Use of a novel cassette to label phenotypically a cryptic plasmid of *Bacillus subtilis* and map loci involved in its stable maintenance

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In order to facilitate studies on the maintenance of cryptic plasmids from Gram-positive bacteria we have constructed a novel cassette cAPG1000 (5.0 kb) which carries both a selectable marker (chloramphenicol resistance from *Staphylococcus aureus* plasmid pC194) and a screenable marker (the *xylE* gene from the TOL plasmid of *Pseudomonas putida* expressed from a cloned promoter of *Bacillus* phage SPO2) and which is flanked by terminators to prevent transcription from the cassette activating or inhibiting loci adjacent to the site of insertion. To demonstrate the usefulness of this cassette we have mapped loci required for stable maintenance of an 8.6 kb cryptic plasmid endogenous to *Bacillus subtilis* (pPOD2000) from the properties of cAPG1000 insertion and insertion/deletion derivatives. We have identified the replication region as well as separate regions required for segregational and structural stability. The segregational mechanism is very efficient since it allows no detectable loss despite the fact that bacteria carrying the plasmid have a greatly increased mean generation time.

Introduction

Bacterial plasmids are widely exploited as vectors for gene cloning and genetic manipulation. Naturally occurring plasmids, although often very stable, are rarely immediately useful as vectors and must themselves be manipulated to produce optimal vector properties. Such manipulation can result in disruption of stable maintenance functions unless these have already been mapped. Analysis of cryptic plasmids requires the addition of phenotypic markers to facilitate detection as well as creation of easily mapped mutations to locate stability functions. To allow phenotypic labelling and mutagenesis to be carried out simultaneously and to simplify testing of mutant stability we have constructed a novel cassette and applied it to the study of a cryptic plasmid endogenous to *Bacillus subtilis*.

B. subtilis has many potential advantages as a host for the industrial exploitation of cloned genes. It is apparently completely harmless to humans, facilitating the preparation of safe pharmaceutical and therapeutic products. Also it secretes proteins efficiently into the

medium, which can limit the intracellular accumulation of gene products toxic to the bacteria and which may enhance the recovery of products in a soluble form (Palva *et al.*, 1983) and reduce downstream processing costs. However a number of factors have limited the use of *B. subtilis* as a host for recombinant DNA. Among these has been the instability, both structural (Gryczan & Dubnau, 1978; Kreft *et al.*, 1982) and segregational (Grandi *et al.*, 1981; Kreft *et al.*, 1982; Yoshimura *et al.*, 1983) of recombinant plasmids in this species. It has been proposed that a major cause of this instability is the fact that the plasmids from *Staphylococcus aureus* which almost exclusively have formed the basis of *B. subtilis* cloning vehicles (Ehrlich 1977, 1978; Gryczan & Dubnau, 1978; Gryczan *et al.*, 1980) replicate via a rolling circle mechanism (te Riele *et al.*, 1986a, b; Gros *et al.*, 1987). In *B. subtilis*, where lagging strand synthesis of the plasmids is not generally initiated efficiently, single-stranded plasmid DNA accumulates (te Riele *et al.*, 1986a) and appears to stimulate deletion formation by slipped mispairing (Ehrlich *et al.*, 1986). However, even with plasmids such as pUB110 which as native plasmids show very little accumulation of single-stranded DNA in *B. subtilis*, insertion of foreign DNA can disrupt the normal replication and stable inheritance processes and lead to both structural and segregational instability (Bron

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& Luxen, 1985; Viret & Alonso, 1987). It is essential therefore to characterize fully a potential plasmid vector system with respect to the effect that insertion of foreign DNA has on plasmid functions and the stability of the cloned DNA.

To provide the basis of an alternative cloning vector system for this species we have initiated studies on the replication and stable inheritance functions of a low-copy-number endogenous plasmid from a strain of *B. subtilis* isolated by Tanaka *et al.* (1977) since such a plasmid should already be well adapted for stable maintenance in its host species. Our initial characterization of the plasmid obtained raised some doubt in our minds as to whether we really had pLS14, the plasmid requested, so we have designated it pPOD2000. It may be related to but is not identical to two plasmids from *Bacillus amyloliquefaciens* which have been partially characterized (Bron *et al.*, 1987; Chang *et al.*, 1987). Plasmid pPOD2000 is maintained at relatively low copy number (approximately five copies per chromosome equivalent) and has no directly selectable markers. In this paper we describe construction of the novel cassette and its use to study the maintenance functions of pPOD2000.

Methods

Bacterial strains and growth conditions. *Escherichia coli* K12 strain C600 (*thr-1 leu-6 thi-1 lacY1 supE44 tonA21 galE⁺T⁺K⁻*) (from M. Rosenberg, SK&F Laboratories, Swedeland, Pennsylvania, USA) and *B. subtilis* 1A289 (*aro-1906 metB5 sacA321 amyE*) (from Bacillus Genetic Stock Center, Ohio State University, Columbus, Ohio, USA), PLS1 (*arg-15 leuA8 thrA stp recE4 r⁻m⁻*) (Ostroff & Pène, 1983) and BD224 (*trpC2 thr-5 recE4*) (1A46 from Bacillus Genetic Stock Center, see above) were used. Bacteria were grown at 37 °C in L broth (Kahn *et*

al., 1979) or on L agar, consisting of L broth supplemented with 1.5% (w/v) agar. Other media are described where appropriate. Antibiotic resistance conferred by plasmids was selected by addition of benzylpenicillin, sodium salt (300 µg ml⁻¹ in solid and 150 µg ml⁻¹ in liquid media) for Pn^R; tetracycline hydrochloride (20 µg ml⁻¹) for Tc^R; chloramphenicol (5 µg ml⁻¹ for *E. coli* and 5–20 µg ml⁻¹ for *B. subtilis*) for Cm^R; and kanamycin sulphate (30 µg ml⁻¹) for Km^R.

Plasmids. Plasmids used in this work and which have previously been described are listed in Table 1. Plasmids constructed during the course of this work are described in the text or the Figures.

DNA isolation, analysis and genetic manipulation. Plasmid DNA isolation on both small and large scales was carried out by the method of Birnboim & Doly (1979). For *E. coli*, lysozyme was omitted from the lysis buffer and alkaline SDS was added immediately after resuspension. For *B. subtilis*, bacteria were incubated with lysozyme for 15 min at 37 °C prior to addition of alkaline SDS. Further purification was achieved by isopycnic centrifugation in CsCl/ethidium bromide gradients. Routine analysis and manipulation of plasmid DNA was carried out as described by Maniatis *et al.* (1982). DNA sequence analysis was carried out as described by Maxam & Gilbert (1980) with modifications as described previously (Smith & Thomas, 1984).

Transformation of bacteria. *E. coli* was made competent for transformation by standard CaCl₂ treatment (Meyer *et al.*, 1977). Transformation of *B. subtilis* protoplasts was achieved by a variation on standard methods developed by A. Mountain. L broth (100 ml) was inoculated with bacteria from a fresh plate and the culture grown to OD₆₅₀ = 0.4. The bacteria were harvested and resuspended in 7.5 ml SPA (5:4:1, by vol, of 2 × SSM, 4 × Penassay broth, 5%, w/v, BSA; 2 × SSM is 1 M-sucrose, 40 mM-maleic acid, 40 mM-MgCl₂). Then 0.4 ml of freshly made and filter-sterilized lysozyme solution (20 mg ml⁻¹ in SPA) was added and the mixture incubated at 37 °C with gentle shaking. Phase-contrast microscopy was used to monitor protoplast formation, which generally took about 60 min. Protoplasts were harvested by centrifugation at 4 °C, resuspended in 7.5 ml SPA by gentle pipetting and then reharvested and resuspended as before. Using 13.5 ml disposable conical tubes (Falcon or Sterilin) 0.5 ml of protoplast suspension was mixed with up to 50 µl DNA solution and 1.5 ml 30% (w/v) PEG 6000. After 2–5 min at 20 °C, 5 ml SPA was added and after mixing by inversion, the protoplasts were harvested by centrifugation at 4300 r.p.m. for 15 min. The SPA/PEG supernatant was removed and the protoplast pellet resuspended in 2 ml SPA and

Table 1. *Previously described plasmids used in this study*

Plasmid	Size (kb)	Replicon	Properties	Reference
pC194	2.9	pC194	Cm ^R	Ehrlich (1977)
pCPP-3	5.6	pMB1/pUB110	Km ^R	Band <i>et al.</i> (1983)
pCT571	9.5	pSC101	Km ^R Tc ^R	Hasnain & Thomas (1986)
pDS3	2.5	p15A	Cm ^R	Thomas (1981)
pHV60	5.55	pMB1	Cm ^R Tc ^R Pn ^R = pHV32	Primrose & Ehrlich (1981)
pKK223-3	4.6	pMB1	Pn ^R	Brosius & Holy (1984)
pPL608	5.0	pUB110	Km ^R Cm ^R	Williams <i>et al.</i> (1981)
pPOD2000	8.6	pPOD2000	Cryptic (pLS14)	Tanaka <i>et al.</i> (1977)
pRK2501	11.0	RK2	Km ^R Tc ^R	Kahn <i>et al.</i> (1979)
pSRW40	6.9	pMB1	Pn ^R	Warne (1986)
pUB110	4.5	pUB110	Km ^R	Gryczan & Dubnau (1978)
pUC7	2.7	pMB1	Pn ^R	Vieira & Messing (1982)

incubated at 37 °C for 2–3 h with gentle shaking to allow expression of antibiotic resistance markers. Aliquots of 0.1 ml were spread on DGSA regeneration/selection plates and incubated at 37 °C for up to 3 d. DGSA consists of, per litre: 280 ml protoplast agar, 230 ml 2 M-sodium succinate pH 7.3, 40 ml 3% (w/v) KH_2PO_4 , 40 ml 7% (w/v) K_2HPO_4 , 320 ml 2.8% (w/v) soluble starch, 20 ml 20% (w/v) glucose, 10 ml 1 M- MgCl_2 , 40 ml 10% (w/v) gelatin, 8 ml 5% (w/v) BSA. Protoplast agar contains, per 280 ml: 10.5 g Difco agar, 4.9 g Difco Casamino acids, 4.9 g Difco Yeast extract. While this method was necessary for transformation with ligated DNA, a scaled-down version using microfuge tubes and 50 μl of protoplast suspension with 2–5 μl DNA, 150 μl PEG and 500 μl SPA could be used simply to transfer plasmids into a new strain.

Estimation of plasmid stability. Stationary-phase cultures of bacteria carrying the plasmid under test were grown with antibiotic selection and then diluted 10^6 -fold into 50 ml nonselective medium in 250 ml conical flasks and grown to saturation with shaking at 37 °C. This gives approximately 20 generations of growth. Dilution and growth to stationary phase were repeated four more times to give 100 generations of unselected growth. Culture samples were withdrawn at convenient points from 0 to 100 generations and spread on nonselective agar after suitable dilution to give approximately 100 colonies per plate. Incubation at 37 °C was continued until colonies were 2–3 mm in diameter, at which stage the plates were sprayed with a 0.5 M-catechol solution (Zukowski *et al.*, 1983). After 5 min the colonies were scored XylE^+ or XylE^- . A selection of colonies of both phenotype (if both were found) were then replica-plated to test for Cm^R .

Determination of XylE activity. Catechol 2,3-oxygenase (XylE) activity was assayed by a modification of the method of Sala-Trepat & Evans (1971). Bacteria from a 30 ml saturated culture were harvested and resuspended in 1.2 ml 0.1 M-sodium phosphate buffer, pH 7.0, 0.15 ml acetone and 0.15 ml lysozyme solution (1 mg ml^{-1}), transferred to a microfuge tube and incubated at 37 °C for 10 min. After disruption by ultrasonic treatment (2 $\times$ 30 s) on ice, cell debris was pelleted by centrifugation at 4 °C in a microfuge. Enzyme activity in the supernatant was assayed by measuring the rate of change of absorbance at 375 nm when a known volume of extract (usually 10 μl) was added to 3.0 ml 0.67 mM-catechol in 0.1 M-sodium phosphate buffer pH 7.0 in a 3 ml cuvette with 1 cm light path. Protein concentration in the extracts was determined by the biuret reaction (Gornall *et al.*, 1949).

Results

Construction of a novel cassette for insertional mutagenesis of plasmids in Gram-positive bacteria

Construction of the cassette is summarized in Fig. 1. The two primary attributes which we required in the cassette were a selectable marker to allow direct selection for the presence of the plasmid into which the cassette was inserted and a screenable marker to facilitate tests on plasmid stability. For these we chose the chloramphenicol resistance gene, *cat* or Cm^R , from *S. aureus* plasmid pC194, which is known to be expressed in *B. subtilis* (Ehrlich, 1977) and expression of the *xylE* gene from the TOL plasmid of *Pseudomonas putida*, whose ribosome-binding site contains strong complementarity to the 16S rRNA of *B. subtilis* and which is known to be expressed

efficiently if transcribed in *B. subtilis* (Zukowski *et al.*, 1983). Since the *xylE* gene comes from the centre of an operon it lacks its own promoter and so to achieve efficient expression, heterologous promoters *tac* and *trp* from *E. coli*, and a SPO2 *Bacillus* phage promoter, all of which are known to function in *B. subtilis* (Penschke *et al.*, 1985; Williams *et al.*, 1981), were inserted upstream of the *xylE* structural gene by linearizing pAPG7 at the *XhoI* site, filling in the sticky ends and ligating to blunt-ended promoter-containing fragments. We found that only the SPO2 promoter gave sufficient *xylE* expression to allow easy screening for the presence of plasmids in *B. subtilis*.

In addition we flanked the cassette with transcriptional terminators to ensure that insertion of the cassette had no deleterious effect other than that due to its disruption of the continuity of the plasmid backbone. The terminators used were the dual *E. coli* *rrnB*_{t1}t₂ and λ t₀, since they have both been shown to function in *B. subtilis* (Penschke *et al.*, 1985). The λ t₀ terminator can be isolated on a *Sau3A* fragment which on insertion at a *Bam*HI site regenerates a *Bam*HI site at the downstream end of the terminator (see pAPG3, Fig. 1). However, downstream of the *rrnB*_{t1}t₂ terminators from pKK223-3 there is no convenient restriction site before the *bla* promoter at position 4189 of the pBR322 sequence (Brosius *et al.*, 1982). To delete this promoter we used *Bal*31 deletion starting from the *PvuI* site in the *bla* gene (see construction of pAPG4 Δ 10, Fig. 1) and inserted a *Bam*HI linker at the end of the deletion. The end-point of the deletion was determined by DNA sequence analysis to confirm the removal of the *bla* promoter. Thus the cassette is flanked by convenient restriction sites and should not carry promoters capable of initiating transcription into surrounding DNA.

To check the behaviour of the cassettes in *B. subtilis* the versions flanked by *Bam*HI were inserted at the *Bgl*II site within the Km^R gene of pUB110. Transformation of *B. subtilis* yielded the expected Cm^RKm^S plasmids but only bacteria carrying plasmids with the cassette in which *xylE* is expressed from the SPO2 promoter (designated cAPG1000) gave colonies that were easily distinguishable from plasmid-negative colonies by the yellow colour they developed when sprayed with a solution of catechol. Quantitative estimates of XylE activity in bacteria carrying pAPG111 and pAPG119 (which contain cAPG1000 in opposite orientations) indicated that the level was independent of the orientation of insertion. By comparisons with previously published data (Zukowski *et al.*, 1983) we estimated that the SPO2 promoter should give enough *xylE* expression for the presence of the cassette to be detectable even if joined to a plasmid with a copy number of five copies per cell.

Fig. 1. Construction of the cassette cAPG1000. Restriction sites are shown as: *Bam*HI, B; *Eco*RI, E; *Hind*III, H; *Pst*I, P; *Pvu*I, Pv; *Sal*I, S; *Sma*I, Sm; *Xho*I, X.

Insertion of the cassette cAPG1000 into the cryptic endogenous B. subtilis plasmid pPOD2000 and approximate location of plasmid regions required for replication

A restriction map of pPOD2000 is shown in Fig. 2, using coordinates originating at the unique *Sal*I site. Previous attempts to insert a selectable *Hind*III fragment into the *Hind*III sites of pPOD2000 gave insertions at all sites except that at coordinate 2.2 kb, suggesting that this site lies within a region essential for replication. The *Eco*RI fragment of pAPG7 carrying cAPG1000 was ligated to the *Eco*RI fragment of pPOD2000 which runs from coordinate 0.9 to 4.7 kb and after transformation of *B. subtilis* 1A289, plasmid DNA from Cm^R XylE⁺ colonies was analysed. Two different plasmids with the two possible orientations of *Eco*RI fragments were found and designated pAPG2001 and pAPG2002. They both appear to have the same copy number as pPOD2000 (estimated as five copies per chromosome equivalent; A. Mountain, unpublished), as estimated by yield of plasmid DNA extracted by the alkaline SDS method. We therefore concluded that a functional replication system with normal control elements is contained within this *Eco*RI fragment. By ligating pPOD2000 DNA which had been partially digested with *Hind*III to *Hind*III-digested pCT571 we obtained clones which contained various subsegments of pPOD2000. Testing these for replication in *B. subtilis* indicated that only the

third- and fourth-largest *Hind*III fragments were necessary to allow autonomous replication, that neither on its own was sufficient and that the two fragments needed to be joined in their natural configuration. This further defines the replication system.

To obtain derivatives with cAPG1000 inserted at a range of positions, pPOD2000 DNA was partially digested with *Sau*3A, separated by agarose gel electrophoresis and the DNA species corresponding to full-length linear plasmid DNA molecules purified. After dephosphorylation the DNA was ligated to purified cAPG1000 DNA produced by cleavage with *Bam*HI. *B. subtilis* was transformed with this DNA and the plasmid DNA from Cm^R XylE⁺ transformants was isolated and characterized by restriction endonuclease digestion and gel electrophoresis. Twenty insertion derivatives were analysed in detail (Fig. 3) and used for further study. Some of these appeared to be simple insertions of the cassette into the full-length plasmid while other insertion events were associated with deletions, some as large as 3.2 kb. One of these deletions extends (in plasmid pPOD2029) from coordinate 3.5 kb to 6.0 kb, removing the *Hind*III site at 3.7 kb and therefore further defining the replication region to between coordinates 1.2 and 3.5 kb. The size of this region, 2.3 kb, is in line with the size of minimal replicons found for many other plasmids. The absence of cassette insertions within this region compared to the disruption of the rest of the plasmid at many sites could be due to a relative deficiency of such sites within this region. However, this region cannot be completely devoid of such sites because it contains a *Pvu*I site and an *Xho*II site, both of which are also *Sau*3A sites. Therefore the lack of insertions in this region suggests that most of the 2.3 kb is important for plasmid replication.

Fig. 2. Physical and preliminary genetic map of pPOD2000. An extensive restriction map of the plasmid is shown; the coordinates of the cleavage sites are given relative to the unique *Sal*I site (0/8.6 kb). This study has identified the replication region (*rep*) (speckled sector), a region whose integrity is required for structural stability (*stab*) (hatched sector), and a region encoding functions involved in the segregational stability (*seg/par*) (solid sector) of pPOD2000.

Stability characteristics of pPOD2000::cAPG1000 hybrids

To locate plasmid regions encoding functions which are important for stable inheritance in *B. subtilis* we screened cultures of Rec⁺ (1A289) *B. subtilis* carrying the derivatives shown in Fig. 3 for loss of plasmid markers during rounds of nonselective growth in batch culture for up to 100 generations. During this screening process we noticed that bacteria carrying hybrid plasmids containing all of the pPOD2000 DNA grew significantly more slowly than bacteria without such plasmids, both in liquid culture and on solid media. Quantification of this effect indicated that bacteria carrying hybrids containing most of pPOD2000 grew with a mean generation time ($\pm$ SEM) of 45 ± 4.5 min compared to 29 min for plasmid-free bacteria. However, plasmids pAPG2001 and pAPG2002 affected bacterial growth rate much less,

Fig. 3. Stability of pPOD2000::cAPG1000 derivatives. The open bar represents the entire pPOD2000 genome (8.6 kb) with the positions of the *Sal*I (S), *Eco*RI (E) and *Hind*III (H) sites indicated. The pPOD2000-cAPG1000 hybrids (pAPG2001 to pAPG2038) are represented as follows: ▲ indicates the insertion position of cAPG1000 into full-length pPOD2000; — indicates the extent of the *in vitro* *Sau*3A deletion from the point of cAPG1000 insertion. The arrows indicate the orientation of cAPG1000 with respect to the direction of *xylE* transcription. For stability test: +, stable; -, unstable; ±, only marginal instability; NT, not tested.

bacteria carrying them having a mean generation time of 33 ± 1.5 min. Since all the plasmids tested contain the cassette cAPG1000 this indicates that the retardation of growth rate is not due to the cassette but must be due to some plasmid-encoded function. Examination of plasmids retaining this growth-retarding effect suggests that the region of the plasmid from 8.3 to 1.0 kb plays some role in conferring this phenotype.

A consequence of this growth disadvantage is that the segregation kinetics for unstable plasmids will be determined by both the rate of appearance of plasmid-free bacteria and the relative rates at which plasmid-positive and plasmid-free bacteria grow. Since this complicates the detailed interpretation of results we initially confined ourselves to determining whether insertions and/or deletions caused structural or segregational instability.

The results of the stability tests are summarized in Fig. 3. A number of classes of phenotype can be identified. First, derivatives with cAPG1000 inserted at coordinates 5.6–6.3 kb and 6.3 kb (pAPG2019 and pAPG2031, respectively) gave no detectable plasmid loss over the 100 generations of the experiment. Thus pPOD2000 is very stably maintained and the cassette can be inserted at certain sites without disrupting its stable inheritance functions. Instability observed with the cassette inserted at other locations must therefore be due to disruption of actively required loci.

Second, an insertion/deletion between coordinates 5.0

and 5.7 kb (pPOD2038) and an insertion at coordinate 5.3 kb (pPOD2016) resulted in plasmids which exhibited a high rate of structural instability but no segregational instability: they gave rise to *XylE*⁻ colonies all of which were Cm^R. This instability was also observed when the experiment was repeated in the *recE* strain BD224 (data not shown), indicating that the structural instability is *RecE*-independent. Since a deletion from 5.6 kb to 6.3 kb has no effect on stability (pAPG2019), the locus affected in pAPG2016 and pAPG2038 must extend no further than coordinate 5.6 kb. However, if there is a specific locus in this region whose inactivation leads to structural instability then the function it provides is either not essential or can be fulfilled by other loci since the plasmid pAPG2029 with a deletion from 3.6 to 6.0 kb resulted in only minor segregational instability and no structural instability.

Third, a series of insertions/deletions in the region of coordinates 6.45–8.25 kb had no effect on structural stability but resulted in significant segregational instability. A majority of these plasmids, which have simple insertions or only small deletions affecting this region (pAPG2003, 2005, 2007, 2008, 2012–2015, 2017, 2032, 2033), resulted in less than 1% plasmid retention after 100 generations. However, hybrids pAPG2009 (deletion from 7.0 to 8.15 kb) and pAPG2036 (deletion from 4.8 to 8.15 kb), while still segregating at a high rate, were significantly less unstable than the majority of these derivatives (about 10% and 4% plasmid retention,

respectively, after 100 generations). This may suggest that partial removal of the functions in this region has a greater effect on plasmid stability than complete removal.

Discussion

We have shown that the cassette cAPG1000 fulfils our predetermined requirements. The cassette carries both a useful selectable marker and a marker whose presence in the bacteria of a colony, even when carried on a relatively low-copy-number plasmid, can easily be detected. Except where the cassette actually disrupts a region important for plasmid stability, insertion of the cassette into pPOD2000 yielded derivatives with no detectable rate of loss. Since the presence of these plasmids appears to increase the mean generation time of host bacteria by about 50%, even a very low rate of segregation of plasmid-free bacteria should have been detectable if it were occurring. Since pPOD2000 can accommodate insertion of at least 5 kb of DNA with no significant destabilization, the stable derivatives described in this paper therefore provide the basis for the development of novel cloning vectors with sites for insertion of foreign DNA within the cassette.

DNA sequence analysis of the *rep* region (A. P. Gleave, unpublished) indicates that pPOD2000 belongs to the pC194 family of plasmids, which replicate by a rolling circle mechanism. Insertions into one of the stability regions identified results in structural instability and by analogy with other members of the pC194 may represent a region involved in lagging strand synthesis during replication (te Riele *et al.*, 1986*a, b*; Gruss *et al.*, 1987; Viret & Alonso, 1987). The accumulation of single-stranded DNA resulting from inactivation of such a locus could stimulate deletion formation (Ehrlich *et al.*, 1986). While we have been unable to detect single-stranded plasmid DNA in cells carrying pPOD2000 (A. P. Gleave, unpublished) we have not yet determined whether or not unstable derivatives of this sort now accumulate single-stranded DNA as would be predicted.

Insertions into a second region result in segregational instability. Given the low copy number of pPOD2000 it is unlikely that all daughter cells would receive plasmid copies by random segregation. Therefore the region is likely to encode some sort of active partitioning mechanism. It is interesting that the data suggest that complete removal of this region results in less instability than the majority of more limited insertions/deletions in this region. Such a phenomenon has previously been observed with the *par* functions of the mini P1 plasmid (Funnell, 1988) and was attributed to the individual subcomponents of the *par* system actively interfering

with the random partitioning of the plasmid DNA molecules. However, this region of pPOD2000 may also provide a function which results in death of bacteria that have lost the plasmid, as has been observed for some low-copy-number plasmids of Gram-negative bacteria (Jaffé *et al.*, 1985; Gerdes *et al.*, 1986). Whatever the underlying mechanism of the stability function(s) of pPOD2000, their efficiency suggests a system not previously described for plasmids of Gram-positive bacteria. Therefore, elucidation of the nature of these functions and their relationship to analogous systems of Gram-negative bacteria will be of considerable fundamental and applied interest.

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