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Title	The isolation of strains of <i>Bacillus subtilis</i> showing improved plasmid stability characteristics by means of selective chemostat culture
Author(s)	Fleming, Gerard T.; Patching, John W.
Publication Date	1988-03-23
Publication Information	FLEMING, G, DAWSON, MT, PATCHING, JW (1988) 'The isolation of strains of <i>Bacillus subtilis</i> showing improved plasmid stability characteristics by means of selective chemostat culture'. <i>Journal Of General Microbiology</i> , 134 :2095-2101.
Link to publisher's version	http://www.ncbi.nlm.nih.gov/pubmed/3150976
Item record	http://hdl.handle.net/10379/4046

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The Isolation of Strains of *Bacillus subtilis* Showing Improved Plasmid Stability Characteristics by Means of Selective Chemostat Culture

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(Received 1 February 1988; revised 23 March 1988)

A pUB110-derived plasmid encoding chloramphenicol resistance, kanamycin resistance and high-temperature α -amylase showed a high degree of segregational instability when inserted into *Bacillus subtilis*. In an attempt to obtain stable derivatives, the organism was grown in chemostat culture in the presence of chloramphenicol. It was periodically found necessary to increase the concentration of chloramphenicol in the medium feed in order to avoid plasmid loss. Strains were isolated after 19 and 160 generations, which showed high levels of plasmid stability. This characteristic appeared to be genotypic. No detectable difference in plasmid copy number was found between the original and the improved strains. The stability characteristics resided in the host, rather than in the plasmid. Stable isolates possessed elevated MICs for both chloramphenicol and kanamycin. Their maximum specific growth rates were higher than that of the original strain, and similar to that of the plasmid-free parent strain.

INTRODUCTION

Bacteria of the genus *Bacillus* are attractive hosts for the manufacture of many important enzymes. Unfortunately, instability of chimeric plasmids encoding commercially valuable foreign proteins may result in loss of the inserted gene during culture. In particular, pUB110-derived plasmids are prone to both structural and segregational instability when introduced into *Bacillus subtilis* (Bron & Luxen, 1985; Joyet *et al.*, 1984; Vehmaanpera & Korhola, 1986; Rabinovich *et al.*, 1985).

Carrier *et al.* (1983) and Doi (1984) have suggested the use of agents, such as antibiotics, which disadvantage plasmid-free organisms in cultures and thus maximize the proportion of recombinant organisms in the population. This technique would be especially effective with continuous cultures, where disadvantaged organisms would tend to wash out. In continuous cultures, such a technique would not only ensure a high proportion of plasmid-containing organisms, but also favour the dominance of stable recombinant clones over less stable ones. This effect may be explained by considering the fate of the daughters of a cell in continuous culture. A simple heuristic model (Kubitschek, 1970) will be used for clarity, though our proposals also apply to the more realistic stochastic model. The heuristic model considers a population in which each cell represents a clone. In the absence of selection pressures, one daughter of each division event will be washed out of the culture vessel, and the other will remain to continue the cell line. In a system where selective pressure is applied against plasmid-free cells, the retained offspring of stable recombinant clones will always be resistant to the selective pressure, and thus able to continue the clone. With increasing degrees of instability, the probability of one or both of the daughters being plasmid-free increases. There will thus be an

Abbreviations: CAT, chloramphenicol acetyltransferase; Cm, chloramphenicol; HT amylase, high-temperature α -amylase; Km, kanamycin.

increasing probability that the retained daughter will be plasmid-free, disadvantaged, and unable to continue the cell line. Hence, as culture proceeds we would expect the spectrum of stabilities of recombinant clones within the system to improve.

This paper describes the use of this method on an unstable *Bacillus subtilis*/chimeric plasmid combination. The inserted plasmid encodes for kanamycin (Km) and chloramphenicol (Cm) resistance, and thus chemostat culture in the presence of chloramphenicol was employed as the selection process. Some properties of the stable isolates so obtained are also discussed.

METHODS

Bacterial strains and plasmids. *Bacillus subtilis* BC1 and BC0 were commercial strains supplied by Bicoon Biochemicals Ltd, Carrigaline, Co. Cork, Ireland. BC0 contained a 6.5 kb cryptic plasmid (pBAA1), and was transformed with plasmid pSA33 (8.35 kb) to form strain BC1. Plasmid pSA33 encodes Cm^r, Km^r and an α -amylase which is active at 100 °C (HT amylase). It is a derivative of pBD64 containing a 3.55 kb *Bacillus licheniformis* HT amylase insert within the unique *EcoRI* site (Ortlepp *et al.*, 1983). Plasmid pBD64 is a 4.5 kb product of a spontaneous deletion event which occurred in a cointegrate of pUB110 and pC194 (Gryczan *et al.*, 1980). *B. subtilis* SO113 (*trpC2 recE4 amy-3*: Ortlepp *et al.*, 1983) was obtained from Bicoon Biochemicals Ltd. Plasmid pMD10 was generated for this study and consists of a 1.85 kb *BclI*-*BglII* genomic fragment from *Bacillus amyloliquefaciens* ligated to pUB110 at the *BamHI* site. It confers Km^r and encodes the *B. amyloliquefaciens* neutral protease. Strains were maintained at -20 °C as cultures in LS broth supplemented with Cm and Km, to which 20% (v/v) glycerol had been added. Antibiotics were omitted for BC0 and SO113. Cm was omitted for organisms containing pMD10. Prolonged storage of BC1 resulted in complete loss of plasmid from the culture. It was thus necessary to reisolate this organism at 80 d intervals using LS agar plates supplemented with Cm and Km.

Media. LS medium contained the following ingredients (l⁻¹): 10 g peptone (Oxoid), 5 g yeast extract (Oxoid), 5 g NaCl, 2 g soluble starch (BDH), and 10 g agar (when necessary: Oxoid no. 1). Antibiotics (Cm and Km) were prepared as stock solutions, filter-sterilized, and added to the medium after autoclaving. Cm and Km (final concentration, 5 mg l⁻¹) were routinely added to plates used for the isolation and enumeration of Cm^r Km^r (plasmid-containing) organisms. Inoculum medium contained (l⁻¹): 50 g soluble starch, 20 g yeast extract, 5 mg Cm and 5 mg Km. Production medium consisted of phosphate buffer (pH 6.7) to which were added (l⁻¹) 34 g corn-steep powder, 22.5 g soya bean meal, 40 g calcium carbonate, 4 g ammonium chloride and 51 g lactose.

Culture conditions. Continuous cultures were grown in a modified 1 l quickfit fermenter vessel (FV1L: Bibby, UK) with a working volume of 500 ml. Cultures were aerated at 900 ml min⁻¹ and stirred by a magnetic follower. Temperature was maintained at 37 ± 0.2 °C. Medium (one-fifth strength LS broth) was added by a peristaltic pump to give a dilution rate of 0.48 h⁻¹. By means of temporary nutrient additions (Goldberg & Er-el, 1981), cultures were shown to be carbon limited. Unless described otherwise, all batch cultures were grown in 80 ml medium in 500 ml baffled flasks incubated at 37 °C and 95 r.p.m. in a rotary incubator.

Assay for plasmid stability. Inoculum medium was inoculated from a single Cm^r Km^r colony or, for experiments on the effects of storage, 1 ml of the stored suspension. After 16 h incubation, 8 ml was used as an inoculum for a 96 h batch culture in production medium. A sample was then plated onto LS agar. One hundred colonies were picked onto LS agar supplemented with Cm and Km and also onto LS agar as a control. The number of colonies growing on the test plate was expressed as a percentage of the number growing on the control plate. This value was taken to represent the proportion of the population which carried the plasmid.

Transformation. The protoplast transformation procedure of Chang & Cohen (1979) was used. Plasmid DNA was prepared by the method of Ish-Horowitz (1982), except that cells were washed twice in TES buffer (50 mM-NaCl, 5 mM-EDTA, 30 mM-Tris: pH 7.5) after harvesting. Plasmid-free hosts were prepared by the method of Edger *et al.* (1981).

Gel electrophoresis. This was done using 0.8% agarose horizontal gels. *EcoRI* restriction enzyme was obtained from Boehringer.

Plasmid copy number. This was determined by a modification of the method of Scheer-Abramowitz *et al.* (1981). Overnight cultures were grown in labelling medium containing Cm and Km (5 mg l⁻¹), diluted to 10⁶ colony-forming units ml⁻¹ in 1.5 ml of the same medium containing 35 μ Ci (1.3 MBq) [³H]thymidine ml⁻¹ and reincubated for 5 h. Cells were harvested, washed twice with TES, resuspended in 200 μ l TES containing 500 μ g lysozyme chloride ml⁻¹ and 50 μ g RNAase A ml⁻¹, and incubated at 37 °C for 20 min. Then 200 μ l of a 1 g l⁻¹ proteinase K solution was added and the lysate incubated at 37 °C for 2 h. The DNA was sheared by vortex mixing for 10 s, and 32 μ l samples were loaded onto horizontal 0.9% agarose gels and run for 20 h at 30 V. The gel was stained in 2 mg l⁻¹ ethidium bromide solution for 30 min followed by destaining in tap water for 20 min. Chromosomal and plasmid bands were detected using long-wave UV light, excised separately and boiled for 10 min in 1 ml distilled water. Radioactivity was measured after the addition of 17 ml liquid scintillant (LKB Optiphase 'safe') and copy number determined by the method of Scheer-Abramowitz *et al.* (1981).

MIC determinations. These were done by the broth dilution method of Waterworth (1978), except that doubling dilutions of antibiotics were started from highest final concentrations of both 50 mg l^{-1} and 40 mg l^{-1} , thus giving closer intervals in the concentration series.

Test for HT amylase activity. Plugs containing isolated colonies and underlying agar were removed from plates by means of a 6 mm diameter cork borer, transferred to 0.5 ml of a solution of (1^{-1}) 0.58 g NaCl, 2.22 g CaCl_2 and 3.28 g sodium acetate, and shaken gently for 1 h. Starch solution (0.5 ml: 0.23%, w/v) was added and the mixture immediately boiled for 15 min. After the addition of iodine (0.5 ml of a saturated solution in 0.24 M-KI), HT-amylase-negative strains produced an intense blue colour and HT-amylase-positive strains produced a straw colour.

Growth rate determinations. Portions (10 ml) of LS broth supplemented with antibiotics (Km and Cm at 5 mg l^{-1} ; omitted when working with BC0) were inoculated from single colonies and incubated with agitation for 12 h. These were used as inocula (8 ml) for batch cultures in LS broth. Optical densities (at 540 nm) of samples were measured at intervals over the first 6 h of culture. No lag phase was observed, and growth rates were determined from plots of log optical density against time.

RESULTS AND DISCUSSION

The original plasmid-containing strain used in this study (BC1) rapidly lost its plasmid-encoded functions when grown in batch culture (Fig. 1). The addition of Cm and Km to the incubation and growth media at a level (5 mg l^{-1}) greater than the MIC for the plasmid-free host BC0 (Table 1) lessened the degree of loss, but 96 h after inoculation the majority of the population (69%) was plasmid-free. Similar results were reported by Pinches *et al.* (1985) for a recombinant strain of *Bacillus subtilis*, 1A297(pVC102).

We have assumed that the appearance of antibiotic-sensitive isolates was due to plasmid loss as a result of segregational instability. Structural instability could also result in the loss of plasmid-encoded functions. Presumptive plasmid-free isolates, identified by their sensitivity to one antibiotic, were cross-checked for their sensitivity to the other. Among 550 Cm^s and 550 Km^s isolates checked, no Cm^sKm^r or Cm^rKm^s segregants were observed. Antibiotic-sensitive isolates were screened for HT amylase activity. Of 2064 isolates checked, 36 were weakly HT amylase positive. This activity was lost on subculture. Agarose gel electrophoresis of DNA extracted from 12 Cm^sKm^s isolates selected at random showed that the chimeric plasmid had been lost. This evidence shows that loss of plasmid-encoded functions from our recombinant strain was essentially due to segregational instability.

As well as the chimeric plasmid pSA33, strain BC1 contains the cryptic plasmid pBAA1. To determine whether interaction between the two plasmids might be responsible for the instability, a combination of the original recipient strain (BC0), which contains pBAA1, and pBD64 was produced by protoplast transformation. Although pBD64 was the immediate precursor of pSA33, it was stably maintained in non-selective batch culture (Table 2). This would suggest that there is no inherent incompatibility between pBAA1 and pUB110-derived plasmids. As a further check, pSA33 was protoplast-transformed into *B. subtilis* SO113, which does not carry the cryptic plasmid. The plasmid was poorly maintained in this host (Table 2). It may be concluded that pBAA1 has no significant role in promoting the instability of pSA33.

In an attempt to obtain more stable host/plasmid combinations, strain BC1 was grown in carbon-limited chemostat culture at a dilution rate of 0.48 h^{-1} and, initially, a Cm concentration of 15 mg l^{-1} in the medium feed. The use of lower antibiotic concentrations resulted in a progressive loss of recombinant organisms over the first five to ten generations, to give a population dominated (>90%) by plasmid-free organisms. The plasmid-encoded Cm^r in BC1 is due to inducible chloramphenicol acetyltransferase (CAT) activity (Shivakumar *et al.*, 1979). Although 15 mg Cm l^{-1} was considerably higher than the MIC for the plasmid-free strain BC0 (Table 1), the concentration of Cm in the culture may have been less than that in the reservoir because of its degradation by the CAT activity of the recombinant clones.

After approximately 20 generations, the proportion of Cm^s Km^s organisms in the population showed a progressive increase (Fig. 2). In order to re-establish selective pressure, the concentration of Cm in the medium was increased to 20 mg l^{-1} . The Cm concentration was further increased whenever a significant proportion (>10%) of the population was found to be Cm^s. At the termination of the chemostat culture (160 generations) the Cm concentration in the

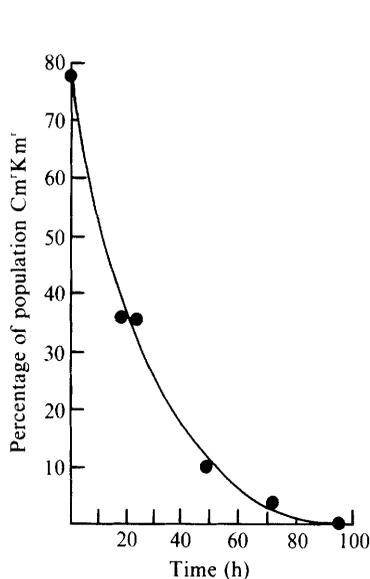


Fig. 1

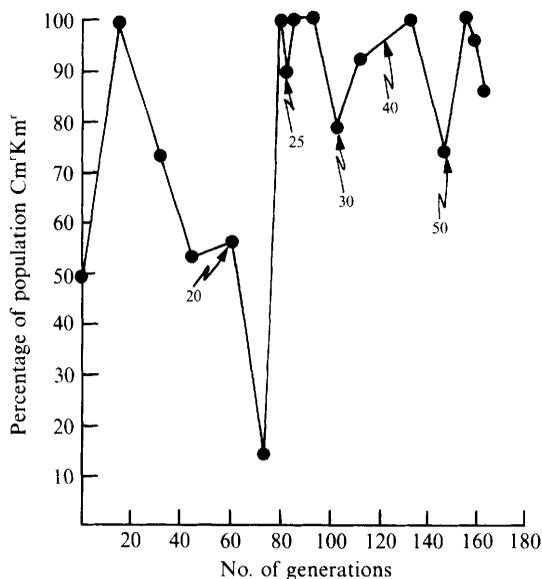


Fig. 2

Fig. 1. Loss of plasmid by *B. subtilis* BC1 in non-selective batch culture. Culture conditions and assay to determine the proportion of Cm⁺Km⁺ organisms in the population were as described in Methods (stability assay), except that antibiotic was omitted from the inoculum medium. Time 0 refers to inoculation of the production medium.

Fig. 2. Proportion of Cm⁺Km⁺ organisms in a Cm-containing chemostat culture of *B. subtilis* BC1. Culture conditions and assay for Cm⁺Km⁺ organisms were as described in Methods. The arrows denote an increase in the Cm concentration in the medium reservoir; the accompanying figures denote the new concentration (mg l⁻¹).

Table 1. *Properties of original and derived strains of Bacillus subtilis* BC1

BC0 is the original host. Other strains were derived from chemostat culture as described in the text. Assay procedures are described in Methods.

Strain	Plasmid stability (%) [*]	MIC (mg l ⁻¹)		μ_{max} (h ⁻¹) [†]	Copy number [†]
		Cm	Km		
BC0	NA	1.6	< 0.8	1.33 ± 0.06 (4)	NA
BC1	3 (4)	12.5	25	0.59 ± 0.06 (4)	6.7 ± 1.5 (5)
GFA-19	94 (16)	25	40	1.05 ± 0.18 (3)	7.1 ± 1.5 (7)
GFB-160	97 (5)	40	40	1.33 ± 0.01 (3)	ND
GFC-160	99 (2)	40	40	1.35 ± 0.02 (3)	6.6 ± 1.5 (5)
GFE-160	96 (4)	50	40	1.49 ± 0.10 (3)	7.1 ± 3.4 (4)
GFG-160	90 (5)	40	25	1.50 ± 0.02 (3)	6.8 ± 2.4 (4)

NA, Not applicable; ND, not determined.

^{*} Proportion of population retaining Cm⁺ and Km⁺ after 96 h non-selective batch culture. Results are means of two to four replicate experiments. Figures in parentheses give the difference between the highest and lowest stabilities observed.

[†] μ_{max} and copy number values are shown ± SE. The number of determinations is given in parentheses.

medium reservoir was 50 mg l⁻¹. A rise in the proportion of Cm^s organisms in the population would suggest that such organisms were no longer disadvantaged. It may be inferred from this that, in spite of increasing concentrations of Cm in the medium supply, microbial activity within the culture was sufficient to periodically decrease its concentration below the MIC for plasmid-free strains, and that changes leading to higher rates of Cm degradation by Cm^r clones were

Table 2. *Plasmid stability characteristics of various host/plasmid combinations*

Stability assays were done as described in Methods except that, when working with pMD10, Cm was omitted from media. The results are presented as the proportion of the population retaining antibiotic resistance after 96 h non-selective batch culture. They are directly comparable with those presented in Table 1. Strains with the prefix GF were derived from chemostat culture as described in the text. GFG-160 was protoplast-cured (Edger *et al.*, 1981) of pSA33 before use as a host.

Host	Plasmid	Plasmid stability (%)
BC0	pBD64	100
SO113	pSA33 from BC1	4
SO113	pSA33 from GFA-19	4
SO113	pSA33 from GFB-160	2
SO113	pSA33 from GFC-160	2
SO113	pSA33 from GFE-160	2
SO113	pSA33 from GFG-160	2
GFG-160	pSA33 from BC1	88
GFG-160	pMD10	100
BC0	pMD10	3

occurring throughout the course of the experiment. Population densities within the culture fluctuated from $10\text{--}12 \times 10^7$ cells ml⁻¹ immediately before a rise in antibiotic concentration in the reservoir to $4\text{--}6 \times 10^7$ cells ml⁻¹ shortly (approximately 6 volume changes) after.

Plasmid-containing strains isolated after 19 and 160 generations of selective chemostat culture were tested for their stability characteristics. In each case, ten isolates were tested. One isolate from the 19-generation strains, and four isolates from the 160-generation strains, showed high levels of plasmid stability (>90% of the population retained the plasmid after 96 h of non-selective batch culture). The rest of the isolates showed low levels of stability (<15% plasmid retention). Whilst the number of isolates tested was insufficient to show the distribution of stability characteristics within the chemostat population, the results show that, after prolonged selective culture, a considerable proportion of plasmid-containing isolates showed a marked improvement when compared with the original strain. The stable strains isolated after 19 generations (GFA-19) and 160 generations (GFB-160, GFC-160, GFE-160, and GFG-160) were retained for further studies. Further tests confirmed the high degree of plasmid stability in these isolates, in comparison with the original strain (BC1: Table 1).

The population changes which occur in prolonged chemostat cultures may be said to represent an adaptation to the culture environment. Characteristics thus developed may rely on the system for their maintenance, and isolates may fail to thrive or revert to their original characteristics when stored or subcultured (Horiuchi *et al.*, 1962). The effects of storage at -20°C on the stability characteristics of three chemostat isolates were examined (Table 3). Two showed good maintenance of their improved stability characteristics over the entire storage period (329 d). The stability characteristic of GFA-19 deteriorated over the first 196 d of storage, but at the end of the storage period the isolate still possessed a stability characteristic superior to that of BC1. No attempt was made to determine the effect of successive subcultures on the stability characteristics of isolates, but since they had undergone at least four subcultures before stability tests, any improvement in plasmid retention would appear to be genotypic, rather than a phenotypic change induced by continuous cultivation.

Agarose gel electrophoresis of DNA extracted from the original and the improved strains showed that the latter had retained both the cryptic and chimeric plasmids. Gel electrophoresis of the DNA after treatment with restriction endonuclease *EcoRI* showed that neither plasmid had undergone any detectable change in size or structure. Removal of the chimeric plasmid from improved strains by the method of Edger *et al.* (1981) resulted in Cm^rKm^s, HT-amy-lase-negative strains. Integration of all or part of the plasmid into the host chromosome may thus be ruled out as the cause of improved marker retention. Determinations of plasmid copy number were carried out on both unimproved and improved strains (Table 1). pSA33 is an 'oligo-copy' plasmid in this host, and no significant change in copy number was detected.

Table 3. *Effect of storage on the stability characteristics of derived strains of Bacillus subtilis BC1*

Strains were derived from chemostat culture as described in the text, and were stored at -20°C in 20% (v/v) glycerol. Stability assays were done as described in Methods and the results are presented as the proportion of the population retaining Cm^r and Km^r after 96 h non-selective batch culture.

Strain	Stability (%) after storage period shown:			
	None	161 d	196 d	329 d
GFA-19	94	84	57	56
GFC-160	99	98	100	96
GFE-160	96	96	96	90

To determine whether improved stability was a function of the plasmid, protoplast transformation was used to introduce plasmids derived from the improved strains into *B. subtilis* SO113. All of the combinations so produced showed a low level of plasmid stability which was similar to that of the original strain (BC1), and of the combination of the SO113 host with pSA33 derived from BC1 (Table 2). Plasmid pSA33 was then obtained from the original strain (BC1) and transformed into a pSA33-free host derived from one of the improved strains (GFG-160). The stability of this combination was high (Table 2), and similar to that of improved strains, suggesting that the factor responsible for improved plasmid stability resides in the host rather than the plasmid. A stability characteristic residing in a host would be of considerable use in providing a general 'recipient' for otherwise unstable chimeric plasmids. The extent of the stabilizing ability of the host derived from GFG-160 has not been fully checked, but it was capable of stably maintaining another pUB110-derived chimeric plasmid (pMD10) which was unstable in BC0 (Table 2).

Several other differences were found between the stable isolates and the original strain BC1 (Table 1). These may have been due to the selective pressure of the Cm in the medium, or to periodic selection events, which are known to occur in long-term chemostat cultures (Dykhuizen & Hartl, 1983). The increased MIC with respect to Cm supports our notion that the selective pressure promoted higher levels of Cm degradation within Cm^r clones. In four out of five isolates tested, an increased MIC with respect to Km was also observed, suggesting an overall increase in the expression of plasmid-encoded genes. A similar correlation has been noted by Lejeune and his coworkers for *B. subtilis* containing a pUB110 hybrid plasmid (pAMY100). Clones selected for enhanced expression of a plasmid-encoded Km^r gene also showed improved plasmid stability, and increased expression of a plasmid-encoded neomycin nucleotidyl-transferase (Lejeune *et al.*, 1984). They also noted an increase in plasmid copy number, which may have been responsible for increases in both stability and expression. No increase in plasmid copy number, however, was detected in our improved strains.

Table 1 compares the maximum specific growth rate (μ_{max}) of the isolates with that of the original strains. Growth rate determinations were done in the absence of antibiotics in order to allow direct comparison between plasmid-free and plasmid-containing strains. Under these conditions the unstable strain (BC1) produced plasmid-free (BC0) clones. The proportion of plasmid-free organisms in cultures of BC1 used for growth rate determinations was always $<20\%$ at the end of the experiment, but in view of the relatively fast growth of BC0, the μ_{max} for BC1 may be overestimated. In the original strain the presence of the plasmid causes a considerable growth rate disadvantage when cultures are grown under non-selective conditions. This phenomenon, reviewed by Imanaka & Aiba (1981), may explain why non-selective batch cultures of BC1 rapidly became dominated by plasmid-free organisms. The stable strains all showed higher μ_{max} values than strain BC1. The loss of the growth rate advantage of plasmid-free clones could be a factor in the improved stability characteristics of the isolates. According to the Monod equation (Monod, 1942) either an increase in μ_{max} or a decrease in K_s (the saturation constant) with respect to the limiting nutrient would provide a clone with a selective advantage in chemostat culture. Dykhuizen & Hartl (1981) studied the evolution of a population of

Escherichia coli in 500 h glucose-limited chemostat cultures and found that, whilst there was an overall tendency for K_s to decrease during the culture period, significant changes in μ_{\max} occurred over the first 100 h of operation.

By their nature, chemostat cultures promote the evolution of microbial populations towards a climax of complete adaptation to the culture environment and maximum use of the limiting nutrient. We believe that the present study has shown the potential of chemostat selection for the improvement of the heritable stability characteristics of host/chimeric plasmid combinations. Initial chemostat enrichment studies on an SO113/pSA33 combination have also produced isolates with improved stability. Experiments with other hosts and plasmids incorporating other selective markers will be necessary to determine the extent to which this technique is applicable.

We are grateful to Michael F. Coughlan for technical assistance, and to Biocon Biochemicals Ltd. for financial assistance and permission to publish this manuscript.

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