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Pulse-Field gel-electrophoretic analysis of the amplification and copy-number stability of an integrational plasmid in *Bacillus subtilis*

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Abstract The stability of integration and amplification of an integrational plasmid in *Bacillus subtilis* was analyzed. A *cat*-containing plasmid was constructed that could be integrated into the *amy* locus to facilitate measurement of excision events. Pulse-field gel electrophoresis was used to measure the copy number in strains that were resistant to different levels of chloramphenicol. The stability of the amplified unit in strains containing from 2 to 18 tandem copies of the amplicon in the presence and absence of chloramphenicol and through different generation times was then determined. Our results demonstrate that, for any given strain, the copy number of the amplicon remains stable. Furthermore, this stability is maintained when a clone containing an amplicon of defined size is cultured through as many as 100 generations in the absence of selective pressure.

Introduction

Bacillus subtilis is extensively used in industry and there is an interest in stably expressing heterologous genes. In *B. subtilis*, replicative plasmids harboring cloned sequences can be prone to either segregational or structural instability (Brön and Luxen 1985).

One strategy employed to avoid the loss of genetic information carried on plasmids is integration into the bacterial chromosome. When a plasmid is integrated by homologous recombination through a single crossover event, a duplication occurs. The region from one segment to the duplicated one is called an amplicon, as it is prone to new recombination events that may lead to its amplification. Strains transformed with an integrational plasmid carrying both an antibiotic-resistance gene and the gene of interest may be subjected to augmented selective pressure by the addition of excess antibiotic. This process leads to the selection of strains in which the amplicon has increased in copy number severalfold. Both chloramphenicol and kanamycin (Piggott et al. 1988; Janniére et al. 1985) have been used to select for amplification of integrated plasmids. The amplicon copy number can be measured in one of two ways. First, DNA from strains exhibiting increased antibiotic resistance is subjected to restriction digestion using an enzyme for which there is a single recognition site within the vector sequence. The digested DNA is then analyzed by Southern hybridization using the vector as probe. The copy number of the amplicon is then calculated on the basis of the relative intensity of the hybridizing band using densitometric analysis. Alternatively, the DNA is digested with an enzyme that does not recognize sequences within the vector and analyzed by pulse-field gel electrophoresis (PFGE) and Southern hybridization. The size of the hybridizing band will be a direct measure of the copy number of the integrated vector. Using this method it has been shown that, within a population of cells for a given strain, there exist amplicons with multiple copy numbers (Piggot et al. 1988). We have observed a similar phenomenon in our studies of the amplification of *cry* genes (Vázquez-Cruz 1993). Although it has been reported that the amplification of integrated plasmids is quite stable in *B. subtilis* (Jannière et al. 1985), it remains to be determined whether the amplified structure of a defined size is also stable or whether its size fluctuates over time. This would not be recognized by densitometric analysis which only gives information on the average copy number in a population. To address the question of amplicon stability, we isolated strains resistant to different antibiotic concentrations, and then

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measured their size by PFGE over time in the presence or absence of selective pressure.

Materials and methods

Bacterial strains, plasmids and growth conditions

B. subtilis strain were PY79 (Youngman et al. 1984) and 1A746 (Bacillus Genetic Stock Center). *Escherichia coli* strain MC1061. Plasmids used in this work were pRHA39 (Albertini and Galizi 1985), kindly provided by Dr. Galizi; pGEM3Z(f)-*cat* (Youngman et al. 1989); pCV4, a PGEM3Zf(+)-*cat* derivative carrying an internal 536-bp fragment of *amyE* derived from pRHA39; pCV5, a pCV4 derivative carrying a *cryIA* gene from *B. thuringiensis* at the *Bam*HI site (Vázquez-Cruz 1993); pBKS(+) (Stratagene).

E. coli, *B. thuringiensis* and *B. subtilis* strains were routinely grown on Luria-Bertani (LB) medium and agar (Sambrook et al. 1989). When present, antibiotics were used at the following concentrations: carbenicillin, 100 μg/ml; erythromycin, 1 μg/ml; chloramphenicol, 5 μg/ml (except where otherwise stated). Doubling times for the cultures were monitored by a Klett Summerson colorimeter (red filter). To monitor stability through different generations, cells were simply diluted out into fresh medium once they reached stationary phase. This procedure was repeated the number of times necessary to reach 25 and 100 generation times.

Plasmids from *E. coli* were prepared by the method of Birnboin and Doly (1979). Transformation into *E. coli* was done by the method of Hannahan (1983). Transformation of *B. subtilis* was done as described by Dubnau and Davidoff-Abelson (1971).

Measure of integration stability

Cells were diluted for single-colony isolation and grown on L-agar plates which had been overlayed with 3 ml LB/agar containing 0.1% potato starch. The plates were incubated overnight and then exposed to iodide vapors. Amy^+ colonies exhibited clear halos as a result of the hydrolyzed starch. The excision frequency was measured by growing cultures of *B. subtilis* strains containing integrated plasmids (Amy-Cm^r) for 20 generations in the absence of chloramphenicol. Dilutions were plated on starch plates and Amy^+ colonies were scored. More than 40 000 colonies were screened. The frequency of excision was calculated as the ratio of the number of Amy^+ colonies to the total number of colonies screened.

Amplification of plasmids integrated into the *B. subtilis* chromosome

B. subtilis was transformed with pCV4 or pCV5 plasmid DNA and transformants were selected on LB/agar plates at 5 μg/ml chloramphenicol after having been induced for 1 h with 0.125 μg/ml antibiotic. Amplification was carried out both on agar plates and in liquid cultures. For amplification on solid media, individual transformants were picked onto LB/agar plates containing different concentrations of chloramphenicol (5, 10, 15, 20, 30, 40 and 60 μg/ml). Most transformants unable to grow on plates containing 10 μg/ml chloramphenicol were determined to contain a single integrated copy of the plasmid by Southern hybridization analysis. The colonies that grew at concentrations of 10 μg/ml and higher were then streaked repeatedly on LB/agar with chloramphenicol (5–60 μg/ml). Colonies that grew reproducibly on the different concentrations of chloramphenicol being tested were chosen as amplificants. To select for amplification in liquid cultures, single-copy transformants were grown on LB with the desired chloramphenicol concentration and the cultures were allowed to grow overnight. Dilution into fresh medium and further growth resulted in enrichment for the desired amplificants. A third dilution was done before streaking on to agar plates containing various chloramphenicol concentrations. All strains were kept in frozen stocks until their analysis.

PFGE analysis and Southern blotting of *B. subtilis* chromosomal DNA

Agarose blocks of *B. subtilis* DNA were prepared essentially as described by Smith et al. (1988). The DNA blocks were equilibrated for 2 h with the appropriate restriction enzyme buffer and incubated with 0.1% bovine serum albumin and 10–20 U restriction enzyme for 18 h. Digested DNA was fractionated 24 h in 1.2% agarose in a pulse-field gel electrophoresis apparatus CMEF-DR®III (BioRad) at a 120° angle, 180 V and pulses of 13 s. Detection of the plasmids integrated into the *B. subtilis* chromosome was done by the method of Southern (1977) as described by Sambrook et al. (1989) modified for use with the non-radioactive digoxigenin technique with alkaline phosphatase (Boehringer). Plasmid pCV5 (Fig. 1) was used as a probe.

Results

Plasmid integration in the *B. subtilis* chromosome and estimation of integration stability through the Amy phenotype

In order to assess the stability of plasmids integrated into the *B. subtilis* chromosome a *cat*-containing plasmid (pCV4) conferring resistance to chloramphenicol was constructed that utilizes an internal 536-bp fragment of *amyE* to provide homology for Campbell-like integration. Plasmid pCV5 was constructed by inserting a *cryIAa* gene derived from *B. thuringiensis*. Integration of pCV4 (or pCV5) results in disruption of the *amyE* gene and an Amy^- phenotype (Fig. 1). The excision of the plasmid results in the restoration of the gene structure and thus in an Amy^+ phenotype. Both events may be easily observed on agar plates by the ability of the resulting colony to hydrolyze starch. Transformants carrying a single copy of the integrated plasmid were analyzed and the stability of the integrated plasmid was measured in the absence of selective pressure (chloramphenicol) and after 20 generations as described in Materials and methods. The percentage of cells that had lost integrated plasmids pCV4 or pCV5 ranged from 0.03% to 0.2%. This is in agreement with previously published studies (Young and Erlich 1989).

Selection and PFGE analysis of strains resistant to various levels of chloramphenicol

For solid-medium amplification, independent transformants of plasmid pCV5 were selected by growth on LB/agar containing chloramphenicol at 5 μg/ml. Cells containing amplified structures were obtained by streaking individual transformants on LB/agar plates containing increasing concentrations of chloram-

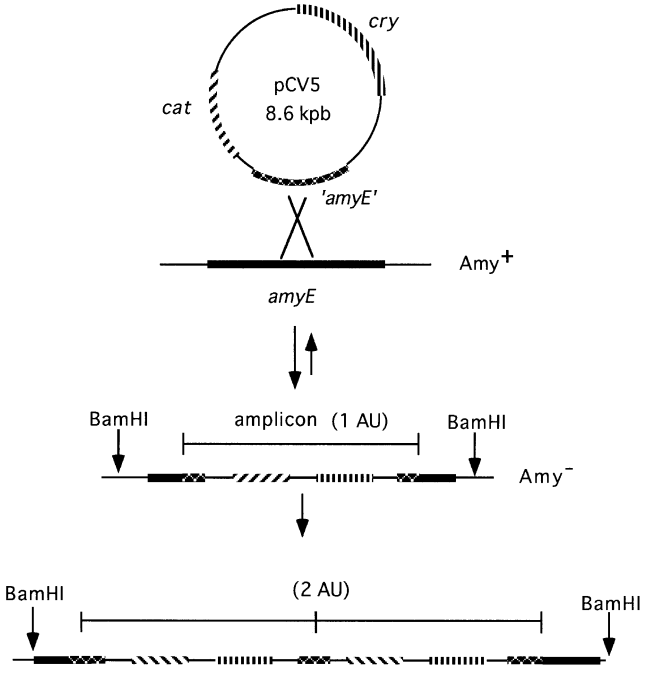

Fig. 1 Diagram of the integration and amplification of pGV5 in the *B. subtilis* chromosome. '*amyE*' an internal 536-bp fragment from *amyE* which provides homology for integration and causes gene disruption and therefore an Amy$^-$ phenotype; *cat* chloramphenicol acetyltransferase gene. Digestion with *Bam*HI produces a 90-kbp fragment in the absence of integrated pCV5; when integrated as a single copy (1 amplification unit, AU) the *Bam*HI fragment size increases by 8 kbp; a 2-AU increase will increase the fragement size by 16 kbp and so on

phenicol (5, 10, 15, 20, 30, 40 and 60 μg/ml). Seven independent colonies were selected as potential single-copy transformants by their inability to grow at chloramphenicol concentrations of 10 μg/ml or higher. Strains capable of growing at 10, 15, 20 and 30 μg/ml chloramphenicol were also obtained. It was observed that it was possible to obtain 20 μg/ml and 30 μg/ml resistant strains in a single step without serial passes through increasing amounts of the antibiotic. For these experiments growth was not observed beyond 30 μg/ml. Amplificants were easier to select in liquid cultures. Fewer streaking passes were required to select the desired amplificants and some strains were selected that could grow on plates containing up to 50 μg/ml.

Thirty-five strains able to grow at different antibiotic concentrations in the range 5–50 μg/ml were analyzed by PFGE and Southern blotting to determine the amplicon copy number or number of amplification units (AU) as described in Materials and methods. Table 1 summarizes the results obtained for these strains. Out of 6 strains resistant to 5 μg/ml chloramphenicol, 5 were confirmed to contain predominantly single-copy amplicon structures; 1 of the 6 strains, however, was unexpectedly found to carry 3 AU (Fig. 2, lane e). Only 1 out of 6 strains resistant to 10 μg/ml chloramphenicol showed amplification. These results are similar to those

Table 1 Analysis of strains resistant to various levels of chloramphenicol. Strains carrying integrational plasmid pCV5 were selected as resisting no more than 5 μg/ml or 10–50 μg/ml chloramphenicol as described in Materials and methods. The degree of amplification of all 35 strains was measured through pulse-field gel electrophoresis/Southern blot. This is represented as the copy number of the amplification unit

Chloramphenicol (μg/ml)	No. of strains analyzed	No. of strains showing some degree of amplification	Degree of amplification (lowest/highest)[a]
5	6	1[b]	1–3
10	6	1	2
15	7	3	4–34
20	8	3	4–34
30	3	2	18–22
40	2	1	12
50	3	2	10–34

[a] The lowest and highest copy number of amplification units among all the strains in the group. For instance, one strain in that group may have 4 copies and another one 34 copies

[b] No amplification is expected at this concentration; however, 1 out of 6 strains tested exhibited 3 amplification units

previously reported by Jannière et al. (1985) who found no amplification in strains resistant 10 μg/ml chloramphenicol. However, 50% of the strains resistant to levels of the antibiotic ranging from 15 μg/ml to 50 μg/ml also showed no amplification. These strains may resist higher levels of chloramphenicol as a result of some permeability defect and were not analyzed further. A total of 12 strains exhibiting different levels of amplification were analyzed. As shown in Table 1, while there is no direct correlation between resistance to chloramphenicol and amplicon copy number, there is a tendency towards selection of higher-copy-number amplicons when higher amounts of chloramphenicol are applied. As shown in Fig. 2, the majority of strains contained only one or a few predominant hybridizing bands. In some cases, however, a faint ladder of amplicon copies increasing in single- or multi-copy steps, or a smear starting up or down from the main hybridization signal was observed. These results suggest that colonies may have a mixture of cells carrying a different copy number of the amplification unit, and that these high-molecular-mass structures are reactive and thus tend to fluctuate in size. In most strains, however, the majority of the total hybridization signal is concentrated in a single band. How stable these structures are in the presence and absence of chloramphenicol and over time was next investigated.

Stability of amplified structures in the presence or absence of selective pressure and over different generation times

Several individual strains containing pCV5 were used to determine whether the amplified structure(s)

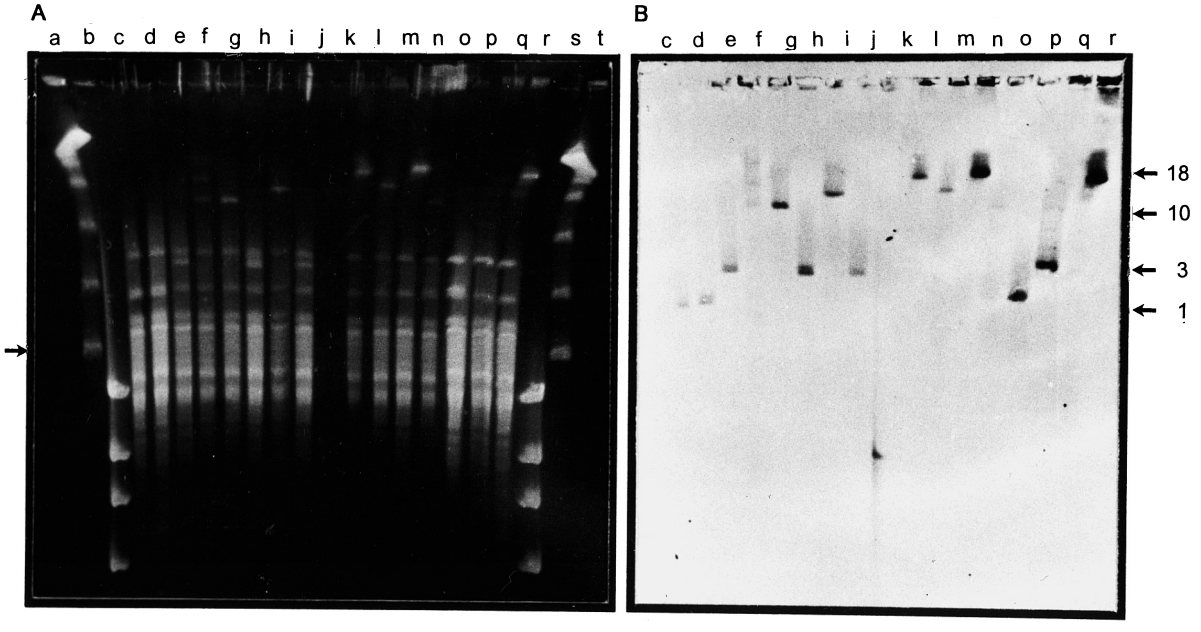

Fig. 2A, B Amplification of the integrated plasmids in strains resistant to different chloramphenicol levels. Cultures from strains carrying the integrated pCV5 plasmid and independently selected as resistant to different chloramphenicol concentrations were grown for six generations and processed in agarose plugs, digested with *Bam*HI and subjected to Pulse-field gel electrophoresis as described in Materials and methods. **A** Ethidium-bromide-stained gel; **B** Southern blot hybridization with digoxigenin-labeled pCV5. Lanes *c* JC5-0; *d* JC5-1; *e* JC5-5; *f* JC15-31; *g* JC15-42; *h* JC15-5; *i* JC20-12; *j* JC20-5; *k* linearized pCV5; *l* JC30-2; *m* JC40-12; *n* JC50-11; *o* JC50-12; *p* C10; *q* C20; *r* C30. The strain nomenclature indicates the level of chloramphenicol resistance by the digits before the dash. *Arrows* at the *right* point to different bands and the calculated AU number of each is given. Molecular mass markers: *a*, *t* successive lambda concatamers, *Arrow* at the *left* points to the 49-kpb lambda monomer; *b*, *s* lambda *Hin*dIII (the four bands observed are: 23.1, 9.4, 6.5 and 4.3 kbp)

observed for each would be stable when grown in the absence of chloramphenicol. Amplificant strains were grown in the presence or absence of chloramphenicol for 25 generations. The cultures were then processed for the analysis of amplicon size by PFGE. The results for some of the strains analyzed are shown in Fig. 3. Although a practically identical pattern is observed when comparing the digested DNA obtained from the strains grown under the two conditions, analyzed by ethidium bromide (Fig. 3A) as well as by Southern blotting (Fig. 3B), a few differences are observed. For instance, for strain JC50-12 (lanes p and q in Fig. 3A, B) the band calculated to be 10-AU copies (see Fig. 2, lane o) seems to be lost after growth in the absence of antibiotic. For strain JC30-2 (lanes i and j in Fig. 3A, B) two faint bands larger than 18 AU almost disappear. Taken together though, these results suggest that maintaining selective pressure is not necessary to conserve a given amplicon copy number. In order to test how stable amplified structures could be during even longer generation times, we analyzed strain JC15-42 grown for 100 generation times. Figure 3C, D (lanes e and f) shows the results of this experiment. Again the 10-AU size in strain JC15-42 is as it was after 6 generations (lane g, Fig. 2), regardless of the addition of antibiotic to the growth medium. A single-copy-number amplicon contained by strain JC5 is also maintained over time (lanes c and d, Fig. 3C, D).

Discussion

From our work in the laboratory with integrational vectors we have seen variations in the size of the amplification units within strains when analyzed by PFGE/Southern blotting. Since the level of amplification is often determined by densitometry of a fragment generated within the amplification unit, which does not reveal such variability, only the average copy number of the amplification unit in the population is quantified. For instance, in a 10-AU strain, half of the population may carry a 5-AU structure and the other half a 15-AU structure. Since however, amplification has been shown to be quite stable, for some applications, this fluctuation may not be important. It may be important when used as a tool for genetic analysis, for instance when used to determine the effect of increasing copy number on the expression of a gene. In this case a heterologous population may give confusing results.

In this work we wanted to determine how stable is an AU of any given size, and whether it shows variation in the absence of antibiotic and over time. We therefore obtained and analyzed several strains carrying an integrational vector and selected for resistance to

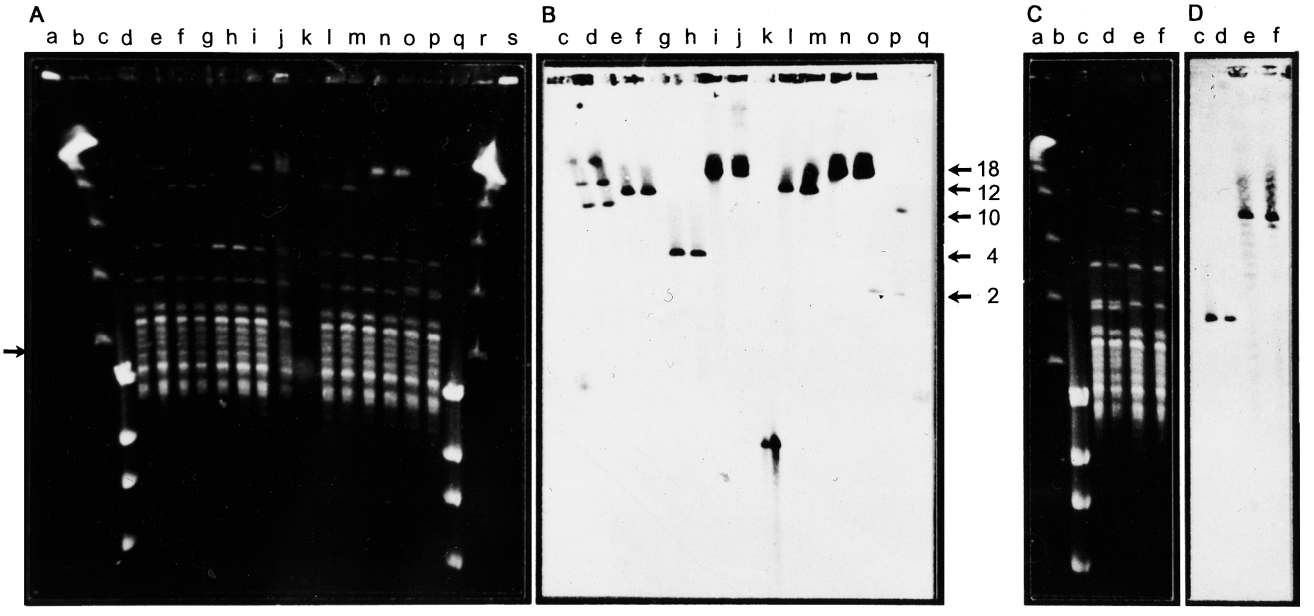

Fig. 3A–D Analysis of the stability of the amplified integrated plasmids. Strains resistant to different chloramphenicol levels were cultured with and without chloramphenicol for 25 (**A**, **B**) and 100 (**C**, **D**) generations. **A**, **C** Ethidium-bromide-stained gels; **B**, **D**, their corresponding Southern-blot hybridizations with digoxigenin-labeled pCV5. **A**, **B** Lanes: *c*, *d* JC15-31; *e*, *f* JC20-12; *g*, *h* JC20-32; *i*, *j* JC30-2; *k* linearized pCV5; *l*, *m* 40-12; *n*, *o* JC50-11; *p*, *q* JC50-12. Samples in lanes *c*, *e*, *g*, *i*, *l*, *n*, *p* are from cells cultured with no chloramphenicol and samples in lanes *d*, *f*, *h*, *j*, *m*, *o*, *q* are from cells grown with antibiotic. **C**, **D** Lanes: *c*, *d* JC5-0; *e*, *f* JC15-42. Samples in lanes *c* and *e* are from cells cultured with chloramphenicol and samples in lanes *d* and *f* are from cells grown with no antibiotic. Molecular mass markers are as in Fig. 2 (lanes *a*, *b*, *r*, *s* in **A** and *a*, *b* in **C**)

different chloramphenicol levels. The selection procedure for amplification was basically as in previous reports (Piggott et al. 1988; Jannière et al. 1985) except that, for some unknown reason, a high background of non-amplificants was obtained. Additionally, we observed that not only was it easier to obtain amplificant strains in liquid cultures as compared to solid medium, but a higher resistance level was achieved. This level of chloramphenicol resistance (50 μg/m) is like that reported by Jannière et al. (1985). The copy number of the amplicons among the strains we analyzed that carried an amplified plasmid ranged typically from 2 to 18 although we would occasionally observe bands of up to 34 copies. We are not certain whether several strains actually exhibited a 34-AU size or if this size represented the resolution limit on the gels.

Amplified strains can be manipulated; if it is of interest to work with strains carrying a single size structure, it is possible to start from a strain carrying several structures and select out single cells to obtain a clonal population. We have prepared competent cells from strains containing 2 or more AU of different size and selected transformants that now carry a single amplified structure (data not shown).

We found that, for each strain analyzed carrying from one to three AU of different size, these were stably maintained in the presence and absence of antibiotic and through 25 generations and even 100 generations. Additionally, a strain carrying a single copy of the integrated plasmid exhibited no detectable amplification after being grown for 100 generations. Thus, our results not only confirm that amplification is maintained in the chromosome of *B. subtilis* but also show that AU of different size are very stable.

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