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Plasmid instability in an industrial strain of *Bacillus subtilis* grown in chemostat culture

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Key words: Plasmid instability; Chemostat culture; *Bacillus subtilis*

SUMMARY

A pUB110-derived plasmid/*Bacillus subtilis* host combination was segregationally unstable when grown in chemostat culture with complex or minimal medium and under starch, glucose or magnesium limitation. The kinetics of plasmid loss were described in terms of the difference in growth rates between plasmid-containing and plasmid-free cells ($d\mu$) and the rate at which plasmid-free cells were generated from plasmid-containing cells (R). Loss of plasmid-containing cells from the population was $d\mu$ dominated. Changes in medium composition and the nature of growth limitation caused variations in both $d\mu$ and R. The plasmid was most stable in glucose-limited chemostat cultures with minimal medium and least stable under starch limitation with complex medium. R and $d\mu$ were smaller for cultures in complex media than those in minimal media. Limitation by starch induced expression of the plasmid-encoded HT α amylase gene and was associated with increased values of R and $d\mu$. Magnesium limitation in minimal medium caused a significant increase in $d\mu$ and a decrease in R.

INTRODUCTION

Plasmid stability has been defined as the ability of transformed cells to maintain plasmid unchanged during their growth [16]. In some cases, instability may reduce the profitability of commercial fermentations to such an extent that they become non-viable [9,10]. Two types of instability exist. Structural instability is due to insertion, deletion or rearrangement of plasmid DNA [24]. Segregational instability arises due to the defective partitioning of plasmid DNA between daughter cells during cell division [24]. There are two basic factors which influence the appearance of plasmid-free segregants during culture. The segregation rate (R) describes the rate at which plasmid-free cells are generated from cells with plasmid [8]. R is affected by mutations in copy number regulatory circuits [14], plasmid multimer formation [23] or the presence or absence of partitioning functions [6]. The difference in growth rate between cells with and without plasmid ($d\mu$) is a measure of the metabolic burden that a plasmid places on the host [8]. Consequently, $d\mu$ describes the competition kinetics between plasmid-free and plasmid-containing cells during culture. This burden is influenced by factors including the expression of plasmid-encoded genes [3], elevated copy number maintenance [26] and the environmental conditions under which the plasmid–host combination is grown [29,30].

Plasmid instability, in particular segregational instability has hampered the use of recombinant *Bacillus* strains for commercial purposes [16]. It is known that pUB110-derived plasmids are prone to instability when introduced into *Bacillus subtilis* [5,32]. *B. subtilis* BC1 is an industrial strain which harbors a pUB-110-derived recombinant plasmid, pSA33. We have previously reported that pSA33 is segregationally unstable in *B. subtilis* BC1 when grown in batch culture under non-selective conditions [11]. Whilst results from batch culture experiments showed that the difference in growth rates between the recombinant *B. subtilis* BC1 and the plasmid-free host strain (*B. subtilis* BC0) contributed to instability, the study did not reveal whether $d\mu$ or R was the dominant factor which determined the instability of the recombinant strain. This paper describes the relative importance of R and $d\mu$ in governing the kinetics of plasmid loss in *B. subtilis* BC1 grown in chemostat culture. The influence on both R and $d\mu$ of using carbon or magnesium limitation in complex or minimal media was also investigated.

MATERIALS AND METHODS

Bacterial strain and plasmid

Bacillus subtilis BC1 was a commercial strain supplied by Biocon Biochemicals Ltd, Carrigaline, Co. Cork, Ireland. The parent strain (*B. subtilis* BC0) was transformed with pSA33 to form strain BC1. Plasmid pSA33 (8.35 kb) encodes for chloramphenicol (Cm^r) and kanamycin (Kan^r)-resistance determinants. The plasmid also codes for the *B. licheniformis* HT α amylase structural gene (3.55 kb) whose product has

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Abbreviations: Cm, chloramphenicol; Kan, kanamycin; Cm^r, cells resistant to chloramphenicol (5 mg L^{-1}); Kan^r, cells resistant to kanamycin (5 mg L^{-1}); Cm^sKan^s, cells sensitive to chloramphenicol and kanamycin.

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activity at 100 °C. The recombinant plasmid is a derivative of pBD64 (4.5 kb), which is a product of a spontaneous deletion event between a cointegrate of pUB110 and pC194 [15]. The construction and properties of pSA33 have been described elsewhere [25]. *B. subtilis* BC1 was maintained at −20 °C as cultures in LS broth [11] (0.8 ml) supplemented with 5 mg L^{-1} Cm and 5 mg L^{-1} Kan, to which 20% (v/v) sterile glycerol had been added. Stock cultures were reactivated by thawing, inoculating them into 80 ml of nutrient broth (Oxoid, NY, USA) containing 5 mg L^{-1} Cm and 5 mg L^{-1} Kan and incubating for 12 h in 500-ml baffled Erlenmeyer flasks (37 °C, 95 r.p.m.).

Media

SM medium (minimal) consisted of Spizizen's minimal medium [1] supplemented with 100 mg L^{-1} glucose for glucose-limited cultures. The glucose was replaced by soluble starch (BDH: 100 mg L^{-1}) to achieve starch limitation. For magnesium-limited cultures, SM was supplemented with 2 g L^{-1} glucose and the magnesium sulphate concentration was reduced to 50 mg L^{-1}.

One-fifth strength Luria broth (complex: LB) contained 2 g L^{-1} bacteriological peptone (Oxoid), 1 g L^{-1} yeast extract (Oxoid) and 1 g L^{-1} sodium chloride. Soluble starch (BDH: 400 mg L^{-1}) was added to provide starch limitation. For glucose-limited studies (complex medium), soluble starch was replaced by 100 mg L^{-1} glucose. Antibiotics were not added to chemostat culture media. Limitations were confirmed by means of temporary nutrient additions [13].

Chemostat 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 was added by a peristaltic pump (Watson-Marlow, UK: 202U) to give a dilution rate of 0.18 h^{-1}. The chemostats were established as follows. A loopful of a colony from a nutrient agar plate containing 5 mg L^{-1} Cm and 5 mg L^{-1} Kan was used to inoculate a 70-ml aliquot of the appropriate full-strength chemostat medium supplemented with 5 mg L^{-1} Cm and 5 mg L^{-1} Kan. This culture was grown in an orbital shaker for 12 h (37 °C, 95 r.p.m.). The entire culture was then transferred to the chemostat vessel and 430 ml of the limiting medium was added. Cultures were allowed to grow in the batch mode until a cell density of 30–43 × 10^6 cells ml^{-1} was reached, which was approximately the cell density at steady-state in all reactors. The medium feed pump was then turned on and nutrient-limited antibiotic-free medium was added to the vessel.

Assay for plasmid retention in chemostat culture

The proportion of the chemostat population retaining pSA33 was determined by stick replica plating at least 100 colonies from a non-selective nutrient agar plate to nutrient agar plates containing: a) 5 mg L^{-1} Cm; b) 5 mg L^{-1} Kan; c) 5 mg L^{-1} Cm and 5 mg L^{-1} Kan; and d) no antibiotic. The number of colonies that grew on the antibiotic-containing plates was expressed as a proportion of those that grew on the antibiotic-free plates.

Determination of $d\mu$ and R parameters

The mathematical equations of Cooper et al. [8] were used to determine values of $d\mu$ and R (with 95% confidence limits) from plots of the natural logarithm of the proportion of plasmid-free cells versus generations elapsed in chemostat culture.

HT α amylase activity

The HT α amylase activity of liquid samples from chemostat broths was measured by the Stanstedt assay [31]. The assay was modified in that incubations were carried out at 100 °C rather than 30 °C. The qualitative HT α amylase activity test was performed as described previously [11].

RESULTS AND DISCUSSION

The use of chemostat cultures provides a controlled and constant environment for studying plasmid instability. Although there are exceptions [18,27], there is usually a decrease in plasmid content with increasing dilution rate [3,28]. To avoid such a phenomenon in the present study, chemostats were operated at a single dilution rate (D = 0.18 h^{-1}). It is known that plasmids may exhibit structural instability when grown under carbon-limiting conditions [17,24]. Samples taken from chemostats in this study were checked for structural instability. During the course of these experiments, cells that were isolated on the basis of their resistance to Cm and/or Kan were screened for the presence of all plasmid-encoded markers (Cm^r, Kan^r, HT α $amylase^+$). No evidence for structural instability was found.

Data obtained from batch studies showed that there was a significant maximum specific growth rate difference (0.74 h^{-1}) between the host and recombinant strains grown on complex medium (LB with 2 g L^{-1} starch). Whilst this growth rate difference was less pronounced on minimal medium (SM with 10 g L^{-1} glucose: 0.31 h^{-1}), it was assumed that $d\mu$ rather than R was the dominant factor describing the appearance of plasmid-less cells during culture. Accordingly, when applying the mathematical equations of Cooper et al. [8] to chemostat data, a null hypothesis was made that $d\mu \gg R$. Other assumptions ($R \gg d\mu$ or $R \approx d\mu$) resulted in meaningless results (e.g. negative values of R).

When *B. subtilis* BC1 was cultured in glucose-limited chemostat culture on minimal (SM) medium, a lag period of 20 generations elapsed before the percentage of the population that was Cm^rKan^r decreased (Fig. 1(A)). Others have interpreted such a lag phase as representing a period of gradually decreasing plasmid copy number in the population [35]. Weber and San [33] have suggested that a plasmid is lost from a population in three stages. It is possible that the lag may delineate a period before stage 2 and 3 kinetics are reached. An exponential increase in the proportion of plasmid-free cells occurred after 20 generations, and the proportion of these cells was greater than 0.99 after

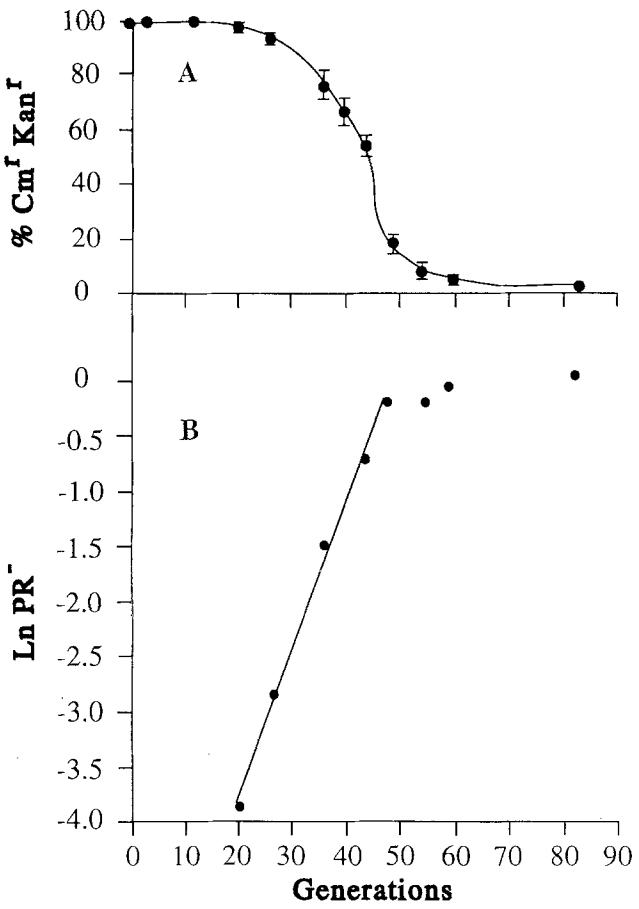

Fig. 1. Stability of *Bacillus subtilis* BC1 in glucose-limited chemostat culture on minimal (SM) medium. (A) % of population with plasmid (Cm^rKan^r) ± SE; (B) Log_e of the proportion of plasmid-free cells (PR^-). Details of culture and assay conditions are given in Materials and methods. D = 0.18 h^{-1}.

83 generations. Values of $d\mu$ and R were determined from Fig. 1(B) and are shown in Table 1. The differential growth rate between the host and recombinant strain ($d\mu$) was about three orders of magnitude greater than the rate at which plasmid-less cells were generated (R). No HT α amylase activity could be detected in samples taken from the culture, which showed that the cloned gene was not expressed.

We have previously reported that pSA33 was maintained at a copy number of 6.7 ± 1.5 when *B. subtilis* BC1 was cultured in SM with glucose [11]. Assuming that a single plasmid is the unit of segregation and plasmid copies segregate randomly to daughter cells, the probability of generating a plasmid-free segregant per cell generation will be between 1.48×10^{-3} and 2.3×10^{-5} according to the binomial probability theory of Meacock and Cohen [21]. The measured rate of segregation for cultures grown in the same medium ($2.14 \pm 0.02 \times 10^{-4}$ gen^{-1}: Table 1) fell within this range. It would appear that under these circumstances, pSA33 segregated randomly to daughter cells and that segregation was not aided by stability-enhancing factors.

The recombinant strain persisted at low frequency after 60 generations (Fig. 1(A)) even though the plasmid-less cells had a growth advantage. The Monod model [22] predicts that, in simple competition between strains for the same limiting nutrient, the disadvantaged strain should be lost from the chemostat. Clearly this was not the case in this study. Other investigations have observed the ability of disadvantaged and unstable recombinant strains to survive for long periods in chemostat culture [12].

When *B. subtilis* BC1 was grown in starch-limited chemostat culture on SM (minimal medium), a lag period of three generations elapsed during which the chemostat populations was 100% Cm^rKan^r (Fig. 2(A)). After this time, the proportion of cells without plasmid increased, and 54% of the population was without plasmid after nine generations. Values of R and $d\mu$ were calculated from Fig. 2(B). R was, again, significantly less than $d\mu$ (Table 1), but the growth rate difference between the host and recombinant was three-fold larger than that observed in the analogous culture limited by glucose (Table 1). The value of R observed under starch limitation was some 35-fold greater than that observed under glucose limitation (both in minimal medium). The

TABLE 1

Kinetic parameters describing the instability of *Bacillus subtilis* BC1 in continuous cultures grown on minimal (SM) or complex (LB) media

Medium	Minimal		Minimal		Minimal		Complex		Complex	
Limitation	Glucose		Starch		Magnesium		Glucose		Starch	
	Value	±CL	Value	±CL	Value	±CL	Value	±CL	Value	±Cl
$d\mu$ ($\times 10^{-1}$ gen^{-1})	1.34	0.02	3.98	0.01	8.71	0.04	1.07	0.02	2.39	0.02
R ($\times 10^{-4}$ gen^{-1})	2.14	0.02	68.6	0.2	1.20	0.04	5.82	0.01	22.8	0.1

$d\mu$ is the growth rate difference between Cm^sKan^s and Cm^rKan^r sub-populations. **R** is the rate at which Cm^sKan^s cells arose from Cm^rKan^r cells. **$d\mu$** and **R** were calculated by the method of Cooper et al. [8] from graphs of Log_n of the proportion of plasmid-free cells (PR^-) against generations elapsed (Figs 1(B)–5(B)). It was assumed that R«$d\mu$ (see Results). All values are expressed with their 95% confidence limits (±CL). All cultures were grown at D = 0.18 h^{-1}.

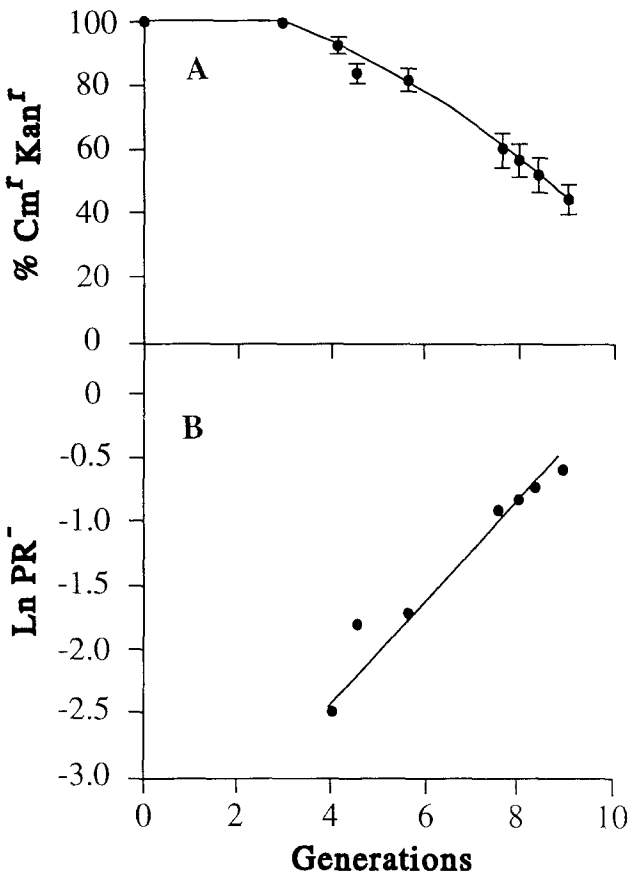

Fig. 2. Stability of *Bacillus subtilis* BC1 in starch-limited chemostat culture on minimal (SM) medium: (A) % of population with plasmid (Cm^rKan^r) ± SE; (B) Log_e of the proportion of plasmid-free cells (PR^-). Details of culture and assay conditions are given in Materials and methods. D = 0.18 h^{-1}.

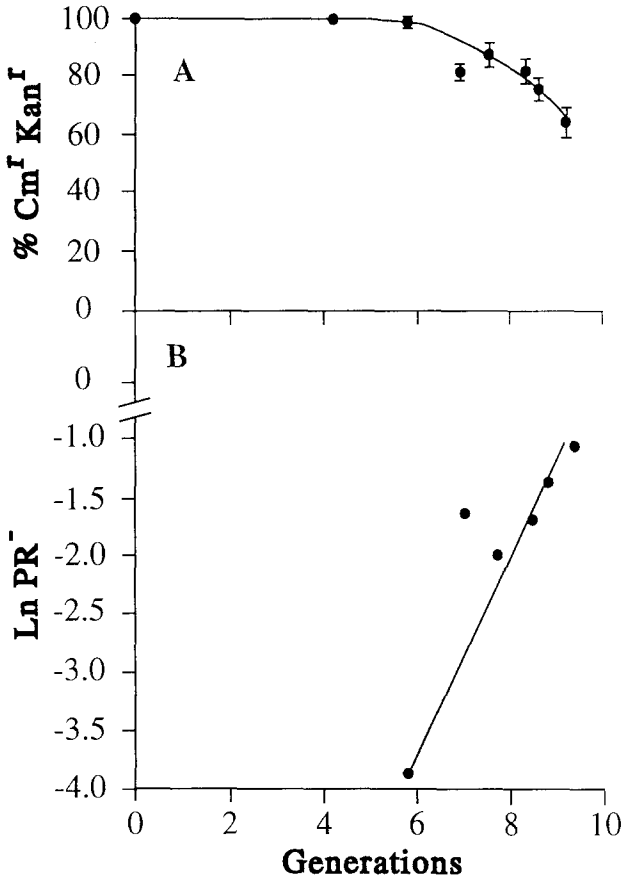

Fig. 3. Stability of *Bacillus subtilis* BC1 in magnesium-limited chemostat culture on minimal (SM) medium: (A) % of population with plasmid (Cm^rKan^r) ± SE; (B) Log_e of the proportion of plasmid-free cells (PR^-). Details of culture and assay conditions are given in Materials and methods. D = 0.18 h^{-1}.

increased $d\mu$ may be explained by the observation that the HT α amylase gene was expressed in the starch-limited culture (35 U ml^{-1}) but not under glucose limitation. The expression of the cloned HT α amylase gene may have lessened the ability of BC1 to compete against plasmid-free cells. This is consistent with other reports which showed that the translation of plasmid-encoded proteins reduced the competitive ability and stability of *B. subtilis* recombinant cells [2,34].

Under magnesium limitation in minimal medium (SM), the chemostat population remained plasmid-bearing for the first four generations (Fig. 3(A)), after which plasmid-less cells began to rapidly take over the culture. Values of $d\mu$ and R were determined from Fig. 3(B) and are shown in Table 1. Again, the differential growth rate between the host and recombinant was the dominant factor which described plasmid instability. The differential growth rate was the highest recorded in this study and was 2.2-fold greater than that found under starch limitation (SM). No HT α amylase activity could be detected in samples taken from the magnesium-limited culture. It is apparent that, in this case, cloned gene expression did not contribute to instability. The larger $d\mu$ under magnesium limitation may have resulted from the increased metabolic cost of plasmid maintenance. It has been established that this may arise from an increased copy number [4,19]. Whilst $d\mu$ was significantly larger for the magnesium-limited culture, this did not result in an increased degree of instability when compared with starch-limitation (Figs 2(A) and 3(A)). This may be explained by the fact that the segregation rate (R) was significantly less for the culture under magnesium limitation (Table 1).

To determine if overall medium composition affected the kinetics of plasmid loss from BC1, further experiments were carried out in complex medium (LB) under glucose or starch limitation. In the glucose-limited culture, the percentage of plasmid-containing cells began to decrease immediately after commencing the medium feed (Fig. 4(A)). Cells without plasmid subsequently took over the culture. They comprised greater than 78% of the population after 20 generations. R and $d\mu$ parameters were calculated from Fig. 4(B). The differential growth rate between host and recombinant ($d\mu$) was significantly greater than R (Table 1). No HT α amylase activity could be detected.

The recombinant plasmid was considerably less stable when grown in complex medium limited by starch (Fig.

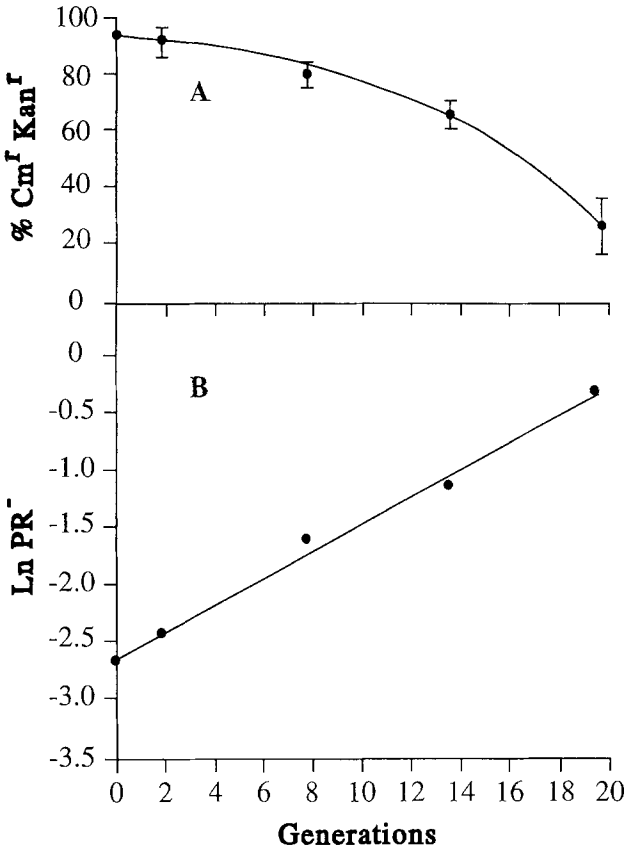

Fig. 4. Stability of *Bacillus subtilis* BC1 in glucose-limited chemostat culture on complex (LB) medium: (A) % of population with plasmid (Cm^rKan^r) ± SE; (B) Log_e of the proportion of plasmid-free cells (PR^-). Details of culture and assay conditions are given in Materials and methods. D = 0.18 h^{-1}.

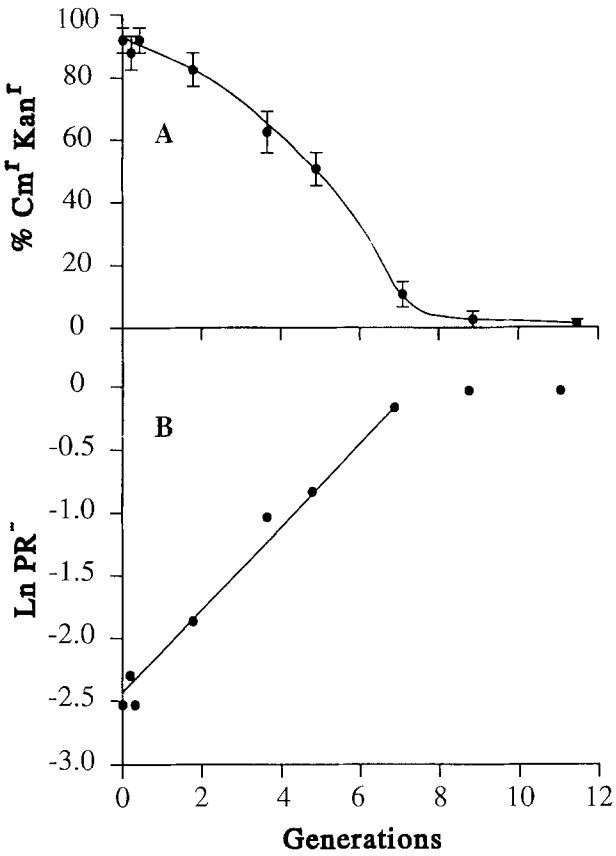

Fig. 5. Stability of *Bacillus subtilis* BC1 in starch-limited chemostat culture on complex (LB) medium: (A) % of population with plasmid (Cm^rKan^r) ± SE; (B) Log_e of the proportion of plasmid-free cells (PR^-). Details of culture and assay conditions are given in Materials and methods. D = 0.18 h^{-1}.

5(A)) than when grown in complex medium limited by glucose (Fig. 4(A)). Values of $d\mu$ and R were calculated from Fig. 5(B) and are shown in Table 1. The difference in growth rate between the host and recombinant ($d\mu$) was the major factor which described the instability. As was found with the minimal medium (SM), starch limitation caused expression of the HT α amylase gene (the culture had an enzyme titer of 179 U ml^{-1}) and an increase in $d\mu$ when compared with that found under glucose limitation. Segregation rate (R) was, again, much larger than that found under glucose limitation. It is not clear from this study why starch limitation promoted relatively high values of R, though some physiologically induced change in copy number may be postulated [18,27].

If cultures grown under the same limitation are compared (Table 1), it can be seen that the value of $d\mu$ was always greater in minimal medium (SM) than in complex medium (LB). This may reflect the greater demand made by the plasmid for cellular pool intermediates when nutrient restriction is more severe [20]. The nutritional stringency of the medium also appears to influence R, but here the effect is less straightforward. Under glucose limitation, R is greatest when the complex medium is employed, whereas the reverse is true under starch limitation.

In summary, the results presented here demonstrate that both R and $d\mu$ may vary considerably with the type of medium and nature of the limiting substrate used. The recombinant plasmid was more stable under glucose limitation than under starch limitation regardless of whether complex or minimal medium was used. The expression of the plasmid-encoded HT α amylase gene contributed to this phenomenon. The difference in growth rate between the host and recombinant was the dominant factor describing instability in all cultures but was greater in minimal medium when compared with complex medium irrespective of the limiting nutrient. In 1985, Caulcott et al. [7] indicated that a relationship might exist between the growth environment and the effect plasmids exert on *Escherichia coli* hosts. In the present study, we have observed such a relationship with a commercially-exploited recombinant strain of *Bacillus subtilis*. It would appear that careful choice of the growth environment may provide a physiological method to improve the stability of unstable host–plasmid combinations.

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REFERENCES

1 Anagnostopolous, C. and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bact. 81: 741–746.
2 Bentley, W.E. and D.S. Kompala. 1989. A novel structured kinetic modelling approach for the analysis of plasmid instability in recombinant bacterial cultures. Biotechnol. Bioeng. 33: 49–61.
3 Bentley, W.E. and D.S. Kompala. 1990. Stability in continuous cultures of recombinant bacteria. Biotech. Lett. 12: 329–334.
4 Betenbaugh, M.J., C. Beaty and P. Dhurjati. 1987. Growth kinetics of *Escherichia coli* containing temperature-sensitive plasmid pOU140. Biotechnol. Bioeng. 3: 1425–1436.
5 Bron, S. and E. Luxen. 1985. Segregational instability of pUB110-derived recombinant plasmids in *Bacillus subtilis*. Plasmid. 14: 235–244.
6 Bron, S., P. Bosma, M. van Belkum and E. Luxen. 1987. Stability function in the *Bacillus subtilis* plasmid pTA1060. Plasmid. 18: 8–15.
7 Caulcott, C.A., G. Lilley, E.M. Wright, M.K. Robinson and G.T. Yarranton. 1985. Investigation of the instability of plasmids during the expression of Met-prochymosin in *Escherichia coli*. J. Gen. Microbiol. 131: 3355–3365.
8 Cooper, N.S., M.E. Brown and C.A. Caulcott. 1987. A mathematical method for analyzing plasmid stability in micro-organisms. J. Gen. Microbiol. 133: 1871–1880.
9 Doi, R.H. 1984. Genetic engineering in *Bacillus subtilis*. In: Biotechnology and Genetic Engineering Reviews (Russell, G.E., ed.), pp. 121–135, Intercept, UK.
10 Ensley, B.D. 1986. Stability of recombinant plasmids in industrial microorganisms. CRC Crit. Rev. in Biotechnol. 4: 263–277.
11 Fleming, G., M.T. Dawson and J.W. Patching. 1988. The isolation of strains of *Bacillus subtilis* showing improved plasmid stability characteristics by means of selective chemostat culture. J. Gen. Microbiol. 134: 2059–2101.
12 Godwin, D. and J.H. Slater. 1979. The influence of the growth environment on the stability of a drug resistance plasmid in *Escherichia coli* K12. J. Gen. Microbiol. 111: 201–210.
13 Goldberg, I. and Z. Er-El. 1981. The chemostat — an efficient technique for medium optimization. Proc. Biochem. 16: 2–8.
14 Gruss, A.D. and S.D. Ehrlich. 1989. The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. Microbiol. Rev. 53: 231–241.
15 Gryczan, T., A.G. Shivakumar and D. Dubnau. 1980. Characterization of chimeric plasmid cloning vehicles in *Bacillus subtilis*. J. Bacteriol. 141: 245–253.
16 Imanaka, T. and S. Aiba. 1981. A perspective on the application of genetic engineering: stability of recombinant plasmids. Proc. N.Y. Acad. Science 10: 1–14.
17 Kadam, K.L., K.L. Wollbirer, J.C. Grosch and Y.C. Jao. 1987. Investigation of plasmid instability in an amylase-producing *Bacillus subtilis* using continuous culture. Biotechnol. Bioeng. 24: 859–872.
18 Koizumi, J., Y. Monden and S. Aiba. 1985. Effects of temperature and dilution rate on the copy number of a recombinant plasmid in continuous culture of *Bacillus stearothermophilus* (pLP11). Biotechnol. Bioeng. 27: 721–728.
19 Lee, S.B., A. Seressiotis and J.E. Bailey. 1985. A kinetic model for product formation in unstable recombinant organisms. Biotechnol. Bioeng. 27: 1699–1709.
20 Matsui, T., H. Sato, S. Mukataka and J. Tanaka. 1990. Effects of nutritional conditions on plasmid stability and production of tryptophan by a recombinant *Escherichia coli*. Agric. Biol. Chem. 54(b): 619–624.
21 Meacock, P.A. and S.N. Cohen. 1980. Partitioning of bacterial plasmids during cell division: a *cis*-acting locus that accomplishes stable plasmid inheritance. Cell 20: 529–542.
22 Monod, J. 1950. La technique de cultière continué: theorie et applications. Ann. Inst. Pasteur Paris 79: 380–410.
23 Novick, R.P., A.D. Gruss, S.K. Highlander, M.L. Gennaro, S.J. Projan and H.F. Ross. 1986. Host-plasmid interactions affecting plasmid replication and maintenance in Gram-positive bacteria. In: Genes, Ecology, Transfer and Expression. Twenty-fourth Branbury report (Levey, S.B. and R.P. Novick, eds), pp. 225–245, Cold Spring Harbor Laboratory Publications, USA.
24 Nugent, M.E., S.B. Primrose and W.C.A. Tacon. 1983. The stability of recombinant DNA. In: Developments in Industrial Microbiology 24 (Nash, C.H. and A. Lelens, eds), pp. 271–285, Soc. for Gen. Microbiol., J.D. Lucas Ltd, USA.
25 Ortlepp, S.A., J.F. Ollington and D.J. McConnell. 1983. Molecular cloning in *Bacillus subtilis* of a *Bacillus licheniformis* gene coding thermostable alpha amylase. Gene 23: 267–276.
26 Park, R. and D.D.Y. Ryu. 1990. Effect of operating parameters on the specific production rate of a cloned gene product and performance of the recombinant fermentation process. Biotechnol. Bioeng. 35: 287–295.
27 Reinikainen, P. and I. Virkajarvi. 1989. *Escherichia coli* growth and plasmid copy numbers in continuous cultivations. Biotechnol. Lett. 11: 225–230.
28 Seo, J. and J.E. Bailey. 1985. A segregated model for plasmid content and product synthesis in unstable binary fission recombinant organisms. Biotechnol. Bioeng. 27: 156–165.
29 Shoham, Y. and A.L. Demain. 1990. Effect of medium composition on the maintenance of a recombinant plasmid in *Bacillus subtilis*. Enzyme Microb. Technol. 12: 330–336.
30 Shoham, Y. and A.L. Demain. 1991. Kinetics of loss of a recombinant plasmid in *Bacillus subtilis*. Biotechnol. Bioeng. 37: 927–935.
31 Stanstedt, R.M., E. Kneen and M.S. Blish. 1939. A standardized Wohlgemuth procedure for alpha amylase activity. Cereal Chem. 16: 712–723.
32 Vehmaanpera, J.O. and M.P. Korhola. 1986. Stability of the recombinant plasmid carrying the *Bacillus amyloliquefaciens* α amylase gene in *Bacillus subtilis*. Appl. Microbiol. Biotechnol. 23: 456–459.
33 Weber, A.E. and K.Y. San. 1990. Population dynamics of a recombinant culture in a chemostat under prolonged cultivation. Biotechnol. Bioeng. 6: 1104–1113.
34 Wei, D., S.J. Parulekar, B.C. Stark and W.A. Weigand. 1989. Plasmid stability and α amylase production in batch and continuous cultures of *Bacillus subtilis* Tn106 (pAT5). Biotechnol. Bioeng. 33: 1010–1020.
35 Wouters, J.T.M., F.L. Driehuis, P.J. Polaczek, M.L. van Oppenraay and J.G. van Andel. 1980. Persistance of the pBR322 plasmid in *Escherichia coli* K12 grown in chemostat cultures. Antonie von Leeuwenhoek 46: 353–362.