Filamentation promotes F'*lac* loss in *Escherichia coli* K12

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The stability of plasmid F'*lac* in *Escherichia coli* strain SP45 (a temperature conditional mutant which grows as spherical cells at 42 °C and as a rod at 30 °C) was studied. F'*lac* elimination was demonstrated when bacteria exposed to subinhibitory concentrations of various chemicals were induced to form filaments. No plasmid loss was found when spherical cells were subjected to the same treatments. Plasmid loss was also observed in *dnaA46* and *lexA41* mutants when cell filamentation was induced at 42 °C, but not when they were cultured at 30 °C. Nalidixic acid promoted F'*lac* elimination at 0.25 µg ml⁻¹ in a *recA13* mutant and at 1.5 µg ml⁻¹ in the *recA*⁺ counterpart. A marked difference was found in the rate of F'*lac* elimination from thermosensitive DNA gyrase mutants [*gyrA43*(Ts) and *gyrB41*(Ts)] between rods and their spherical (*rodA51*) derivatives growing at semipermissive temperature (36.5 °C). Plasmids carrying the *ccd* segment of F in DNA gyrase mutants were lost after 2.5 generations from rods and after 6 generation from spherical cells. Plasmid segregation into non-viable minicell-like elements was found after induction of filaments. These data suggest that plasmid stability is correlated with cell shape and that curing is more easily achieved when bacteria can elongate normally.

Introduction

A large number of chemical and physical agents (curing agents) promote the loss of plasmids from several bacterial hosts (Stanisich, 1988). Studies using DNA gyrase inhibitors have demonstrated the role of this enzyme in plasmid replication and thus in plasmid maintenance (Hooper *et al.*, 1984; Weisser & Wiedeman, 1985; Michel-Briand *et al.*, 1986). Although these agents have been used to enhance the recovery of plasmid-free derivatives of various bacteria, the precise mechanism of plasmid elimination is not understood.

Novick *et al.* (1980) demonstrated the loss of high copy number plasmids during protoplast formation and regeneration in *Staphylococcus aureus*, providing evidence for a relationship between cell envelope and maintenance of plasmids. The first proposal that the bacterial cell envelope is involved in plasmid maintenance came from Jacob *et al.* (1963) who suggested that plasmids in *Escherichia coli* are attached to the cell membrane, and that this attachment is necessary for both replication and partition. Indeed, membrane-associated proteins appear to be involved in both plasmid and chromosome partition as reviewed recently by Williams & Thomas (1992).

In agreement with this interpretation, Rashtchian *et al.* (1986) demonstrated that plasmid segregation into minicells depends on the spatial location of these replicons in the host cells and on their association with membrane attachment sites. *E. coli* minicell mutant strains are characterized by aberrant division events leading to the appearance of small spherical chromosome-less cells together with filamentous cells of variable lengths (Adler *et al.*, 1967).

Non-nucleate or minicell-like elements also appear in certain *E. coli* temperature-sensitive cell-division mutants at the non-permissive temperature (Hirota *et al.*, 1968; Howe & Mount, 1975; Gudas, 1976; Mulder & Woldringh, 1989). This also occurs after exposure of DNA-repair mutants to nalidixic acid, especially in those defective in the *recA* function (Inouye, 1971). Segregation of plasmids into these non-viable cells has never been evaluated.

Exposure of *E. coli* to the majority of curing agents, including DNA gyrase inhibitors, generally leads to the formation of filamentous cells (Diver & Wise, 1986). Since this very feature might modify the spatial location of the plasmids, such morphological abnormalities may well be involved in plasmid loss.

The present study was undertaken to investigate the stability of the F'*lac* episome in *E. coli* cells induced either to form filaments, or to grow as cocci. The experimental approach exploits the properties of *E. coli*

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strain SP45, a temperature-conditional mutant for the synthesis of *pbpA45*(Ts) (Spratt *et al.*, 1980). Inactivation of *pbpA45*(Ts) at the nonpermissive temperature results in the growth of bacteria as large spherical cells that cannot elongate even if exposed to beta-lactam antibiotics or agents affecting DNA metabolism which generally cause filamentation in bacteria (Begg & Donachie, 1985). At the permissive temperature, SP45 behaves as a normal rod which changes its shape only if subjected to the treatments mentioned above.

The role of the cell envelope in *F'*lac maintenance was also evaluated in experiments carried out with *E. coli* strains defective in *dnaA46*(Ts), *lexA41*(Ts) and *recA13* functions. The *dnaA46*(Ts) mutant stops DNA synthesis at 42 °C, but continues to divide, and forms anucleate cells (Hirota *et al.*, 1968; Mulder & Woldringh, 1989). The *lexA41*(Ts) mutant expresses SOS response and forms non-septated filaments at 42 °C even though DNA synthesis is normal (Gudas, 1976).

Finally, the stability of *F'*lac was investigated in *E. coli* *gyrA43*(Ts) and *gyrB41*(Ts) mutants and in their *rodA51* derivatives. These thermosensitive DNA gyrase strains exhibit a phenotype reminiscent of minicell-producing strains. At the non-permissive temperature these *gyr* mutants generate minicells and anucleate rods of variable lengths and show an irregular nucleoid distribution in filaments (Orr *et al.*, 1979; Jaffé *et al.*, 1988; Mulder *et al.*, 1990). In contrast, the *rodA51* derivatives grow as spherical cells in all experimental conditions. These experiments were prompted by the observations of Wolfson *et al.* (1982) who described the elimination of two plasmids from *E. coli* with a thermolabile gyrase at semipermissive temperature, establishing the requirement of a functioning gyrase B subunit for plasmid maintenance.

The stability of plasmids carrying (pXX333) and lacking (pXX332) the *ccd* function of the F plasmid (Ogura & Hiraga, 1983; Jaffé *et al.*, 1985) was also evaluated in the thermolabile gyrase A and B subunit mutants and in their *rodA51* derivatives.

Methods

Bacterial strains. Bacterial strains used in this study are listed in Table 1. The P1 transduction protocol used in strain construction was described by Miller (1972). The *rodA51* gene was transduced using selection for mecillinam resistance (20 µg ml⁻¹). The *sulB25* gene was transduced into *lexA41*(Ts) from a spontaneous azide-resistant PM161 strain, and thermoresistant mutants from among azide-resistant transductants.

Plasmid *F'*lac (F'128) was one of the F' kit kindly provided by B. Bachmann; a Tn5-carrying derivative was constructed in order to simplify its identification in different hosts and selection of transconjugants in mating experiments. The transposon Tn5 encodes kanamycin-neomycin resistance. Plasmids pXX332 and pXX333 were described and kindly supplied by A. Jaffé (1985). Both plasmids are unable to replicate at 42 °C and carry a gene conferring chloramphenicol resistance. pXX333, but not pXX332, carries the *ccd* function of the mini-F plasmid pSC138 (Timmis *et al.*, 1975). These plasmids were introduced into bacterial cells by transformation.

Susceptibility tests. The Minimum Inhibitory Concentration (MIC) of each compound employed in this study was performed in microtitre trays using Mueller-Hinton Broth (Difco), adjusted to pH 7.6 when necessary, as previously described (Debbia *et al.*, 1988).

Methods for plasmid elimination. Bacteria were grown overnight in Mueller-Hinton Broth (MHB) containing kanamycin (25 µg ml⁻¹) to ensure *F'*lac presence. Under these conditions the percentage of plasmid-free cells was lower than 0.1%. After the drug was removed by washing the cells in saline buffer, about 5 × 10³ bacteria were cultured in a series of tubes containing 1 ml MHB and various concentrations of the curing agent. A tube of drug-free MHB was used as a control.

After incubation for 18–20 h at the required temperature, a direct microscopical observation of bacterial cultures was done. Suspensions

Table 1. *E. coli* strains used

Strain	Main characteristics	Source or reference
SP45	<i>pbpA45</i> (Ts), <i>trp</i> (Am), <i>tyr</i> (Am), <i>ilv</i> , <i>supD126</i> (Ts).	B. G. Spratt (1980)
CTR4610	<i>dnaA46</i> (Ts), <i>leu</i> , <i>thy</i> , <i>thi</i> .	Y. Hirota (1968) via G. Satta (1978)
DM936	<i>lexA41</i> (Ts), <i>ara-14</i> , <i>argE3</i> , <i>leuB6</i> , Δ (<i>gpt-proA</i>)62, <i>thr-1</i> , <i>recA1</i> , <i>rpsL31</i> , <i>galK2</i> , <i>kdgK51</i> , <i>lexA3</i> ?	D. Mount (1975) via B. J. Bachmann
PAM161	<i>ara-14</i> , <i>sulB25</i> , <i>leuB6</i> , Δ (<i>gpt-proA</i>)62, <i>thr-1</i> , <i>rpsL31</i> , <i>galK2</i> , <i>lon-22</i> , <i>hisG4</i> , <i>metF24</i> , <i>thi-1</i> , <i>lacY1</i> ,	B. F. Johnson (1977) via B. J. Bachmann
db1413	same as DM936 but <i>sulB25</i>	This work
AB1157	<i>thr-1</i> , <i>leuB6</i> , <i>proA2</i> , <i>argE3</i> , <i>his-4</i> , <i>thi-1</i> , <i>lacY1</i> , <i>galK2</i> , <i>rpsL31</i>	P. Howard-Flanders (1966) via C. Monti-Bragadin
AB2463	<i>recA13</i> derivative of AB1157	P. Howard-Flanders (1966) via C. Monti-Bragadin
S1	<i>proA2</i> , <i>rodA51</i> , <i>purB15</i> , <i>hisG4</i> , <i>lacY1</i> , <i>galK2</i> , <i>xyl-5</i> , <i>rpsL35</i>	H. Matsuzawa (1973) via B. J. Bachmann
N4177	<i>galK</i> , <i>gyrB41</i> (Ts)	Menzel & Gellert (1983) via B. J. Bachmann
db1589	same as N4177 but <i>rodA51</i>	This work
KNK453	HF4704 <i>gyrA43</i> (Ts), <i>thyA</i> , <i>polA</i> , <i>uvrA</i> , <i>phx</i>	K. Kreuzer (1979) via C. Monti-Bragadin
db1588	same as KNK453 but <i>rodA51</i>	This work
pop3208	<i>araD139</i> , Δ (<i>argF-lac</i>)205, <i>flbB5301</i> , <i>ptsF25</i> , <i>relA1</i> , <i>rpsL150</i> , <i>lamB204</i> <i>deoC1</i>	B. J. Bachmann
db1590	same as pop3208 but <i>rodA51</i>	This work
J-53	<i>pro22</i> , <i>metF63</i>	B. J. Bachmann

showing bacteria with morphological changes were then diluted 100-fold in pre-warmed drug-free medium and incubated for 90 min at the appropriate temperature. Controls were diluted similarly. Bacteria were diluted further in saline buffer and plated on drug-free Mueller-Hinton agar (MHA). After incubation for 18–20 h, colonies were replicated onto drug-free and antibiotic-containing MHA using velvets. Growth on antibiotic-containing media indicated the presence of the plasmid encoding that resistance. In some cases the presence of *F'*lac was detected by determining the susceptibility of the cells to the sex-specific phage MS2 or by scoring the lactose-positive colonies on McConkey agar plates. The number of colonies on drug-containing plates was expressed as a fraction of the number on drug-free plates.

Cultures of *gyrA43*(Ts), *gyrA43*(Ts)(*rodA51*), *gyrB41*(Ts) and *gyrB41*(Ts)(*rodA51*) carrying either pXX332 or pXX333 were incubated overnight at 28 °C in the presence of chloramphenicol (20 µg ml⁻¹). They were then diluted 1000-fold in fresh prewarmed LB medium [per litre: tryptone (Difco), 10 g; yeast extract, 5 g; NaCl, 5 g] and transferred to the semipermissive temperature (36.5 °C). Control cultures were incubated at 28 °C. Samples were taken at appropriate intervals and spread onto LB agar plates. Colonies were scored after incubation at 28 °C. The presence of plasmids in bacteria was detected by replica plating onto chloramphenicol-containing LB agar.

Separation of non-nucleated cells. Non-nucleated cells were prepared by differential centrifugation and sucrose gradient (Levy, 1970). Briefly, 400 ml culture were centrifuged at 1500 *g* for 5 min. The supernatant was sedimented at 10000 *g* for 15 min. The pellet was resuspended in 4 ml 0.05 M-sodium phosphate, pH 7. Samples (2 ml) were layered on top of a 25 ml sucrose gradient prepared by dispensing 25 ml of 20% (w/v) sucrose solution into a 30 ml glass centrifuge tube, freezing the gradient at -20 °C and then thawing it at 4 °C overnight. The gradients were centrifuged for 40 min at 2500 *g*. The non-nucleated cells appeared as a broad band in the upper half of the tube. These were recovered, collected by centrifugation and further purified by a second sucrose gradient. The final band of non-nucleated cells was treated with ampicillin (200 µg ml⁻¹) to eliminate contaminating nucleated cells.

Plasmid transfer. General mating conditions for evaluating plasmid transfer followed those outlined by Willetts (1988). Plasmid transfer from minicell-like elements was carried out in liquid medium. Donors (0.2 ml) were mixed with 0.2 ml culture (2 × 10⁸ cells per ml) of a nalidixic-acid-resistant derivative of strain J-53. After 90 min of incubation at the appropriate temperature, the mixture was plated on selective media. The number of viable bacteria was determined by plating the diluted culture on LB agar. The number of non-viable cells was estimated by microscopical observation in a Petroff-Hausser chamber.

Results

*Evaluation of F'*lac elimination from SP45 strain grown at permissive and non-permissive temperatures

In preliminary tests, SP45 harbouring the *F'*lac plasmid, grown at the permissive temperature, was exposed to subinhibitory concentrations of various chemicals, including the well-known curing agents acridine orange and ethidium bromide. Among those that, irrespective of their mode of action, produced morphological alterations in bacteria, some representative compounds were chosen for further testing. Piperacillin, chlorohexidine, polymyxin B, and sodium azide were studied because their

ability to cure plasmids has not been previously described. Nalidixic acid, ciprofloxacin and acridine orange were tested because they are considered selective inhibitors of plasmid DNA replication, and mecillinam because it induces spherical forms.

After incubation at 30 °C for 18 h, a microscopic examination of the culture revealed the presence of elongated or round forms in test tubes containing drug concentrations near the MIC values (0.5–0.25 × MIC depending on the compound tested). Filaments produced by piperacillin were very long in comparison to those produced by the other compounds which were equivalent in length to 3–4 cells. As expected, spheres were present in the samples containing mecillinam (Spratt *et al.*, 1980).

Survivors from those concentrations affecting bacterial shape were then plated on antibiotic-free medium and tested for plasmid traits. The number of microorganisms in control cultures ranged from 4 × 10⁸ to 1.3 × 10⁹ c.f.u. ml⁻¹ (18 to 21 generations of growth) and in the treated cultures from 4 × 10⁸ to 9 × 10⁸ c.f.u. ml⁻¹ (18–20 generations of growth).

The results of these experiments (Table 2) demonstrate that the chemical agents used, with the exception of mecillinam, were able to promote the loss of *F'*lac from the cells. When the same experiments were carried out at 42 °C, bacteria were pre-incubated for 90 min to allow the cells to undergo a rod-sphere transition. Following this, they were treated with the same compounds used at 30 °C. The resident *F* plasmid was not cured from SP45 at 42 °C even if the host was treated with the same amounts of chemicals which cause *F'*lac loss from the same strain at the permissive temperature. These data suggest that plasmid maintenance in bacteria might be compromised by agents that cause elongation of the cell envelope. In contrast, the selective block of lateral wall extension seems not to influence the stability of the plasmid.

*Evaluation of F'*lac elimination from various hosts

Further evidence for the role of the cell envelope in plasmid maintenance was obtained in experiments carried out with strains defective in *dnaA*, *lexA* or *recA* functions and carrying *F'*lac (Table 3). Plasmid curing was observed with the *dnaA46*(Ts) mutant after incubation of the culture at 42 °C for 3 h but not when this strain was grown at the permissive temperature. *F'*lac curing was also observed in the *lexA41*(Ts) mutant after 3 h at the non-permissive temperature, but not in the control sample, or in its *sulB25* derivative, under the same experimental conditions, or after incubation at 42 °C for a further 18 h (data not shown). Furthermore, when a *recA13* mutant was treated with nalidixic acid, filaments

Table 2. *Effect of various chemicals on F'lac stability and on bacterial growth in E. coli SP45*

Compound	MIC ($\mu\text{g ml}^{-1}$)	Bacterial shape at the indicated sub-MIC ($\mu\text{g ml}^{-1}$)*	Cured cells (%) obtained from bacteria with altered shape at temperature†		Number of cells examined
			30 °C	42 °C	
Piperacillin	1	long filaments (0.25)	27	0	937
Chlorohexidine	0.25	short filaments (0.12)	21	0	1361
Polymyxin	0.12	short filaments (0.08)	15	0	1107
Sodium azide	50	short filaments (12.5)	16	0	867
Nalidixic acid	4	long filaments (1.5)	24	0	2036
Ciprofloxacin	0.01	long filaments (0.005)	47	0	1483
Acridine orange	20	short filaments (10)	22	0	856
Mecillinam	2	cocci (0.5)	0	0	1214
None		rods	0	0	1664

* At the permissive temperature.

† Data are means of 3 experiments.

Table 3. *Effect of bacterial mutations on the stability of F'lac and susceptibility of some strains to nalidixic acid (nal)*

Mutation	Treatment		Cured cells (%)**	MIC ($\mu\text{g ml}^{-1}$)	Number of cells examined
	Temp. (°C)	Drug ($\mu\text{g ml}^{-1}$)			
<i>dnaA46</i> (Ts)	32	—	0	—	762
<i>dnaA46</i> (Ts)	43	—	23	—	938
<i>lexA41</i> (Ts)	32	—	0	—	1074
<i>lexA41</i> (Ts)	43	—	27	—	1248
<i>lexA41</i> (Ts) <i>sulB25</i>	32	—	0	—	654
<i>lexA41</i> (Ts) <i>sulB25</i>	43	—	0	—	1332
<i>recA13</i>	37	0	13	—	595
<i>recA13</i>	37	nal(0.25)	49	1	826
<i>recA</i> **	37	nal(1.5)	28	4	1388

* Control strain AB1157.

† Data are means of 3 experiments.

appeared, but septation and division occurred at a higher rate than in either *dnaA46*(Ts) or *lexA41*(Ts) mutants (Inouye, 1971). The *recA13* strain lost *F'lac* when treated with a drug concentration which did not significantly affect bacterial growth ($0.25 \times \text{MIC}$). Spontaneous plasmid loss was also observed with the *recA13* strain.

Surprisingly, the curing effect could be achieved with different concentrations of nalidixic acid. *F'lac* was eliminated from strain SP45 at 30 °C at the same nalidixic acid concentration ($1.5 \mu\text{g ml}^{-1}$) as that used to obtain the equivalent rate of curing in the control strain AB1157(*F'lac*). No curing was observed in the *pbpA45*(Ts) mutant strain at 42 °C in the presence of $1.5 \mu\text{g}$ nalidixic acid ml^{-1} , but *F'lac* was lost from the *recA13* mutant in the presence of $0.25 \mu\text{g ml}^{-1}$ of the drug. If the mechanism by which nalidixic acid promotes the loss of the episome from the cells is the preferential inhibition of plasmid replication, curing might have been expected under all the experimental conditions

employed, and at the lowest drug concentration. Since this was not the case, factors other than plasmid replication might be involved in curing by nalidixic acid.

Further clues, which seem to exclude the possibility that this DNA gyrase inhibitor affects plasmid replication, comes from experiments performed with Hfr strains selected by integrative suppression in a *dnaA* mutant. In these Hfr strains at high temperature, chromosome replication and cell division are under control of the *F* plasmid. Nishimura *et al.* (1971) and Bazzicalupo & Tocchini-Valentini (1972) have shown that acridine orange and rifampin, respectively, inhibit cell division at 43 °C in these strains, providing evidence that the *F* replication system is sensitive to these chemicals. In contrast, when the same experiments were carried out with nalidixic acid, no changes were found in the MIC values of the Hfr strains at both 30 and 42 °C (Table 4).

Table 4. Effect of nalidixic acid (Nal) on Hfr strains*, selected by integrative suppression in a *dnaA46* mutant sensitive to both acridine orange (AO) and rifampicin (Rif)

	30 °C					43 °C				
	Control	AO (20 µg ml ⁻¹)	Rif (1 µg ml ⁻¹)	Nal		Control	AO (20 µg ml ⁻¹)	Rif (1 µg ml ⁻¹)	Nal	
				(2 µg ml ⁻¹)	(4 µg ml ⁻¹)				(2 µg ml ⁻¹)	(4 µg ml ⁻¹)
Hfr 1	+	+	+	+	—	+	—	—	+	—
Hfr 2	+	+	+	+	—	+	—	—	+	—
Hfr 3	+	+	+	+	—	+	—	—	+	—
Hfr 4	+	+	+	+	—	+	—	—	+	—
Hfr 5	+	+	+	+	—	+	—	—	+	—

* Representative of 27 Hfr strains isolated.

Evaluation of *F'*lac elimination from bacteria temperature-sensitive in the A and B subunits of DNA gyrase

KNK453 [*gyrA43*(Ts)], db1588 [*gyrA43*(Ts)(*rodA51*)], N4177 [*gyrB41*(Ts)] and db1589 [*gyrB41*(Ts)(*rodA51*)] strains carrying *F'*lac were cultured at permissive (28 °C) and semipermissive (36.5 °C) temperatures. In KNK453 and N4177, cured cells were found after 1 generation, and at the end of the experiment they represented 75 and 95% respectively of the total bacteria. Viable plasmid-free segregants were also detected after 1 generation in the cultures of db1588 and db1589, but their number was about 20% of the total bacterial population after 8 generations (Fig. 1). This finding indicated that plasmid stability might be affected by thermal inactivation of gyrase A and B subunits, but the elongation of the bacterial envelope induced by these mutations played a determinant role in the *F'*lac curing process. When filament formation was prevented by the *rodA51* mutation, plasmid maintenance was improved.

Evaluation of pXX332 and pXX333 elimination from bacteria temperature-sensitive in the A and B subunits of DNA gyrase

As established by Jaffé *et al.* (1985) the *ccd* (coupled cell division) function of F plasmid inhibits the segregation of viable plasmid-free cells in *E. coli*. The *ccd* locus in fact causes filamentous growth of the *F*⁻ derivative and so causes cell death. The similarities between the morphological abnormalities in cell growth induced by the *ccd* function and the agents or the mutations that promote plasmid loss prompted an investigation of the stability of pXX332 (*ccd*⁻) and pXX333 (*ccd*⁺) in KNK453 and N4177, and in their *rodA51* derivatives db1588 and db1589 respectively.

In kinetic studies performed at 36.5 °C (Fig. 2a), both pXX332 and pXX333 were eliminated after 2 generations from KNK453 and after 5 generations from

db1588. The growth rates of rods were 54 min per generation (pXX332) and 58 min per generation (pXX333), and those of cocci 58 min per generation (pXX332) and 60 min per generation (pXX333). Plasmid-free segregants were not found in rod and spherical cells grown at 28 °C after the same number of generations (data not shown).

Fig. 1. Kinetics of elimination of *F'*lac from DNA gyrase mutants and their *rodA51* derivatives. (a) ■, KNK453(*F'*lac); ●, db1588(*F'*lac) grown at 36.5 °C; and ▲, KNK453(*F'*lac); ○, db1588(*F'*lac) grown at 28 °C. (b) ■, N4177(*F'*lac); ●, db1589(*F'*lac) grown at 36.5 °C; and ▲, N4177(*F'*lac); ○, db1589(*F'*lac) grown at 28 °C.

Fig. 2. Kinetics of elimination of pXX332 (filled symbols) and pXX333 (open symbols) from DNA gyrase mutants and their *rodA51* derivatives. (a) ■, □, KKN453; and ●, ○, db1588 grown at 36.5 °C. (b) ■, □, N4177; and ●, ○, db1589 grown at 36.5 °C. (c) ■, □, pop3208; and ●, ○, db1590 grown at 42 °C. Control cultures grown at permissive temperature were also studied (data not shown).

Results obtained with N4177 (Fig. 2b) demonstrated plasmid elimination after 2.5 generations from *gyrB41*-(Ts), and after 6 generations from the *gyrB41*-(Ts)-(*rodA51*) double mutant incubated at 36.5 °C. After 9 generations, the percentage of cured cells was greater

than 99.9% in the rods and about 55% in the spherical cells. No marked difference was found in the curing rate between mini-F plasmids carrying (pXX333) and lacking (pXX332) the *ccd* genes. The generation times calculated for all the cultures were 48 min per generation (pXX332) and 50 min per generation (pXX333) in rods, and 51 min per generation, (pXX332) and 55 min per generation (pXX333) in *rodA51* mutants. Plasmid-free bacteria were not found in spherical cells at 28 °C, while about 7% of plasmid-free segregants were noted in rods after the same number of generations (data not shown).

A direct microscopical observation of the cultures grown at 36.5 °C revealed the presence of elongated forms after about 2 h incubation in NKN453 and N4177 carrying either pXX332 or pXX333.

To determine whether, under the same growth conditions, pXX332 and pXX333 are stably inherited in strains other than DNA gyrase mutants, pop3208 and its *rodA51* derivative (db1590) harbouring the above plasmids were grown at 36.5 °C. Plasmid-free segregants were not found after 9 generations in any of the cultures (data not shown).

These tests were also performed at the restrictive temperature (42 °C) for the replication of the plasmids. The kinetics of elimination of pXX332 and pXX333 from pop3208 and db1590 cultured at 42 °C revealed that pXX332-free cells first appeared after 2 generations and their number increased rapidly. After 9 generations, more than 99% of the bacterial population had lost the plasmid (Fig. 2c). When the cultures of *rodA*⁺ and *rodA51* strains harbouring pXX333 were grown under the same conditions, plasmid-free segregants appeared after 4 generations and although the number of colony formers remained nearly constant, at the end of the experiment the fraction of viable plasmid-carrying cells was about 60% (pop3208) and 64% (db1590) of the survivors.

Therefore pXX333 was eliminated at about the same rate from rods and cocci at the non-permissive temperature. As expected (Jaffé *et al.*, 1985), the number of pXX333-free cells was lower than that observed with pXX332, because the *ccd* function of F produced lethal effects on both rods and coccal plasmid-free segregants.

Segregation of *F'*lac in minicell-like elements

As reported above, cell elongation *per se* seems to play an important role in plasmid elimination. Since filament formation is followed by the production of DNA-less cells especially in *dnaA46*(Ts) mutants and in cells exposed to nalidixic acid, segregation of plasmids in these non-viable elements was evaluated.

These non-viable cells were sought in bacterial cultures processed for plasmid elimination employing

Table 5. *Transfer of F'lac from the ampicillin fraction of treated and untreated cultures*

Donors	Treatment	Total viable cells (c.f.u. ml ⁻¹)	Non-viable elements	Recombinants
<i>dnaA46</i> (Ts)	none	9×10^3	$< 5.4 \times 10^4$	< 10
<i>dnaA46</i> (Ts)	43 °C	4×10^3	3.2×10^7	753
AB1157	none	$< 1 \times 10^3$	$< 5.4 \times 10^4$	< 10
AB1157	nal	8.8×10^4	4.3×10^7	2850
AB1157*	none	5×10^4	—	< 10

* Original culture diluted at the indicated cell number per ml.

sucrose gradient followed by ampicillin enrichment. AB1157(F'lac) was exposed to 1.5 µg nalidixic acid ml⁻¹, and *dnaA46*(Ts)(F'lac) was cultured at 42 °C for 3 h. The final pellet from treated and control cultures, obtained after ampicillin enrichment, was resuspended in 2 ml buffer. Recovery of biologically functional F factor from these preparations in the absence of viable bacterial cells would provide the most compelling evidence for the presence of plasmids in minicell-like elements. The purified minicell suspensions were mated with a recipient strain and exconjugants were sought by plating the mixture on appropriate selective media. As reported in Table 5, plasmid transfer occurs from both treated cultures. No recombinants were obtained under these experimental conditions from the suspensions of the control strains. In addition, the evaluation of viable and non-viable bacteria in all the samples showed that they contained about the same number of survivors. A great number ($3.2\text{--}4.3 \times 10^7$) of non-viable elements was found in the suspensions of treated cultures when compared to the control ($< 5.4 \times 10^4$).

The possibility that donor ability resided in viable bacteria was also explored using an appropriate dilution of the original culture of AB1157(F'lac). The results indicate that this was not so. No plasmid was transferred to the recipient strain from a low number of donor cells equivalent to that of survivors after the purification step. These findings indicate that treated cultures contain a number of non-nucleated cells carrying the F' factor which are not present in the control strains.

Discussion

Plasmid elimination as the direct consequence of the elongation of bacterial cells appears to be the most striking observation in this study. The effect of shape alterations on plasmid stability was demonstrated in experimental conditions under which elongated or spherical cells resulted either from the presence of a bacterial mutation or the mode of action of a drug. No curing was found under these conditions in spherical

cells or in bacteria where filamentation was prevented. In contrast, a high percentage of plasmid-free bacteria was obtained from cells capable of undergoing filament transition.

The asymmetrical cell division that follows the elongation of the cells can be envisaged to be the second factor playing an important role in the curing process. This paper demonstrates, in fact, that the curing process is the result of plasmid segregation into minicell-like elements.

It has been reported that curing is achieved in experimental conditions which still permit cell growth (Weisser & Wiedemann, 1985; Michel-Briand *et al.*, 1986). These observations suggest that the effects caused by the curing agents are rapidly overcome by bacteria which are thus able to divide (Engle *et al.*, 1982). During filament formation, chromosomal and plasmid DNA might be found at opposite extremities of the elongated cells. If septa are then formed plasmid and chromosomal DNA will segregate in different cellular units.

The proposed plasmid-curing process presents many analogies with that concerning the segregation of plasmid into minicells (Rashtchian *et al.*, 1986). In particular, some investigators have demonstrated that plasmids during part of their life cycle might be bound to the bacterial membrane or might be associated with the folded chromosome or may reside in both positions (Jacob *et al.*, 1963; Kline & Miller, 1979; Rashtchian *et al.*, 1986). This suggests that the ability of plasmids to segregate into minicells is related to their spatial location. Therefore, the probability that a curing agent has of eliminating an extrachromosomal element from its host might depend on the plasmid being bound to the membrane or associated with the folded chromosome. In particular, the former localization will favour plasmid curing, while the latter will not. On the other hand, multicopy plasmids or those located in both positions should have a lower tendency to be lost. This model explains how different chemical compounds that induce filament formation determine plasmid loss, and why the same effect is not achieved when treated bacteria are induced to a coccal shape. Preliminary results obtained

in this laboratory have shown that plasmids R387 (IncK), RN3 (IncN), R386 (IncFI), R391 (IncJ), R16 (IncO), R621a (IncI), but not RP4 (IncP-1) were lost from SP45 after filament induction, as described here, but not from the spherical cells.

In this context, it is worth noting that exposure of bacteria to DNA gyrase inhibitors leads to the induction of the *recA* gene product (Gudas & Pardee, 1976) which is responsible for the elongation of the bacterial cells (Satta & Pardee, 1978), a condition in which curing occurs. Thus, all the curing events induced by agents whose mechanisms of action are not known should be reconsidered, especially if morphological alterations are produced.

In considering *F'**lac* and pXX332 or pXX333 elimination mechanisms from DNA gyrase mutants and other bacterial hosts it is necessary to take into account the following observations: (i) *F'**lac* and pXX332 or pXX333 at 36.5 °C are stably inherited in strains other than DNA gyrase mutants; (ii) the phenotype of *gyrB41*(Ts) and *gyrA43*(Ts) at 36.5 °C carrying or not carrying plasmids is reminiscent of that of some minicell-producing mutants; (iii) the rate of pXX332 or pXX333 elimination at 36.5 °C from DNA gyrase mutants is dependent on host shape and is not affected by the presence of the *ccd* function of F; (iv) the number of pXX332- and pXX333-free segregants at 42 °C from strains other than DNA gyrase mutants is dependent on the presence of the *ccd*⁺ gene in the plasmid and is not related to host shape; (v) filaments induced by the *ccd* function of F are present in plasmid-free segregants only when the replication of plasmid is blocked by the temperature; (vi) the mutation which renders bacteria able to grow as cocci does not affect plasmid stability *per se*.

Taken together these observations suggest that plasmid elimination from DNA gyrase mutants at semi-permissive temperature occurs because, like minicell producing mutants, they present abnormalities in the division process which lead to the production of anucleated rods and minicells into which plasmids can segregate (Kass & Yarmolinsky, 1970). The fact that in *rodA51* derivatives, plasmid elimination is strongly reduced but not totally abolished, also suggests that DNA gyrases play a role in plasmid maintenance. Thus, cell elongation or the inactivation of these enzymes or both, influence plasmid stability in DNA gyrase mutants.

Another important factor known to influence plasmid maintenance in bacteria is the system for regulation of DNA replication (Scott, 1984). It has been demonstrated that acridine orange cures F factor from cells by inhibiting plasmid replication (Hohn & Korn, 1969; Wechsler & Kline, 1980). However, the presence of elongated forms has also been noted here in acridine-

orange-exposed cells, suggesting a possible involvement of bacterial shape as cofactor in this process.

The observations that nalidixic acid was unable to promote plasmid loss from round cells at the same concentration effective in curing plasmids in rods, and that it was not able to inhibit Hfr strains that grow under the control of F replication system are of special interest. This behaviour strongly suggests that the mechanism by which nalidixic acid eliminates plasmids does not primarily affect plasmid replication.

Studies from this laboratory have shown that the replication of plasmid pSUP5011 (Simon, 1984) in starved cells is blocked by a concentration of cinoxacin two orders of magnitude greater than that required to inhibit cell growth (Debbia & Schito, 1986). This is far higher than the concentration effective in plasmid curing under usual experimental conditions.

Differences in the susceptibility to oxolinic acid between plasmid and chromosome replication were also reported in other studies (Drlica, 1984). Additional experiments therefore seem to be required to elucidate the role of these DNA gyrase inhibitors in plasmid maintenance.

Finally, the above results might be of clinical significance. During antibiotic therapy, conditions for plasmid curing are easily achieved because bacteria are exposed to variable concentrations of drugs, some of these being more effective in curing than in others. Plasmids could therefore segregate *in vivo* into non-viable elements. Since these minicell-like units are no longer affected by antibiotics but are able to transfer plasmids, they might represent one factor contributing to plasmid dissemination.

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