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Effect of growth rate on plasmid maintenance by *Escherichia coli* HB101(pAT153)

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The effects of changing the composition of the growth medium, the dilution rate and the source of the bacterial host on maintenance of the plasmid pAT153 in *Escherichia coli* HB101 have been studied. In a medium supplemented with Casamino acids, the plasmid was maintained longer during phosphate-limited growth at a dilution rate of 0·3 h^{-1} than at 0·15 h^{-1}. In contrast, phosphate-limited growth was not achieved when the Casamino acids were replaced by proline, leucine and thiamin to satisfy the auxotrophic requirements of the host. Although 100% of the bacteria were still ampicillin resistant after 72 generations of growth at a dilution rate of 0·15 h^{-1}, the original plasmid had almost totally been replaced by a structurally modified plasmid which lacked a functional *tet* gene. Further experiments confirmed that neither the host nor the plasmid was retained unchanged in the minimal medium. The changes were highly reproducible and reflected periodic selection of sub-populations which were either plasmid-free or carried a structurally modified plasmid, which had reverted to Leu^+ or Pro^+, or had acquired other chromosomal mutations which gave them a selective advantage. We conclude that in complex media the plasmid is maintained longer by *E. coli* HB101 at a high than at a low growth rate and that different results reported from different laboratories are largely due to differences in analytical techniques and the growth medium rather than to differences in the bacterial host or the plasmid used. A fermenter-adapted strain was isolated which reproducibly maintained the plasmid longer during phosphate-limited continuous growth than the original strain which had been cultured on laboratory media.

Introduction

Genetic factors which promote structural or segregational instability of plasmids used as cloning vectors are well documented (Stueber & Bujard, 1982; Skogman *et al.*, 1983; Summers & Sherratt, 1984; Austin, 1988; Gerdes, 1988). The frequency at which bacterial variants arise due to structural or segregational instability can therefore be decreased substantially by designing the vector carefully. Nevertheless, the generation of plasmid-free variants which outcompete plasmid-containing bacteria during prolonged growth cannot totally be prevented (Caulcott *et al.*, 1985).

Far less information is available about how plasmid maintenance is influenced by the physiology of the bacterial host. Although nutritional factors have been shown to influence competition between plasmid-containing bacteria and their plasmid-free derivatives (and hence the ability of plasmid-containing bacteria to be maintained) in a chemostat, conflicting conclusions were reached even for the same combination of bacterial host and cloning vector. For example, in several studies the maintenance of the plasmid pBR322 or its derivatives by continuous cultures of *Escherichia coli* K12 has been studied (Jones *et al.*, 1980; Wouters *et al.*, 1980; Noack *et al.*, 1981; Jones & Melling, 1984; Caulcott *et al.*, 1985). Chew *et al.* (1988) reported that continuous cultures maintained the plasmid pAT153 longer at higher than at lower growth rates irrespective of whether the limiting nutrient was the carbon, nitrogen, phosphorus or sulphur source. Similar results were obtained by Wouters *et al.* (1980) with carbon- and nitrogen-limited cultures of *Escherichia coli* (pBR322) and *Klebsiella aerogenes* (pBR322). Surprisingly, however, the rate of plasmid loss in phosphate-limited cultures increased with increasing dilution rate of *K. aerogenes* (pBR322) but not with *E. coli* (pBR322) (Sterkenburg *et al.*, 1984). Noack *et al.* (1981) reported that although pBR325 was maintained for more generations at high growth rates in carbon-limited cultures, it was more stable at low growth rates in ammonium-limited cultures.

Although *E. coli* HB101(pAT153) was used as a model

system in several of the studies cited above, three differences between the experiments of Chew *et al.* (1988) and others were identified as possible reasons for the different results obtained. First, the auxotrophic growth requirements of *E. coli* HB101 were satisfied in the experiments of Chew *et al.* (1988) by adding excess Casamino acids to the growth medium; in contrast, the defined media of Jones & Melling (1984), Caulcott *et al.* (1985) and Warnes & Stephenson (1986) were supplemented with proline, leucine and thiamin. Secondly, the source of the *E. coli* host, which is believed to vary between laboratories, was different. (This point has not been documented in the literature.) Finally, different criteria were used to measure plasmid maintenance by individual bacteria. Consequently, it is not at present possible to specify general principles for designing media and growth conditions that will minimize the ability of plasmid-free variants to grow faster than plasmid-containing bacteria in large fermenters. The aims of this paper are to determine how the maintenance of plasmid pAT153 in *E. coli* HB101 is affected by changes in the growth rate or medium composition during phosphate-limited growth, to document the changes which occur in both the plasmid and the host chromosome, and to determine whether the changes observed are random or predictable. Such results would indicate basic principles for designing host–vector combinations which can be maintained sufficiently long for the reproducible expression of a cloned gene in a large fermenter.

Methods

Bacterial strain, plasmid and growth conditions. The bacterial host, *Escherichia coli* HB101 (F⁻ *pro leu thi lacY rpsL31 hsdR recA*; Boyer & Roulland-Dussoix, 1969) was obtained from two sources: Searle Research and Development, High Wycombe, UK; and the Centre for Applied Microbiology and Research, Porton Down, Salisbury, UK. The 3657 base pair plasmid, pAT153, carries the pMB1 replication origin. It is a derivative of pBR322 from which the 706 base pair *Hae*II B and G fragments were deleted *in vitro* (Twigg & Sherratt, 1980). This deletion results in the loss of the Rom protein and increases the copy number approximately threefold.

Bacteria were grown either in an MBR Minibioreactor (Sulzer Bros., Farnborough, Hants., UK) with a working volume of 1 litre, pH 7·0, air supply 1 litre min^{-1} and an agitation rate of 1000 r.p.m.; or in a Bioflo C30 fermenter (New Brunswick Scientific Co.) with a working volume of 350 ml, pH 7·0, air supply 350 ml min^{-1}. In all of the experiments to be described, the dissolved oxygen concentration remained above 90% saturation.

The medium supplemented with Casamino acids contained, per litre of distilled water: 5 g glucose, 2·7 g Casamino acids, 7·94 g $(NH_4)_2SO_4$, 0·06 g KH_2PO_4, 1·07 g KCl, 0·4 g $MgSO_4.7H_2O$, 11 mg $CaCl_2.2H_2O$, 7·2 mg $FeCl_3.6H_2O$, 10 mg thiamin hydrochloride and 1 ml of the trace elements solution described by Brown *et al.* (1985). The phosphate-limited defined medium contained, per litre of distilled water: 5·4 g glucose, 1·42 g Na_2SO_4, 5·35 g NH_4Cl, 0·31 g NaH_2PO_4, 0·19 g KCl, 2 mg Na_2MoO_4, 0·254 g $MgCl_2.6H_2O$, 0·42 g citric acid, 0·32 g leucine, 0·32 g proline, 32 mg thiamin hydrochloride, 11 mg $CaCl_2.2H_2O$ and 1 ml of the trace element solution described by Evans *et al.* (1970).

At the end of each experiment, components of the medium were injected into the fermenter to identify the growth-limiting nutrient. The biomass concentration (OD_{650}) was measured at 1 h intervals for a further 6 h. Injection of the growth-limiting nutrient resulted in an increase in the growth rate and biomass.

Screening for plasmid-containing bacteria and reversion of host chromosomal mutations. Samples taken at regular intervals from the fermenter were diluted aseptically into minimal salts solution and plated onto nutrient agar. After 24 to 48 h at 37 °C, colonies were replicated onto nutrient agar supplemented with either 100 mg sodium ampicillin l^{-1} or 50 mg tetracycline hydrochloride l^{-1}.

Reversion to Pro⁺ or Leu⁺ was detected by replica plating the original colonies onto minimal agar supplemented with 0·4% (w/v) glucose, appropriate amino acids (25 mg l^{-1}) and thiamin (1 mg l^{-1}).

Preparation of plasmid DNA samples and detection of structural instability in the plasmid. Plasmid DNA was isolated by the acid-phenol whole cell lysis method (Zasloff *et al.*, 1978). Bacteria equivalent to 1·0 ml of culture at an OD_{650} of 2·0 were pelleted in a minifuge, washed in 0·5 ml sterile saline and gently resuspended in 100 µl of the lysis solution. Bacteria were lysed for 30 min at 65 °C. Acid phenol was then added. The tube was shaken to mix the contents and then centrifuged for 6 min. The clear upper aqueous layer was removed and the phenol layer was back-extracted twice to optimize the yield of the plasmid. The DNA was concentrated and precipitated with 2·5 vols ethanol plus 0·1 vol. 3 M-sodium acetate to remove inorganic salts. The DNA was collected by centrifugation, lyophilized and resuspended in 35 µl TE buffer (10 mM-Tris/HCl pH 7·0 containing 1 mM-EDTA). The plasmid preparation was linearized by adding 4 µl REact 3 buffer (BRL) and 1 µl *Eco*RI. The mixture was incubated at 37 °C for 2 h. The resulting samples were separated by electrophoresis on a 1% (w/v) agarose gel using linearized pAT153 as a standard. The gels were stained with ethidium bromide (1 µg ml^{-1}) for 20 min and destained for 5 min. The DNA bands were visualized with a UV transilluminator and photographed using an orange filter and type 55 Polaroid film (Kodak). The negative was scanned with an LKB Ultroscan XL laser densitometer to determine the amount of pAT153 in each lane. A calibration curve was constructed for each gel by plotting the area under the densitometer trace for known amounts of a pAT153 standard against the quantity of standard DNA loaded onto the gel. The volume of each sample loaded was adjusted to ensure that only the linear portion of this standard curve was used to determine the amount of plasmid in each lane.

To determine the restriction map of a plasmid, DNA fragments were separated on either a 1% (w/v) agarose gel or a 7·5% (w/v) polyacrylamide gel containing 15% (w/v) glycerol. The gels were stained for 5 min and visualized as above.

Results

Effect of dilution rate on plasmid retention in a phosphate-limited medium supplemented with Casamino acids

Initial experiments were designed to confirm the results of Chew *et al.* (1988) that the plasmid pAT153 is maintained longer by phosphate-limited continuous cultures of *E. coli* HB101(pAT153) at a dilution rate of

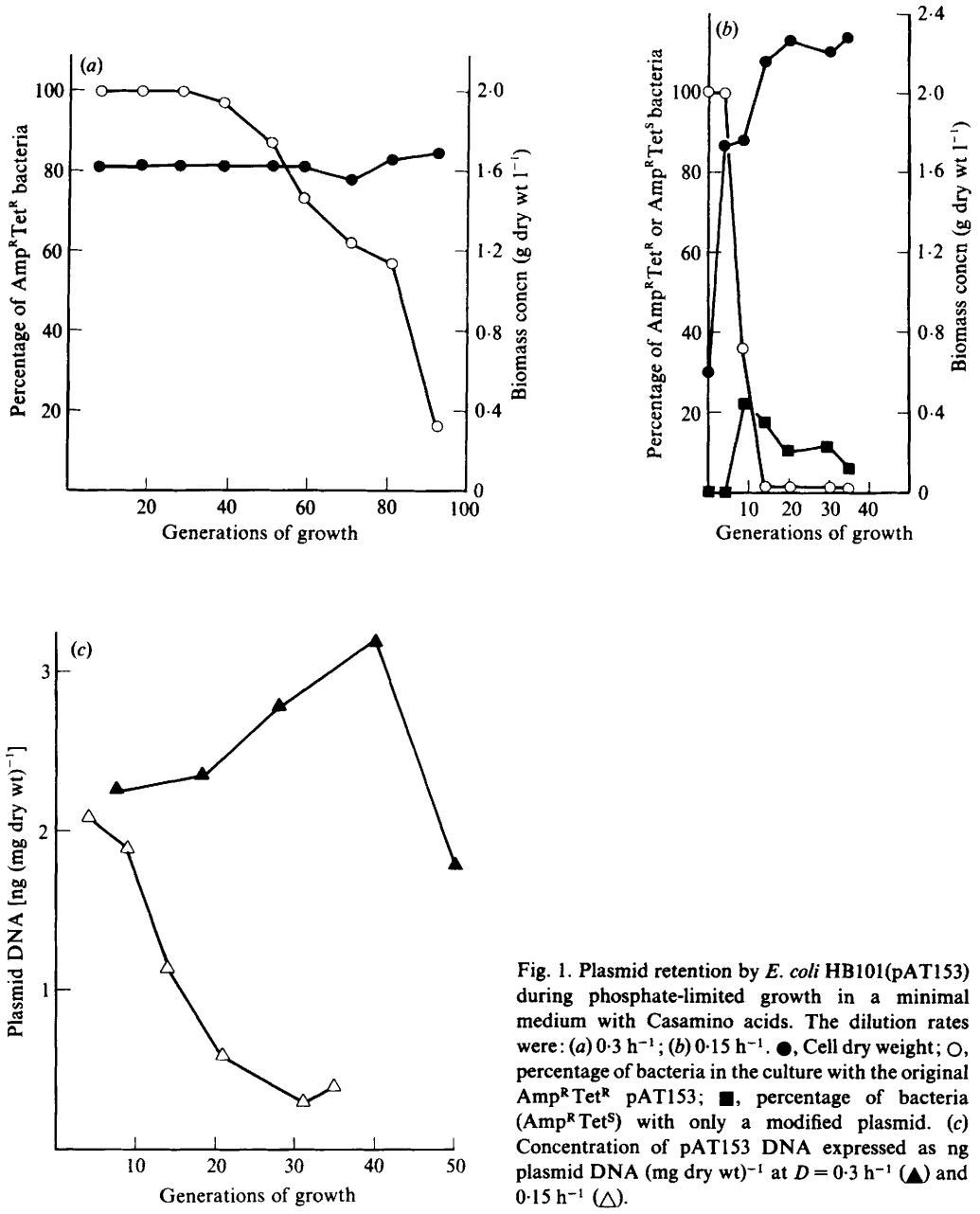

Fig. 1. Plasmid retention by *E. coli* HB101(pAT153) during phosphate-limited growth in a minimal medium with Casamino acids. The dilution rates were: (*a*) 0·3 h^{-1}; (*b*) 0·15 h^{-1}. ●, Cell dry weight; ○, percentage of bacteria in the culture with the original $Amp^R Tet^R$ pAT153; ■, percentage of bacteria ($Amp^R Tet^S$) with only a modified plasmid. (*c*) Concentration of pAT153 DNA expressed as ng plasmid DNA (mg dry wt)$^{-1}$ at $D = 0{\cdot}3\ h^{-1}$ (▲) and 0·15 h^{-1} (△).

0·3 h^{-1} than at a dilution rate of 0·15 h^{-1}. For these experiments, the bacterial host was obtained from CAMR, Porton Down, but the phosphate-limited medium of Chew *et al.* (1988) was used in which the auxotrophic requirements of the host were satisfied by Casamino acids in an otherwise minimal, defined medium.

At the higher dilution rate of 0·3 h^{-1}, no plasmid-free bacteria (< 0·5%) were detected for at least 30 generations (Fig. 1*a*) and the content of plasmid DNA increased by about 40% during the first 40 generations of steady-state growth (Fig. 1*c*). Subsequently, plasmid-free bacteria gradually accumulated in the culture to 38% of the population after 70 generations and 84% after 92 generations. The yield of bacteria was constant at 1·6 g l^{-1} throughout this experiment and no $Amp^R Tet^S$ bacteria, indicative of structural instability of the plasmid, were detected. After 92 generations, excess phosphate was added directly to the culture vessel to a final concentration of 5 mM. The cell density increased rapidly by more than 50% during the following 4 h, confirming that phosphate had been the growth-limiting

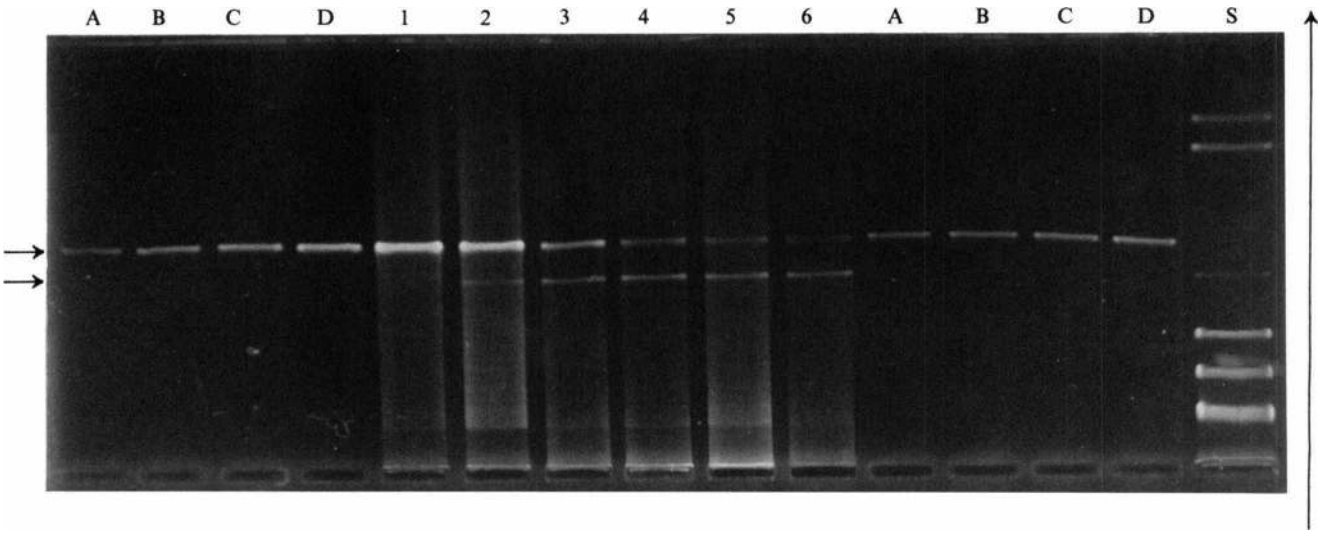

Fig. 2. Plasmid species detected by agarose gel electrophoresis in samples taken from the chemostat after different periods of continuous growth. Plasmid DNA was extracted from bacteria equivalent to 1 ml of an 0·8 g l^{-1} culture by the acid-phenol whole cell lysis method, linearized with *Eco*RI and separated on a 1% agarose gel. Lanes 1 to 6: DNA from samples of the culture after 4, 9, 14, 20, 30, and 35 generations of continuous growth, respectively. Lanes A, B, C and D were loaded with 0·08, 0·16, 0·24 and 0·32 ng, respectively, of purified, linearized pAT153 standard DNA. Lane S contains λ DNA fragments after digestion with *Hin*dIII; sizes of fragments are 23·13, 9·42, 6·56, 4·36, 2·33, 2·03 and 0·54 kilobases. The arrows on the left indicate bands of pAT153 (top arrow) and pAT153::IS*1* DNA; the vertical arrow on the right indicates the direction of DNA migration.

nutrient before the pulse. This increase in biomass was consistent with that expected on the basis of the Casamino acids content of the initial medium.

At the lower dilution rate of 0·15 h^{-1}, plasmid-free variants were detected soon after steady-state growth had been achieved (after five generations; Fig. 1*b*). After nine generations, 22% were $Amp^R Tet^S$ due to the presence of a larger plasmid similar to the pAT153::IS*1* plasmids characterized by Chew *et al.* (1988) (Figs 1*b* and 2). A further 42% were $Amp^S Tet^S$: repeated attempts to isolate structurally modified plasmids from these colonies were unsuccessful, confirming that these bacteria were plasmid-free derivatives resulting from segregational instability of pAT153. The content of plasmid DNA also decreased steadily throughout this experiment, even before plasmid-free bacteria were detected (Fig. 1*c*). The biomass was constant only between generations 5 and 10, but increased by about 25% as plasmid-free bacteria accumulated in the culture, consistent with yield data reported previously for phosphate-limited cultures (Chew *et al.*, 1988). The biomass again increased rapidly in response to a pulse of excess phosphate at the end of the experiment, confirming that the culture had been phosphate-limited.

Virtually identical results were obtained in duplicate experiments at the dilution rate of 0·15 h^{-1}, with only slight differences between experiments in the rate of loss of pAT153 and the extent of its replacement by a structurally modified derivative with an insert in the *tet* gene of the plasmid (data not shown). Neither Pro^+ nor Leu^+ bacteria were found in any of these experiments. In summary, the conclusion of Chew *et al.* (1988) was confirmed that, in a phosphate-limited medium supplemented with Casamino acids, plasmid pAT153 is retained longer by continuous cultures of *E. coli* HB101(pAT153) at a dilution rate of 0·3 h^{-1} than at 0·15 h^{-1}.

Plasmid instability and periodic selection in a defined, minimal medium

In subsequent experiments, the effect of dilution rate on the maintenance of pAT153 by *E. coli* HB101 obtained from CAMR, Porton Down, in a totally defined medium supplemented with proline, leucine and thiamin was determined. At a dilution rate of 0·3 h^{-1}, no ampicillin- or tetracycline-sensitive bacteria were found during the first 30 generations of continuous growth (Fig. 3*a*). Although the concentration of biomass was constant during this period, the yield of only 0·47 g dry weight l^{-1} was well below that expected from the published yield coefficient of 40 g bacteria per g of phosphorus (Elsworth *et al.*, 1968), suggesting that some other factor was limiting.

After 30 generations, changes in both the host and the plasmid became apparent as sub-populations were successively selected. The decreased yield after 40 generations correlated with structural and segregational

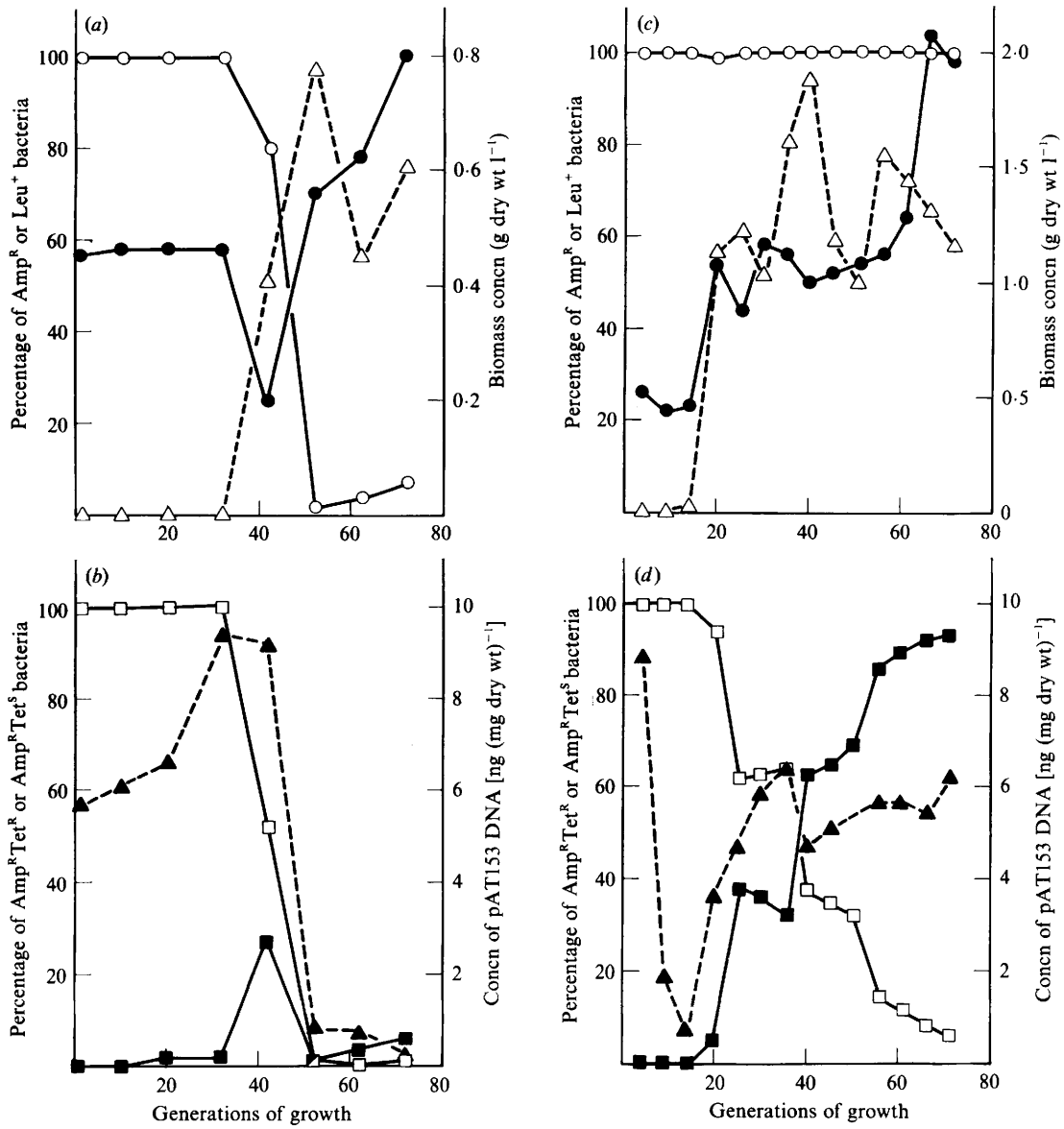

Fig. 3. Periodic selection and plasmid instability in a defined, minimal medium. The dilution rates during growth in the phosphate-limited medium were (*a*, *b*) 0·3 h^{-1}; (*c*, *d*) 0·15 h^{-1}. ●, Biomass; ○, percentage of bacteria that were Amp^R; □, percentage of bacteria that were $Amp^R Tet^R$ due to the retention of the original plasmid, pAT153; ■, percentage of $Amp^R Tet^S$ bacteria that contained only a modified plasmid with a defective *tet* gene; △, percentage of Leu^+ revertants; ▲, pAT153 DNA concentration in ng DNA (mg dry wt)$^{-1}$.

instability of the plasmid (Fig. 3*b*): at this time, only 53% of the bacteria were $Amp^R Tet^R$, 20% were $Amp^S Tet^S$ and 27% were $Amp^R Tet^S$ due to the presence of a structurally different plasmid. During the subsequent 10 generations of growth, bacteria carrying either the modified plasmid or pAT153 (or both) were out-grown by the plasmid-free cells, and the yield then increased towards the expected value (Fig. 3*a*, *b*).

Genetic changes in the host bacteria were also apparent in this experiment, Leu^+ derivatives of the original Leu^- strain being detected after 40 generations (Fig. 3*a*). The percentage of Leu^+ cells continued to increase until 50 generations of growth, but then decreased again, presumably because an alternative mutation had given a Leu^- cell an even greater selective advantage under the imposed growth conditions.

After 70 generations, the experiment was terminated by pulsing the culture with excess phosphate. The yield

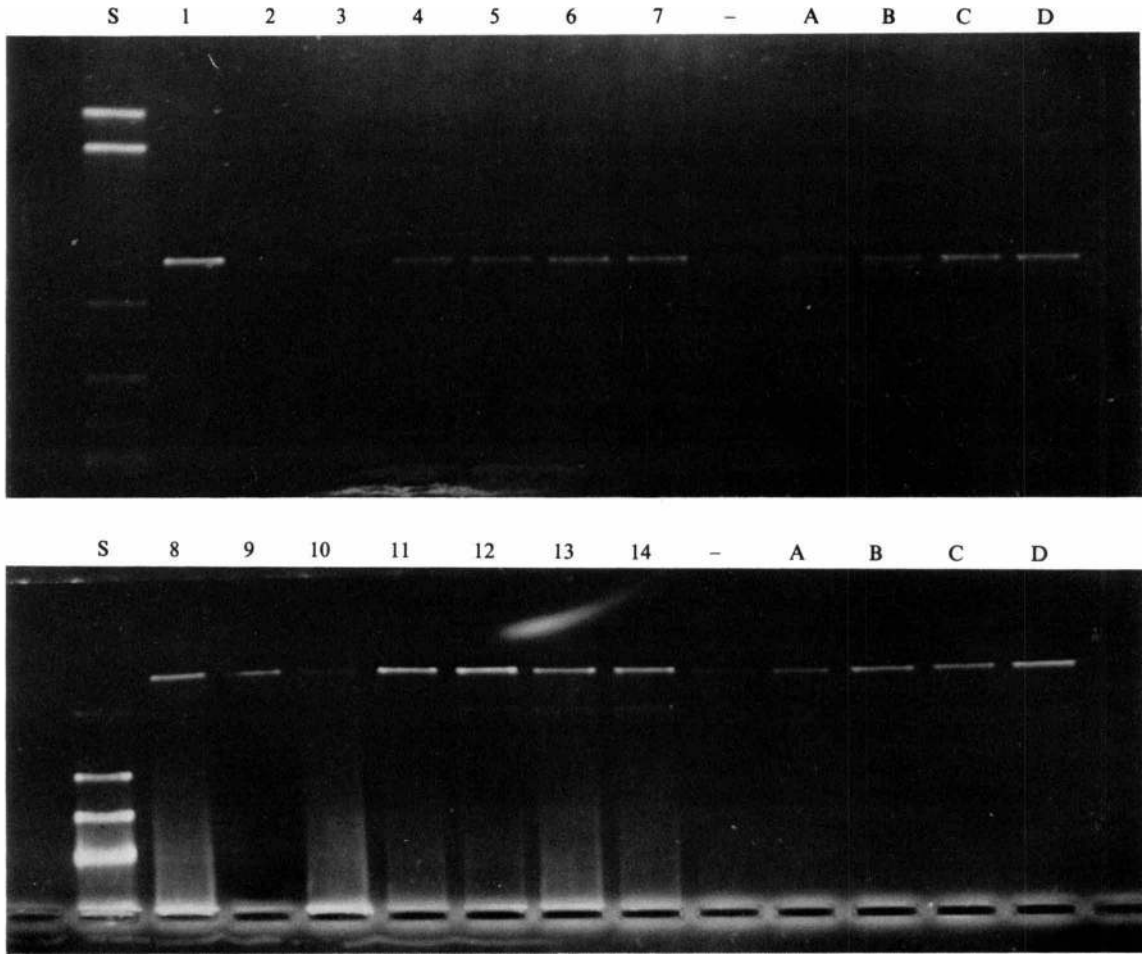

Fig. 4. Plasmid instability during continuous growth in the defined, minimal medium at a dilution rate of 0·15 h^{-1}. DNA in lanes 1 to 14 was isolated from samples taken from the culture after 4, 9, 14, 20, 25, 31, 36, 40, 45, 50, 56, 61, 67 or 71 generations of continuous growth. Other symbols are as in Fig. 2.

of biomass increased steadily by more than 60% during the subsequent 6 h, confirming that phosphate had been the growth-limiting nutrient before the pulse.

In summary, at least four types of genetic instability were detected during the first 60 generations of growth at a dilution rate of 0·3 h^{-1}. These were structural and segregational instability of the plasmid, reversion of the host of Leu^+ and the selection of an uncharacterized but fitter variant of the original Leu^- host.

At a dilution rate of 0·15 h^{-1} in the defined medium supplemented with leucine, proline and thiamin instead of Casamino acids, the initial yield of biomass, 0·41 g l^{-1}, was again well below that expected for a phosphate-limited culture (Fig. 3*c*). The yield had increased to 1·0 g l^{-1} after 20 generations, and further erratic increases occurred until the experiment was terminated after 70 generations. At the end of the experiment, all of the colonies formed by samples removed from the culture had retained a plasmid, as judged by their ability to grow on nutrient agar + ampicillin. This result was misleading, however, because further tests on previous samples taken throughout the experiments revealed that 1% of the bacteria after 20 generations were $Amp^S Tet^S$ and a further 5% were $Amp^R Tet^S$ (Fig. 3*d*). The proportion of Tet^S colonies had increased erratically to 94% of the sample after 70 generations of continuous growth. Clearly a transient loss of plasmid due to segregational instability had occurred as well as structural changes resulting in the loss of tetracycline resistance (Fig. 4).

A further source of instability in the culture at a dilution rate of 0·15 h^{-1} was again the reversion of the host to Leu^+ after 20 generations, which coincided with an almost threefold increase in bacterial yield (Fig. 3*c*). This indicated that the growth medium had originally been leucine-limited rather than phosphate-limited. If so, most of the leucine supplied must have been catabolized rather than used solely for protein synthesis, because batch culture control experiments had indicated

that the medium used was leucine-sufficient. Even after 70 generations no further increases in biomass occurred in response to a pulse of excess phosphate, so phosphate-limited growth had not been established at this low dilution rate. A possible explanation for this is that the culture had been growth-limited by more than one substrate at the lower dilution rate of 0·15 h^{-1}, but not at 0·3 h^{-1}, as described by Egli & Quayle (1986) and by Egli & Schmidt (1989).

In further experiments using the same minimal growth medium and a dilution rate of 0·15 h^{-1}, the fermenter was inoculated with *E. coli* HB101(pAT153) obtained from another source, G. D. Searle (Chew *et al.*, 1988). Although no $Amp^S Tet^S$ bacteria were found in samples taken after five generations of continuous growth, 13% had lost the original plasmid after 10 generations and 78% has lost it after 25 generations. During this period, the proportion of both $Amp^S Tet^S$ and $Amp^R Tet^S$ bacteria increased to 40% and 38%, respectively. Once again Leu^+ host cells were selected and dominated the culture after 25 generations: the mutation responsible for the Leu^+ population clearly occurred in a cell that had retained the original pAT153 plasmid because the increase in Leu^+ bacteria coincided with a sudden increase in the percentage of $Amp^R Tet^R$ bacteria and decreases in the proportions of both $Amp^S Tet^S$ and $Amp^R Tet^S$ bacteria. After this reversion to Leu^+, pAT153 was subsequently lost by this sub-population and was partially replaced by a smaller, structurally modified plasmid which rendered the host Tet^S. This plasmid, pLAB446, was isolated and used in a subsequent series of experiments. A further source of instability in this culture was the reversion of the host to Pro^+, despite the fact that the *proA2* mutation carried by strain HB101 is a deletion. It is known, however, that *proAB* mutants can 'revert' to Pro^+ by acquiring secondary leaky mutations in the *argD* gene (Itakawa *et al.*, 1968).

In a final experiment in this series, pAT153 obtained from G. D. Searle was transformed into the CAMR, Porton, culture of *E. coli* HB101 and a purified transformant was grown in the defined medium for 40 generations at a dilution rate of 0·15 h^{-1}. As in the previous three experiments, loss of pAT153 and its partial replacement by a structurally modified plasmid was detected within 15 generations. After 24 generations, 38% of the population were Leu^+, but none were Pro^+. After 31 generations, more than 90% were Leu^+ and 35% were Pro^+: this demonstrated that the cultures of the bacterial host obtained from two different sources were indistinguishable in their ability to revert to Pro^+ or Leu^+ and in their ability to retain the plasmid pAT153 during continuous growth in a defined medium.

Molecular basis for the structural instability of plasmid pAT153

Chew *et al.* (1988) showed that $Amp^R Tet^S$ derivatives of *E. coli* HB101(pAT153) in continuous cultures were frequently the result of the spontaneous transposition of IS*1* from the host chromosome into the N-terminal segment of the plasmid *tet* gene. Less frequently, even larger plasmids were found with an IS*5* insertion.

Plasmids larger than pAT153 were again found in some of the $Amp^R Tet^S$ bacteria in the current series of experiments: in each case examined by restriction mapping of the isolated DNA, the higher molecular mass was due to an insertion of IS*1* into the *Eco*RI–*Bam*HI fragment of pAT153. Other types of modified plasmid were also detected, however: these were either smaller than pAT153 as in plasmid pLAB446, or identical in size as judged by the sizes of restriction fragments revealed by agarose and polyacrylamide gel electrophoresis (plasmid pLAB135; Fig. 5). Plasmid pLAB446 is 446 base pairs smaller than pAT153 due to a spontaneous deletion extending from base pair 35 in the leader transcript of the *tet* gene to base pair 480 in the *tet* structural gene (Brownlie *et al.*, 1990). *E. coli* HB101(pAT153) is resistant to 50 mg tetracycline l^{-1}. In contrast, bacteria transformed with the smaller plasmids were unable to grow on nutrient agar + 0·1 mg tetracycline l^{-1}, which is the lowest concentration that was tested. Transformants with the modified plasmid which was the same size as pAT153 grew well on nutrient agar + 0·1, 1, 12 or 24 mg tetracycline l^{-1} but were sensitive to 50 mg l^{-1}.

Maintenance of structurally altered derivatives of pAT153 during phosphate-limited continuous growth

The $Leu^+ Amp^R Tet^S$ colony from the previous experiment and from which plasmid pLAB446 was isolated was used to study the maintenance of pLAB446 during phosphate-limited growth at three dilution rates, 0·15, 0·3 and 0·5 h^{-1}. No loss of plasmid was detected in any of these experiments (data not shown).

The stable maintenance of pLAB446 could have been due either to the loss of expression of the *tet* gene resulting from the deletion in the plasmid, or to mutations in the bacterial chromosome which might have generated a fitter, fermenter-adapted host better able to retain the plasmid. Seven attempts to isolate a plasmid-free culture of this putative 'fitter' host were unsuccessful because pLAB446 was retained by 100% of the bacteria during more than 500 generations of phosphate-limited growth. To resolve whether changes in the host or the plasmid were the cause of the increased stability, a stock culture of the original *E. coli* HB101 obtained from CAMR, Porton, was transformed with

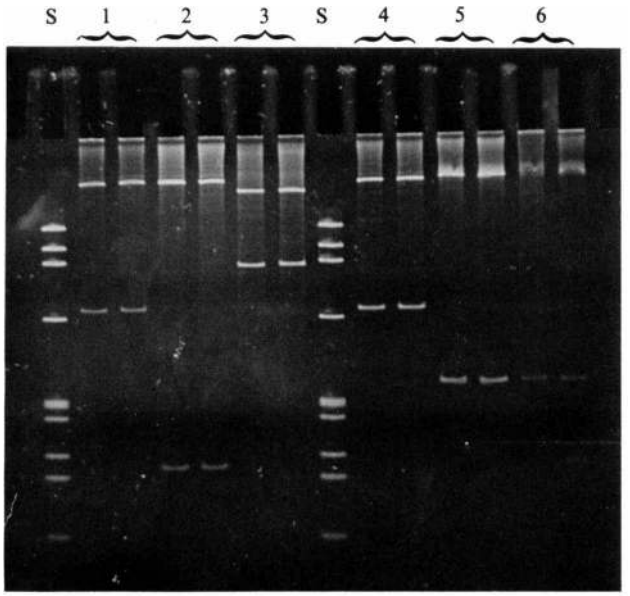

Fig. 5. Polyacrylamide gel electrophoresis of restriction fragments from plasmid pAT153 and its derivatives which were isolated from chemostat samples. S, φX174 size standard; the sizes of the fragments are 1353, 1078, 872, 603, 310, 281 (plus 271; unresolved doublet), 234, 194, 118 and 72 bases. Tracks labelled 1 to 6 are duplicate samples of: pAT153 cut with *Eco*RI and *Sal*I; 2, pLAB446 cut with *Eco*RI and *Sal*I; 3, pLAB446 cut with *Eco*RI and *Ava*I; 4 and 5, a plasmid with a point mutation, pLAB135, cut with *Eco*RI and *Sal*I or *Eco*RI and *Bam*HI, respectively; 6, pAT153 cut with *Eco*RI and *Bam*HI.

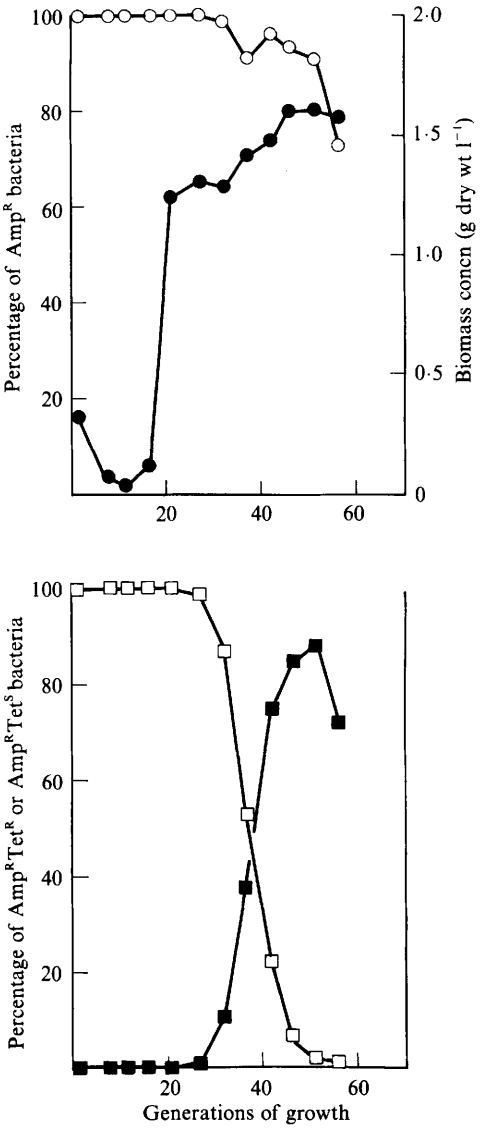

Fig. 6. Retention of plasmid pAT153 during phosphate-limited growth of a fermenter-adapted host. The dilution rate was 0·15 h^{-1}. The growth medium and other details, including the symbols used, were as in Fig. 3.

pLAB446 and a purified transformant was grown in the minimal medium at a dilution rate of 0·15 h^{-1}. Even in this host which was not adapted to continuous growth, plasmid pLAB446 was maintained unchanged for at least 70 generations (data not shown). This is further evidence that the loss of a functional *tet* gene was far more important than host chromosomal mutations in determining the rate of selection of plasmid-free bacteria in cultures of *E. coli* HB101 (Lee & Edlin, 1985; Chew *et al.*, 1986; Lodge *et al.*, 1989).

Stability of pAT153 in a fermenter-adapted bacterial host

As an alternative strategy for isolating a fermenter-adapted bacterial host in which the plasmid would be retained longer, a Leu$^+$ derivative of *E. coli* HB101 carrying pAT153 was isolated after 62 generations from the experiment in which the bacterial culture was obtained from G. D. Searle. This strain was then grown in the phosphate-limited minimal medium at a dilution rate of 0·15 h^{-1} (Fig. 6). In contrast to the original experiment in which 13% of the bacteria has lost pAT153 after five generations, no Amp^{S}Tet^S colonies were detected after 27 generations of growth of the fermenter-adapted host and only 1% of the colonies were Amp^{R}Tet^S due to the presence of a structurally modified plasmid. The original plasmid was then lost rapidly and was only transiently replaced by the modified plasmid which was found in 27% of the colonies after 42 generations of continuous growth.

In a second, identical experiment to confirm that the increased plasmid retention by the fermenter-adapted host was reproducible, pAT153 was maintained unchanged by 100% of the population for at least 30 generations before plasmid-free segregants were detected (data not shown).

Discussion

Preliminary experiments were designed to determine how the growth rate and composition of the growth medium influence the maintenance of the plasmid pAT153 in phosphate-limited cultures of *E. coli* HB101. These experiments were necessary because different results were obtained by Chew *et al.* (1988) and by Jones & Melling (1984) or by Caulcott *et al.* (1985) using the same host–vector combination.

The results clearly established that the original pAT153 was maintained longer at a growth rate of 0·3 h^{-1} than at 0·15 h^{-1} in a medium supplemented with Casamino acids. In contrast, the most striking results from the series of experiments with the defined salts medium supplemented with proline, leucine and thiamin were the diversity and reproducibility of the genetic variants selected during continuous growth. Neither the plasmid nor the bacterial host was maintained unchanged even for 20 generations and, despite contrary results from preliminary control experiments with batch cultures, the growth-limiting nutrient was not phosphate, at least during the initial stages of the experiments and at the lower dilution rate. The results obtained were determined not by the source of the plasmid or the host but by the composition of the growth medium. Other apparent differences in results obtained in different laboratories can readily be explained by differences in criteria used to detect plasmid loss. If, for example, only the ampicillin resistance marker is used to detect the presence of a plasmid, no information will be obtained about structural instability which so frequently results in the selection of Tet^S variants (Chew *et al.*, 1988; Figs 1 to 4). Furthermore, the concentration of tetracycline used in this laboratory to check for retention of pAT153 was 50 mg l^{-1}, higher than the 10 to 25 mg l^{-1} that is often used. Although one of the derivative plasmids isolated in the present series of experiments was the same size as pAT153, bacteria transformed with this plasmid were still resistant to 12 or 24 mg tetracycline l^{-1} but were unable to grow on agar supplemented with 50 mg tetracycline l^{-1}. We have described elsewhere the molecular basis for this loss of resistance which was a point mutation in the transcribed but untranslated leader mRNA (Brownlie *et al.*, 1990).

In addition to the point mutation in pAT153 mentioned above, two other types of structural instability were repeatedly detected. These were deletions in the plasmid *tet* gene and the transposition of IS*1* from the chromosome into the plasmid. Whatever the structural basis of the instability, the selective advantage of the resulting $Amp^R Tet^S$ bacteria enabled them to outcompete their $Amp^R Tet^R$ parents. This interpretation was supported by results of a subsequent series of experiments in which no loss of plasmid was detected in over 500 generations of phosphate-limited continuous growth of bacteria transformed with one of the modified plasmids. Similar experiments to document that a functional *tet* gene is a major cause of plasmid loss from continuous cultures were reported by Lee & Edlin (1984) and Chew *et al.* (1986).

The rapid loss of pAT153 from continuous cultures at a dilution rate of 0·15 h^{-1} correlated closely with a rapid decrease in the concentration of plasmid DNA relative to biomass, even before plasmid-free segregants were detected in the culture (Figs 1*b*, 1*c* and 3*d*). In contrast, the concentration of plasmid DNA per unit of biomass increased slightly during the initial stages of continuous growth at a dilution rate of 0·3 h^{-1} (Figs 1*c* and 3*b*). The rapid appearance of plasmid-free segregants at low growth rates therefore appears to be a consequence of a decrease in plasmid copy number in these cultures.

Although early descriptions of bacterial growth in a chemostat were based on the assumption that the culture contained only a single type of organism, two-component systems have also been modelled successfully (San & Weber, 1989, and references cited by them). Clearly, however, the data for cultures in the defined medium presented in this paper established that the genetic heterogeneity of these plasmid-containing bacteria was too great even before steady-state growth was achieved for the stringent assumptions of the mathematical models to be satisfied (San & Weber, 1989). Consequently, the kinetics of plasmid loss (and apparent recovery) bear little resemblance to those in published model systems. Nevertheless, this paper has illustrated several basic principles, apart from the need to avoid a functional *tet* gene, for achieving adequate stability in processes in which plasmid-containing bacteria are grown for many generations, as would be required for an industrial fermenter. Emphasis on plasmid design must clearly be balanced by careful selection of a genetically stable host which has been pre-adapted to growth under process conditions. This will decrease, but not eliminate, instability from periodic selection of host variants (see Fig. 6). Equally important, however, is the physiology of the host which will respond to changes in the medium composition and growth rate. The dramatic effect on growth and plasmid retention of a trivial modification to the growth medium in which Casamino acids were replaced by proline, leucine and thiamin to satisfy the auxotrophic requirements of the host were surprising (Figs 1 and 3). As previously advocated by Cole (1985) and Chew *et al.* (1988), high growth rates which favour a high productivity also decrease problems of any unavoidable segregational instability of the plasmid due to the selective advantage of the plasmid-deficient host. Furthermore, Chew *et al.* (1988) showed that any selective

disadvantage of plasmid-containing bacteria increases progressively as the growth-limiting nutrient is changed from sulphur to phosphorus, nitrogen or carbon.

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