Efficient cloning in *Bacillus megaterium*: comparison to *Bacillus subtilis* and *Escherichia coli* cloning hosts

Michael A. Von Tersch * and Helen Loidl Robbins

Ecogen Inc., Langhorne, PA, U.S.A.

Received 23 February 1990
Revision received 9 April 1990
Accepted 20 April 1990

Key words: Quantitative cloning; Transformation; Plasmid selection; Plasmid stability

1. SUMMARY

Quantitative cloning efficiencies for *B. megaterium*, *B. subtilis*, and *E. coli* were compared. Transformation of *B. megaterium* is less efficient than transformation of *B. subtilis* or *E. coli*. The frequency of recombinant clones was equal in *E. coli* and *B. megaterium*; both somewhat higher than in *B. subtilis*. Equivalent average insert sizes were found in *B. megaterium* and *E. coli* clones, but significantly smaller inserts were obtained in *B. subtilis* clones. Clones obtained and propagated in *B. megaterium* were structurally stable when grown under plasmid selection.

2. INTRODUCTION

*Bacillus megaterium* has been used extensively for studies of bacterial sporulation and germination, and industrial interest in this organism has focused on strains that produce vitamins, amino acids, and commercially valuable enzymes. Cloning of genes from *B. megaterium* has typically involved initial isolation in *E. coli* [1–9] or *B. subtilis* [10], and in some cases, subsequent introduction into *B. megaterium* [7,10,11]. Primary gene isolation in *B. megaterium* [12,13] became feasible with the development of plasmid-free host strains [13,14] and efficient protoplast transformation systems [15,16]. *B. megaterium* has been employed for expression studies of heterologous *B. thuringiensis* δ-endotoxin genes [9,17] that are in some cases poorly maintained (M. Von Tersch and A. Macaluso, unpublished) or poorly expressed in *B. subtilis* [9]. Difficulties relating to molecular cloning in *B. subtilis* are well known and extensively studied [18–21].

To study the potential of *B. megaterium* as a possible alternative host to *B. subtilis* for primary gene isolation, we quantitatively compared cloning efficiencies in *B. megaterium*, *B. subtilis*, and *E. coli* and analyzed the properties of recombinant plasmids isolated in each host.

3. MATERIALS AND METHODS

3.1. Bacterial strains

*B. megaterium* VT1660 has been described [13]. *E. coli* HB101 [22] was obtained from Life Technologies Inc. Gaithersburg, Maryland. *Bacillus*
**subtilis** BD170 [23] was kindly provided by D. Dubnau. *B. thuringiensis* var. *kenyae* strain EG2170 was kindly provided by H. Dulmage.

### 3.2. Enzymes and reagents

Restriction enzymes and T4 DNA ligase were obtained from Life Technologies Inc., Gaithersburg, MD, and were used under recommended conditions. Agarose, egg white lysozyme, and antibiotics were from Sigma Chemical Co., St. Louis, MO.

### 3.3. Construction of *E. coli, Bacillus* shuttle plasmid pEG7

Shuttle vector plasmid pEG7 (Fig. 1) was constructed by ligation of *Bacillus* plasmid pNN101 [24] and *E. coli* plasmid pBR322 [25] at unique *BamHI* sites. Plasmid pEG7 encodes ampicillin resistance (Ap'), tetracycline resistance (Tc'), and chloramphenicol resistance (Cm') in *E. coli*, Tc' and Cm' in *Bacillus* hosts, and contains a unique *HpaI* site within the tetracycline resistance gene that can be insertionally inactivated in all three host systems.

### 3.4. Transformation of *E. coli* and *B. subtilis

Competent *E. coli* HB101 were obtained from Life Technologies Inc., Gaithersburg, MD, and transformed with an aliquot of the ligation reaction containing 0.5 ng vector pEG7 as described by the supplier. *Bacillus subtilis* BD170 protoplasts were transformed with 0.2 µg of ligated pEG7 by the method of Chang and Cohen [26].

### 3.5. Transformation of *B. megaterium*

*B. megaterium* VT1660 was transformed with 0.2 µg of ligated pEG7 essentially as described [27], with the following modifications. Cells were grown at 30°C, 200 rpm until a Klett-Summerson turbidity reading of 15 (red filter) was obtained. The concentration of lysozyme was reduced to 25 µg per ml during protoplasting, and 200 µl of washed protoplasts were used per transformation. The stock solution of 30% polyethylene glycol (PEG), average molecular mass 8000, was freshly prepared in RHAF broth [27] then filtered, prior to use. Regeneration efficiencies were determined by plating transformed protoplasts on RHAF plates. To maximize the transformation levels, protoplasts were plated on RHAF plates containing minimally selective levels of antibiotics (1.0 µg per ml chloramphenicol or 5.0 µg per ml tetracycline). Putative transformants were confirmed by growth on NSM [28] plates containing 10 µg per ml of chloramphenicol or tetracycline.

### 3.6. Size determinations of plasmids

Plasmids present in 50 randomly-chosen Tc' transformants of each host were examined by electrophoresis in 0.5% agarose gels after lysis of cells in the wells of the gels (Eckhardt lysates) [27]. Size determinations were made by comparison to the mobilities of plasmids from *B. megaterium* strain VT1405 [27]. VT1405 contains 5 plasmids that have been previously sized by electron microscopy, agarose gel mobility, and for some marker plasmids, restriction analysis.

### 3.7. Stability of recombinant and vector plasmids

To assess both segregational and structural stability of recombinant plasmids isolated and propagated in each background, five different pEG7 recombinant clones of each host, the host strains with pEG7, and the *Bacillus* strains with pNN101 were inoculated 1:100 into 2.0 ml of LB [29] (*E. coli*) or NSM (*Bacillus* hosts) broth with and without antibiotic selection for the plasmid (50 µg/ml ampicillin for *E. coli* and 5.0 µg/ml chloramphenicol for *Bacillus*). After overnight growth the cultures were serially diluted 1:100 into the same media for two additional subculturings, then streaked for single colonies. Colonies isolated after growth without antibiotic were tested for growth on LB plates with ampicillin (*E. coli*) or NSM plates with chloramphenicol (*Bacillus* hosts) to determine the degree of plasmid curing. Ten or twelve colonies isolated from each of five recombinant pEG7 derivatives after growth in broth containing antibiotic were examined on Eckhardt gels to assess gross plasmid structural stability.

### 4. RESULTS AND DISCUSSION

#### 4.1. Comparative cloning efficiencies in *E. coli, B. megaterium* and *B. subtilis*

We constructed plasmid pEG7 (Fig. 1) then ligated *HpaI*-cleaved genomic DNA from *B.
Table 1
Comparative transformation and cloning efficiencies in *E. coli*, *B. megaterium* and *B. subtilis*

<table>
<thead>
<tr>
<th>Host</th>
<th>Transforming DNA</th>
<th>Ap⁺ or Cm⁺ transforms/μg pEG7</th>
<th>Fraction Tc⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>pEG7</td>
<td>1.9 × 10⁷</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pEG7 ligation mixture</td>
<td>2.5 × 10⁶</td>
<td>0.18</td>
</tr>
<tr>
<td><em>B. megaterium</em></td>
<td>pEG7</td>
<td>6.0 × 10⁸</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pEG7 ligation mixture</td>
<td>1.3 × 10⁷</td>
<td>0.16</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>pEG7</td>
<td>2.5 × 10⁸</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>pEG7 ligation mixture</td>
<td>7.1 × 10⁷</td>
<td></td>
</tr>
</tbody>
</table>

* Ligation product of *HpaI*-cleaved *pEG7* and *HpaI*-cleaved genome DNA from *B. thuringiensis* var. *kennae*.

*thuringiensis* EG2170 to the unique *HpaI* site of pEG7. Each host was transformed with an aliquot of the ligation reaction and an equivalent amount of undigested pEG7. Transformed cells of *E. coli* were recovered by Ap⁺ selection and transformed *Bacillus* cells were recovered by Cm⁺ selection. Inactivation of the tetracycline resistance gene was used to estimate the percent of recombinant clones recovered in each host. The results (Table 1) indicated that *B. megaterium* was transformed at 4 to 5 times lower efficiency than *B. subtilis* for both the ligation product and native pEG7. The transformation efficiency of *E. coli* was significantly higher than for either *B. megaterium* or *B. subtilis*, although the actual number of transformants was higher in the *Bacillus* hosts because more DNA was used in the *Bacillus* transformations. The percent of tetracycline-sensitive recombinant clones was about the same for *E. coli* and *B. megaterium* and somewhat lower for *B. subtilis*.

Table 2
Analysis of plasmids in tetracycline sensitive transformants of *E. coli*, *B. megaterium* and *B. subtilis* after transformation with pEG7 ligation mixture

<table>
<thead>
<tr>
<th>Host</th>
<th>No. of transformants</th>
<th>Average insert size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>35</td>
<td>3.2</td>
</tr>
<tr>
<td><em>B. megaterium</em></td>
<td>29</td>
<td>3.0</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>32</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Estimated by subtracting the size of pEG7 (10 kb) from the average sizes of plasmids larger than pEG7.

Analysis of plasmids in transformed clones (Table 2) indicated that each host yielded approximately equal numbers of recombinant plasmids larger than pEG7. The average size of recombinant plasmids was similar in *B. megaterium* and *E. coli*, but significantly smaller in *B. subtilis*. To confirm the size estimates of insert fragments deduced from Eckhardt gels, we prepared miniprep plasmid DNA from 23 of the 29 Tc⁺ *B. megaterium* pEG7 recombinants shown in Table 2. We digested the plasmids with *HpaI*, then measured the sizes of insert fragments by comparison of their mobilities in 0.7% agarose gels to *HindIII* digested Lambda fragments. The average insert fragment found in pEG7 recombinant plasmids was 2.9 kb by restriction analysis. By Eckhardt gel analysis this group of 23 clones showed an average insert size of 3.0 kb. We therefore concluded that Eckhardt gels yielded accurate estimates of the sizes of plasmids recovered in each host. The range of insert sizes was the same for both *B. megaterium* and *B. subtilis*, from 0.3 to 6.3 kb, but 5 of 29 *B. megaterium* recombinants contained inserts larger than 5.0 kb, while only 1 of 32 *B.

---

*Fig. 1. E. coli, Bacillus* shuttle vector plasmid pEG7 (16.6 kb). The bold line indicates DNA from pBR322 and the light line indicates DNA from pNN101. Restriction endonuclease cleavage sites are abbreviated as follows: A. *Aval*; B. *BamHI*; C. *ClaI*; D. *PstI*; E. *EcoRI*; F. *Scal*; G. *SphI*; H. *HpaII*; J. *BstEII*; K. *EcoRV*; L. *SulI*; M. *HpaI*; N. *NcoI*; O. *HaeIII*; P. *PstII*; S. *SspI*; U. *NruI*; X. *XbaI*. 
**E. coli** recombinants contained an insert larger than 5.0 kb. The inserts among the *E. coli* recombinants ranged from 0.2 to 6.7 kb except for one plasmid with an insert of 9.7 kb. Eight of 35 *E. coli* recombinants contained inserts greater than 5.0 kb in size.

We also observed some plasmids equal in size or slightly smaller than pEG7 in each of the three hosts. Restriction analysis of some of these plasmids from *B. megaterium* showed loss of the *Hpa* site or small deletions encompassing the *Hpa* site. This suggests that the ligation mixture contained some vector pEG7 plasmids that were damaged at the *Hpa* ends at some point in the cloning experiments.

Bron et al. [20] have hypothesized that a strong bias against the recovery in *B. subtilis* of recombinant clones with inserts greater than 3.0 kb may be a consequence of the increased segregational instability of pUB110-based plasmids with large inserts. Because pBC16 (the parent *Bacillus* replicon in pNN101 and pEG7) is highly homologous to and incompatible with pUB110 [30], a similar mechanism may account for the significantly smaller inserts in pEG7 clones in *B. subtilis*. In addition, host restriction has also been implicated in the poor recovery of clones with large inserts in *B. subtilis* [21], and may also contribute to the results for pEG7 clones. In contrast, our results do not show a significant bias against recovery of large inserts in pEG7 in *B. megaterium* compared to *E. coli*.

### 4.2. Stability studies of plasmids recovered and propagated in each host

Segregational stability studies showed that recombinant pEG7 derivatives, isolated and propagated in *E. coli*, are stable when grown without plasmid selection. In contrast, we found that recombinant pEG7 derivatives, isolated and propagated in either *Bacillus* host, as well as shuttle vector plasmid pEG7, were extensively cured (> 90%) when grown without plasmid selection. *Bacillus* plasmid pNN101, however, was not cured (< 2% loss) in either *B. megaterium* or *B. subtilis*. These data indicated that the segregational instability of recombinant pEG7 derivatives in both *Bacillus* hosts may be due to the presence of pBR322 sequences, rather than heterologous inserts in pEG7. Further, we have observed that other heterologous inserts into the *Bam*H1 site of pNN101 are not cured in *B. megaterium* hosts in the absence of selective pressure (M. Von Tersch, unpublished).

We found no evidence of structural instability for pEG7 recombinants isolated and propagated both in *E. coli* and in *B. megaterium* when cultured under plasmid selection. Most *B. subtilis* pEG7 recombinants were also structurally stable when grown under plasmid selection, although one of the five isolates yielded some deletion derivatives. Structural instability of recombinant plasmids in *B. subtilis* has been well documented [18].

Our results demonstrated that clone banks prepared in *B. megaterium* compare favorably to those prepared in *E. coli* and in some cases may afford significant advantages over banks prepared in *B. subtilis*. *B. megaterium* may be particularly appropriate for cloning strategies that involve the isolation of larger fragments or that involve developmentally regulated genes that may be poorly expressed when cloned in high copy in *E. subtilis* [9].

**ACKNOWLEDGEMENTS**

We thank M. Cynthia Gawron-Burke and Anthony Macaluso for helpful discussions and careful review of the manuscript. We thank William F. Burke Jr. for providing plasmid pNN101. This work was supported in part by Small Business Innovative Research Award ISI 8560276 from the National Science Foundation.

**REFERENCES**