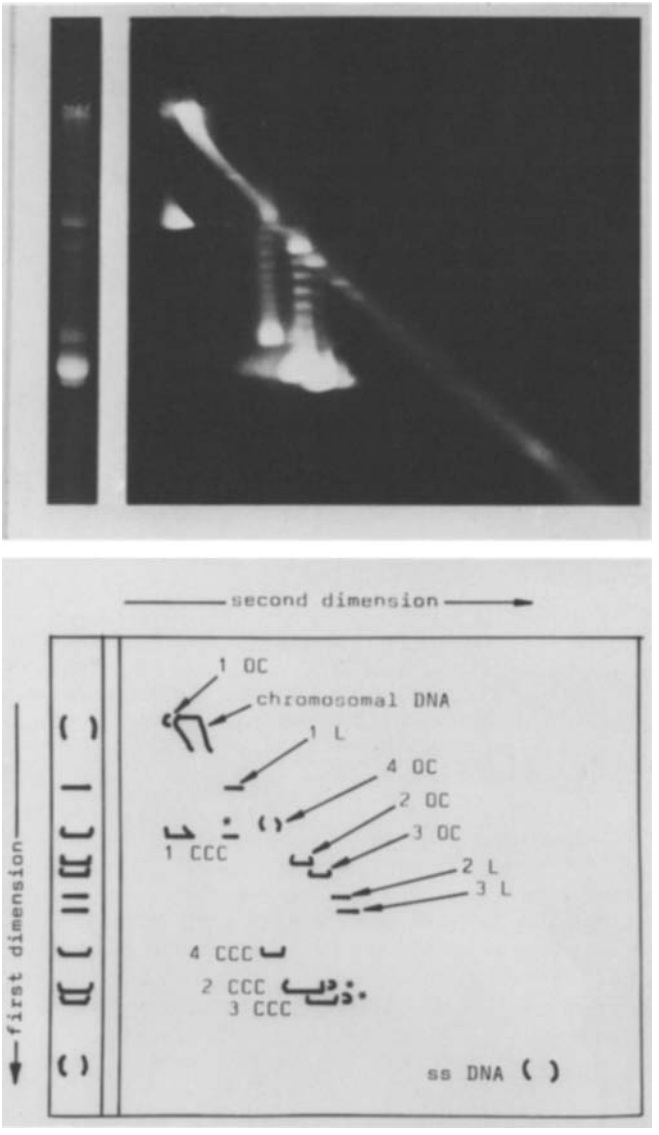

Fig. 1. Two-dimensional agarose gel electrophoresis (see Hintermann et al. 1981) of plasmid DNA isolated from strain 2908. After running in the first dimension (0.7% agarose, 90 mM Tris-borate, 10 h at 1.5 V/cm) the gel was stained with 0.5 μg/ml ethidium bromide for 2 h and photographed under 300 nm UV illumination. In the presence of ethidium bromide, DNA is nicked by the UV, causing supercoiled (CCC) molecules to relax to the open circular (OC) form but inducing insignificant changes in OC or linear DNA. The gel track was cut out, cast into a new agarose gel and run in the second dimension (0.8% agarose, 90 mM Tris-borate, 8 h at 2.5 V/cm). The increase in the agarose concentration and in the voltage makes the linear DNA molecules run slightly faster relative to the circular form. This allowed the separation of the OC form of plasmid pIJ101 (1 OC) from the chromosomal DNA. On the second-dimension gel several DNA species can be identified. Four supercoiled species (1–4 CCC), recognisable by their slower migration speed compared to the first dimension, represent plasmids pIJ101-4 respectively. The corresponding OC forms have run the same distance as the UV-nicked supercoiled molecules. The bands between each CCC form and its OC form, not included in the diagrammatic representation of the gel, are molecules with different numbers of supercoil turns. The other bands represent linear forms of the plasmids (L marks the linear species that were in the original preparation, and (*) marks the linear forms generated by nicking from the CCC forms). The fastest running band possibly represents single-stranded DNA (ssDNA)

DNA in large amounts. Plasmid samples prepared from both cultures by the alkaline lysis procedure gave a large number of bands, some very intense, when fractionated by agarose gel electrophoresis. Two-dimensional separation by the method of Hintermann et al. (1981) revealed supercoiled DNA species of four different sizes, present as molecules with various numbers of supercoil turns and as linear forms (Fig. 1). The plasmids were of sizes 8.9 kb (pIJ101), 4.2 kb (pIJ102), 3.9 kb (pIJ103) and 4.9 kb (pIJ104). In strain 2896, pIJ101 and pIJ102 were the most abundant plasmids, while in strain 2908, pIJ102 and pIJ103 predominated. Incompletely supercoiled DNA was most abundant in young, fast growing cultures; DNA from older or slow growing cultures showed fewer such forms. In extreme cases, especially with larger in vitro constructed derivatives of pIJ101 (see below), the fully supercoiled form of the plasmid was not more abundant than the species with fewer supercoil turns; on agarose gels this sometimes resulted in a smear that was not easily recognised as plasmid DNA.

Transfer of Plasmids to S. lividans 66 and Relationships Between the Plasmids of Different Size

When either strain 2896 or strain 2908 was grown in mixed culture with strain CT2, a streptomycin-resistant derivative of *S. lividans* 66, and the resulting spores were plated on streptomycin-containing plates with an added excess of strain CT2, numerous zones of lethal zygosis (pocks) were seen. Such pocks are characteristic of the presence of conjugative plasmids (Bibb et al. 1977, 1978, 1981; Bibb and Hopwood 1981). Pocks were also observed after transformation of *S. lividans* protoplasts with plasmid DNA preparations from strains 2896 or 2908. From the pocks, *S. lividans* 66 progeny were isolated that reproducibly gave the lethal zygosis (Ltz^+) phenotype on *S. lividans* 66 derivatives. They were found to contain either the largest plasmid (pIJ101) alone or various mixtures of the original plasmids. Plasmid DNA isolated from strains 2930 (containing pIJ101), T208 (containing mainly pIJ102 together with a small proportion of pIJ101), and 2890 (containing mainly pIJ103 with a minority of pIJ101) was digested with a series of restriction endonucleases. All three plasmids were cleaved once by *Bam*HI, *Kpn*I, *Sst*I and *Xho*I and double digests showed that the four sites were arranged identically in the three plasmids. None of the plasmids was cleaved by *Eco*RI, *Cla*I, *Hin*dIII, *Hpa*I or *Xba*I. Digestion with each of the enzymes *Bcl*I, *Bst*EII, *Sma*I and *Sst*II produced fragments nearly all of which were identical in size for all three plasmids; for each enzyme, one fragment was particular to each of the smaller plasmids. This suggested that the smaller plasmids were each related to the largest member of the group, pIJ101, by a simple deletion (the deletion would fuse parts of two restriction fragments of pIJ101 to generate a unique fragment for each derivative, as observed). Homology between the three plasmids was further tested by comparing the fragment pattern generated by *Mbo*I or *Taq*I on polyacrylamide gels. *Mbo*I cleaved pIJ101 at $\geqq 36$ sites and pIJ102 and pIJ103 at $\geqq 23$ sites, with the largest fragment being ca. 0.6 kb. *Taq*I cleaved pIJ101 at $\geqq 23$ sites and the two smaller plasmids at $\geqq 10$, the largest fragment being 0.9 kb. Again, all except one fragment of the smaller plasmids was identical in size to fragments generated from the larger plasmids. This extensive sequence homology (more than 40 sites conserved) suggests a recent origin of the smaller plasmids from pIJ101. Since no strain was found that contained pIJ104 as the major species, only a limited amount of information about it could be obtained from digests of samples containing all four plasmids, and these data suggested that pIJ104 is also a derivative of pIJ101.

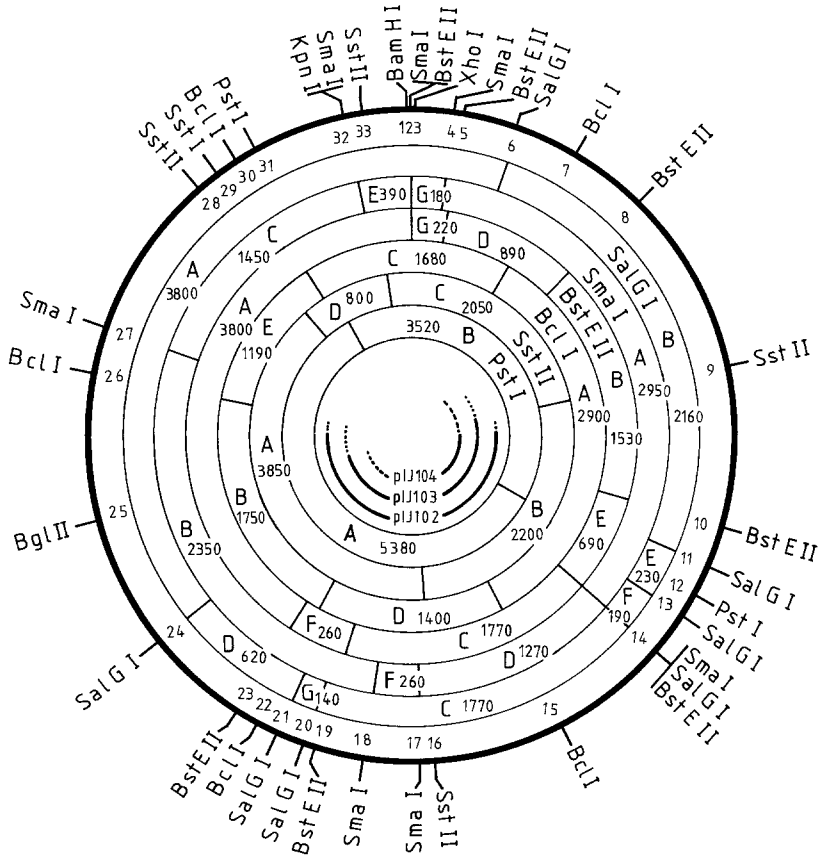

Fig. 2. Restriction endonuclease cleavage map of pIJ101 (8.9 kb). The restriction sites are numbered for easier reference. pIJ101 is cut once by *Bam*HI, *Bgl*II, *Kpn*I, *Sst*I and *Xho*I. Estimates of restriction fragment sizes produced by endonucleases with two or more recognition sites on the plasmid are given to the nearest 10 base pairs. In the centre of the diagram are shown the approximate limits of the deletions presumed to have given rise to plasmids pIJ102–104

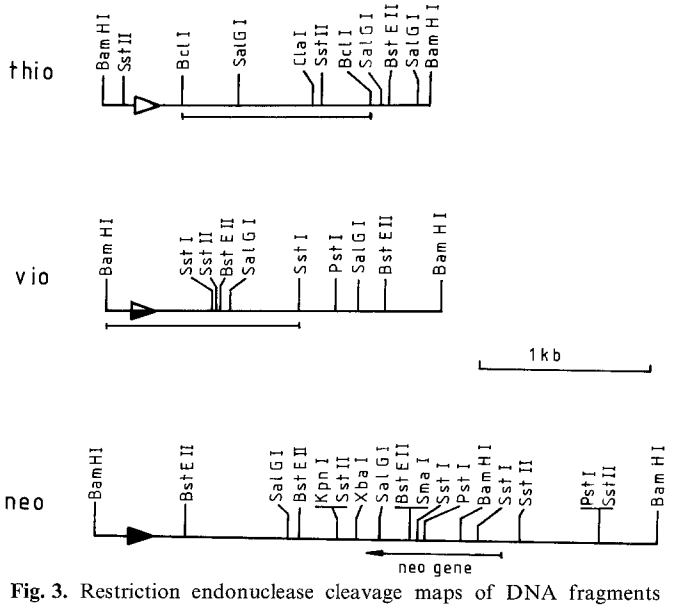

Fig. 3. Restriction endonuclease cleavage maps of DNA fragments carrying thiostrepton resistance (*tsr*) from *S. azureus*, viomycin-resistance (*vph*) from *S. vinaceus* and neomycin (aminoglycoside) resistance (*aph*) from *S. fradiae*. The *tsr* and *vph* genes are located within the limits of the bars. The *aph* gene has been tentatively located by DNA sequencing (C.J. Thompson and G. Gray, unpublished results), as shown. (Data from Thompson et al. 1982b)

Restriction Endonuclease Cleavage Map of pIJ101

The restriction map of pIJ101 is in Fig. 2. The arrangement of cleavage sites for the enzymes *Bam*HI, *Kpn*I, *Bgl*II, *Sst*I, *Xho*I (single sites) and *Pst*I (two sites) was determined by measuring the fragment sizes generated by digests of pIJ101 with each combination of two enzymes. The arrangement of the fragments generated by *Sal*GI (8 sites), *Sma*I (7 sites), *Bst*EII (7 sites), *Bcl*II (5 sites) and *Sst*II (4 sites) was deduced from size measurements of single digests with each enzyme and double digests, using the *Bam*HI, *Kpn*I, *Bgl*II, *Pst*I and *Xho*I cleavage sites as reference points. Information from insertion and deletion mutants of pIJ101 (see below) was also used. Thus, for positions 16 to 23 (Fig. 2), measurements from the single *Cla*I site in the thiostrepton fragment in plasmid pIJ328 were used as an additional criterion. The positioning of the fragments *Sal*GI-F and -G, *Sma*I-F and *Bst*EII-F depends entirely on data from deletion and insertion mutants. The fragment *Sal*GI-H (80 bp) was not positioned and is therefore not included in the map. In the centre of the map the approximate limits of the deletions in plasmids pIJ102, pIJ103 and pIJ104 are shown, deduced from a comparison of the sizes of restriction fragments.

Construction in vitro of Insertion and Deletion Variants of pIJ101

Analysis of the biological functions of pIJ101 was hampered by the lack of a selectable marker apart from pock formation. Use was therefore made of cloned DNA fragments carrying antibiotic resistance genes, for neomycin (*aph*), thiostrepton (*tsr*) and viomycin (*vph*), which had been cloned on to plasmid SLP1.2 (Thompson et al. 1980, 1982a, 1982b). These fragments (from pIJ2, pIJ6 and pIJ13 respectively) are flanked by *Bam*HI sites (Fig. 3) and can therefore be ligated with cohesive ends in pIJ101 generated by *Bam*HI, *Bcl*I, *Bgl*II or *Mbo*I.

Partial digestion of pIJ101 with *Bcl*I or *Mbo*I and ligation with the above resistance fragments was successful. The resistant transformant colonies isolated from the primary protoplast regeneration plates generally contained a large majority of wild-type plasmids and only few copies of the hybrid plasmids which

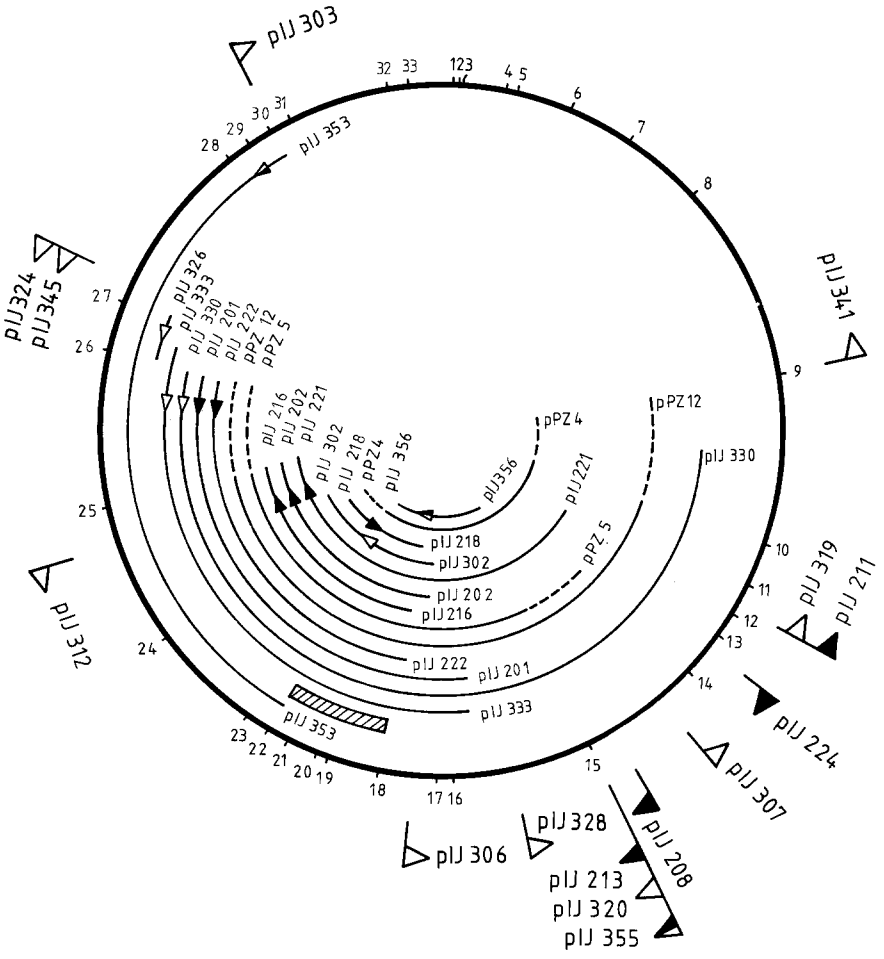

Fig. 4. Positions and orientations on the map of pIJ101 of insertions (▷) and deletions (—▷) involving the *tsr* fragment (▷), *vph* fragment (▶) and *aph* fragment (▶) shown in Fig. 3. Numbers on the circumference of the circle are restriction enzyme sites in pIJ101 defined in Fig. 2. The shaded bar indicates the overlap region of all single deletions giving rise to the Ltz⁻ phenotype. (See Table 1 for a list of the plasmids)

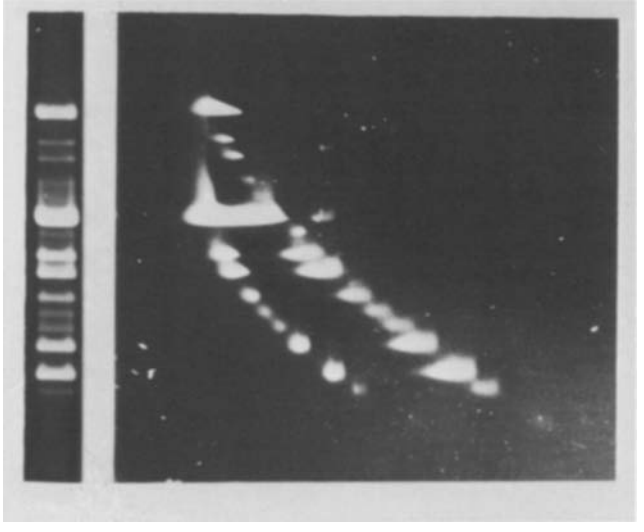

Fig. 5. Two-dimensional gel analysis (see Fig. 1 for details) of plasmid DNA from strain TK103 carrying plasmid pIJ353. Note the numerous in vivo derived deletions of the parent plasmid. (Note that CCC DNA was only partially nicked)

in most cases were only faintly visible, or even invisible, on gels. Retransformation, combined with early selection of resistant individuals by overlaying the protoplasts with soft agar containing antibiotic to reduce plasmid spread, was used successfully to purify strains carrying individual resistance plasmids, and a large series was collected. As expected, most contained an insert of DNA corresponding to the resistance fragment used in the ligation, often together with a deletion of part of the host plasmid. The position and orientation of each insert and the extent of the deletions were determined by agarose gel electrophoresis of appropriate restriction digests. Figure 4 summarises the results of this analysis. Derivatives that could not be explained as simple insertion or deletion variants of pIJ101 were excluded. (Included in Fig. 4 are the plasmids pIJ353, a viomycin and neomycin resistant derivative (see below) of pIJ208, and pPZ4, pPZ5 and pPZ12, which are derivatives of pIJ303 that had undergone in vivo deletions after transformation into *S. rimosus* (see below).)

The positions of the inserts and the range of deletions found seem to be non-random. All the deletions (with the exception of pIJ353 which carries two resistance fragments and pIJ326) overlap in the region between positions 18 and 22, marked with a shaded bar in Fig. 4. This non-random distribution could be due either to preferential cutting of pIJ101 DNA by *Mbo*I or to the biological properties of pIJ101, which may render certain derivatives unable to survive or to compete with other plasmids.

Some derivatives of pIJ101, after purification by retransformation, gave strains containing several plasmids. An extreme example is strain TK103 which, apart from plasmid pIJ353, contained eight smaller CCC species (Fig. 5). Although no plasmids smaller than 3.9 kb (the size of pIJ103) were generated in vivo it was possible to produce in vitro stable derivatives of pIJ102 consisting of less than 3 kb of the plasmid sequence (e.g. pIJ58, pIJ350 and pIJ364: see later). Structural instability was observed only with derivatives of pIJ101, never with derivatives of pIJ102 or pIJ103 (see below).

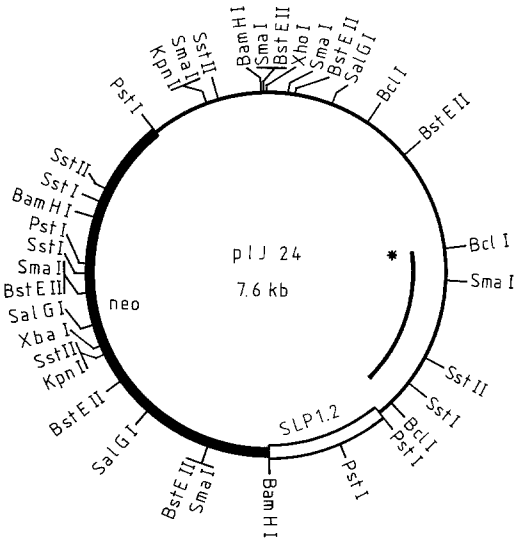

Fig. 6. Restriction enzyme cleavage map of pIJ24. The bar (*) indicates the *Bcl*I fragment deleted to give rise to pIJ363

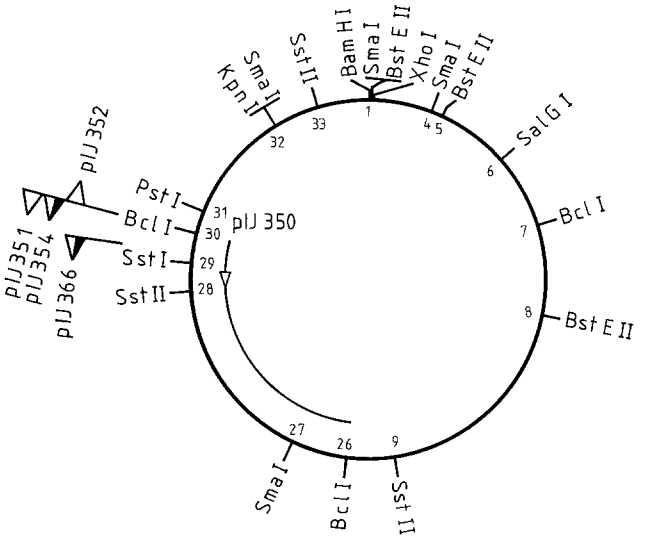

Fig. 7. Restriction enzyme cleavage maps of plasmids derived from pIJ102 by insertion of *tsr* or *vph* fragments (in the case of pIJ350, together with a deletion). For meaning of symbols, see Fig. 4

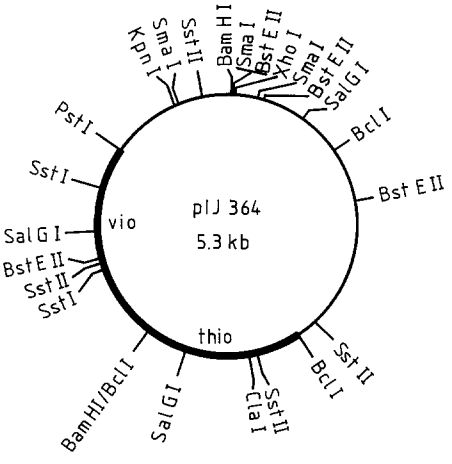

Fig. 8. Restriction enzyme cleavage map of pIJ364

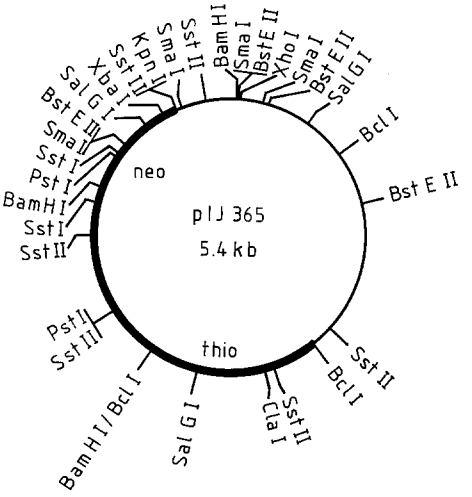

Fig. 9. Restriction enzyme cleavage map of pIJ365

Derivatives of pIJ102 and pIJ103

pIJ24 (Fig. 6) was constructed by inserting a *Pst*I fragment from a partial digestion of the neomycin-resistant SLP1.2 derivative pIJ2 (Thompson et al. 1980, 1982b) into the *Pst*I site of pIJ103. pIJ363 (Fig. 6) was generated from pIJ24 by in vitro deletion of a *Bcl*I fragment (corresponding to *Bcl*I-E in pIJ101 (Fig. 2)). pIJ24 and pIJ363 carry 0.81 kb of the dispensable region of SLP1.2 in addition to the neomycin resistance (*aph*) fragment of pIJ2 originating from *S. fradiae*.

pIJ350, pIJ351 and pIJ352 (Fig. 7) were generated by ligating pIJ102 DNA partially digested with *Bcl*I and a 1.1 kb *Bcl*I fragment (Fig. 3) from pIJ6 (Thompson et al. 1980, 1982b) carrying the thiostrepton resistance determinant (*tsr*): pIJ351 and pIJ352 are simple insertions, while pIJ350 carries a deletion. pIJ366 (Fig. 7) carries the 1.9 kb *Bam*HI fragment (Fig. 3) coding for viomycin resistance (*vph*) from pIJ13 (Thompson et al. 1982c) inserted into a *Mbo*I site of pIJ102. pIJ354 carries the same *vph* fragment inserted into a *Bcl*I site of pIJ102.

pIJ364 (Fig. 8) was constructed by combining pIJ350 digested partially with *Bcl*I and completely with *Pst*I with the *Bam*HI – *Pst*I *vph* fragment from pIJ36 (pBR322 with a *Bam*HI viomycin resistance fragment inserted at its *Bam*HI site: Thompson et al. 1982b). This procedure eliminated a 90 bp fragment from pIJ350.

pIJ365 (Fig. 9) was constructed by first inserting the *Cla*I fragment of pIJ41 (Thompson et al. 1982b), carrying the *aph* gene and part of the *tsr* gene, into the *Cla*I site of pIJ350 in the orientation that allowed restoration of thiostrepton resistance. From this intermediate plasmid a *Kpn*I fragment was eliminated, deleting all SLP1.2 sequences, a 0.51 kb fragment of pIJ350 (corresponding to positions 30 – 32 of pIJ101), and part of the neomycin fragment without destroying the *aph* gene.

pIJ360 (Fig. 10) was constructed by inserting a *Pst*I fragment carrying the *vph* gene from plasmid pIJ36 into the single *Pst*I site of pIJ350. pIJ360 contains a 1.125 kb sequence of pBR322. pIJ361 (Fig. 11) is a combination of pIJ351 (Fig. 7) digested with *Sst*I and pIJ36 partially digested with *Sst*I. pIJ361 contains the complete sequence of pBR322 and replicates both in *Streptomyces* and in *E. coli*. In *Streptomyces*, thiostrepton and viomycin-resistances are expressed and in *E. coli* ampicillin and viomycin-resistances can be used for selection.

pIJ58 (Fig. 12) was constructed by ligating pIJ350 and pIJ41

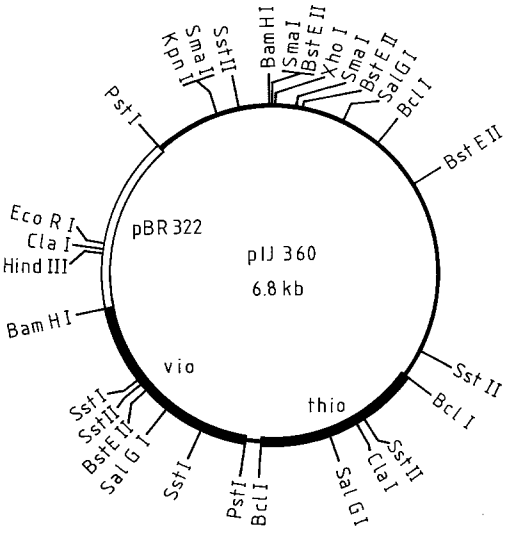

Fig. 10. Restriction enzyme cleavage map of pIJ360 (the indicated segment of pBR322 lacks replication functions; therefore this plasmid does not replicate in *E. coli*)

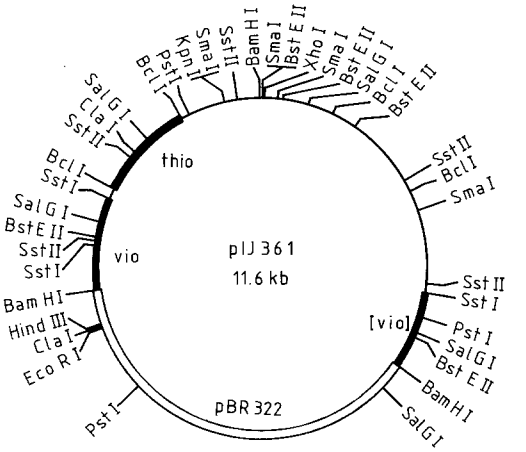

Fig. 11. Restriction enzyme cleavage map of pIJ361. This plasmid contains the whole of pBR322 and is therefore capable of replicating in *E. coli* as well as in *Streptomyces*. (The fragment indicated [vio] is derived from the vio fragment in Fig. 3 but lacks the *vph* gene; the fragment indicated vio carries the *vph* gene, which is expressed in both *E. coli* and *Streptomyces*)

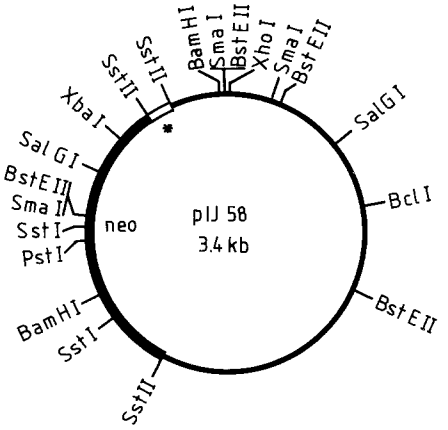

Fig. 12. Restriction enzyme cleavage map of pIJ58. *Indicates an extra *Sst*II fragment, probably from pIJ41

DNA, both completely digested with *Sst*II; it consists of three *Sst*II fragments, one carrying the *aph* gene, another all pIJ101 functions needed for replication, and a third (0.1 kb) probably coming from pIJ41.

The plasmid preparations of the pIJ102 and pIJ103 derivatives showed no evidence of the presence of any other plasmid species when present in *S. lividans* 66 or *S. coelicolor* A3(2) derivatives. This, and the high transformation frequency obtained with plasmid pIJ350 (up to 5×10^6 transformants per μg plasmid DNA: E.T. Seno, personal communication), suggests that these plasmids can survive independently. Furthermore, it is very unlikely that pIJ361 could have been transferred from *S. lividans* to *E. coli* and back to *S. lividans* if a second, "helper", plasmid were needed to compensate for the deleted sequences of pIJ101.

All functions necessary for plasmid survival must be located in the 2.1 kb *Sst*II fragment of pIJ58, between positions 9 and 33 of pIJ101. In fact the minimum replicon must be slightly shorter than this because the *Sst*II site corresponding to position 9 of pIJ101 is deleted in pIJ103 (Fig. 2).

Probing of the BglII site of pIJ101

Attempts to insert the *Bam*HI fragments carrying the *aph, tsr* or *vph* genes into the *Bgl*II site of pIJ101 failed; this could have been due to technical problems (e.g. non-specific nuclease contamination in the enzyme preparation or masking of the cohesive ends by the *Bgl*II enzyme) or to a biological problem.

Technical problems were excluded by ligating *Bam*HI-digested pIJ36 with *Bgl*II-digested pIJ302, pIJ218 or pPZ4 (Fig. 4) and transforming both *E. coli* (selecting for ampicillin-resistance and screening for tetracycline-sensitive colonies) and *S. lividans* (selecting for viomycin-resistance). No viomycin-resistant *S. lividans* transformants were found, but analysis of the tetracycline-sensitive *E. coli* transformants showed that most of them actually contained hybrid plasmids, indicating proper ligation of the *Bgl*II-cut DNA.

In a second experiment, the *E. coli* plasmid pACYC184, digested with *Bam*HI, was ligated to the plasmids pIJ355 and pIJ356 (Fig. 4) digested with *Bgl*II. (This construction leaves the *vph* determinant in a position in pIJ101 where it is known to be expressed in *S. lividans*.) Chloramphenicol-resistant, tetracycline-sensitive *E. coli* transformants with the two plasmids joined in both orientations were identified. These plasmids, also, failed to transform *S. lividans* protoplasts (selection was for viomycin-resistance or chloramphenicol-resistance). Certain *E. coli* transformant strains contained plasmids composed of two copies of pIJ355 in tandem and one copy of pACYC184. These did transform *S. lividans*, excluding the possibility of a restriction system in *S. lividans* that efficiently eliminates pIJ101 DNA propagated in *E. coli*. Plasmid DNA isolated from these viomycin-resistant *S. lividans* transformants no longer showed the tandem duplication, probably because of homologous recombination between the two pIJ355 parts of the hybrid plasmids. Restriction specifically active against the pACYC184 sequence is unlikely to be the cause of the failure to transform the simple hybrid plasmids into *S. lividans* since pIJ366-pACYC184 hybrids grown in *E. coli* could be transformed into *S. lividans* (see below).

pIJ355 derivatives with pACYC184 inserted in one orientation allowed growth of the *E. coli* host on 1,000 μg/ml viomycin,

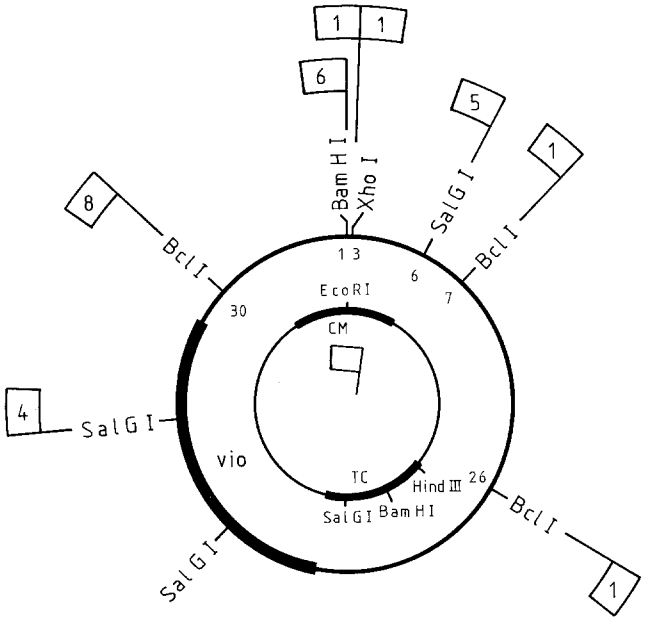

Fig. 13. Positions of insertions of pACYC184 into restriction enzyme sites on pIJ366. The sites are numbered according to the map of pIJ101 in Fig. 2. The map of pACYC184 is shown in the centre of the figure; the flags show the orientation of this plasmid in the various sites on pIJ366 and the numbers in the flags indicate the frequency of occurrence of each class of insertion

whereas hybrid plasmids with pIJ356 in either orientation did not.

The above experiments suggest that, unexpectedly, the unique *Bgl*II site of pIJ101 is not available for cloning in *Streptomyces*.

Probing of the BamHI, SalGI, XhoI and BclI sites in the "core region" of pIJ101

For the probing of the *Bam*HI, *Xho*I, *Sal*GI and *Bcl*I sites (positions 1, 3, 6 and 7 on the map of pIJ101) the viomycin-resistant pIJ102 derivative pIJ366 (Fig. 7) was combined with the *E. coli* plasmid pACYC184 linearised with either *Bam*HI (for *Bam*HI and *Bcl*I) or *Sal*GI (for *Sal*GI and *Xho*I). pIJ366 was chosen for this experiment because viomycin-resistance has the potential of being expressed in both *E. coli* and *Streptomyces* and because pIJ366 carries the *Bcl*I site from position 30 in pIJ101 which was known to be available for cloning (see below) and could serve as a control. pACYC184 was chosen because its chloramphenicol resistance has the potential of being expressed in *Streptomyces* (Schottel, Bibb and Cohen 1981). The hybrid plasmids found in *E. coli* are indicated in Fig. 13. It is noticeable that only for the *Xho*I site were plasmids joined in both orientations found. This could be due either to the ligase being responsive to the DNA sequence around the joining point, or perhaps the pIJ366 DNA, depending on its orientation, could affect the viability of the transformed *E. coli* cells. There is also a non-random distribution of the frequency of inserts found in the different *Bcl*I and *Sal*GI sites, probably at least in part due to preferential cleavage of certain sites of pIJ366 DNA by the two enzymes.

Not unexpectedly, because they lie in the region of only 2.1 kb deduced to contain the replication functions of pIJ101, insertion into the *Bam*HI, *Xho*I, *Sal*GI and *Bcl*I sites corresponding to positions 1, 3, 6 and 7 in pIJ101 resulted in plasmids that could not be transformed into *S. lividans*. Insertion into one of the *Sal*GI sites in the viomycin fragment possibly inactivated the *vph* gene and therefore prevented the detection of viomycin-resistant *S. lividans* transformants. Plasmids were found, however, that consisted of one molecule of pACYC184 and two molecules of pIJ366 joined as direct repeats at either the *Bam*HI or the *Sal*GI site at position 6. These plasmids gave rise to viomycin-resistant *S. lividans* transformants. When the simple hybrid plasmids consisting of only one copy each of pACYC184 and pIJ366 joined at the *Bam*HI or *Sal*GI(6) sites were digested with *Bam*HI or *Sal*GI and religated, viomycin-resistant transformants were obtained. This proves that insertion into these sites, and not other alterations, either destroyed the ability of these plasmids to replicate in *S. lividans* or prevented expression of viomycin-resistance. Insertion into the two remaining *Bcl*I sites (26 and 30) gave plasmids that could replicate in both *E. coli* and *S. lividans*. No increase in chloramphenicol resistance was observed when these plasmids were transformed into chloramphenicol sensitive *S. lividans* strain TK126, which does not grow on MM containing 8 μg/ml chloramphenicol. Plasmid DNA isolated from *S. lividans* and *E. coli* gave fragments of identical size when digested with *Sal*GI and was therefore assumed to be unaffected by the interspecific transformation. Viomycin-resistance in *E. coli* was detected only with the hybrid plasmids joined at the *Bcl*I site at position 30 and for these plasmids direct selection of viomycin-resistant transformants in *E. coli* was possible. Hybrid plasmids joined at the *Bcl*I site at position 26, or at the *Sal*GI site in the viomycin fragment, or clockwise at the *Xho*I site, gave no detectable increase in viomycin resistance. All the other plasmids led to a slight increase in viomycin-resistance which was too small to allow direct selection.

Estimates of Plasmid Copy Number

The method depended on a comparison of densitometer traces of chromosomal DNA and of plasmid DNA bands. Assuming 10^4 kb for the size of the chromosomal DNA (Benigni et al. 1975) between 40 and 300 (average of 170) copies per chromosome were found for pIJ101 and six of its derivatives (pIJ208, pIJ303, pIJ312, pIJ350, pIJ355, pIJ366), all in *S. lividans* 66. The copy number of plasmid pIJ350 in 24 and 48 h old cultures was found to be 70 and 250 respectively. This suggests that the observed wide range in copy number may mainly reflect different physiological ages of the cultures rather than differences between the plasmids studied.

Plasmid Stability

The resistances conferred on *S. lividans* strains by the various derivatives of pIJ101 were found to be inherited stably without the need for selection. However, the 0.5 kb sequence between the *Pst*I site at position 31 and the *Sst*II site at position 33 (Fig. 2) appeared to be necessary for stable plasmid inheritance since pIJ58 (Fig. 12) and pIJ365 (Fig. 9), with deletions in this region, were markedly unstable (13% and 55% loss after one round of growth on antibiotic-free medium).

Lethal Zygosis and Transfer Phenotypes of pIJ101 and Its Derivatives

Strains carrying pIJ101 alone produced large distinct pocks on plasmid-free *S. lividans* 66 derivatives and on strains containing one or more of the plasmids SCP1, SCP2*, SLP1, SLP2, SLP3 or SLP4 (Table 1). Thus, pIJ101 determines an Ltz^+ phenotype of different specificity from that shown by these other plasmids. Since strains carrying pIJ101 were resistant to this Ltz reaction, pIJ101 also carries an Ltz^R determinant. In contrast, all strains

containing a derivative of pIJ102 or pIJ103 as the only detectable plasmid showed no lethal zygosis on plasmid-free strains (they were Ltz^-) and were fully sensitive to the lethal zygosis elicited by pIJ101 (they were Ltz^S), suggesting that both Ltz^+ and Ltz^R determinants are carried on that part of pIJ101 deleted in pIJ102 and pIJ103.

In an attempt to localise the Ltz^+ and Ltz^R determinants on pIJ101, the lethal zygosis phenotype of each of the antibiotic resistant derivatives shown in Fig. 4 was determined. All the derivatives carrying deletions (except pIJ326 which lacks a very small segment of DNA from the region between restriction sites 26 and 27) were Ltz^-, as was just one of the insertion derivatives, pIJ306. Strains carrying the remaining insertion derivatives all produced pocks, but these were of two distinct sizes. Plasmids with insertions in the "upper" half of pIJ101 (Fig. 4) running clockwise from site 27 to site 12 (i.e. pIJ324, pIJ345, pIJ303, pIJ341, pIJ319 and pIJ211) yielded pocks identical in size to those of pIJ101 (Fig. 14). The remaining plasmids, with insertions in the "lower" half of pIJ101 (i.e. pIJ224, pIJ307, pIJ208, pIJ213, pIJ320, pIJ355, pIJ328 and pIJ312), gave rise to much smaller pocks, usually less than one quarter of the diameter of those of pIJ101 (Fig. 14); their lethal zygosis phenotype was designated $Ltz^{+/-}$ (Table 1). These results confirmed that DNA in the segment of pIJ101 missing from pIJ102 is responsible for the lethal zygosis phenotype.

The difference between the Ltz^+ and $Ltz^{+/-}$ phenotypes was found to reflect a difference in the distance to which the two classes of plasmids migrated from the centre of the pocks which they initiated. Pocks produced by pIJ303 (*tsr*, Ltz^+) and pIJ224 (*aph*, $Ltz^{+/-}$), each in a strain carrying a chromosomal mutation (*str* and *spc* respectively), within a lawn of a plasmid-free strain carrying a different chromosomal marker (*spc* and *str* respectively), were replica plated to two media, selecting the antibiotic resistance determined by the plasmid (thiostrepton or neomycin), or the chromosome of the plasmid's original host (spectinomycin or streptomycin). The resistance carried by the plasmid was found in an area exactly corresponding to the visible manifestation of the pock: large in the case of the Ltz^+ plasmid, small in that of the $Ltz^{+/-}$ plasmid. In both cases the chromosomal marker of the plasmid's original host was detectable only in the centre of the pocks (Figs. 15 and 16). In strains containing both a Ltz^+ and a Ltz^- derivative of pIJ101, the markers of the Ltz^- plasmid did not spread in lawns of plasmid-free strains. Similarly, $Ltz^{+/-}$ plasmids were not induced to spread over a greater distance by the presence of a Ltz^+ plasmid. Thus, both functions, plasmid transfer and plasmid "spread", acted in *cis*.

The Ltz^R or Ltz^S reaction of each of the Ltz^- derivatives of pIJ101, when tested by a strain carrying the Ltz^+ derivative pIJ303, was less clearly determined. A few (notably the only Ltz^- *insertion*, pIJ306) appeared to be fully resistant, but the reaction of many of the Ltz^- *deletions* was ambiguous and in several cases an intermediate reaction was apparent. Thus the determinant(s) of the Ltz^R phenotype could not be localised precisely.

Genetically marked derivatives of *S. lividans* 66 undergo a low but unambiguously detectable level of chromosomal recombination (Bibb et al. 1981). This is due, at least primarily, to the presence of an endogenous plasmid, SLP2, perhaps with a contribution from a second plasmid, SLP3; pairs of $SLP2^-$ $SLP3^-$ strains yield no detectable recombinants when crossed (Kieser et al., to be published). The availability of such "cured" strains allowed the self-transfer properties of derivatives of pIJ101, and their possible mobilisation by other known plasmids, to be determined. Each plasmid was transformed into an $SLP2^-$ $SLP3^-$ strain and the resulting cultures were crossed with an $SLP2^-$ $SLP3^-$ strain carrying *str-6*. Plating the products of the crosses on streptomycin plus the antibiotic appropriate to each pIJ101 derivative (neomycin, thiostrepton or viomycin) and comparing the colony counts with those on streptomycin alone allowed the apparent transfer frequency of each plasmid to be estimated. This frequency, expressed as a proportion of the recipient genotype, was always at least 20% and often approached 100% for the Ltz^+ and $Ltz^{+/-}$ derivatives, but was only 5×10^{-6}–3×10^{-8} (and usually towards the lower end of this range) for the Ltz^- plasmids.

When one of the plasmids SCP2*, SLP2 or SLP1.2 (the latter as one of the two derivatives pIJ2 or pIJ6) was present *in one or other* of the parents in the cross, the transfer frequency of the Ltz^- plasmid marker was raised by at least one and usually two to three orders of magnitude to 5×10^{-3}–5×10^{-5}, suggesting mobilisation of the Ltz^- plasmids by any one of these three conjugative plasmids. Mobilisation was studied further in crosses between a series of *S. lividans* 66 strains with the chromosomal markers *his-2 leu-2 ura-6 ath-8 ilv-3 str-6*, and each carrying a pIJ101 Ltz^- derivative (pIJ350, pIJ351, pIJ353 or pIJ356), and strains with the marker *spc-1* carrying SCP2*, SLP2 or pIJ2/pIJ6. In each cross, selection was made for ilv^+ and *str* (as a measure of chromosomal recombination) and ilv^+ and resistance to the antibiotic relevant to each pIJ101 derivative (neomycin, thiostrepton or viomycin) as a measure of the transfer of the marked plasmid into the recipient. Colony counts on these two media were mostly approximately equal and never differed by more than about one order of magnitude (in either direction), suggesting that mobilisation of the Ltz^- plasmid and that of chromosomal markers occurred at similar frequencies. Plasmid DNA was isolated from samples of progeny with the chromosomal genotype of the recipient (*spc*) and having the antibiotic resistance of the appropriate pIJ101 derivative. CCC DNA was detected in all 80 isolates studied. In all cases it had the size expected for the particular pIJ101 derivative used in the crosses (in some isolates, deleted forms of pIJ353, present also in the donor culture, were detected). This result suggests that the Ltz^- plasmids are usually transferred, under the influence of a mobilising plasmid, in their normal autonomous form.

The Ltz^+ and $Ltz^{+/-}$ derivatives of pIJ101 were found to promote chromosomal recombination at high frequency. In the example in Table 2, a frequency of about 0.4% for a single class of recombinants was observed when the plasmid (in this case pIJ303) was present in one or both parents (Table 2). A cross in *S. coelicolor*, between strains 3083 (*hisA1 uraA1 strA1* pIJ224) and M124 (*proA1 argA1 cysD18*), was analysed by the characterisation of all possible classes of progeny recovered non-selectively and yielded 110 recombinants out of 2203 progeny (5.0%), indicating that an $Ltz^{+/-}$ derivative (pIJ224) was also a very efficient fertility plasmid.

In vivo Recombination Between pIJ101 Derivatives and Correlation of Genetic and Physical Maps

In vivo recombination between pairs of pIJ101 derivatives could readily be detected. Strains carrying pIJ303 or pIJ211 (inserts of thiostrepton resistance and neomycin resistance respectively into pIJ101) were mixed and spore progeny were plated on thiostrepton plus neomycin. Plasmid DNA from a purified member of the progeny was analysed by two-dimensional gel analysis. In addition to CCC DNA corresponding to pIJ303 (10.8 kb) and pIJ211 (12.2 kb), CCC species of $\geq$14 kb and 8.9 kb were seen. These are approximately the sizes expected of reciprocal

Table 1. Plasmids referred to in the paper

Plasmid	Size (kb)	Antibiotic resistance(s)[a]	Origin[c]	Reference[d] or Fig. in this paper	Phenotype in *S. lividans*	
					Ltz	Tra
SCP1	?	Mmy	Autonomous plasmid of *S. coelicolor* A3(2)	A	+	+
SCP2	31	–	Autonomous plasmid of *S. coelicolor* A3(2)	B	+	+
SCP2*	31	–	Spontaneous variants of SCP2	B	+	+
SLP1.2	14.5	–	Integrated in *S. coelicolor* A3(2); becomes autonomous in *S. lividans* 66	C	+	+
SLP2	?	–	Autonomous plasmid of *S. lividans* 66	D	+	+
SLP3	?	–	Autonomous plasmid of *S. lividans* 66	D	+	?
SLP4	?	–	In *S. coelicolor* A3(2); detected in *S. lividans* 66	D	+	+
pIJ2	17.8	Neo	*aph* from *S. fradiae* cloned in *Bam*HI site of SLP1.2	E	+	+
pIJ6	21.3	Thio	*tsr* from *S. azureus* cloned in *Bam*HI site of SLP1.2	E	+	+
pIJ13	16.4	Vio	*vph* from *S. vinaceus* cloned in *Bam*HI site of SLP1.2	F	+	+
pIJ24	7.4	Neo	*Pst*I *aph* fragment from pIJ2 cloned in *Pst*I site of pIJ103	Fig. 6	–	–
pIJ36	6.3	(Vio)	*Bam*HI *vph* fragment from pIJ13 cloned in *Bam*HI site of pBR322	G	Does not replicate	
pIJ37	13.8	Neo	*Sal*GI fragment deleted from pIJ2	G	+	+
pIJ41	14.8	Neo, Thio	*Bcl*I *tsr* fragment from pIJ6 cloned in *Bam*HI site of pIJ37	G	+	+
pIJ58	3.3	Neo	Combination of *Sst*II replicon fragment of pIJ350 with *Sst*II *aph* fragment of pIJ2	Fig. 12	–	–
pIJ101	8.9	–	Autonomous plasmid in *S. lividans* ISP5434	Fig. 2	+	+
pIJ102	4.2	–	Autonomous plasmid in *S. lividans* ISP5434	Fig. 2	–	–
pIJ103	3.9	–	Autonomous plasmid in *S. lividans* ISP5434	Fig. 2	–	–
pIJ104	4.9	–	Autonomous plasmid in *S. lividans* ISP5434	Fig. 2	?–	?–
pIJ201	9.5	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	–	–
pIJ202	10.5	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	–	–
pIJ208	12.2	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+/–	+
pIJ211	12.2	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+	+
pIJ213	12.2	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+/–	+
pIJ216	10.4	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	–	–
pIJ218	11.1	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	–	–
pIJ221	8.7	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	–	–
pIJ222	9.9	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	–	–
pIJ224	12.2	Neo	*Bam*HI *aph* fragment from pIJ2 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+/–	+
pIJ302	9.6	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	–	–
pIJ303	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+	+
pIJ306	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	–	–
pIJ307	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+/–	+
pIJ312	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+/–	+
pIJ319	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+	+
pIJ320	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+/–	+
pIJ324	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+	+
pIJ326	10.6	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	+	+
pIJ328	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+/–	+
pIJ330	6.1	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	–	–
pIJ333	8.0	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (and deletion)	Fig. 4	–	–
pIJ341	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+	+
pIJ345	10.8	Thio	*Bam*HI *tsr* fragment from pIJ6 cloned in *Mbo*I site of pIJ101 (no deletion)	Fig. 4	+	+
pIJ350	4.1	Thio	Replacement of *Bcl*I fragment (sites 26–30) of pIJ102 by *Bcl*I *tsr* fragment of pIJ6	Fig. 7	–	–
pIJ351	5.3	Thio	*Bcl*I *tsr* fragment from pIJ6 cloned into *Bcl*I site 30 of pIJ102	Fig. 7	–	–
pIJ352	5.3	Thio	*Bcl*I *tsr* fragment from pIJ6 cloned into *Bcl*I site 30 of pIJ102	Fig. 7	–	–
pIJ353	11.1	Neo, Vio	Replacement of *Bcl*I fragment (sites 22–30) of pIJ208 by *Bam*HI *vph* fragment of pIJ13	Fig. 4	–	–
pIJ354	5.3	Vio	*Bam*HI *vph* fragment from pIJ13 cloned into *Bcl*I site 30 of pIJ102	Fig. 7	–	–
pIJ355	10.8	Vio	*Bam*HI *vph* fragment from pIJ13 cloned into *Bcl*I site 15 of pIJ101	Fig. 4	+/–	+
pIJ356	9.4	Vio	Replacement of *Bcl*I fragment (sites 15–22) of pIJ101 by *Bam*HI *vph* fragment of pIJ13	Fig. 4	–	–
pIJ360	6.8	Thio, Vio	*Pst*I *vph* fragment from pIJ36 cloned into *Pst*I site of pIJ350	Fig. 10	–	–
pIJ361[b]	11.6	Thio, Vio	pIJ36 cloned with *Sst*I into *Sst*I site 29 of pIJ351	Fig. 11	–	–
pIJ363	6.4	Neo	Deletion of *Bcl*I fragment (sites 26–30) of pIJ24	Fig. 6	–	–
pIJ364	5.3	Vio, Thio	Replacement of *Bcl*I-*Pst*I fragment (sites 26–31) of pIJ350 with *Bam*HI-*Pst*I *vph* fragment from pIJ36	Fig. 8	–	–
pIJ365	5.4	Neo, Thio	*Cla*I *aph* fragment (with part of *tsr*) from pIJ41 cloned into *Cla*I site of pIJ350, followed by deletion of *Kpn*I fragment	Fig. 9	–	–
pIJ366	5.3	Vio	*Bam*HI *vph* fragment from pIJ13 cloned into *Mbo*I site of pIJ102 (no deletion)	Fig. 7	–	–

Table 1 (continued)

Plasmid	Size (kb)	Antibiotic resistance(s)[a]	Origin[c]	Reference[d] or Fig. in this paper	Phenotype in *S. lividans*	
					Ltz	Tra
pPZ4	7.7	Thio	In vivo deletion from pIJ303 in *S. rimosus*	Fig. 4	–	–
pPZ5	6.6	Thio	In vivo deletion from pIJ303 in *S. rimosus*	Fig. 4	–	–
pPZ12	6.2	Thio	In vivo deletion from pIJ303 in *S. rimosus*	Fig. 4	–	–

[a] Mmy, methylenomycin; Neo, neomycin; Thio, thiostrepton; Vio, viomycin
[b] Replicates in *E. coli* as well as in *Streptomyces*
[c] *aph*, neomycin (aminoglycoside) phosphotransferase; *tsr*, thiostrepton resistance methylase; *vph*, viomycin phosphotransferase
[d] A, Vivian 1971; B, Bibb et al. 1977; C, Bibb et al. 1981; D, Kieser et al., to be published; E, Thompson et al. 1980; F, Thompson et al. 1982a; G, Thompson et al. 1982b

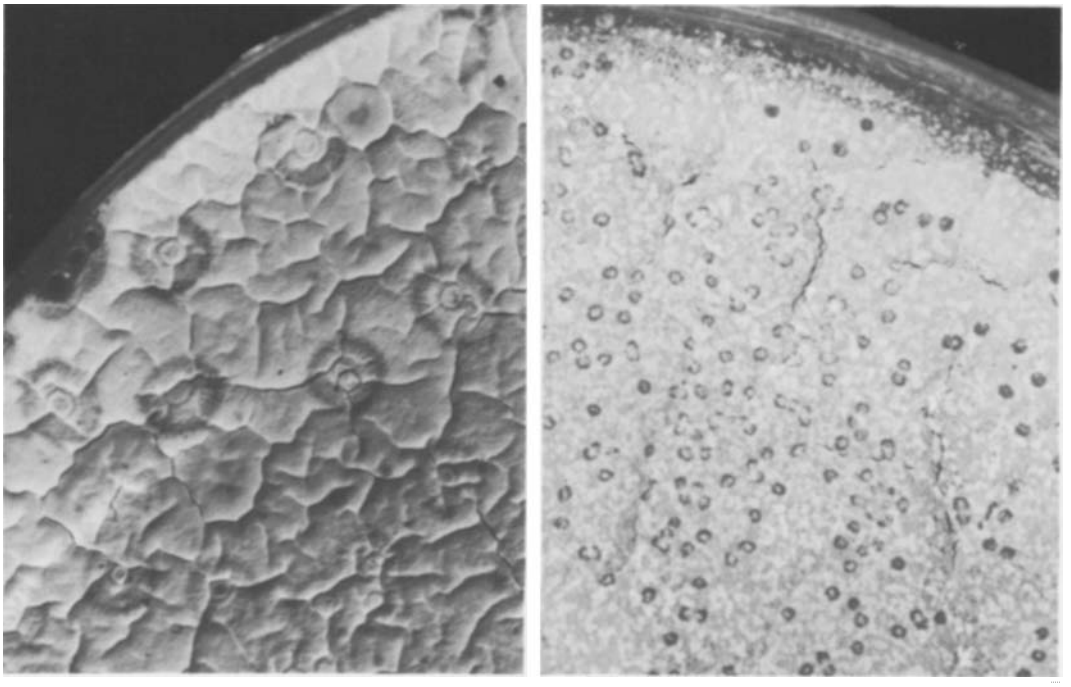

Fig. 14. Pocks produced on a plasmid-free lawn of *S. lividans* 66 by Ltz$^+$ plasmid pIJ303 (left) and Ltz$^{+/-}$ plasmid pIJ224 (right)

recombinant molecules containing both the thiostrepton and neomycin inserts and neither insert (the reconstituted pIJ101) respectively. A second cross, between strains carrying pIJ303 and pIJ24 (pIJ103 with a neomycin insert, 7.4 kb) generated molecules of $\leqq$6 kb, the size expected for recombinants in which crossing-over between the homologous regions of pIJ101 and pIJ103 had generated a pIJ103 replicon carrying the thiostrepton insert instead of the neomycin insert. (The reciprocal recombinant, pIJ101 carrying the neomycin insert (12.2 kb), was not unambiguously detected.)

These results led to crosses between pairs of *S. lividans* strains carrying Ltz$^{+/-}$ or Ltz$^-$ derivatives of pIJ101 in order to test the consistency of the physical map by looking for Ltz$^+$ recombinant progeny on lawns of *S. lividans* inoculated with spores from each cross (Fig. 17). All 36 pairwise crosses between the set of 9 insertion variants in the "lower" half of the map (pIJ224 – pIJ312 running in a clockwise direction) were made. Since all the insertions (except pIJ213, pIJ320 and pIJ355) had been mapped to different positions, recombination between all pairs was expected (except those involving these three variants). This result was found, except that no recombinants were detected between pIJ208 and pIJ320 or between pIJ208 and pIJ355; perhaps the resolution of the test was not high enough to detect recombinants in the short interval involved. However, unexpectedly, recombinants did occur between members of two of the three pairs involving inserts at apparently the same site (between pIJ213 and pIJ320 and between pIJ213 and pIJ355, but not between pIJ320 and pIJ355); perhaps this indicates some small undetectable error in the physical map of one of the variants.

A second series of crosses involved the deletion variants. Since they were all Ltz$^-$ (and therefore transfer-defective) meaningful crosses could not be made between pairs of deletions (unless strains carrying pairs of variants had been laboriously made by transformation), nor between the Ltz$^-$ insertion (pIJ306) and the set of deletions. The results of the matrix of crosses between the 8 Ltz$^{+/-}$ insertions and the set of deletions are in Table 3. They are consistent with the physical map in Fig. 4.

That recombination, rather than complementation, was responsible for the origin of the Ltz$^+$ progeny in these crosses was demonstrated by purifying Ltz$^+$ strains from three sample crosses (pIJ224 × pIJ201, pIJ224 × pIJ216 and pIJ224 × pIJ222).

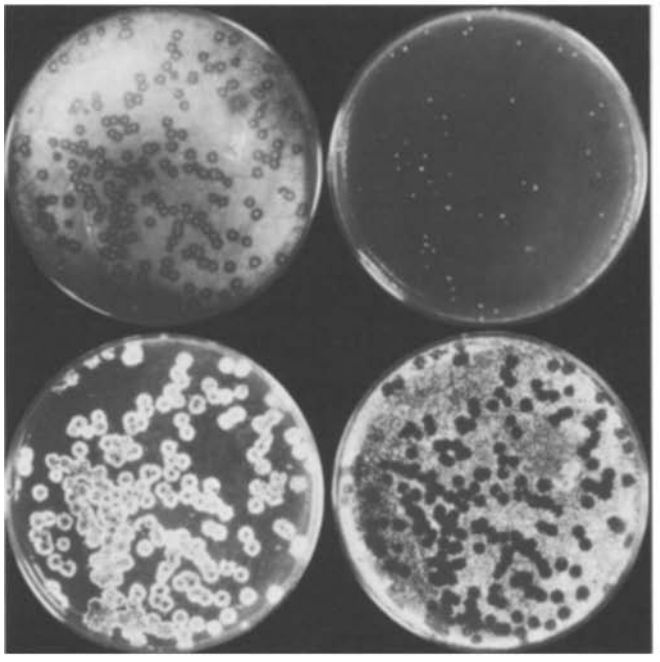

Fig. 15. Top left: Ltz^+ pocks produced by strain 3058 (*str-6* pIJ303) in a lawn of strain TK23 (*spc-1*). Top right: replica on streptomycin, selecting the chromosomal marker of the strain initiating the pocks. Bottom left: replica on thiostrepton, selecting the marker of Ltz^+ plasmid pIJ303. Bottom right: replica on spectinomycin, selecting the chromosomal marker of the background lawn; the positions of the pocks can be seen as areas of reduced growth

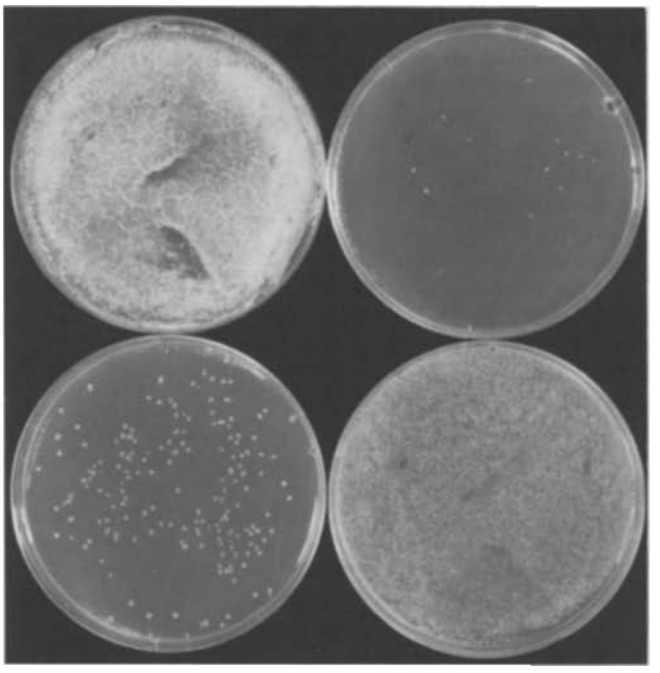

Fig. 16. Top left: $Ltz^{+/-}$ pocks (scarcely visible after replica plating) produced by strain 3062 (*spc-1* pIJ224) in a lawn of TK24 (*str-6*). Top right: replica on spectinomycin, selecting the chromosomal marker of the strain initiating the pocks. Bottom left: replica on neomycin, selecting the marker of the $Ltz^{+/-}$ plasmid pIJ224. Bottom right: replica on streptomycin, selecting the chromosomal marker of the background lawn

Table 2. Chromosomal recombination in crosses between *S. lividans* strains, mediated by pIJ303

Cross No.	Plasmid status of parent strains		Average recombinants[a] ml^{-1}	Parental genotypes ml^{-1}		Average recombinants/ sum of parentals
	his leu spc	*pro*		*his leu spc*	*pro*	
F3061	–	–	1.0×10^{2} [b]	2.4×10^{8}	1.5×10^{7}	4.0×10^{-7}
F3074	pIJ303	–	4.1×10^{6}	1.4×10^{8}	5.0×10^{8}	6.4×10^{-3}
F3075	–	pIJ303	5.5×10^{5}	7.2×10^{7}	1.9×10^{8}	2.1×10^{-3}
F3076	pIJ303	pIJ303	5.3×10^{6}	5.8×10^{8}	6.5×10^{8}	4.3×10^{-3}

[a] Selecting *his*$^+$ *spc* or *leu*$^+$ *spc*

[b] 0.1 ml was plated, yielding 10 colonies (which were of genotypes that could have resulted from mutation)

All the cultures were neomycin-sensitive (as expected of strains carrying pIJ101 recombinants) and yielded CCC DNA of a size identical to pIJ101.

Host Range of pIJ101

In attempts to transfer pIJ101 to other species of *Streptomyces*, *S. lividans* strain 2930 (containing pIJ101 alone) was mixed with streptomycin-resistant mutants of several strains (or with wild-type strains sufficiently resistant to streptomycin to allow counterselection of the *S. lividans* parent) and spore progeny were spread on streptomycin-containing plates with an added excess of the other partner in the cross. Pocks were visible in crosses involving *S. coelicolor* A3(2) and *S. parvulus* ATCC 12434 derivatives; the transfer of pIJ101 into these species was confirmed by gel electrophoresis.

In a further series of crosses, pIJ303 was transferred in crosses from *S. lividans* 66 to *S. acrimycini* IPV1610 and *S. griseus* ATCC 10137, by selecting for streptomycin-resistance from the recipient and thiostrepton resistance from the plasmid donor. The plasmid was also transferred, in a similar fashion, from *S. griseus* to a spectinomycin-resistant mutant of *S. coelicolor* A3(2).

Since negative results in this series of crosses could have been due to growth inhibition of one species by antimicrobial activities produced by the other, attempts were made to *transform* pIJ303 and/or pIJ211 into a series of streptomycetes, with selection for thiostrepton or neomycin resistance. Thiostrepton resistance was found to be particularly useful, since all strains tested (except the thiostrepton-producer *S. azureus*) were sensitive to this antibiotic; a much higher proportion of strains were neomycin-resistant. It was found (Table 4) that one or both of the pIJ101 derivatives could be maintained in 13 of the 18 strains tested (transformation was verified in every case by isolation of CCC DNA of appropriate size from at least one putative transformant of each type).

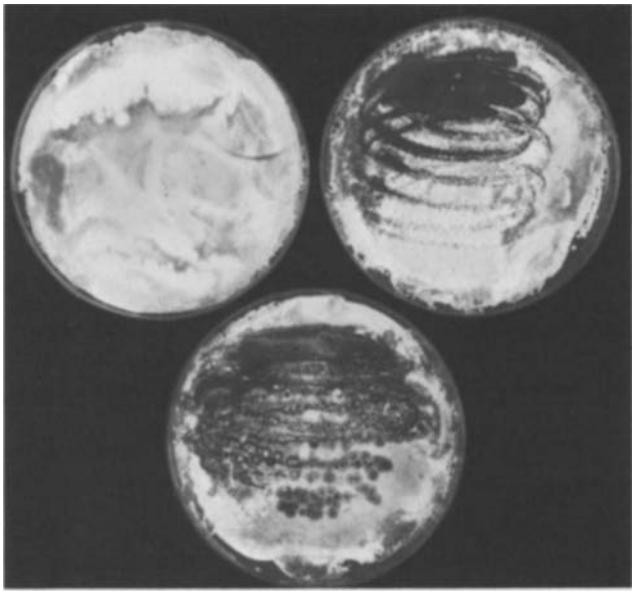

Fig. 17. Recombination between Ltz$^-$ and Ltz$^{+/-}$ plasmids. Top left: spores from a strain carrying Ltz$^-$ plasmid pIJ306 streaked in a lawn of plasmid-free *S. lividans* 66 (no pocks visible). Top right: spores from a strain carrying Ltz$^{+/-}$ plasmid pIJ224 streaked in a lawn of plasmid-free *S. lividans* 66 (Ltz$^{+/-}$ pocks visible). Bottom: spores from a cross between strains carrying pIJ306 and pIJ224 streaked in a lawn of plasmid-free *S. lividans* 66 (mixture of Ltz$^{+/-}$ and recombinant Ltz$^+$ pocks visible)

Transformants of most of the species showed an Ltz$^+$ reaction (pocks, sometimes small and indistinct) on lawns of the corresponding strain (Table 4). This suggested that the plasmids would be transferable by conjugation in most of the species and, with one exception, this proved to be true when pairs of auxotrophs or antibiotic-resistant mutants were available in order to provide a counter-selection for the plasmid-carrying strain: the apparent transfer frequencies, per recipient, ranged from 2–100%. The one exception was *S. rimosus* NRRL 2234, in which no transfer could be detected. That this was not due to the origin of a transfer-defective mutant of pIJ303 in this strain was shown by transforming plasmid DNA from *S. rimosus* back to *S. lividans* 66, where again 100% transfer was observed.

The apparent stability (that is the proportion of thiostrepton- or neomycin-resistant colonies after a round of growth on an antibiotic-free slant, varied widely in different hosts (Table 4)). Whereas no antibiotic-sensitive derivatives of *S. lividans* 66 or *S. coelicolor* A3(2) carrying pIJ303 or pIJ211 (or any of the many other pIJ101 variants studied, except pIJ58 and pIJ365: see above) were found, this was not true in some other strains.

While no CCC DNA molecules of clearly different sizes were detected in any of the strains in Table 4 which were (cursorily) examined, in vivo deletion variants of pIJ303 in another strain of *S. rimosus* were readily found (I. Hunter and M. Warren, personal communication). Three of these (pPZ4, pPZ5 and pPZ12) were included in the physical and genetic analysis described earlier (Fig. 4 and Table 1).

Discussion

The copy number of plasmids of the pIJ101 family (40–300) appears to be amongst the highest (without artificial amplification) so far described in any bacterium. The high copy number may account for the fact that the smaller plasmids (pIJ102–104), apparently derived by deletion from pIJ101, co-exist with it stably (they were found in two cultures of *S. lividans* ISP5434 from different collections). When introduced alone into other strains, pIJ101 derivatives were structurally rather stable, but sometimes gave rise to deletion variants, as in the cited examples in *S. lividans* 66 and *S. rimosus*. Interestingly, a plasmid (pS10147) whose physical map, insofar as the same endonucleases were studied, appears to be almost identical with that of pIJ101 (it lacked the single *SstI* site) has recently been described, as the sole CCC DNA species, in *S. coelicolor* ATCC 10147 (Pernodet and Guerineau 1981).

Analysis of the phenotypes of the in vivo deletions and in vitro derivatives of pIJ101 described here has begun to define some functions of parts of the plasmid. These are summarised in Fig. 18. The discovery that the smallest member of another family of plasmids (the SLP1 series), only 9.4 kb in size, is self-transmissible had already suggested that the process of plasmid-mediated conjugation in *Streptomyces* is genetically (and there-

Table 3. Production of Ltz$^+$ recombinants in pairwise crosses between Ltz$^{+/-}$ insertion derivatives of pIJ101 and Ltz$^-$ deletion derivatives (see Fig. 4 for physical analysis)

Deletion derivative	Insertion derivative							
	pIJ312	pIJ328	pIJ213	pIJ320	pIJ355	pIJ208	pIJ307	pIJ224
pIJ353	−	+	+	−[b]	−[b]	−[a]	+	+
pIJ333	−	+	+	+	+	+	+	+
pIJ330	−	−	−	−	−	−	−	−
pIJ201	−	+	+	+	+	+	+	+
pIJ222	−	+	+	+	+	+	+	+
pPZ12	−	−	−	−	−	−	−	−
pPZ5	−	−	−	−	−	−	−	+
pIJ216	−	+	+	+	+	+	+	+
pIJ202	−	+	+	+	+	+	+	+
pIJ221	−	−	−	−	−	−	−	−
pIJ302	−	+	+	+	+	+	+	+
pIJ218	−	+	+	+	+	+	+	+
pPZ4	+	−	−	−	−	−	−	−
pIJ356	+	−	−	−	−	−	+	+

[a] Expected since pIJ353 is derived from pIJ208

[b] Expected from finding of no recombinants between pIJ208 and pIJ320 or pIJ355 (see text)

Table 4. Transformation of pIJ101 derivatives into various streptomycetes

Strain	Transformation by		Stability		Transfer frequency	Ltz reaction (pocks)
	pIJ303[a]	pIJ211[a]	pIJ303	pIJ211		
S. acrimycini IPV 1610	+	+	451/697 (65%)	17/290 (6%)	13%	+
S. albus G	+	+	109/410 (27%)	497/500 (99%)	2%	+
S. albus P CMI 52766	+	ND	77/78 (98%)	ND	13%	?+[b]
S. azureus ATCC 14921	NA	+	NA	ND	ND	+
S. coelicolor A3(2)	+	+	503/503 (100%)	ND	100%	+
S. fradiae NRRL B3357	+	ND	109/215 (51%)	ND	ND	−
S. glaucescens ETH 22794	+	+	370/370 (100%)	62/109 (57%)	100%	+
S. griseus ATCC 10137	+	NA	53/170 (31%)	NA	41%	−
S. lividans 66	+	+	196/196 (100%)	223/223 (100%)	100%	+
S. parvulus ATCC 12434	+	+	ND	ND	ND	+
S. pristinaespiralis ATCC 25486	+	ND	151/151 (100%)	ND	5%	?+[b]
S. rimosus NRRL 2234	+	NA	239/252 (95%)	NA	$<10^{-6}$	−
S. rimosus JI 2246	+	NA	387/387 (100%)	NA	ND	−
S. albogriseolus NCIB 9604	−	NA	NA	NA	NA	NA
S. fradiae ATCC 10745	−	NA	NA	NA	NA	NA
S. kanamyceticus NRRL B2535	−	NA	NA	NA	NA	NA
S. violaceoruber NCIB 9622	−	NA	NA	NA	NA	NA
S. vinaceus NCIB 8852	−	NA	NA	NA	NA	NA

[a] NA = not applicable because host was naturally resistant to thiostrepton or neomycin; ND = not done
[b] = v. small pocks

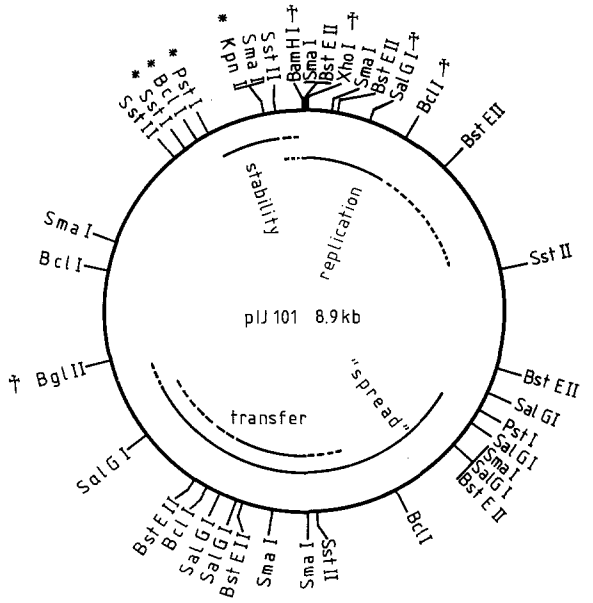

Fig. 18. Approximate locations of functions involved in conjugal transfer and "spread", stability and replication of pIJ101 deduced from the properties of in vitro and in vivo derived variants. Restriction enzyme sites indicated (†) are unavailable for the insertion of foreign DNA. Those marked (*) have been shown to be available for such cloning in pIJ101 or one of its derivatives

fore perhaps structurally) simpler than that in Gram-negative bacteria such as *E. coli* (Bibb et al. 1981). pIJ101 confirms this conclusion. The parental, self-transmissible plasmid is 8.9 kb in size; insertions and deletions in only a fraction of the molecule abolish self-transfer. The maximum size of this transfer region (assuming only one contiguous segment) is the distance between the sites of insertion of foreign DNA in the transfer-proficient plasmids pIJ328 and pIJ312 (Fig. 4), a length of 2.1 kb. Another segment of DNA, adjacent to this region, although apparently not required for transfer between mating individuals, can be postulated to control what may be a special property of *Streptomyces* plasmids: their ability to "spread" quite extensively within a mycelial culture, giving rise to the reaction seen on agar media as pocks. Conceivably, this reflects at least some element of intra- rather than intermycelial colonization. In the $Ltz^{+/-}$ variants, this property, but not transfer itself, is curtailed, or perhaps abolished. The function responsible for plasmid transfer in $Ltz^{+/-}$ variants and the function that allows plasmid spread in Ltz^{+} variants are *cis*-acting. Ltz^{-} derivatives of pIJ101 could, however, be mobilized at a comparatively low frequency, similar to the frequency of mobilization of chromosomal markers, by unrelated plasmids.

The stable inheritance of plasmid copies by daughter individuals appears to depend on a specific region of the plasmid (Fig. 18). Whether this is due to a partition function of the type identified on the low copy number *E. coli* plasmid pSC101 (Meacock and Cohen, 1980) and postulated for the low copy number *Streptomyces* plasmid SCP2* (Bibb et al. 1980) remains to be seen.

The region of pIJ101 essential for maintenance and replication has so far been narrowed down to a 2.1 kb segment. Consistent with this conclusion is the finding that insertion of foreign DNA at four sites in this segment (†, Fig. 18) was found to destroy the ability of the plasmid to exist in *Streptomyces*. Apart from these, the only other "unavailable" site tested is the unique *Bgl*II site. This site lies within a long segment of DNA inessential for maintenance and replication, and absent from many in vivo and in vitro deletions. It seems that insertion of DNA at this site produces derivatives that cannot be maintained in viable *Streptomyces* hosts. The reason for this is not clear. Conceivably insertion into the *Bgl*II site inactivates a plasmid function that is necessary to counteract another function that is lethal to the cell on its own. This postulated lethal function is not identical with the genes involved in plasmid transfer since insertion into the *Bgl*II site of non-transmissible derivatives of pIJ101 still inactivated these plasmids.

Some applications of various types of plasmid vectors to problems amenable to study by endogenous DNA cloning in *Streptomyces* have been discussed elsewhere (Chater and Hop-

Table 5. Some possibilities for DNA cloning into pIJ101 derivatives

Site	Plasmids with single sites	Comments
*Bgl*II	pIJ101 and all its derivatives unless the *Bgl*II site is deleted	Site not available (but segment containing it can be deleted or replaced)
*Cla*I	All derivatives with thiostrepton resistance	Insertion probably inactivates thiostrepton resistance
*Eco*RI	pIJ360, pIJ361	
*Hind*III	pIJ360, pIJ361	
*Kpn*I	All plasmids without neomycin resistance	Site possibly in a function necessary for plasmid stability
*Pst*I	pIJ102, pIJ103, pIJ330, pIJ350, pIJ351, pIJ352, pIJ364, pPZ4, pPZ12	pIJ350 has been used successfully for shotgun cloning with *Pst*I (J. Gil, personal communication)
*Sst*I	pIJ101, pIJ102, pIJ103 and all derivatives with thiostrepton resistance only (except pIJ350)	
*Xba*I	All derivatives with neomycin resistance	

wood 1982; Chater et al. 1982; Hopwood and Chater 1982). Several properties of the pIJ101 series of plasmids are useful for incorporation into such vectors. These include high copy number (making for ease of study of the DNA and, probably, useful amplification of some products of cloned genes); broad host range within *Streptomyces* (potential hosts outside this genus have not been investigated); and the ability to manipulate the plasmid to give conjugal or non-conjugal vectors. The non-conjugative property is advantageous in the use of these high copy number plasmids for shotgun cloning since presumptive clones are harder to purify in the presence of a conjugative plasmid which can superinfect the clones on the protoplast regeneration plates.

For DNA cloning it is desirable to have single sites in non-essential regions of the vector plasmids. For most purposes sites for enzymes that generate staggered cuts are best. The presence of *Bam*HI, *Bcl*I, *Bst*EII, *Sal*GI, *Sst*II and *Xho*I sites in the region essential for plasmid replication is rather unfortunate. In pIJ101 and its derivatives described in this paper there are, however, several other sites useful for DNA cloning (Table 5). Furthermore, it should be relatively easy to use plasmids with only two sites for a given enzyme by using partial digestion. pIJ353, pIJ58 and pIJ365 have one *Bam*HI site in the neomycin resistance gene which is inactivated by insertion into this site (Thompson et al. 1982b). pIJ363, pIJ364 and pIJ365 have two *Bcl*I sites, one of which is expected to be available for cloning, and pIJ361 has two *Cla*I sites. The *Cla*I site in the thiostrepton resistance determinant is methylated in *dam*$^+$ strains of *E. coli* while the other *Cla*I site (in the pBR322 sequence) is not (Mayer et al. 1981; J.S. Feitelson, personal communication).

The construction of plasmid chimaeras able to function in more than one class of host, such as pIJ361 for *E. coli* and *Streptomyces*, should also have particular advantages, in allowing the transfer of some well-developed *E. coli* techniques – such as transposon mutagenesis and the exploitation of suppressor systems – to *Streptomyces*. The availability of the viomycin phosphotransferase gene, which was found to be expressed both in *Streptomyces* and *E. coli* (presumably by read-through from *E. coli* promoters in certain positions in chimaeric plasmids) could be particularly useful.

The finding that conjugative derivatives of pIJ101 are very efficient fertility factors for promoting chromosomal recombination may also have applications. Recombinants constitute more than one percent of the total progeny of matings when a pIJ101 derivative is present in one or both parents. Although this property has so far been tested only in *S. lividans* 66 and *S. coelicolor* A3(2), it is likely that introduction of these plasmids into other strains of interest would result in frequent recombination and therefore would facilitate chromosomal mapping.

In short, the pIJ101 series appears to represent an important new class of plasmids with implications both for the understanding of plasmid-mediated conjugation in *Streptomyces* and for the genetic manipulation of this important genus of industrial microorganisms by in vitro and in vivo approaches.

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Notes Added in Proof

Plasmid Stability. It has recently been found that a plasmid resembling pIJ58, but lacking the small extra *Sst*II fragment, is much more stably inherited than pIJ58 itself (only 0.5–5% loss after one round of growth on antibiotic-free medium). This suggests that the region of pIJ101 between sites 31 and 33 may not contain a sequence specifically required for stability but that certain physical rearrangements in this segment of the plasmid may have effects on plasmid maintenance.

Host Range of pIJ101. A small derivative of pIJ101 has been successfully transformed into two out of three of the strains which had failed to be transformed with pIJ303 (*S. fradiae* ATCC 10745 and *S. vinaceus* NCIB 8852 but not *S. violaceoruber* NCIB 9622). This indicates that the host range of pIJ101 derivatives is even wider than at first thought; probably restriction barriers are responsible for the failure of some strains to be transformed.