Plasmid maintenance in *Bacillus stearothermophilus* is strain-dependent

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SUMMARY

We studied the segregational stability of plasmids based on pTB913, a 4.5-kb rolling-circle plasmid derived from the thermophilic *Bacillus* plasmid pTB19. In *Bacillus stearothermophilus* the stability of pTB913 derivatives appeared to be strain-dependent. In strain CU21 large amounts of single-stranded pTB913 DNA were found and the plasmid was highly unstable at 57°C. In strain NUB3621, however, very low amounts of single-stranded plasmid DNA were formed and pTB913-based replicons were only slightly unstable at 57°C. The NUB3621/pTB913 host-vector system seems appropriate for molecular cloning. A RepA-based replicon, also derived from pTB19 but replicating by a theta mechanism, was highly unstable in *B. stearothermophilus* NUB3621.

2. INTRODUCTION

The stable maintenance of a plasmid in a bacterial cell is of prime importance in the development of efficient cloning vectors. Loss of recombinant plasmids has, however, frequently been observed in bacilli (for reviews see [1,2]). Plasmid maintenance is governed by at least three factors: replication, partitioning and differences in growth rate between plasmid-free and plasmid-harbouring cells. Most small plasmids used for cloning in Gram-positive bacteria generate single-stranded DNA (ssDNA) intermediates by rolling-circle replication (RCR), which is considered to decrease the levels of structural and segregational plasmid stability [1–3]. Such plasmids do not contain known partitioning functions and are believed to segregate randomly among daughter cells, after which the copy number is restored through replication control mechanisms. As a consequence, the segregational stability of these plasmids will at least in part depend on plasmid copy numbers.
Extremophilic bacilli, such as thermophiles, have a high potential for fundamental and applied use. Their use requires the availability of efficient and stable cloning systems. So far, only a few studies on plasmid stability in thermophilic bacilli have been reported [4–8]. In these studies, plasmid maintenance was not tested for more than 20–25 generations, which is too short for many applications. Plasmid maintenance appeared to be highly dependent on the growth temperature. For instance, pUB110 was rapidly lost from cultures grown at temperatures exceeding 55°C [4–7]. In one study, this was thought to result from the loss of DNA superhelicity [6]. In another study [7], thermosensitivity of kanamycin nucleotidyltransferase, the pUB110-encoded enzyme causing resistance to kanamycin, was held responsible. Segregational plasmid instability might also result from a decrease in copy number at higher temperatures, as was suggested from studies involving pTHT15 [8].

pTB19 is a 26.5-kb composite plasmid isolated from a thermophilic Bacillus [9]. It consists of two inactive RCR plasmids [10–12] and a RepA replication determinant which probably confers theta replication [13]. One of the integrated plasmids, pTB913 (4.5 kb), can be excised from pTB19 [9] and can replicate autonomously by a rolling-circle mechanism [10]. pTB913 appeared to be very similar to pUB110, a well-known plasmid from Staphylococcus aureus [10,14,15]. Since pTB19 was derived from a thermophilic Bacillus, we analysed in the present studies the properties of pTB913 and the RepA replication determinant as potential cloning vectors for thermophilic bacilli, in particular Bacillus stearothermophilus strains CU21 and NUB3621. Analogous studies have been carried out by Zhang et al. [7] using B. stearothermophilus strain SIC1.

3. MATERIALS AND METHODS

3.1. Bacterial strains, plasmids and media

Strains and plasmids are listed in Table 1. LB medium was according to Maniatis et al. [16]. B. stearothermophilus NUB3621 was grown in supplemented LB medium and plated on MPM agar [8,17]. Solid media contained either 1.5% agar for the plating of cells, or 0.8% agar for the regeneration of protoplasts. Kanamycin (Km), phleomycin (Phleo) and chloramphenicol (Cm) were used at final concentrations of 8, 20 and 8 μg/ml, respectively, unless stated otherwise.

Table 1

<table>
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<tr>
<th>Bacterial strains and plasmids</th>
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<td><strong>Properties</strong></td>
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<tr>
<td>Strains</td>
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<td>B. stearothermophilus CU21</td>
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<td>B. stearothermophilus NUB3621</td>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pTB913</td>
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<tr>
<td>pTB913-1C</td>
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<tr>
<td>pTB3</td>
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<tr>
<td>pTB3-1C</td>
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<td>pTB19C</td>
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<td>pUC7C</td>
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Table I

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<th>Strains or reference</th>
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<tr>
<td>Bacterial strains and plasmids</td>
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<tr>
<td>SmR, spontaneously cured of plasmid pBS01</td>
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<tr>
<td>RifR, Hsr-, Hsm-</td>
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<tr>
<td>RepB, PhleoR, 5.8 kb, pTB913 containing a random 1.3-kb E. coli DNA fragment, denoted 1C</td>
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<tr>
<td>RepB, KmR, PhleoR, ΔpalU, 4.3 kb</td>
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<tr>
<td>RepB, PhleoR, 5.6 kb, pTB3 containing the 1.3 kb 1C fragment</td>
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<td>RepA determinant of pTB19 on an EcoRI fragment ligated to the cat gene of pUC7C</td>
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<tr>
<td>pUC7 containing the cat gene of pC194 in its PstI site</td>
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3.2. Transformation of *B. stearothermophilus*

Protoplasts of *B. stearothermophilus* NUB3621 were prepared and transformed as described by Wu and Welker [8], except that whole cells and protoplasts were collected by centrifugation at 7500 × g and 4000 × g. Protoplasts of *B. stearothermophilus* CU21 were prepared and transformed as described previously [4]. Regeneration plates contained 50 μg/ml of Km.

3.3. Isolation and characterization of total DNA

Total DNA lysates were prepared as described by Bron [1]. After electrophoresis of 10-μl samples in 0.6% agarose gels, DNA was transferred to GeneScreen Plus filters by Southern blotting [18]. Probe labelling, DNA hybridization conditions and washing steps were performed using the ECL gene detection system (Amersham International plc, Amersham, UK).

3.4. Assays of segregational plasmid stabilities

Stability assays were performed in liquid cultures as described by Bron and Luxen [19]. Usually, the segregation kinetics of plasmid-free cells were followed during 100 generations of growth under non-selective conditions. *B. stearothermophilus* NUB3621 cultures were diluted in MPM media [17] and grown on MPM plates containing 1.5% agar.

4. RESULTS AND DISCUSSION

4.1. pTB913 derivatives and their introduction into *B. stearothermophilus*

Like other RCR plasmids, pTB913 contains a minus origin (MO) of replication, denoted as palU [10], which serves as the initiation site for complementary strand synthesis. In general, the absence of a functional MO in RCR plasmids results in the accumulation of ssDNA plasmid forms [3]. The removal of palU from pTB913 resulted in pTB3 [10]. To examine the effect of plasmid size and the MO on the segregational stability, we cloned the 1.3-kb *Escherichia coli* 1C DNA fragment [19] into the unique BglII-site of

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**Fig. 1.** Segregational stability and plasmid pattern of pTB913 in *B. stearothermophilus* CU21. Panel A shows the segregational stability at 57°C; panel B the plasmid patterns in total DNA lysates at 48°C (a) and 57°C (b), each sample without (−) or after (+) treatment with endonuclease S1. Plasmid pTB913 DNA was used as a probe in the hybridizations. The positions of ssDNA (ss) and high-molecular mass (HMW) DNA are indicated.
pTB913 and pTB3. The resulting plasmids were designated pTB913-1C and pTB3-1C. These plasmids could all be introduced into *B. stearothermophilus* NUB3621. We were also able to introduce pTB913 in *B. stearothermophilus* CU21, but did not succeed in obtaining transformants with the other three plasmids. *B. stearothermophilus* NUB3621 was also transformed with plasmid pTB19C, which is based on the theta type RepA replicon of pTB19. As shown before [9], strain CU21 could not be transformed with plasmids based on the RepA replication determinant of pTB19.

### 4.2. Segregational stabilities and plasmid patterns in *B. stearothermophilus* CU21

The segregational stability of the parental plasmid pTB913 in strain CU21 was tested at 48°C (the lowest temperature at which this strain can grow) and at 57°C. The results obtained at 57°C are shown in Fig. 1A; similar results were obtained at 48°C (data not shown). It is clear that pTB913 segregated very rapidly from the cell population: only about 1% of the cells contained the plasmid after 20 generations of growth. This renders this host/vector system unattractive for molecular cloning. In addition, the presence of pTB913 appeared to reduce the growth rates of CU21 cells considerably. To test whether the instability was associated with the accumulation of either ssDNA replication intermediates or high-molecular mass plasmid DNA forms, as has been observed with other RCR plasmids in *Bacillus subtilis* [3], the plasmid patterns in total DNA lysates were studied. The results (Fig. 1B) showed that CU21 cells containing pTB913 produced large amounts of ssDNA, both at 48°C and 57°C: densitometric scanning of photographic negatives indicated that more than 40% of all plasmid molecules were in the single-stranded form (data not shown). This indicates that *palU* functioned poorly in the CU21 strain. Since *palU* was functional in *B. subtilis* at 47°C [21], it appeared that *palU* activity was dependent on the host rather than on the temperature. As suggested for other RCR plasmids [1,20], the absence of a functional MO and the accumulation of ssDNA may well account for the high levels of plasmid instability observed with this host/vector system. Low amounts of plasmid DNA were also present at positions which could contain high-molecular mass DNA.

### 4.3. Plasmid patterns in *B. stearothermophilus* NUB3621

In an attempt to search for *B. stearothermophilus* strains that would maintain pTB913 derivatives more stably, we assayed strain NUB3621 [17]. Analysis of total DNA lysates (Fig. 2) revealed that NUB3621 cells containing pTB913 or pTB913-1C did not produce detectable amounts of ssDNA at 57°C. As was to be expected, some ssDNA (10–15% of the plasmid molecules) was present in the absence of the MO (cells containing pTB3; Fig. 2, lane C). Therefore, in contrast to the situation in strain CU21, *palU* appeared to be functional in *B. stearother-
mophilus NUB3621, indicating that palU activity in B. stearothermophilus was strain-dependent.

4.4. Segregational plasmid stabilities in B. stearothermophilus NUB3621

Assays of the segregational stability of pTB913 clearly indicated that its maintenance in strain NUB3621 was superior to that in strain CU21 (compare Figs. 3 and 1A). This is presumably due to the more efficient conversion of single-stranded replication intermediates in the former strain. The insertion of the 1.3-kb 1C E. coli DNA fragment in pTB913 (plasmid pTB913-1C) somewhat reduced the stability. This decrease in stability upon enlarging the plasmid is in accordance with results obtained previously with B. subtilis and may well be due to a decrease in the copy number of the circular plasmid forms [19–21]. In the present study we also observed (data not shown) that the copy numbers (per chromosome equivalent) of the circular plasmid forms were reduced from about 75 (pTB913) to about 50 (pTB913-1C). The insertion of the 1C fragment also resulted in relatively large amounts of plasmid DNA at the high-molecular mass DNA position. In analogy with what has been speculated for other RCR plasmids [22,23], we speculate that the formation of the high-molecular mass DNA may interfere with plasmid stability in this system. Even with the recombinant pTB913-1C plasmid the levels of instability were relatively low: more than 90% of the cells still contained the plasmid after 40 generations of growth under non-selective conditions. This indicates that the NUB3621/pTB913 host/vector pair is suitable for cloning, at least of relatively small DNA fragments. At least one other B. stearothermophilus strain (SIC1) was reported to maintain pTB913 relatively stable at 60°C [7]. Since this strain was not available, it could not be tested in the present experiments. A further advantage of NUB3621 is that this strain is deficient in restriction and, therefore, a good host for foreign DNA.

Unexpectedly, the levels of instability were increased only very slightly when palU was removed (pTB3 and pTB3-1C). We attribute this marginal effect to the fact that even in the absence of the MO the levels of ssDNA accumulation were not high (10–15% of the plasmid molecules). Apparently, these amounts of ssDNA were not harmful to NUB3621 cells.

Plasmid pTB19C, based on the RepA replication determinant of pTB19, is considered to replicate by a theta mechanism and does not produce single-stranded plasmid DNA [13]. Moreover, in B. subtilis, RepA-based replicons are structurally highly stable and allow the cloning of large DNA inserts [13]. The results presented in Fig. 3 show that pTB19C was segregationally highly unstable in B. stearothermophilus NUB3621. This plasmid is, therefore, less suited than pTB913 as a cloning vector in strain NUB3621. The reasons for the instability of pTB19C in strain NUB3621 are not clear. Instability of RepA-based replicons was also observed in strain SIC1 at 60°C [7].

In conclusion, the results presented in this study show that the maintenance of the rolling-circle plasmid pTB913 in B. stearothermophilus is strain-dependent. Even in the absence of selective pressure, this plasmid is relatively stably maintained in strain NUB3621. The NUB3621/pTB913 host/vector pair is suitable for cloning.
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