Stability of a Catabolic Plasmid in Continuous Culture

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A wild-type strain of *Pseudomonas putida* PPK1, carrying a non-conjugative TOL plasmid, was grown in continuous culture under carbon-limitation with *m*-toluate as growth substrate. When the medium was changed to benzoate a plasmid-free strain appeared after about 100 h. After this event the proportion of plasmid-containing bacteria declined rapidly but in several experiments this was followed by oscillations in the relative frequencies of the plasmid-free and plasmid-containing populations. About 1% of the total population retained the plasmid after 600 h growth under benzoate-limitation. When the input medium was returned to *m*-toluate the plasmid-free bacteria disappeared and the TOL⁺ population recovered to 100%. The plasmid in the wild-type strain was stably maintained during 500 h of chemostat growth under succinate-limitation. Tol⁺ strains re-isolated from continuous culture under benzoate-limitation retained their TOL plasmids for longer periods. It is suggested that plasmid loss is related to a failure in the control of partitioning at cell division but that this is not absolute and allows the maintenance of a residual low level of plasmid-containing bacteria in the population.

INTRODUCTION

Many bacteria contain plasmids coding for such characters as drug resistance or the ability to utilize certain organic compounds which are of advantage to the host only under particular growth conditions. Nevertheless, it is clearly advantageous to a natural population of bacteria to retain such plasmids in order to survive in a changing environment. In some cases a plasmid may provide both catabolic genes and drug resistance genes (Kanemitsu *et al.*, 1980) or a plasmid may render its host more resistant to UV irradiation or some other toxic hazard (Jacoby, 1974). The disadvantage of possessing plasmids has been considered to be that they place a significant burden on the bacterial host (Dykhuizen & Hartl, 1983). The implication of this view was that under non-selective growth conditions the plasmid-free bacteria would have a growth advantage and outgrow the original population. It was also predicted that the size of the plasmid would be directly related to its effect on the growth rate of the bacterial host. Recent work has shown that this simple relationship does not always hold (Dykhuizen & Hartl, 1983; Nordstrom, 1983; Zund & Lebek, 1980). The stability of drug resistance plasmids in continuous culture has been examined by Melling *et al.* (1977) and Godwin & Slater (1979). We have studied the stability of a catabolic plasmid in continuous culture. TOL plasmids are widely distributed in nature and strains carrying these plasmids can be isolated by selective enrichment on *m*-toluate (Williams & Worsey, 1976). The original TOL plasmid was identified in *Pseudomonas putida* (*arvilla*) strain mt-2 by Williams & Murray (1974). The TOL plasmid of strain mt-2 (PaW1) carries genes for the catabolism of toluene, *m*- and *p*-xylene, and their intermediates, via the *meta* cleavage pathway. Benzoate is catabolized via the *meta* pathway in strains carrying TOL plasmids but cured strains break down benzoate by the *ortho* cleavage pathway coded by chromosomal genes. Strain PaW1, and some strains carrying other TOL plasmids, can be cured by growth on benzoate (Williams & Murray, 1974; Williams & Worsey, 1976). Extensive rearrangements of the TOL plasmid DNA may take place under selection for growth on benzoate and the chlorobenzoates (Jeenes *et al.*, 1982).

In this study we have examined the stability of a TOL plasmid of a natural isolate of *P. putida* in chemostat culture with *m*-toluate, benzoate or succinate as the growth-limiting substrates. The strain used was a recent isolate so as to avoid as far as possible the changes that might result from prolonged laboratory cultivation. The plasmid was non-conjugative in batch culture so it was unlikely that the results would be complicated by plasmid transfer from TOL⁺ to TOL⁻ bacteria during chemostat growth.

METHODS

Bacterial strains. The strains are listed in Table 1. *P. putida* strain PPK1 was isolated from enrichment culture on *m*-toluate of a soil sample from the London area. Cured strains were isolated from batch or continuous culture and auxotrophic and drug-resistant mutants were isolated by standard methods. The sources of other strains are given in Table 1.

Growth media. Nutrient broth and agar were prepared with Oxoid Nutrient Broth no. 2. The minimal salts medium was based on that described by Brammar & Clarke (1964) and the specified carbon source was added to give the required concentration for each experiment. Dilution buffers and media for batch experiments were sterilized by autoclaving at 121 °C for 15 min. The 20 litre vessels used for media reservoirs for continuous culture experiments were autoclaved for 40 min.

Batch growth. Small volumes of culture (about 5 ml) were grown in universal screw-capped bottles. Larger volumes were grown in conical flasks (250–1000 ml). Cultures were incubated at 30 °C (or 37 °C) on a mechanical shaker, either reciprocating or orbital in operation. In some experiments a chemostat vessel was used for batch culture with appropriate rates of forced aeration.

Continuous culture. Two types of vessels were used. Some experiments were carried out with a New Brunswick BioFlo model C30 with a culture volume of 300 ml stirred at 400 r.p.m. and sparged with water-saturated air to prevent loss of water vapour during aeration. Other experiments were carried out with a glass bioflow fermenter with a working volume of 150 ml designed at University College London. Sampling points were located in the overflow lines of each fermenter for the removal of large samples (30 ml) which were directed into sterile bottles cooled on ice. Small samples of about 1 ml were taken directly from the fermenter vessels by means of sterile syringes.

For each experiment the culture vessel was inoculated with 10% (v/v) of an overnight culture and when the culture was approaching the end of the exponential phase of growth, continuous flow operation was started. The dilution rate was kept constant at 0.2 h⁻¹. The concentrations of carbon sources were *m*-toluate 5 mM, benzoate 5.5 mM and succinate 10 mM, as the sodium salts. Polypropylene glycol antifoam was added to the medium (0.1 ml l⁻¹). When steady state conditions had been established for growth on *m*-toluate the medium was changed to benzoate or succinate.

Bacterial counts and growth phenotypes. The bacterial concentration of batch and chemostat cultures was obtained by measuring the optical density at 675 nm with a Pye Unicam SP600 spectrophotometer. Total counts were obtained by plating suitable dilutions of cultures on nutrient agar plates and samples of the same, or other, dilutions were spread on minimal agar plates containing *m*-toluate, succinate or benzoate as the sole carbon source. Plates were incubated at 30 °C until colonies were large enough to be counted. The apparent loss of the TOL plasmid was calculated from the number of colonies on *m*-toluate plates compared with the numbers on nutrient,

Table 1. *Bacterial strains and plasmids*

Strain	Relevant phenotype†	Plasmid	Origin	Reference
<i>P. putida</i>				
PPK1	Tol ⁺	pTK0	Isolate from <i>m</i> -toluate	This paper
PPK1*	Tol ⁺	pTK0	see text and Fig. 1(a)	This paper
PPK2	Tol ⁻	None	see text and Fig. 1(b)	This paper
PPK2 <i>str</i> -204	Tol ⁻ Str ^r	None	PPK2	This paper
PPK6	Tol ⁺	PTK0	see text and Fig. 3	This paper
PPK1(pMO61)	Tol ⁺ Cb ^r Km ^r	pTK0, pMO61	Conjugal transfer	This paper
PaW1	Tol ⁺	pWW0	Wild-type	Williams & Murray (1974)
PaW17	Tol ⁻ Str ^r	None	Benzoate curing	Williams & Murray (1974)
<i>P. aeruginosa</i>				
PAC21	Tol ⁻ Lys ⁻	None	PAC1	Laboratory collection
PAO127	Tol ⁻	pMO61	PAO1	Morgan (1982)

† Phenotype designations: Tol⁺, ability to grow on *m*-toluate; Lys⁻, lysine auxotrophy; Str^r, resistance to streptomycin (200 µg ml⁻¹); Cb^r, Km^r, resistance to carbenicillin (500 µg ml⁻¹) and kanamycin (250 µg ml⁻¹) coded on plasmid pMO61.

benzoate or succinate agar plates and was expressed as percentage loss. The colonies from nutrient agar plates (or succinate agar plates) were also replicated onto *m*-toluate plates. Cured Tol⁻ strains were selected in this manner. Isolates were tested for growth on *m*-xylene and toluene.

Isolation of streptomycin-resistant mutants. The strains used in mating experiments were selected on nutrient agar plates containing streptomycin and were resistant to 200 µg streptomycin ml⁻¹.

Conjugation experiments. Tests for conjugal transfer were carried out by plate mating or by membrane mating (Austen & Dunn, 1977).

Plasmid mobilization. Plasmid pMO61 derived by Morgan (1982) from the IncP-1 plasmid R68.45 was used for mobilizing the TOL plasmid pTK0 using the method of Austen & Dunn (1977). Plasmid pMO61 was transferred into PPK strains from *P. aeruginosa* strain PAC21 *lys*(pMO61) by plate mating on *m*-toluate minimal agar plates supplemented with 400 µg kanamycin ml⁻¹.

Cell-free extracts. Samples from the continuous cultures were centrifuged and the bacteria were stored at -20 °C until required. The bacterial pellet was resuspended in 0.1 M-phosphate buffer pH 7.6, supplemented with 10% (v/v) acetone, and disrupted by sonication. The bacterial debris was removed by centrifugation at 15000g for 15 min and the supernatant kept on ice until enzyme assays were completed.

Enzyme assays. Spectrophotometric assays were carried out at room temperature using a Pye Unicam SP1700 spectrophotometer. Reactions were started by the addition of 0.1 ml of the crude extract to the cuvette containing the reaction mixture. Units were defined as the amount of enzyme converting 1 nmol of substrate to the product in 1 min under the assay conditions used. Catechol 1,2-oxygenase (pyrocatechase) was measured by the increase in absorbance due to the production of *cis,cis*-muconate (Hegeman, 1966). Catechol 2,3-oxygenase was measured by the methods described by Sala-Trepat & Evans (1971) following the increase in absorbance due to the production of 2-hydroxymuconic semialdehyde.

Plasmid isolation. Samples for plasmid isolation containing glycerol (16%, v/v) were preserved at -70 °C. The methods of Wheatcroft & Williams (1981) were used to isolate plasmid DNA.

Characterization of m-toluate isolate PPK1. Electron microscopy showed that the PPK1 bacteria were short rods with single polar flagella; the Gram strain was negative; PPK1 grew at 4 °C and not at 37 °C or 41 °C; optimal growth was at 30 °C. In growth habit it behaved as an aerobe and colonies on succinate (and other) minimal salts media were faintly yellow with slight fluorescence. Other comparisons were limited to the nutritional criteria of Stanier *et al.* (1966) and indicated that PPK1 resembled most closely *P. putida* biotype A. Compounds utilized were: D-glucose, D-fructose, acetate, citrate, DL-lactate, DL-malate, malonate, propionate, pyruvate, succinate, glycerol, ethanol, *n*-propanol, *n*-butanol, *iso*-butanol, L-alanine, L-aspartate, L-histidine and L-phenylalanine. Compounds not utilized were D-galactose, inositol, methanol, L-tryptophan, anthranilate and acetamide. Strain PPK1 grew on benzoate, toluene and *m*-xylene as well as on *m*-toluate.

RESULTS

Plasmid stability in continuous culture with benzoate as carbon-limiting growth substrate

The experiments were all set up in the same way and growth conditions were kept as constant as possible. Strain PPK1 and its variants were grown in batch culture until a value of 0.5–0.6 for OD₆₇₅ had been reached before switching to continuous operation at a dilution rate of 0.2 h⁻¹. Each experiment comprised four separate growth stages: (A) growth on *m*-toluate and establishment of steady state conditions (40–100 h); (B) transition from *m*-toluate to benzoate; (C) growth on benzoate and establishment of steady state conditions; (D) transition from benzoate to *m*-toluate and re-establishment of steady state conditions. Experiments were maintained for up to 800 h and samples taken for determinations of bacterial concentration, plasmid frequency and catechol oxygenase activity. In Table 2 the growth times are given for stages A + B, C and D for the experiments shown in Figs 1–4. At the dilution rate used in these experiments the time for complete medium replacement during stage B was 5 h.

Fig. 1(a, b) shows the results obtained for two experiments with PPK1 for growth during stage C. Zero time is 5 h after the switch from *m*-toluate to benzoate. In the experiment shown in Fig. 1(a) there was a perturbation in optical density after about 80 h growth and this was paralleled by an apparent loss of plasmid reaching 92% after 118 h. The optical density began to change at about 160 h and the culture eventually returned to a steady state of growth and reached a maximum of 98% plasmid loss. In the experiment shown in Fig. 1(b) there was only a slight perturbation in optical density at about the time that plasmid loss was first detected. In other experiments with PPK1 the extent of the perturbations in optical density observed at about the time at which the plasmid level began to drop was intermediate between the two shown in Fig. 1.

Table 2. *Plasmid stability in continuous culture with benzoate as carbon-limiting growth substrate*

The continuous culture vessel was operated at a dilution rate of 0.2 h^{-1} with 5 mM-*m*-toluate as carbon source for at least 40 h (stage A) before the medium was switched to 5.5 mM-benzoate (stage B, transition to benzoate). At the end of benzoate-growth (stage C) the medium was switched back to *m*-toluate (stage D). See text for details.

Strain	Growth period (h)				Stage C				Stage D	
					Plasmid loss first detected		Maximum plasmid loss		Plasmid	Plasmid recovery
	A + B	C	D	Total	(h)	(%)	(h)	(%)	$t_{\frac{1}{2}}$ (h)	(h) (%)
PPK1	46	644	164	854	90	6	410	98	96	22 100
PPK1	50	333	26	409	47	3	142	98	92	26 100
PPK1*†	49	310	26	385	85	32	150	99	90	30 100
PPK6‡	93	527	51	671	115	16	312	99	130	22 100

† PPK1*: inoculum derived from the experiment shown in Fig. 1(a).

‡ PPK6: inoculum derived from the experiment shown in Fig. 3.

Values for plasmid loss were based on the numbers of Tol⁺ and Tol⁻ bacteria and in later experiments we found that plasmid DNA could be isolated from Tol⁺ bacteria but not from Tol⁻ bacteria (see Fig. 4). Table 2 gives the times at which plasmid loss was first detected in these and other experiments and the times at which plasmid loss was maximal. Each experiment on population changes in continuous culture is unique (Dykhuizen & Hartl, 1983) and we shall return to this point later. It was however essential to have some way of comparing one experiment with another and we have used the term plasmid $t_{\frac{1}{2}}$ for this comparison. This is the time from zero, during growth on benzoate, at which 50% of the chemostat population had become Tol⁻. The values for the experiments discussed in this paper are given in Table 2. For the PPK1 experiment shown in Fig. 1(a) the $t_{\frac{1}{2}}$ value was calculated from the time at which the plasmid loss had reached 92% at the first perturbation rather than the 98% reached later. In a series of experiments with PPK1 the values were 90–96 h. Values for the PPK1* and the PPK6 experiments (Figs 3 and 4a) were also calculated from the first period of rapid plasmid loss.

At the end of stage C growth on benzoate the chemostats were returned to *m*-toluate as the carbon source. In all cases the optical density fell rapidly but then recovered and the Tol⁺ population reached 100% in 20–30 h. Fig. 2 shows the optical density, apparent plasmid levels and enzyme activities during growth stage D, for a typical experiment. The kinetics of growth were consistent with the establishment of a Tol⁺ population from the residual plasmid-containing bacteria. We have taken stage D of the experiment shown in Fig. 1(a) as an example but similar recovery rates occurred in all the experiments (see also Fig. 4a).

One of the objectives of these experiments was to select strains with increased plasmid stability. It was thought that the Tol⁺ survivors during growth on benzoate might be more stable than the original strain. Fig. 3 shows an experiment inoculated with PPK1* isolated from a sample taken from the continuous culture shown in Fig. 1(a) after 300 h growth on benzoate. After about 150 h the apparent plasmid loss was 99%. After 250 h there was a marked recovery and the plasmid-free population then dropped to 70% by 320 h. This experiment demonstrated the spontaneous recovery of plasmid-containing bacteria even under cultural conditions that were expected to provide a strong selection pressure for plasmid loss.

The key *meta* pathway enzyme coded by the TOL plasmid is catechol 2,3-oxygenase and in each of these experiments the plasmid loss calculated from the ratio of Tol⁻ to Tol⁺ colonies was associated with a decrease in the specific activity of this enzyme. At about the time at which the specific activity of catechol 2,3-oxygenase decreased there was a slight increase in the activity of the *ortho* pathway enzyme, catechol 1,2-oxygenase (Figs 1–3). (Catechol 1,2-oxygenase is induced by its product, *cis,cis*-muconate, and would not be expected to reach high levels of activity in cultures grown under carbon-limitation.) The *meta* pathway enzymes were not investigated in detail, but Tol⁺ and Tol⁻ colonies were tested for growth on other aromatic

Fig. 1. Growth of *P. putida* strain PPK1 in continuous culture with benzoate as growth-limiting carbon source. Cultures were grown to steady state with *m*-toluate (5 mM) and the incoming medium was switched to benzoate (5.5 mM). The elapsed time is shown for growth on benzoate (stage C). ●, Bacterial concentration as OD₆₇₅; ○, percentage plasmid loss; □, catechol 1,2-oxygenase; ■, catechol 2,3-oxygenase. The experiments shown in (a) and (b) were carried out under identical conditions of growth.

Fig. 2

Fig. 3

Fig. 2. Growth of *P. putida* strain PPK1 in continuous culture following transition from benzoate (5.5 mM) to *m*-toluate (5 mM). Elapsed time is shown for growth on *m*-toluate during stage D, continued from the experiment shown in Fig. 1(a) (the designation of the symbols is the same).

Fig. 3. Growth of *P. putida* strain PPK1* in continuous culture with benzoate as growth-limiting carbon source. Cultures were grown to steady state with *m*-toluate (5 mM) and the incoming medium was switched to benzoate (5.5 mM). The elapsed time is shown for growth on benzoate (stage C). ●, Bacterial concentration as OD₆₇₅; ○, percentage plasmid loss; □, catechol 1,2-oxygenase; ■, catechol 2,3-oxygenase.

compounds. All the *m*-toluate-negative strains obtained were also unable to grow on toluene and *m*-xylene. The appearance of the cultures growing on *m*-toluate was yellowish green and this changed to deep green when the medium was switched to benzoate. At the time of rapid plasmid loss, at about 90 h in most cases, the colour changed gradually from deep to very pale green.

Variants with altered plasmid stabilities

Chemostat culture has been widely used to select mutant strains producing altered enzymes or gene duplications (Rigby *et al.*, 1974; Inderlied & Mortlock, 1977). It was thought that it might be possible to select strains which retained their TOL plasmids for longer periods. There were slight oscillations in plasmid levels in the PPK1 experiment shown in Fig. 1(a). Strain PPK1* was isolated from a sample taken after about 300 h growth on benzoate during stage C of this experiment and was used as the inoculum for the experiment shown in Fig. 3. Although plasmid

Fig. 4. Variations in plasmid stability of strain PPK6 in continuous culture with benzoate as the growth-limiting carbon source. Cultures were grown to steady state on *m*-toluate (5 mM) and the incoming medium was switched to benzoate (5.5 mM). The elapsed time is shown for growth on benzoate (stage C). ●, Bacterial concentration as OD₆₇₅; ○, percentage plasmid loss; □, catechol 1,2-oxygenase; ■, catechol 2,3-oxygenase. Both culture vessels (a) and (b) were inoculated with PPK6 isolated from stage C of growth on benzoate as shown in Fig. 3. Fig. 4(a) includes the transition from benzoate to *m*-toluate and the recovery of the Tol⁺ phenotype. Samples were used for isolation of plasmid DNA; the times at which plasmid bands were detected are shown at the top of the figure (+, plasmid band on gel; -, no plasmid band).

loss reached 99% after about 150 h growth on benzoate there was a later recovery of Tol⁺ bacteria in the population. In another experiment with PPK1* as the inoculum (not shown) the oscillations were greater. The apparent plasmid loss was 44% after 142 h and then fluctuated between 40% and 14%. During 240 h growth with benzoate-limitation there was no time at which the plasmid loss was greater than 44%.

In an attempt to continue this selection for increased stability, Tol⁺ bacteria were isolated from the PPK1* experiment (Fig. 3) as PPK6. Two chemostats were inoculated with PPK6 and run in parallel under identical conditions (Fig. 4a, b). With one of these there were marked oscillations in apparent plasmid levels. Plasmid loss was detected after 115 h growth on benzoate and reached 87% after 180 h. Thereafter there was a rapid recovery of Tol⁺ bacteria followed by a dramatic decline to give 99% loss at 312 h. The optical density of the culture and the enzyme activities also fluctuated and the typical changes of colour from pale to deep green and back were observed during the periods of oscillation of plasmid levels in the chemostat culture. Fig. 4(a) also shows the usual rapid plasmid recovery during stage D after the return to growth on *m*-toluate. On the other hand, in the parallel experiment (Fig. 4b) there was no plasmid loss during 500 h growth on benzoate.

Stability of the PPK1 TOL plasmid in continuous culture with succinate as carbon-limiting growth substrate

Succinate is a catabolite repressor for many induced enzymes of *Pseudomonas* species and in some cases its role as a preferred growth substrate is manifested in diauxic growth. Continuous culture experiments were set up as previously with cultures established in steady state growth on *m*-toluate before switching to growth on succinate. There were no significant fluctuations in optical density after the transition to succinate and the cultures remained in steady state growth on succinate for periods of 300–600 h with no detectable plasmid loss.

Tol⁻ isolates from chemostat culture

The fluctuations in apparent plasmid levels could have been due to total loss of plasmid, to repression of plasmid genes or to inactivation by integration of the plasmid into the chromosome followed by intermittent release. If the latter had occurred it was possible that Tol⁻ bacteria isolated from benzoate growth, stage C, would be able to recover the Tol⁺ character spontaneously. A chemostat was set up with benzoate as the carbon-limiting growth substrate and inoculated with strain PPK2, a Tol⁻ isolate from the experiment shown in Fig. 1(b). The culture was maintained in steady state with benzoate for 47 h and then switched to *m*-toluate. The optical density dropped from 0.55 to 0.06 in 5 h and after 21 h was 0.01. The medium feed was turned off and the culture left for a further 20 h. There was no growth and no Tol⁺ bacteria were detected. The curing appeared to be irreversible.

Isolation of plasmid DNA from chemostat cultures

A single plasmid band, running more slowly than pWW0 from strain PaW1 (used as control), was detected in samples from growth on *m*-toluate and during growth on benzoate before apparent plasmid loss. No plasmid DNA could be detected in the cured strain PPK2 or in samples taken from chemostat runs during stage C at times at which it appeared that plasmid loss had reached about 98%. Plasmid DNA was detected in all samples removed from the PPK6 experiment shown in Fig. 4(b). The times at which plasmid bands could be detected in samples from the PPK6 experiment shown in Fig. 4(a) corresponded to the times at which plasmid loss had been inferred from the observations made on growth phenotype and enzyme activities. In the sample taken at 244 h (55% loss), a plasmid band was seen but was fainter than in the samples taken during the earlier stages up to 100 h. No differences in mobility were detected, so the plasmid did not appear to have lost or gained any significant amounts of DNA during the experimental period. Although small changes could not be ruled out it was concluded that the observations on growth phenotype and enzyme activities were related to retention or loss of the entire plasmid. The size was estimated from the mobilities of the plasmid bands and from restriction enzyme digests (not shown) to be approximately 150 kb. The TOL plasmids isolated by Williams & Worsey (1976) ranged in size from 70 to 200 kb.

*Non-transmissibility of plasmid pTK0 from *P. putida* PPK1*

No recombinants were obtained in plate matings with PPK1 as donor and PPK2 (cured strain) or PaW17 as recipients but the pTK0 plasmid from strain PPK1 could be mobilized by the IncP-1 plasmid pMO61. Although the plasmid pTK0 of strain PPK1 was not self-transmissible it was thought that some of the variants isolated from the chemostats might be altered in this respect. However, none gave transconjugants in plate matings with any of the potential recipients.

The further possibility remained that during the rapid recovery of the Tol⁺ character after the chemostat culture had been returned from benzoate to *m*-toluate at stage D, the transfer functions were derepressed with consequent rapid spread of the plasmid through the population. Samples removed from the chemostat at this stage were tested for donor activity by patch plate mating and by membrane mating. No donor activity was detected and it was concluded that conjugal transfer of pTK0 played no part in the changes in the growth phenotype of the chemostat population at any growth stage.

Fig. 5. Rates of loss of TOL plasmids from *P. putida* strains growing with benzoate as growth-limiting carbon source. The data are taken from the experiments shown in Figs 1, 3 and 4(a) and the curves are plotted for the period during which the numbers of plasmid-containing bacteria declined most rapidly. (a) PPK1 from Fig. 1(a); (b) PPK1 from Fig. 1(b); (c) PPK1* from Fig. 3; (d) PPK6 from Fig. 4(a); (e) is the theoretical washout curve at the dilution rate of 0.2 h^{-1} .

Kinetics of plasmid loss in continuous culture

P. putida strain PPK1 retained its TOL plasmid for about 100 h when grown in continuous culture under benzoate-limitation and thereafter the numbers of plasmid-containing bacteria declined to about 1% of the total population. Strains PPK1* and PPK6, isolated from samples taken after prolonged growth on benzoate in continuous culture, retained their plasmids for longer periods. In one experiment with strain PPK6 as the inoculum there was no plasmid loss during 500 h continuous culture on benzoate (Fig. 4b).

In Fig. 5 we have plotted the rates of plasmid loss over the period of rapid decline in numbers for the experiments shown in Figs 1, 3 and 4(a). We have also included the theoretical curve for washout of the plasmid-containing bacteria from the chemostat population (Fig. 5e).

The classical explanation for the decline in numbers of plasmid-containing bacteria is that plasmid-free bacteria grow faster. This would give simple competition kinetics and the rate of replacement would depend on the growth advantage of the plasmid-free strain (Powell, 1958; Dykhuizen & Hartl, 1983). Assuming a linear correlation of $\ln[X_1(t)/X_2(t)]$ with time, with $X_1(t)$ and $X_2(t)$ representing the relative proportions of the two competing strains at time t , it can be calculated from the data shown in Fig. 5(a-d) that it would be necessary to have a growth advantage of between 0.13 and 0.25 h^{-1} to account for the observed rates of decline in plasmid-containing bacteria. From these values it would be predicted that there would be marked differences in the growth rates of the Tol⁺ and Tol⁻ bacteria, and the plasmid-containing strain would be expected to disappear completely with time, but this never occurred in any of our experiments. After an initial period of plasmid loss there was often a marked recovery; in one experiment the plasmid level did not drop below 44% and in another the plasmid was retained throughout the experimental period.

We need to account for the event leading to the initial loss of plasmid from one or more of the original population. Among other possibilities is that this event is due to a failure in partitioning (Nordstrom *et al.*, 1980a, b). If we assume a negligible difference in the growth rates of the Tol⁺ and Tol⁻ bacteria then, using the data shown in Fig. 5, the percentage loss of plasmid per generation varies from 20% to 40%, which could mean that correct partitioning of plasmid copies into both daughter cells occurs in between 20% and 60% of the dividing cells. Assuming no further plasmid replication after the initial catastrophe, and initially one plasmid copy per cell, a change from 32% to 88% plasmid loss would take about 8.7 h. In the experiment with PPK1* (Fig. 5c) this took place in 11 h so that the rate of plasmid loss is approaching that for washout (Fig. 5e). We have taken the extreme case but we know that limited plasmid replication and segregation does occur since some plasmid-containing bacteria remain in the population.

DISCUSSION

The maintenance of plasmids in bacterial cells presents a paradox. Under certain growth conditions plasmid-containing strains are at an advantage compared with plasmid-free strains. On the other hand it may be disadvantageous for an organism to carry DNA that is not required for immediate needs. This argument holds less force now that it is known that plasmids are present in many organisms, that bacteria may carry genes for cryptic pathways and that genes for multiple catabolic pathways may exist in some organisms. The identification of several plasmids, some very large, in species of *Rhizobium* and *Pseudomonas* (Rosenberg *et al.*, 1982) suggests that the maintenance of plasmids is a complex and well-regulated process. However, it is also a common observation that bacteria isolated for their interesting catabolic activities may be rather unstable in laboratory culture.

In our experiments the TOL plasmid of PPK1 was lost during benzoate-limited growth. Although each chemostat experiment is a unique exercise in the evolution of a bacterial population it has frequently been observed that periodic selection events are relatively reproducible (Dykhuizen & Hartl, 1983). Starting with the wild-type strain PPK1 the time at which the plasmid-free strains appeared was around 100 h. Even in experiments in which the frequency of the plasmid-free population remained constant at about 98% for long periods the plasmid-containing strain was never completely lost and could recover to become 100% of the population when the selection pressure was reimposed.

When PPK1 was grown under succinate-limitation its TOL plasmid was stably maintained for up to 600 h. Under these conditions the enzymes of the aromatic pathways are not induced and one possible interpretation is that the plasmid is more stable when the catabolic genes are not expressed. This implies that replication of the plasmid DNA is not a disadvantage to the host bacterium but that gene expression may be. Our experiments with the variant strains selected from continuous growth on benzoate indicate that some plasmids can be maintained for long periods even when there is good gene expression. In one experiment the plasmid of PPK6 was stably maintained in continuous culture with benzoate for over 500 h. In this case the plasmid-coded catabolic genes were fully expressed. The increased stability in PPK6 could have been due to either plasmid or chromosomal factors but was not an irreversible change since in another experiment, set up at the same time and with the same inoculum, the plasmid-containing bacteria eventually dropped to 1% of the population.

The possession of catabolic plasmids, or drug resistance plasmids, provides bacteria with genes that may be useful under particular growth conditions. The TOL plasmids have the unusual property of replacing a catabolic pathway coded by chromosomal genes by a pathway coded by plasmid genes. With the TOL plasmid present benzoate is degraded by the *meta* cleavage pathway and in its absence by the *ortho* cleavage pathway. It has been suggested that the TOL plasmid is lost in batch culture because the *ortho* cleavage pathway allows faster growth of *P. putida* (arvilla) mt-2 on benzoate (Williams & Murray, 1974) and we had thought that we might observe this in continuous culture with strain PPK1. If the growth rate on benzoate via the *ortho* cleavage pathway were to be significantly higher than that via the *meta* cleavage pathway, that could account for the replacement of plasmid-containing strains by plasmid-free strains but not for plasmid recovery. However, the growth rates of strains PPK1 and PPK2 in batch culture do not differ significantly and considerable growth rate differences would be required to account for the very rapid loss of plasmids observed in our continuous culture experiments.

Competition in chemostat culture could also be affected by differences between the activities of the chromosomal and plasmid benzoate oxidases, the 1,2- and 2,3-catechol oxygenases and other enzymes of the two pathways. Since the synthesis of the plasmid-coded enzymes is regulated by a different mechanism from that controlling the chromosomally-coded enzymes, both the substrate affinities and the amounts of the enzymes synthesized could be important. Also, many aromatic substrates and their metabolic intermediates are toxic at relatively low concentrations. Benzoate was present in the incoming medium at a growth-limiting concentration (5.5 mM) but there could have been transient accumulation of toxic intermediates

of the *meta* pathway. This might give an advantage to the plasmid-free bacteria. We do not rule out the possibility that these factors may contribute to competition between the plasmid-free and plasmid-containing strains.

We observed oscillations in the frequencies of the plasmid-free and plasmid-containing bacteria which varied in extent in different experiments and were more marked with strains isolated after prolonged growth under benzoate-limitation. Helling *et al.* (1981) observed oscillations in chemostat culture of an *Escherichia coli* strain carrying a small non-conjugative plasmid and they suggested that the frequency of the plasmid-free population increased principally because the bacteria had a higher growth rate but also because of occasional defective segregation. The spontaneous mutations resulting in a faster growth rate were expected to occur more frequently among the plasmid-containing bacteria since these constituted the bulk of the population. However in our experiments the oscillations, and the increase in frequency of the plasmid-containing bacteria, appeared at times at which the plasmid-free population was dominant.

It has now become possible to relate some changes in plasmid properties to specific alterations in DNA structures. Genes carried on transposons may be exchanged between different replicons; genes may be inactivated by the insertion of IS sequences; single-site mutations or duplication of gene sequences may alter gene expression (see Bukhari *et al.*, 1977). A duplicated sequence in the drug resistance plasmid R68.45 is thought to account for its acquisition of chromosome-mobilizing ability and further modifications confer the ability to generate R-primes (Willets *et al.*, 1981; Morgan, 1982). The DNA of TOL plasmids has been shown to be capable of extensive rearrangement during selection for new activities (Jeenes *et al.*, 1982; Kunz & Chapman, 1981).

We suggest that it may be possible to relate our observations on the stability of the TOL plasmid of PPK1 in continuous culture to factors affecting the control of plasmid replication and segregation. Nordstrom *et al.* (1980*a, b*), working with drug-resistance plasmids, concluded that plasmid partitioning is tightly controlled. They found that plasmid R1*drd*-19 was stably inherited while certain mini-plasmid derivatives with *par* deletions were less stable and they concluded that plasmid R1*drd*-19 carries a region controlling the plasmid segregation at cell division. Meacock & Cohen (1980) identified a *cis*-acting locus, *par*, that accomplishes stable inheritance of pSC101. Similar findings on the importance of *par* functions for accurate partitioning of plasmids allowing stable inheritance have been reported by other workers (Haakkart *et al.*, 1982; Jones *et al.*, 1980; Seelke *et al.*, 1982). The mechanism of partitioning is not yet understood although the *par* region of pSC101 has now been sequenced (Meacock & Cohen, 1980; Miller *et al.*, 1983). Austin & Wierzbicki (1984) identified two proteins encoded by the *par* sequence of mini-F plasmids that were essential for equipartition at cell division.

The regulation of partitioning is clearly important for plasmid maintenance in a population. It could also be an evolutionary advantage to have a means of slippage so that catabolic plasmids could be maintained in a small percentage of a natural population in the absence of any selection pressure.

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