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Gyrase-dependent stabilization of pSC101 plasmid inheritance by transcriptionally active promoters

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The pSC101 plasmid encodes a *cis*-acting genetic locus termed *par* that ensures the stable inheritance of plasmids in a population of dividing cells. In the absence of selection, *par*-defective plasmids are lost rapidly from the bacterial population. We report here that the stability of *par*-deleted pSC101 derivatives is restored by introducing certain adventitious bacterial promoters onto the plasmid. Stabilization requires active transcription from the inserted promoter and is affected by the site and orientation of the insertion, the length of the nascent transcript and DNA gyrase activity. While a promotor-associated overall increase in negative superhelicity of plasmid DNA was observed, stabilized inheritance appeared to be dependent on localized rather than generalized supercoiling. Our demonstration that promoter-induced DNA supercoiling can mimic the effects of the pSC101 *par* locus provides evidence that the previously reported superhelicity-generating effects of *par* are intrinsic to its function.

Key words: DNA gyrase/DNA topology/partitioning/supercoiling/transcription

Introduction

The oligocopy pSC101 plasmid encodes a *cis*-acting genetic determinant termed *par* that accomplishes stable inheritance of the plasmid in populations of actively dividing bacterial cells in the absence of selective pressure (Meacock and Cohen, 1980). Regions functionally analogous to the pSC101 *par* locus have been identified on other bacterial plasmids, including NR1 (Miki *et al.*, 1980), R1 (Nordström *et al.*, 1980), F (Ogura and Hiraga, 1983) and P1 (Austin and Abeles, 1983), and on the yeast plasmid pSR1 (Jearnpipatkul *et al.*, 1987). In contrast to certain of these other partition loci, the *par* locus of pSC101 does not include a protein-coding sequence (Miller *et al.*, 1983).

The pSC101 *par* locus is contained on a 375 bp DNA fragment adjacent to the plasmid's replication origin; while *par* is not required for pSC101 DNA replication, it appears to enhance replication capabilities (Tucker *et al.*, 1984) and to have an effect on plasmid copy number (Manen *et al.*, 1990) which varies with the stage of cell growth (H.Ingmer and S.N.Cohen, unpublished). Three partition-related (PR) segments, two of which are largely identical, and one of which is an inverted repeat of the two directly repeated segments, have been implicated in its function (Tucker *et al.*, 1984). Deletion of all three PR segments leads to extreme instability of the plasmid, while deletion of any two of the three segments yields a less unstable replicon. Loss of only one of the PR segments results in a plasmid that is stably maintained when present alone, but is defective in its ability to compete with a wild-type pSC101-derived replicon present in the same host [i.e. the competition-minus (Cmp^-) phenotype, Tucker *et al.*, 1984].

Recent work indicates that DNA superhelicity is centrally involved in the partitioning mechanism (Miller *et al.*, 1990). pSC101 derivatives lacking the *par* locus show decreased overall superhelical density as compared with DNA of wild-type pSC101. Moreover, partition-defective plasmids that utilize the unrelated pSC101, p15A, F or *ori*C replication systems are stabilized in *Escherichia coli* by *topA* gene mutations, which increase negative DNA supercoiling. Conversely, mutations in DNA gyrase and DNA gyrase inhibitors increase the rate of loss of *par*-defective pSC101 derivatives and convert ordinarily stable replicons that have minimal *par* region deletions into unstable plasmids (Miller *et al.*, 1990). Possibly relevant to these observations is the finding that a strong binding site for DNA gyrase overlaps the PR segments within the *par* region (Wahle and Kornberg, 1988).

During the construction of certain pSC101 derivatives, we observed that certain antibiotic-resistant genes, when introduced in one orientation at specific plasmid sites, stabilized the inheritance of plasmids deleted for the entire *par* locus. Our subsequent investigations have shown that the observed stability, which may be accompanied by detectable changes in the superhelical density of plasmid DNA, is dependent on transcriptional activity within the insert and on normal cellular concentrations of DNA gyrase. Our experiments demonstrating these gyrase-dependent effects of transcription on plasmid inheritance are reported here.

Results

par-deleted pSC101 derivatives are stabilized by insertions of the Tn5-derived aph gene

Whereas the wild-type pSC101 plasmid is stably maintained in *E.coli* cells for at least 100 generations of growth in the absence of selection (Meacock and Cohen, 1980), pSC101 derivatives that lack the entire *par* locus (i.e. are deleted for PR segments a, b and a') are lost rapidly from dividing cell populations (Tucker *et al.*, 1984). The stability properties of one such deletion mutant plasmid (pZC56, Figure 1A) in the wild-type bacterial host strain PM191 (Meacock and Cohen, 1979) are shown in Figure 2. As has been found earlier for other *par*-deleted plasmids (Tucker *et al.*, 1984), pZC56 was retained by only 12% of the population after 40 generations of growth in non-selective medium and was present in < 1% of cells after 60 generations of growth.

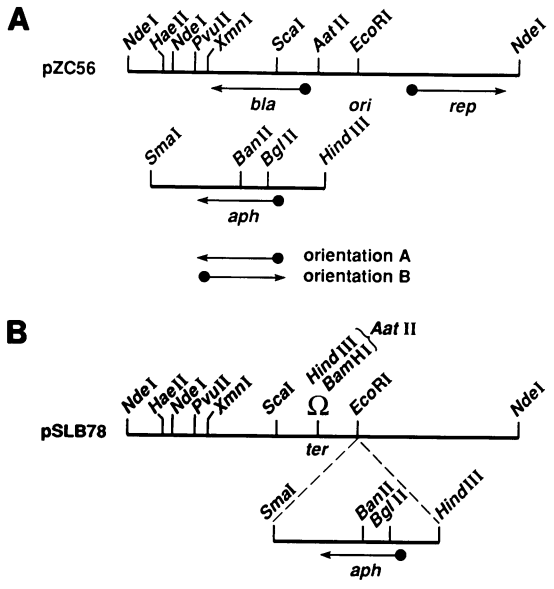

Fig. 1. (**A**) Map of plasmid pZC56 showing relevant restriction sites and loci. The plasmid is derived from pZC20 (Biek and Cohen, 1986) with the *par* region (a 375 bp *Eco*RI–*Ava*I) deleted leaving the *Eco*RI site intact. Different sequences have been cloned into various sites as indicated in Table I in the orientation A or B as indicated. The black dots indicate the promoters and transcripts are represented by lines, their direction of transcription by the arrows. The origin region has been delineated by Linder *et al.* (1985). (**B**) The transcription termination signal from gene 32 of bacteriophage T4 (*ter*) was isolated from pWTT552 as a 170 bp *Eco*RI–*Hin*dIII fragment. To determine the length of the transcript necessary for stabilization deletions were made from the sites in the Tn*5 aph* gene to the terminator.

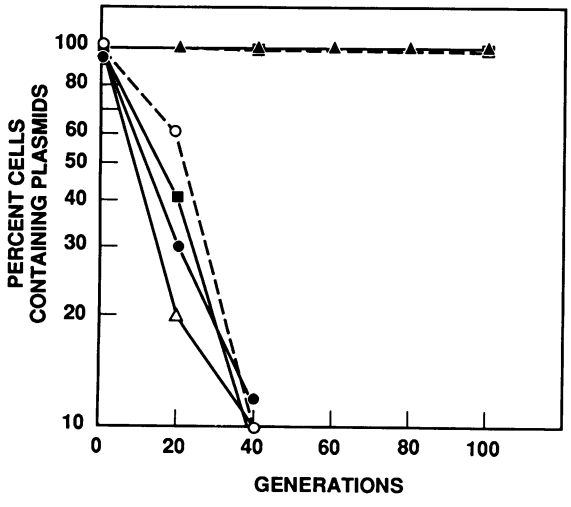

Fig. 2. Stability of pSC101 derivatives. Plasmid stability was measured in liquid cultures grown without selection. In this figure the plasmids that show no loss are graphed as one line. The plasmids used were pSLB56 (Km), pSLB57 (Km), pSLB98 (Km), pSLB87 (Km), pSLB871 (Km), pSLB204 (Km), pSLB208 (Km) ▲; pCM67 –△–; pZC56 (Ap) ○; pSLB67 (Km) ●; and pSLB671 (Km) ■ pSLB127 (Km) △.

However, plasmid instability was reversed by adding to pZC56 a *Hin*dIII–*Sma*I DNA fragment containing the Tn*5*-derived *aph* gene, which encodes the enzyme aminoglycoside 3′-phosphotransferase and specifies resistance to kanamycin (Km) (Berg *et al.*, 1975, 1978; Jorgensen *et al.*, 1979; Beck *et al.*, 1982). The stabilized plasmid had a copy number similar to that of the parental

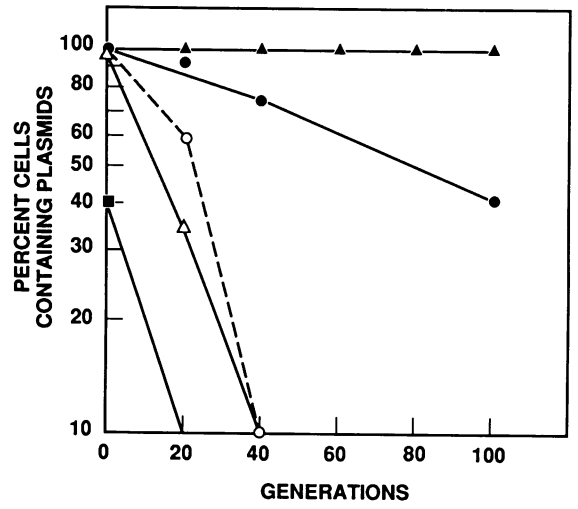

Fig. 3. Stability of pSC101 derivatives. Plasmid stability was measured in liquid cultures grown without selection. In this figure the plasmids that show no loss are graphed as one line. The plasmids tested were pSLB62 (Cm), pSLB80 (Tc), pSLB120 (Tc), pSLB121 (Tc), pSLB57 (Ap,Km) ▲; pZC56 (Ap) ○; pSLB60 (Km) ●; pSLB122 (Ap) ■; pCM57 (Ap) △.

replicon pZC56. Stabilization occurred when the *aph* gene was introduced at the pZC56 *Hae*II site (yielding plasmid pCM67), *Sca*I site (pSLB56) or *Eco*RI site (pSLB57) (Figure 2) where insertions were observed only in orientation A. When the *aph* gene fragment was substituted in either orientation for a 400 bp *Nde*I fragment (pSLB67 and pSLB671 plasmids) the plasmid was not stably maintained (Figure 2). The lack of stability did not reflect essentiality of specific sequences within the *Nde*I fragment, since absence of this fragment did not prevent stabilization by the *aph* gene inserted at the *Sca*I site (pSLB98, Figure 2). Moreover, plasmids that lack the 400 bp *Nde*I fragment but contain the *par* locus are also fully stable (pSLB87 and pSLB871, Figure 2).

Insertions of the *aph* gene in orientation B at the *Hae*II, *Sca*I or *Eco*RI sites could not be obtained in *par*-deleted plasmids unless a transcription terminator was placed between the insert and the replication origin, but were obtainable in par^+ replicons (e.g. pSLB204 and pSLB208). This observation is consistent with earlier evidence that transcriptional read-through into the origin region can interfere with plasmid replication (Gentz *et al.*, 1981; Stueber and Bujard, 1982; Bujard *et al.*, 1983), and that the *par* locus can serve as a terminator of transcription *in vivo* (Tucker *et al.*, 1984). Unlike plasmids containing insertions in orientation A, replicons containing these terminator-dependent insertions in orientation B were not stably inherited (pSLB127, Figure 2).

Stabilization of par-deleted plasmids by other bacterial gene inserts

To investigate the basis for plasmid stabilization by the *aph* gene insertion at specific plasmid sites, other DNA fragments were tested at the same loci. The Tn*9*-derived *cat* gene (Chang and Cohen, 1978) (plasmid pSLB62), and the pBR322 *tetA* gene (Bolivar *et al.*, 1977) (plasmid pSLB80) also stabilized plasmid inheritance (Figure 3) when introduced in orientation A at the *Sca*I site of pZC56, as did insertion of *tetA* at the *Eco*RI site (pSLB120) or *Hae*II site

(pSLB121). However, insertion of the *aph* gene of Tn*903*, which differs in its control region and primary sequence from the *aph* gene of Tn*5*, resulted in only partial stabilization (pSLB60, Figure 3). In addition to restoring full stability to the *par*-deleted pZC56 plasmid, the *tetA* gene insertion at the *Sca*I site yielded the Cmp^+ phenotype (plasmid pSLB80, Table II), whereas insertion of the same DNA fragment at the plasmid's *Eco*RI or *Hae*II site resulted in the Cmp^- phenotype (pSLB120 and pSLB121, Table II). Collectively, these experiments and the ones described in the previous section show that the ability of DNA inserts to compensate for the absence of the pSC101 *par* region depends on (i) properties inherent to the DNA fragment being inserted, (ii) the site of the insertion, and (iii) the orientation of the insertion. Moreover, the effects of two separate inserts were additive: plasmids stabilized by insertion of the *aph* gene into the *Sca*I site (pSLB56) or insertion of the *cat* gene into the *Eco*RI site (pCM62) were Cmp^-. However, a *par*-deleted plasmid containing both of the inserted DNA fragments in orientation A (pSLB96) showed not only the Par^+, but also the Cmp^+ phenotype (Table II).

Stabilization of par-deleted pSC101 derivatives results from transcription within the DNA insert

Examination of published sequences of the various stabilizing inserts showed no commonality within the protein-encoding segments of the genes, and also no similarity to the sequence of the pSC101 *par* locus. To investigate the basis for stabilization of *par*-deleted plasmids by such disparate DNA inserts and to identify more precisely the *aph* insert segment responsible for stabilization, we introduced either the promoterless protein-coding segment or the upstream promoter region into the *Eco*RI site of pZC56, yielding the unstable pSLB59 and stable pSLB63 plasmids respectively (Table I). The segregation properties of these constructs (Figure 4) indicated that the stabilizing effects of *aph* gene fragments are associated with the DNA segment containing the promoter.

The possibility that the observed stabilization of inheritance of pSC101 derivatives is related specifically to promoter activity was tested by an experiment using a construct in which *aph* gene expression was abolished by deletion of 2 bp at an *Hae*II site within the promoter region. As shown in Figure 3, this manipulation eliminated the stabilizing effect of the promoter-containing insert (plasmids pSLB57 versus pCM57). Similarly, the plasmid-stabilizing effect of the *tetA* gene was eliminated by insertion of 2 bp at a *Cla*I site identified previously as being in the promoter sequence (Pruss and Drlica, 1986) (pSLB120 versus pSLB122, Figure 3).

We found also that transcript length has a prominent role in promoter stabilization of plasmid inheritance. When the bacteriophage T4 gene 32 terminator (Prentki and Krisch, 1984) was placed at different distances downstream from the *aph* gene insert, the following results were obtained (Figure 4). Insertion of the termination signal at the *Aat*II site had no effect on the ability of the *aph* insert at the *Eco*RI site to stabilize plasmid inheritance (pSLB78 plasmid, Table I). A negligible effect on *aph*-induced stabilization was observed when the terminator was brought closer to the promoter by deleting the segment between a *Bam*HI site 9 bp upstream from the terminator and a *Ban*II site 166 bp downstream from the transcription initiation site (pSLB95). However, extending the deletion to a *Bgl*II site 55 bp downstream from the site of initiation of transcription (pSLB89) eliminated the stabilizing effect of the insert (Figures 1B and 4). Thus, transcription in orientation A of 175 bp of DNA inserted at the *Eco*RI site was sufficient to stabilize this *par*-deleted pSC101 derivative, but transcription of 55 bp was insufficient.

Table I. Plasmid constructions

Plasmid	Immediate precursor	Relevant manipulation
pZC20		wild-type *par*
pZC56	pZC20	delete *par* *Eco*RI–*Ava*I
pSLB56	pZC56	*aph* (Tn*5*) in *Sca*I in orientation A
pSLB57	pZC56	*aph* (Tn*5*) in *Eco*RI in orientation A
pSLB59	pSLB57	delete *aph* promoter *Hin*dIII–*Bgl*II
pSLB60	pZC56	*aph* (Tn*903*) in *Eco*RI in orientation A
pSLB62	pZC56	*cat* (Tn*9*) in *Sca*I in orientation A
pSLB63	pSLB57	delete *aph* structural gene *Bgl*II–*Sma*I
pSLB67	pZC56	*aph* (Tn*5*) to replace *Nde*I fragment in orientation A
pSLB671	pZC56	*aph* (Tn*5*) to replace *Nde*I fragment in orientation B
pSLB78	pSLB57	insert terminator at *Aat*II
pSLB80	pZC56	*tet* from pBR322 in *Sca*I in orientation A
pSLB87	pZC20	*aph* (Tn*5*) to replace *Nde*I fragment in orientation A
pSLB871	pZC20	*aph* (Tn*5*) to replace *Nde*I fragment in orientation B
pSLB89	pSLB78	delete *Bgl*II to terminator
pSLB95	pSLB78	delete *Ban*II to terminator
pSLB96	pSLB56	*cat* (Tn*9*) in *Eco*RI in orientation A
pSLB98	pSLB56	delete *Nde*I fragment
pSLB120	pZC56	*tet* from pBR322 in *Eco*RI in orientation A
pSLB121	pZC56	*tet* from pBR322 in *Hae*II in orientation A
pSLB122	pSLB120	*Cla*I site filled in to Tc^S
pSLB127	pSLB204	replace *par* with terminator
pSLB204	pZC20	*aph* (Tn*5*) in *Sca*I in orientation B
pSLB208	pZC20	*aph* (Tn*5*) in *Sca*I in orientation A
pCM57	pSLB57	*Hae*II site deleted to Km^S
pCM62	pZC56	*cat* (Tn*9*) in *Eco*RI in orientation A
pCM67	pZC56	*aph* (Tn*5*) in *Hae*II in orientation A

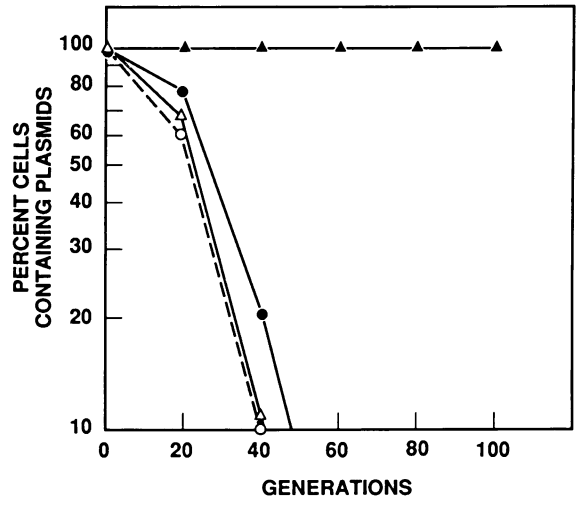

Fig. 4. Plasmid stability was measured in liquid cultures grown without selection. In this figure the plasmids that show no loss are graphed as one line. The plasmids tested were pSLB57 (Ap, Km), pSLB78 (Ap), pSLB95 (Ap), pSLB63 (Ap) ▲; pZC56 (Ap) ○; pSLB89 (Ap) △; pSLB59 (Ap) ●.

The stabilizing effects of inserted promoters are associated with transcriptionally related DNA supercoiling

Recent work has shown that transcriptionally active DNA fragments can produce regions of negative superhelicity in plasmid DNA (Pruss and Drlica, 1986; Wu *et al.*, 1988; Figueroa and Bossi, 1988; Tsao *et al.*, 1989; Rahmouni and Wells, 1989). As already noted, other studies have demonstrated that the degree of DNA superhelicity has a profound effect on the stable maintenance of pSC101 and other plasmids (Miller *et al.*, 1990). Since DNA gyrase mutations can affect the extent of DNA supercoiling, we investigated the effect of gyrase deficiency on the promoter-induced stabilization of pSC101 derivatives. In these experiments, plasmid stability was assessed at both the permissive (30°C) and semi-permissive (35°C) temperatures in an *E.coli* strain (i.e. LE316, Orr *et al.*, 1979) that conditionally expresses the *gyrB* gene.

While the stability of pSC101 plasmids carrying a *bona fide par* locus is not reduced significantly in gyr^- hosts (Leonard *et al.*, 1985; Miller *et al.*, 1990), plasmids fully stabilized by promoter-containing DNA insertions in the wild-type host were stabilized only partially in gyr^{ts} bacteria (strain LE316) at both permissive (30°C) and semi-permissive (35°C) temperatures (Table II). Interestingly, the promoter-stabilized plasmid least affected by the *gyr* mutation was the construct containing the *tetA* gene insertion at the *Sca*I site of pZC56 (i.e. the pSLB80 plasmid); this was one of the few insertion-stabilized plasmids that yielded the Cmp^+ phenotype in the gyr^+ strain PM191 (see above). Introduction of the *tetA* gene at the *Eco*RI site (pSLB120) or *Hae*II site (pSLB121) yielded plasmids that were fully stable in PM191 but nevertheless showed the Cmp^- phenotype in that strain; they were highly unstable in the gyr^{ts} host at 35°C (Table II), consistent with earlier observations (Miller *et al.*, 1990) showing that Cmp^- pSC101 derivatives are lost rapidly from bacteria deficient in DNA gyrase. As seen in Table II, the *par*-deleted plasmids stabilized in wild-type hosts by introduction of transcriptionally active DNA fragments showed instability in LE316, even at 30°C.

In all instances that we have examined, promoter-induced stabilization of plasmid inheritance was associated with an increase in the overall superhelical density of plasmid DNA, as determined by electrophoresis in chloroquine–agarose gels. As seen in Figure 5, the overall negative supercoiling of *par*-deleted pSC101 derivatives containing a transcriptionally active *aph* gene insert in the *Hae*II, *Sca*I or *Eco*RI sites was increased markedly as compared with the supercoiling of a similarly sized plasmid containing an *aph* gene in which the promoter had been inactivated by a 2 bp deletion (lanes 1, 2 and 3 versus lane 4). Similar results were obtained for *tet* gene inserts containing a transcriptionally active promoter versus an inactive promoter. However, a general increase in superhelicity did not always result in plasmid stability; the phenotypically Par^- pSLB67 plasmid, in which an *aph* gene insertion replaces an *Nde*I DNA fragment, has the same superhelical density as the similarly sized Par^+ pSLB98 plasmid, which also has the *Nde*I fragment deleted but contains its *aph* gene insertion at the *Sca*I site (Figure 5, lanes 5 and 6). Additionally, the overall superhelicity of the plasmid carrying an *aph* gene insertion in orientation B at the *Sca*I site (plasmid pSLB127) was the same as the superhelicity resulting from insertion of the same gene, in orientation A at the *Eco*RI site (plasmid pSLB78, Figure 5, lanes 7 and 8)—but pSLB127 was highly unstable (Figure 2) while pSLB78 was stably inherited. Given the finding that the stabilizing effects of promoter insertions require normal DNA gyrase activity, these observations suggest that the extent of supercoiling at one or more specific plasmid sites, rather than the overall superhelical density of the plasmid DNA, determines promoter-induced stabilization of plasmid inheritance.

Table II. Correlation of Cmp phenotype and stability in limiting gyrase

Plasmid	Cmp phenotype	Stability in LE 316 gyr^{ts}	
		30°C	35°C
pZC20	+	100/100	99/100
pZC56	–	40/0, 1/40	0/40
pSLB56	–	96/100	26/40
pSLB208	+	99/100	93/100
pSLB80	+	99/100	94/100
pSLB120	–	94/100	71/100
pSLB121	–	26/40	0/40
pCM62	–	86/100	12/40
pSLB57	–	85/100	3/40
pSLB96	+	92/100	38/100

Stability measurements are expressed as percent of cells containing plasmids over the number of generations grown at the permissive temperature (30°C) or non-permissive temperature (35°C) without selection. All the plasmids were stable in the parent of LE316, LE234, except for the pZC56 plasmid which was unstable. The Cmp phenotype was determined by transformation of the test plasmid into strain PM191 alone and containing a wild-type Par^+ plasmid, either pSC101 or pPM20. If the number of transformants was reduced by > 100-fold by having a wild-type plasmid present the test plasmid was phenotypically Cmp^- (Tucker *et al.*, 1984).

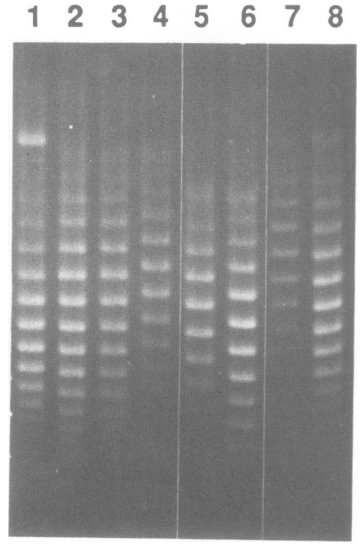

Fig. 5. Plasmid DNA from LE234 grown at 30°C to OD 0.4 was isolated and run on chloroquine–agarose gels to compare the superhelical density. Lane 1 is plasmid DNA from pSLB56, lane 2 is from pSLB57, lane 3 is pCM67 and lane 4 is pCM57. Also compared are plasmid DNA from pSLB98 in lane 5 and lane 6, pSLB67; lane 7 is plasmid DNA from pSLB78 compared with lane 8 pSLB127.

Discussion

Our results show that orientation-dependent insertions of promoters at certain DNA sites can compensate for the absence of the *par* region on the pSC101 plasmid. The observed stabilization of *par*-deleted pSC101 derivatives is a consequence of the transcription process itself, rather than the transcription of particular DNA sequences. Inactivation of the promoter abolished its stabilizing effect, as did the placement of a transcription terminator a short distance downstream from the point of initiation of transcription.

Earlier observations have shown that transcriptionally induced negative DNA supercoiling is affected by the strength of the promoter (Wu *et al.*, 1988; Figueroa and Bossi, 1988) and the length of the transcript (Lodge *et al.*, 1989); we observed analogous effects on plasmid stability. The extent of reversal of the effects of *par* region deletions varied with different inserts and with the site of insertion; the constructs that yielded the Cmp^+ phenotype in a wild-type host were stably inherited in a *gyr* or a gyrase-inhibited host (cf. Miller *et al.*, 1990). However, plasmids that were Cmp^- in wild-type bacteria were highly unstable in *gyr*-defective hosts.

The multi-faceted interrelationship between transcription and DNA supercoiling has been well established: superhelicity of the DNA template can affect promoter activity (Menzel and Gellert, 1983; Franco and Drlica, 1989; Hulton *et al.*, 1990) and conversely, transcription beginning at a strong promoter can result in localized and transient supercoiling of the template DNA (Pruss and Drlica, 1986; Wu *et al.*, 1988; Liu and Wang, 1987; Figueroa and Bossi, 1988; Tsao *et al.*, 1989; Rahmouni and Wells, 1989). It has been postulated that such supercoiling results from movement of the transcription complex along the topologically constrained template, causing the DNA segment ahead of the complex to become positively supercoiled and the segment behind the site being transcribed to become negatively supercoiled. Such localized supercoiling is influenced by the length of the nascent RNA chain (Lodge *et al.*, 1989) and can lead to alteration of the overall superhelical density of plasmid DNA as examined by electrophoresis in chloroquine–agarose gels (Pruss and Drlica, 1986; Wu *et al.*, 1988; Liu and Wang, 1987; Figueroa and Bossi, 1988; Tsao *et al.*, 1989; Rahmouni and Wells, 1989).

As the observed promoter-induced stabilization of plasmid inheritance was gyrase dependent, it appears to be mediated through changes in DNA superhelicity. However, insertion of the same promoter at different locations or in different orientations yields the same increase in overall supercoiling but different phenotypic effects on plasmid stability, implying that stabilization requires supercoils at a particular DNA site or sites; supercoiling induced by promoter insertions at some plasmid sites fails to stabilize plasmids, even though it may produce the same overall superhelical density as promoter insertions at other sites. Such findings are consistent with evidence that plasmids having the same overall superhelical density can show supercoiling differences within different segments (Rahmouni and Wells, 1989).

Collectively, the results reported here and those obtained previously (Miller *et al.*, 1990) suggest a model in which the plasmid DNA conformation induced by a particular extent of negative supercoiling facilitates protein–DNA interactions that enable partitioning to occur. Our observation that stabilized maintenance of pSC101 is seen only when a transcriptional promoter is introduced in the orientation that would result in the transient build up of negative supercoils at the replication origin points to the origin region as a possible target of the superhelicity-generating effects of transcriptional activity. Evidence that the *par* locus itself can both increase negative supercoiling of pSC101 DNA (Miller *et al.*, 1990) and enhance protein–DNA complexing at the pSC101 replication origin (H.Ingmer and S.N.Cohen, in preparation) is consistent with this notion. Earlier work has suggested that DNA–protein interactions at the pSC101 replication origin can also be enhanced by DNA bending induced by the binding of integration host factor (Stenzel *et al.*, 1987). The ability of *topA* mutations, which can circumvent the effects of *par* region deletions in pSC101, to stabilize also the inheritance of partition-defective *mini*F, p15A and *ori*C plasmids (Miller *et al.*, 1990), Par^- P1 derivatives (S.Austin, personal communication), and Par^- derivatives of the broad host range plasmid, RK2 (R.Roberts and D.Helinski, personal communication), suggests that a common conformationally related mechanism may be involved in the inheritance of these widely diverse replicons.

Materials and methods

Bacterial strains and plasmids

E.coli K-12 strains PM191 (*deoC thr fhuA thi deoB supE recA*56) (Meacock and Cohen, 1979), LE234 (*metB argE ilv tna supE*) (Orr *et al.*, 1979), LE316 [LE234 *ts* (coumermycin resistant)] (Orr *et al.*, 1979).

Enzymes and chemicals

Restriction endonucleases were purchased from New England Biolabs. T4 DNA polymerase and T4 DNA ligase were obtained from Bethesda Research Laboratories. The Klenow fragment of *E.coli* DNA polymerase I and calf intestine alkaline phosphatase were supplied by Boehringer Mannheim. All enzymes were used as recommended by the manufacturer.

Antibiotics such as ampicillin (Ap), kanamycin monosulfate (Km), tetracycline (Tc) and chloramphenicol (Cm) and the chemicals ethidium bromide and chloroquine diphosphate were provided by Sigma.

General procedures

LB medium (Miller, 1972) was used for growth of liquid cultures. Antibiotics were used at the following concentrations in both solid and liquid media: Ap, 20 μg/ml; Km, 30 μg/ml; Tc 10 μg/ml; Cm, 20 μg/ml.

DNA fragments resulting from digestion with restriction endonucleases were purified on agarose gels and isolated according to the freeze and squeeze method (Tautz and Renz, 1983).

All routine DNA manipulations, transformations and isolation of plasmid DNA, were done as reviewed in Maniatis *et al.* (1982). DNA used for topoisomer analysis (Biek and Cohen, 1989) from logarithmically growing cells was prepared by rapid lysis.

DNA topoisomers of similarly sized pairs of plasmids were compared by electrophoresis in 1% agarose gels (30 cm long) containing a given concentration of chloroquine (15 μg/ml) equilibrated in a Loening buffer containing the same concentration of chloroquine. After electrophoresis for 17 h at 3 V/cm in the presence of circulating buffers, gels were soaked in water for 1 h, treated for 1 h in 10 mM magnesium chloride, rinsed with water for 1 h, and stained with ethidium bromide for 30 min. Gels were photographed after destaining for 1 h in water.

Plasmid constructions

The Tn*5*-derived *aph* gene was purified as a *Hin*dIII–*Sma*I fragment from pZT331. The *aph* gene from Tn*903* was purchased from Pharmacia. The *tetA* gene from plasmid pBR322 was purified as an *Ava*I–*Eco*RI fragment. The *cat* gene was isolated from pACYC184 as a *Hae*II–*Hae*II fragment. These genes were made blunt-ended with either T4 DNA polymerase or the Klenow fragment of *E.coli* DNA polymerase I, or by mung bean nuclease, and inserted at the *Sca*I, *Eco*RI, *Hae*II or *Nde*I sites of either pZC56 or pZC20 to produce the plasmids listed in Table I. The transcription termination signal from gene 32 of bacteriophage T4 was isolated from pWTT552

as a 170 bp *Eco*RI−*Hin*dIII fragment. The terminator was treated with Klenow and inserted by blunt end ligation at the *Ava*I site of pSLB204 to generate pSLB125 which upon deletion of the *par* region as an *Eco*RI−*Bam*HI fragment and recircularization yielded pSLB127.

Assays for plasmid stability and competition

Plasmid stability tests were performed as described previously (Meacock and Cohen, 1980). Alternatively, in experiments evaluating the effects of gyrase limitation on plasmid stability, media containing the appropriate antibiotic(s) were inoculated with cells from a single colony grown on selective agar and incubated at 30°C to a cell density of 10^9 per ml. After dilution into non-selective medium, plasmid stability was determined at both 30 and 35°C.

To compare the relative stability of various plasmids in strain LE316, experiments for stability of maintenance were carried out simultaneously in the same water bath under strict temperature control. Each stability determination reported was performed in triplicate to ensure accuracy and reproducibility under the described conditions.

Competition tests were performed by transformation of calcium chloride-treated competent cells (PM191) containing or lacking the wild-type pSC101 plasmid, with the mutant plasmid to be tested, as described previously (Tucker *et al.*, 1984). Host strains harboring both plasmids, generated colonies on double selective agar.

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