Analysis of the site for second-strand initiation during replication of the *Streptomyces* plasmid pIJ101

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The indigenous plasmid pIJ101 is the parent of many cloning vectors used in *Streptomyces*. One early pIJ101 derivative, pIJ702, has been particularly widely used. pIJ702 lacks *sti:cop/korB* and accumulates single-stranded DNA (ssDNA). The 1.2 kb *BclI*–*BclI sti:cop/korB* and 0.7 kb *SpeI*–*BclI sti* regions were isolated from pIJ101 and cloned into pIJ702 at the *PstI* site in both orientations. No ssDNA was detected in constructs containing *sti* present in its correct orientation with respect to the basic replicon, with or without *cop/korB*. Constructs which contained *sti* in the reverse orientation did accumulate ssDNA. Thus, *sti* is only active as the site for second-strand synthesis in its natural orientation. Furthermore, *sti* inserted in either orientation into the structurally unstable pIJ702–pUC8 shuttle vectors prevented them from rearranging in *S. lividans*. The *sti* function was defined to a 0.53 kb *SpeI*–*SacII* fragment and the probable site for second-strand initiation (*ssi*) was identified.

Introduction

The *Streptomyces* plasmid pIJ101 (8.9 kb) is a high-copy-number (40–300 copies per chromosome), broad-host-range plasmid (Kieser *et al.*, 1982). Kendall & Cohen (1988) have determined the complete nucleotide sequence of pIJ101 and have analysed it for open reading frames (ORFs) with respect to the available information about the genetic properties of the plasmid. Currently, more is known about the biology of pIJ101 than any other *Streptomyces* plasmid.

pIJ101 probably replicates using a rolling-circle mechanism via a single-stranded DNA (ssDNA) intermediate (Pigac *et al.*, 1988; Gruss & Ehrlich, 1989). ssDNA intermediates corresponding to one strand of a plasmid monomer have also been discovered in plasmids isolated from *Bacillus subtilis* (te Riele *et al.*, 1986a, b; Devine *et al.*, 1989), *Staphylococcus aureus* (te Riele *et al.*, 1986a, b; Gros *et al.*, 1987; Gruss *et al.*, 1987; Boe *et al.*, 1989; Novick, 1989) and *Streptococcus pneumoniae* (del Solar *et al.*, 1987). Such plasmids which replicate using a

rolling-circle mechanism can be classified according to the homologies of their replication (Rep) proteins and the position of the plus origin with respect to the replication (*rep*) gene (Gruss & Ehrlich, 1989). Gruss & Ehrlich (1989) found that the predicted amino acid sequence of the pIJ101 Rep protein active site is similar to those found in several other plasmid-encoded Rep proteins which bind to pC194-like plus origins. They also suggested that the pIJ101 plus origin lies upstream of the *rep* ORF where there is structural similarity to the *Staph. aureus* pC194 plus origin, although it is now known that the pIJ101 plus origin is not located here (Zaman *et al.*, 1993).

Derivatives of pIJ101 have been constructed for cloning purposes and one, pIJ702 (Katz *et al.*, 1983), has been used extensively as a cloning vector in *Streptomyces* (for reviews see Tomich, 1988; Chater, 1990). pIJ702 contains the 2.0 kb *BalI*–*SacII* region of pIJ101 found to be essential for maintenance and replication in *S. lividans* (Zaman *et al.*, 1993). pIJ702 is known to accumulate ssDNA molecules (Deng *et al.*, 1988; Pigac *et al.*, 1988), which are the presumed intermediates in plasmid replication (Pigac *et al.*, 1988; Gruss & Ehrlich, 1989). Thus, pIJ702 probably lacks the primary site for second-strand initiation (*ssi*) but contains one or more sequences which can act as weak or inefficient sites for second-strand synthesis, thereby allowing double-stranded pIJ702 to form but also keeping a pool of ssDNA present in the cell. Such ssDNAs are likely to be highly

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Abbreviation: ssDNA, single-stranded DNA.

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Description*	Reference
<i>Escherichia coli</i> JM107	$\Delta(lac-proAB) endA1 gyrA96 thi hsdR17 supE44 relA1 \lambda^- [F' traD36 proAB^+ lacI^q \Delta M15]$	Yanisch-Perron <i>et al.</i> (1985)
<i>Streptomyces lividans</i> TK24	<i>str-6</i> SLP2 ⁻ SLP3 ⁻	Hopwood <i>et al.</i> (1983)
pUC8 (2.7 kb)	Ap ^r LacZ'	Vieira & Messing (1982)
pUC19 (2.7 kb)	Ap ^r LacZ'	Yanisch-Perron <i>et al.</i> (1985)
pBluescript II (3.0 kb)	Ap ^r LacZ'	Stratagene
pIJ101 (8.9 kb)	<i>sti</i> ⁺ <i>cop/korB</i> ⁺	Kieser <i>et al.</i> (1982)
pIJ303 (10.8 kb)	Tsr ^r <i>sti</i> ⁺ <i>cop/korB</i> ⁺	Kieser <i>et al.</i> (1982)
pIJ702 (5.8 kb)	Tsr ^r Mel ⁺ <i>sti</i> ⁻ <i>cop/korB</i> ⁻	Katz <i>et al.</i> (1983)
pQR200 (3.9 kb)	1.2 kb <i>BclI</i> - <i>BclI</i> <i>sti</i> : <i>cop/korB</i> fragment in pUC8 <i>Bam</i> HI site	This work
pQR410a (9.7 kb)	pQR200 in pIJ702 <i>PstI</i> site (<i>sti</i> ^{rev} <i>cop/korB</i> ⁺)	This work
pQR410b (7.0 kb)	1.2 kb <i>BclI</i> - <i>BclI</i> fragment in pIJ702 <i>PstI</i> site (<i>sti</i> ^{rev} <i>cop/korB</i> ⁺)	This work
pQR411a (9.7 kb)	pQR200 in pIJ702 <i>PstI</i> site (<i>sti</i> ⁺ <i>cop/korB</i> ⁺)	This work
pQR411b (7.0 kb)	1.2 kb <i>BclI</i> - <i>BclI</i> fragment in pIJ702 <i>PstI</i> site (<i>sti</i> ⁺ <i>cop/korB</i> ⁺)	This work
pQR417 (3.4 kb)	0.7 kb <i>SpeI</i> - <i>BclI</i> <i>sti</i> fragment in pUC19 <i>XbaI</i> / <i>Bam</i> HI sites	This work
pQR421a (9.2 kb)	pQR417 in pIJ702 <i>PstI</i> site (<i>sti</i> ⁺ <i>cop/korB</i> ⁻)	This work
pQR421b (6.5 kb)	0.7 kb <i>SpeI</i> - <i>BclI</i> fragment in pIJ702 <i>PstI</i> site (<i>sti</i> ⁺ <i>cop/korB</i> ⁻)	This work
pQR422a (9.2 kb)	pQR417 in pIJ702 <i>PstI</i> site (<i>sti</i> ^{rev} <i>cop/korB</i> ⁻)	This work
pQR422b (6.5 kb)	0.7 kb <i>SpeI</i> - <i>BclI</i> fragment in pIJ702 <i>PstI</i> site (<i>sti</i> ^{rev} <i>cop/korB</i> ⁻)	This work
pQR437 (3.3 kb)	0.6 kb <i>SpeI</i> - <i>SacI</i> <i>sti</i> fragment in pUC19 <i>XbaI</i> / <i>SacI</i> sites	This work
pQR438 (3.23 kb)	0.53 kb <i>SpeI</i> - <i>SacII</i> <i>sti</i> fragment in pUC19	This work
pQR441a (9.1 kb)	pQR437 in pIJ702 <i>PstI</i> site (<i>sti</i> ⁺ <i>cop/korB</i> ⁻)	This work
pQR441b (6.4 kb)	0.6 kb <i>SpeI</i> - <i>SacI</i> fragment in pIJ702 <i>PstI</i> site (<i>sti</i> ⁺ <i>cop/korB</i> ⁻)	This work
pQR442a (9.1 kb)	pQR437 in pIJ702 <i>PstI</i> site (<i>sti</i> ^{rev} <i>cop/korB</i> ⁻)	This work
pQR443a (9.0 kb)	pQR438 in pIJ702 <i>PstI</i> site (<i>sti</i> ⁺ <i>cop/korB</i> ⁻)	This work
pQR443b (6.33 kb)	0.53 kb <i>SpeI</i> - <i>SacII</i> fragment in pIJ702 <i>PstI</i> site (<i>sti</i> ⁺ <i>cop/korB</i> ⁻)	This work
pQR444a (9.0 kb)	pQR438 in pIJ702 <i>PstI</i> site (<i>sti</i> ^{rev} <i>cop/korB</i> ⁻)	This work

* Ap^r, ampicillin resistance; Tsr^r, thiostrepton resistance; Mel, tyrosinase determinant; LacZ, β -galactosidase determinant. *sti*, strong incompatibility (Deng *et al.*, 1988), shown in this work to contain the site for second-strand initiation (*ssi*); *sti*⁺, *sti* in the correct orientation with respect to the basic replicon; *sti*^{rev}, *sti* in the reverse orientation with respect to the basic replicon; *sti*⁻, absence of *sti*. *cop/korB*, repressor of *kilB* (Kendall & Cohen, 1987; Stein *et al.*, 1989; Stein & Cohen, 1990; Zaman *et al.*, 1992); *cop/korB*⁺, presence of *cop/korB* gene; *cop/korB*⁻, absence of *cop/korB* gene.

recombinogenic and it may be this property which leads to the rapid breakdown of some pIJ702-based vectors (Pigac *et al.*, 1988). In support of this hypothesis, Pigac *et al.* (1988) showed that a shuttle vector consisting of the *S. lividans* plasmid pIJ350 and the *Escherichia coli* plasmid pBR322 was structurally unstable and accumulated ssDNA in *S. lividans*. Similarly, Lee *et al.* (1986) stated that a shuttle vector consisting of pIJ702 and the *E. coli* plasmid pUC12 was structurally unstable and induced deletions in *S. lividans*. Inefficient conversion of ssDNA intermediates has also been reported to underlie plasmid structural instability in other organisms. For example, in *Staph. aureus* pT181-like plasmids, *palA* is needed for normal replication and stability since rearrangements affecting *palA* result in ssDNA accumulation and plasmid structural instability (Gruss *et al.*, 1987; Novick, 1989). Similarly, deletions

affecting the *ssi* region of the streptococcal plasmid pLS1 cause ssDNA accumulation and plasmid structural instability (del Solar *et al.*, 1987).

Deng *et al.* (1988) identified a non-coding region of pIJ101 DNA, called *sti*, which causes strong incompatibility when present in its natural orientation with respect to the basic replicon. A pair of plasmids can co-exist in the same host if they both possess *sti* in the correct orientation (*Sti*⁺), or both possess *sti* in the reverse orientation (*Sti*^{rev}) or both lack *sti* (*Sti*⁻). *Sti*⁺ and *Sti*⁻ plasmids cannot co-exist in the same cell: if they occur together the *Sti*⁺ plasmid is retained while the *Sti*⁻ plasmid is eventually lost. *Sti*⁻ plasmids accumulate more ssDNA than *Sti*⁺ plasmids, implicating *sti* as the site where second-strand synthesis is initiated. *Sti* therefore contains the minus origin. Deng *et al.* (1988) suggested that Cop, a protein which is encoded by a gene adjacent

to *sti*, is a *trans*-acting negative regulator which decreases the copy number of *Sti*⁺ plasmids by inhibiting the initiation of second-strand synthesis at *sti*. Mapping studies indicate that *cop* occupies the same site on pIJ101 as another characterized gene called *korB* (Kendall & Cohen, 1988). *KorB* repress the transcription of *kilB*, a gene necessary for normal intra-mycelial plasmid transfer (Kendall & Cohen, 1987; Stein *et al.*, 1989; Stein & Cohen, 1990; Zaman *et al.*, 1992). This paper describes the characterization of the primary site for second-strand synthesis of pIJ101 and shows that *Cop* (*KorB*) does not regulate the conversion of ssDNA intermediates to the double-stranded plasmid form.

Methods

Bacterial strains and plasmids. Table 1 contains the description of bacterial strains and plasmids used in this study.

Media and growth conditions. *E. coli* JM107 was grown on nutrient agar plates or in nutrient broth overnight at 37 °C. These media were supplemented with ampicillin (500 µg ml⁻¹ in solid media or 50 µg ml⁻¹ in liquid media), IPTG (40 µg ml⁻¹) and X-Gal (80 µg ml⁻¹) when necessary. *S. lividans* TK24 was grown on R2YE agar (Hopwood *et al.*, 1985) or malt extract/yeast extract agar (2.4%, w/v, Difco malt extract; 0.5%, w/v, Oxoid yeast extract; 2%, w/v, Bactoagar) at 30 °C until sporulation (typically one week). For liquid cultivation, *S. lividans* TK24 was grown in YEME (Hopwood *et al.*, 1985) for 2–3 d or in malt extract/peptone medium (2%, w/v, glycerol; 1%, w/v, Oxoid malt extract; 1%, w/v, Difco Bacto-peptone; 0.714%, w/v, K₂HPO₄) overnight at 30 °C with good aeration. These media were supplemented with thiostrepton (50 µg ml⁻¹ in solid media and 5 µg ml⁻¹ in liquid media) when required. Thiostrepton was a kind gift from S. J. Lucania (E. R. Squibb & Sons Inc., NJ, USA).

DNA manipulations. *E. coli* plasmid DNA was isolated by alkaline lysis (Birnboim & Doly, 1979). *Streptomyces* plasmid DNA isolation was based on the alkaline lysis method developed by Kieser (1984). Restriction enzymes and T4 ligase were either made by Dr L. Wallace (University College London) or purchased from Anglian Biotechnology. All restriction digestions and ligations were performed as described by Maniatis *et al.* (1982). *E. coli* plasmid transformation was done as described by Morrison (1979), with the cells grown in nutrient broth supplemented with 20 mM-MgCl₂ as described by Hanahan (1983). Preparation and transformation of *S. lividans* TK24 protoplasts were done as described by Bibb *et al.* (1978) and Thompson *et al.* (1982).

ssDNA detection. Total DNA was isolated from *Streptomyces* as described in Hopwood *et al.* (1985). Agarose gels (0.7%, w/v) were blotted directly onto Bio-Rad Zeta-probe nylon membranes as described by Southern (1975), without prior denaturation of the gel. The Amersham nick-translation kit was used to obtain [α -³²P]dCTP-labelled DNA probes with a specific activity of > 1 × 10⁸ c.p.m. (µg DNA)⁻¹. Labelled probe was boiled for 5 min and placed in a heat-sealable plastic bag containing the baked nylon membrane and prehybridization solution (50%, v/v, formamide; 4 × SSC; 1%, w/v, SDS, 5 × Denhardt's solution, 500 µg denatured salmon sperm DNA ml⁻¹) to a volume of 150 µl per cm of filter [1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0; 1 × Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone]. The bag was then placed in a 55 °C oven overnight with shaking. The membrane was removed from the bag, rinsed briefly with 2 × SSC, and then washed successively in each of the following solutions, for 15 min

at room temperature: 2 × SSC, 0.1% (w/v) SDS; 0.5 × SSC, 0.1% SDS; and finally 0.1 × SSC, 0.1% SDS. The membrane was then wrapped in Saran wrap and exposed to Fuji RX X-ray film.

Results and Discussion

Cloning of *cop/korB* and *sti* into pIJ702

The *cop/korB:sti* region was isolated from pIJ101 on a 1.2 kb *Bcl*I fragment and cloned into pUC8 cut with *Bam*HI, producing pQR200. In order to insert both the *cop/korB* and *sti* determinants into pIJ702, pQR200 was cut with *Pst*I and ligated to pIJ702 also cut with *Pst*I. Recombinant plasmids containing pIJ702 in both orientations were selected in *E. coli* and named pQR410a and pQR411a respectively (Fig. 1). We have previously found that pIJ702-pUC8 shuttle vectors constructed in our laboratory were structurally unstable (R. Barallon, personal communication). The 2.7 kb pUC8 sequence was therefore excised from pQR410a and pQR411a by an *Eco*RI–*Hind*III digestion. The remainder of the plasmid was religated using a 58 bp *Eco*RI–*Hind*III polylinker isolated from pUC19, producing pQR410b and pQR411b (Fig. 1). pQR410a, pQR411a, pQR410b and pQR411b all successfully transformed *S. lividans* to thiostrepton resistance and were structurally stable from restriction map analysis.

In order to subclone only the *sti* determinant, pQR200 was cut with *Eco*RI and *Spe*I and the 0.7 kb fragment containing *sti* was cloned into pUC19 cut with *Eco*RI and *Xba*I, producing pQR417. To reinsert the *sti* region into pIJ702, pQR417 was ligated to pIJ702 via the *Pst*I site. Again, constructs in both orientations were found in *E. coli*, producing pQR421a and pQR422a respectively (Fig. 1). To remove the pUC19 component, pQR421a and pQR422a were digested with *Eco*RI and *Hind*III, and religated to the *Eco*RI–*Hind*III pUC19 polylinker, producing pQR421b and pQR422b respectively (Fig. 1). pQR421a, pQR422a, pQR421b and pQR422b all successfully transformed *S. lividans* to thiostrepton resistance and were structurally stable from restriction map analysis.

Analysis of ssDNA from plasmids containing *sti* and *cop/korB*

Total DNA was prepared from all eight constructs described above (that is, pQR410a, pQR410b, pQR411a, pQR411b, pQR421a, pQR421b, pQR422a and pQR422b), electrophoresed on an agarose gel (together with lysates containing pIJ702 and pIJ303 as positive and negative controls respectively), and blotted directly onto a nylon membrane without a prior denaturation step. The membrane was then probed with [α -³²P]dCTP-labelled pIJ702 (Fig. 2). ssDNA was detected in lysates containing pQR410a/b and pQR422a/b but not in

Fig. 1. Construction of pQR410a/b, pQR411a/b, pQR421a/b and pQR422a/b. The straight arrows indicate the orientation of *sti* with respect to the pIJ702 replicon (*rep*; indicated by a curved broad arrow). These plasmid constructs represent *sti* in both orientations with respect to the basic replicon, with and without *cop/korB*. Details of plasmid constructions are given in the text. *tsr*, thiostrepton resistance gene; *mel*, tyrosinase gene.

Fig. 2. Detection of ssDNA in *S. lividans*. (a) Agarose gel electrophoresis of *S. lividans* whole-cell lysates; the upper band is the chromosomal DNA and the lower, faster-migrating band is the plasmid DNA. (b) Southern blot (without pretreatment) of the same gel, probed with [α - 32 P]dCTP-labelled pIJ702 showing ssDNA accumulation for lysates in lanes 2, 3, 4, 9 and 10. Lanes: 1, pIJ303; 2, pIJ702; 3, pQR410b; 4, pQR410a; 5, pQR411b; 6, pQR411a; 7, pQR421b; 8, pQR421a; 9, pQR422b; 10, pQR422a. Details of ssDNA isolation and detection are given in Methods.

Fig. 3. Construction of pQR441a/b and pQR443a/b to delineate the upper limit of *sti*. The straight arrows indicate the orientation of *sti* with respect to the pIJ702 replicon (*rep*; indicated by a curved broad arrow). Details of plasmid constructions are given in the text. *tsr*, thiostrepton resistance gene; *mel*, tyrosinase gene.

lysates containing pQR411a/b or pQR421a/b. Thus, if *sti* is reinserted into pIJ702 in the correct orientation with respect to the basic replicon, a decrease in ssDNA production occurs, as seen in lysates containing pQR411a/b and pQR421a/b, regardless of the presence or absence of *cop/korB*. This suggests that *sti* alone is sufficient for the efficient initiation of second-strand synthesis to form double-stranded plasmid derivatives, since no ssDNA was detected in pQR421a/b lysates.

If Cop/KorB is involved in copy number regulation as suggested by Deng *et al.* (1988), it would have to negatively control plasmid copy number in one of two ways: it could bind to the double-stranded form of *sti* and thus block the production of ssDNA intermediates by Rep; or it could bind to the single-stranded form of *sti* and thus inhibit the conversion of ssDNA to double-stranded plasmid DNA (which would lead to a pool of ssDNA in the cell). The former situation has been shown not to occur as Cop/KorB does not bind to double-stranded *sti* DNA in gel-retardation assays (Zaman, 1991; Zaman *et al.*, 1992). The latter case also probably does not occur since there is no accumulation of ssDNA in lysates containing pQR411a/b (Fig. 2). Furthermore, from yields of plasmid DNA obtained for *Sti*⁺ plasmids

with and without Cop/KorB, it would seem that Cop/KorB does not significantly alter plasmid copy number.

If *sti* is reinserted in the reverse orientation with respect to the basic replicon (pQR410a/b, pQR422a/b), then ssDNA accumulates in the cell, presumably because *sti* cannot be recognized as the site for second-strand synthesis in its inactive orientation. It should be noted that Deng *et al.* (1988) have claimed that the *SpeI* site (used to sub-clone *sti* in pQR417) lies within the *sti* determinant. However, our results suggest this is not the case since cleavage at the *SpeI* site does not disrupt *Sti* function and ssDNA is not detected in lysates containing pQR421a/b.

These results firstly confirm that *sti* is the site for second-strand initiation (*ssi*) and is active only in its natural orientation with respect to the basic replicon. Secondly, they show that Cop/KorB does not seem to play a role in the control of plasmid copy number through the *sti* locus since it does not bind either to the double-stranded form of *sti*, which would inhibit second strand initiation (Zaman, 1991; Zaman *et al.*, 1992), or to the single-stranded form of *sti*, which would inhibit ssDNA conversion to double-stranded plasmids (this

Fig. 4. Potential secondary structure of the *sti* region of pIJ101. The 0.53 kb *SpeI*–*SacII* *sti* region was scanned for stable inverted repeats. The most stable stem-loop structure found within this defined *sti* region lies between nucleotides 7310 and 7385 on the pIJ101 sequence (Kendall & Cohen, 1988). The identified stem-loop structure has a stability of $\Delta G = 64 \text{ kcal mol}^{-1}$ as defined by Tinoco *et al.* (1973). Filled circles indicate bases that are homologous to the TAGCGT consensus sequence found in *ssi* regions of several plasmids and phages (see Table 2).

work). Thirdly, they reveal that *sti* in either orientation and in the absence of Cop/KorB seems to stabilize the pIJ702-pUC8 shuttle vectors, since no rearrangements were detected in *S. lividans*.

Stability of *Sti*⁺ and *Sti*[−] plasmids in *S. lividans*

The term plasmid instability is used for both structural instability (rearrangements of plasmid DNA) and segregational instability (loss of the plasmid during cell division). In this work, we have shown that *sti* in either orientation structurally stabilizes the pIJ702-pUC8 shuttle vectors which would otherwise rearrange and accumulate ssDNA in *S. lividans* (R. Barrallón, personal communication).

Plasmid segregational instability in unicellular bacteria is normally followed by plating samples on agar to reveal

the proportion of colony-forming units that exhibit a plasmid-borne phenotype, e.g. antibiotic resistance. Since *Streptomyces* are filamentous micro-organisms and almost always form microscopically-sized clumps in most commonly used liquid media, a simple plating method does not work. This is because a colony on a plate is not derived from a single cell but from a clump in which only a small proportion of the mycelial filaments need to have a plasmid for a colony to be formed on selective agar. Since segregational instability is often preceded by a decrease in plasmid copy number, we have developed a method to measure plasmid copy number and therefore monitor plasmid stability throughout growth (Wrigley-Jones *et al.*, 1992). The method is designed to measure all topoisomers of double-stranded DNA. Using this method, we have measured the copy number of the *Sti*⁺ plasmid pIJ303 (pIJ101 with a thiostrepton resistance gene) and found it to increase from less than 200 to more than 400 copies per chromosome between the initial rapid growth phase and stationary phase (Wrigley-Jones *et al.*, 1993). In comparison, the copy number for the *Sti*[−] plasmid pIJ702 increased from 100 to a maximum of 200 copies per chromosome but then declined to fewer than 100 copies per chromosome during stationary phase (Wrigley-Jones, 1991). These results show that pIJ702, which lacks the primary site for second-strand initiation, has a lower copy number than its parent and is therefore more likely to be lost during cell division. A similar decline in copy number during stationary phase was observed for two further *Sti*[−] plasmids, pMT605 and pMT608, both derivatives of pIJ702 with the agarase gene from *S. coelicolor* (Wrigley-Jones, 1991). This suggests that *Sti*[−] plasmids have a lower copy number than *Sti*⁺ plasmids because they cannot efficiently convert the pool of single-stranded intermediates to double-stranded plasmids.

Location of the *sti* function

Several further constructs were made in order to define more precisely where the *sti* determinant was located on the 0.7 kb *SpeI*–*BclI* fragment (Fig. 3). pQR417 was cut with *SacI* and *HindIII*, and the 0.6 kb *sti* fragment ligated to pUC19 cut with the same enzymes (thereby removing approximately 100 bp from the original *sti* fragment), creating pQR437. pQR437 and pIJ702 were digested with *PstI*, ligated, and used to transform *E. coli*. Constructs containing pIJ702 in both orientations were identified and named pQR441a and pQR442a, respectively. Only pQR441a was used in subsequent experiments as it possessed *sti* in the same orientation with respect to the basic replicon (Fig. 3). To remove the pUC19 component, pQR441a was digested with *EcoRI*–*HindIII* and ligated to the 58 bp *EcoRI*–*HindIII* pUC19 poly-

Table 2. Nucleotide sequence comparison of DNA regions containing *ssi* sites of phages and plasmids

The consensus sequence is shown in bold.

Plasmid/phage	<i>ssi</i> nucleotide sequence	Host	Reference
pE194	GCGAAAGTAG TAGCGA CAGCTATTAA	<i>Staphylococcus</i>	del Solar <i>et al.</i> (1987)
pC221	AGCGGTCAGAT TAGCGT CAGCTATCAA	<i>Staphylococcus</i>	
pC194	AATGTCGGCAT TAGCGT GAGCTATTAA	<i>Staphylococcus</i>	
pLS1	CGAAAGGCTT TAGCGT TCGGACGGA	<i>Streptococcus</i>	
pLS1 (<i>att</i>)	TTCTTATCGT TAGCGT GCTGTCATTC	<i>Streptococcus</i>	
φX174	GTAAAAATTT TAATTT TTGCCGCTGA	<i>E. coli</i>	Bahk <i>et al.</i> (1988)
M13	GGGCTCCCTT TAGGGT TCCGATTAG	<i>E. coli</i>	
pACYC184	TCGAACGACCG GAGCGT AGCGAGTCAG	<i>E. coli</i>	
p15A	TCGAACGACCG GAGCGT AGCGAGTCAG	<i>E. coli</i>	
pBR322	CCGAACGACCG GAGCGC AGCGAGTCAG	<i>E. coli</i>	
pIJ101	GCGAAGACAG GAGCGT CGGCCGGGCC	<i>S. lividans</i>	This work
Consensus	TAGCGT		

linker, producing pQR441b (Fig. 3). pQR441a and pQR441b both successfully transformed *S. lividans* to thiostrepton resistance and were structurally stable from restriction map analysis.

pQR437 was digested with *SacI* and *SacII* (removing a further 65 bp from the original *sti* fragment) and the 3.23 kb fragment was ligated to the 14 bp *SacI*–*SacII* pBluescript polylinker, creating pQR438. pQR438 was ligated to pIJ702 via the *PstI* site. Again constructs containing pIJ702 in both orientations were found in *E. coli* and named pQR443a and pQR444a, respectively. Only pQR443a was studied since it possessed *sti* in the same orientation with respect to the basic replicon (Fig. 3). To remove the pUC19 component, pQR443a was digested with *EcoRI* and *HindIII* and ligated to the *EcoRI*–*HindIII* pUC19 polylinker, producing pQR443b (Fig. 3). Both pQR443a and pQR443b successfully transformed *S. lividans* to thiostrepton resistance and were structurally stable from restriction map analysis.

Total DNA was prepared from pQR441a/b and pQR443a/b, run on an agarose gel (with pIJ702 and pIJ303 as controls), and blotted directly onto a nylon membrane. The membrane was probed with [α - 32 P]dCTP-labelled pIJ702 (data not shown). No ssDNA was detected in lysates containing pQR441a/b or pQR443a/b, indicating that the deletions made to the original 0.7 kb *SpeI*–*BclI* *sti* fragment were located outside the functional site for second-strand synthesis. Thus, this 0.53 kb *SpeI*–*SacII* fragment defines the upper limit of the *sti* determinant.

Almost all *ssi* sequences contain potential stem and loop structures and both the nucleotide sequence and secondary structure may play critical roles in the functional activity of these signals (Marians *et al.*, 1982; van der Ende *et al.*, 1983; Stuitje *et al.*, 1984; del Solar *et al.*, 1987; Gruss *et al.*, 1987; Bahk *et al.*, 1988; Boe *et al.*, 1989). Several potential stem-loop structures were identified within the 0.53 kb *SpeI*–*SacII* *sti* sequence,

the most stable having a ΔG of $-64 \text{ kcal mol}^{-1}$ (-268 kJ mol^{-1}), which could form the stem-loop structure *in vivo* shown in Fig. 4. This stem-loop structure contains five out of six bases which appear in a hexanucleotide consensus sequence (TAGCGT) found in *ssi* sites of staphylococcal (Gruss *et al.*, 1987), streptococcal (del Solar *et al.*, 1987), and *E. coli* (Bahk *et al.*, 1988) plasmids. A comparison of the hairpin loop and the sites of second-strand synthesis of these plasmids is shown in Table 2. Our results suggest that the DNA sequence shown in Fig. 4 may therefore be the *ssi* sequence of pIJ101.

In conclusion, functional *ssi* sites are essential for replication and may be important for maintaining plasmid structural stability by preventing the accumulation of large amounts of highly recombinogenic ssDNA. The observations reported in this paper may contribute to the development of efficient and stable cloning vectors based on pIJ702 for use in *S. lividans*.

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