An improved β-galactosidase α-complementation system for molecular cloning in Bacillus subtilis

(Recombinant DNA; translation efficiency; chromosomal integration; lacZ gene; structural plasmid stability)

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SUMMARY

The recently described β-galactosidase α-complementation system for molecular cloning in Bacillus subtilis [Haima et al., Gene 86 (1990) 63-69] was optimized in several ways. First, the efficiency of translation of the lacZΔM15 gene was improved. Second, the plasmid-borne lacZΔM15 gene was segregationally stabilized by integration into the B. subtilis chromosome. Third, a new lacZα-complementing cloning vector was constructed, containing more unique target sites. It was shown that large heterologous DNA fragments (up to at least 29 kb) could be cloned with lacZα-complementing vectors carrying the replication functions of the cryptic B. subtilis plasmid pTA1060, and that these inserts were structurally stably maintained for at least 100 generations of growth.

INTRODUCTION

Since natural plasmids in Bacillus subtilis are usually cryptic, molecular cloning in this organism has mainly been carried out with high-copy-number plasmids from Staphylococcus aureus, such as pC194 (CmR), pE194 (ErR) and pUB110 (KmR). Although vectors based on such plasmids have successfully been used in some cases, frequently observed problems are structural and segregational instability (Ehrlich et al., 1986; Bron et al., 1985; 1988), and low efficiencies obtained in random cloning of foreign DNA. The latter is manifested in low numbers of clones and limited sizes of the DNA inserts (Michel et al., 1980; Gryczan et al., 1982; Jannière et al., 1990). Recently, it was postulated that the inferior cloning properties of the staphylococcal plasmids might be a consequence of their mode of replication (Jannière et al., 1990); all these plasmids appear to replicate via ssDNA intermediates, probably by rolling-circle replication (for a review, see Gruss and Ehrlich, 1989). Plasmids based on the ssDNA-generating replicon pC194 appeared to be structurally unstable and showed inferior cloning properties as compared to plasmids which replicate without generating ssDNA, like pAMβ1 (Jannière et al., 1990).

We recently described cloning vectors based on the B. subtilis low-copy-number plasmid pTA1060 (Uozumi et al., 1980), which showed important advantages in comparison to the commonly used staphylococcal plasmids (Bron et al., 1987; Haima et al., 1987). First, it was shown that the segregational stability of pTA1060-derived repli-
cons was hardly affected by plasmid size (Bron et al., 1987). In contrast, the S. aureus plasmid pUB110 showed size-dependent effects on copy numbers (Bron et al., 1985; 1988) resulting in dramatic levels of segregational instability. Second, pTA1060-derived vectors showed superior cloning efficiencies as compared to the staphylococcal plasmids (Haima et al., 1987).

In a follow-up study we extended the applicability of the pTA1060-derived vectors by developing a βGal α-complementation cloning system for B. subtilis (Haima et al., 1990). This system was based on two unrelated compatible plasmids. The host contained the pWVO1-based (Kok et al., 1984) plasmid pGHS1, which carries the constitutively expressed lacZΔM15 gene. The cloning vector was the pTA1060-based plasmid pHPS9, which carries a constitutively expressed translational fusion of the Bacillus

Fig. 2. Structural stability of recombinant pHPS9 plasmids. Cultures started from single colonies of B. subtilis 6GM15 containing recombinant pHPS9 plasmids (pH DNA) were grown overnight at 37°C in 10 ml of Cm-containing TY medium. The cultures were diluted 10^5- to 10^6-fold in the same medium after every cycle of approximately 20 generations until approximately 100 generations of growth. Plasmid DNAs extracted from the overnight cultures at the start (a) and after 100 generations of growth (b) were analyzed by BamHI digestion and 0.8% agarose/EtBr gel electrophoresis. Numbers 7.2, 29.1 and 16.8 correspond to the total size of the DNA inserts (kb). Note that the 29.1-kb insert consists of two BamHI fragments of 16.8 and 12.3 kb. BamHI-cleaved λ DNA served as reference markers (lane R; fragment sizes are indicated in kb on the left margin).

(Fig. 1)
This cloning system offered: (1) the direct selection of recombinants; (2) the cloning of large heterologous DNA fragments with high efficiency; and (3) the availability of six unique target sites: SphI, NdeI, NheI, BamHI, SmaI and EcoRI.

The aim of the present studies was to improve the βGal α-complementation cloning system in two ways: to stably integrate the lacZΔM15 gene into the B. subtilis chromosome and to improve the efficiency of translation of this gene.

RESULTS AND DISCUSSION

(a) Integration of the lacZΔM15 gene into the Bacillus subtilis chromosome

A drawback of the previously described βGal α-complementation system (Haima et al., 1989) was the segregational instability of the lacZΔM15-carrying plasmid pGHS1, which is based on the lactococcal plasmid pWVO1 (Kok et al., 1984). Plasmid pGHS1 appeared to be extremely unstable during competence development of B. subtilis, resulting in about 97% plasmid-free cells at the state

TABLE 1

Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant properties</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>B. subtilis</td>
<td>trpC2 tyr met his ura rib r64 m44 lacZΔM15 KmR</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>araD139_d(araABC-leu)7679 galU galK lacX74 rpsL shi supE shi d(lac-proAB) [F' traD36 proA+B+ lacIΔZΔM15]</td>
<td></td>
</tr>
<tr>
<td>M13mp19</td>
<td>lacZα</td>
<td></td>
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<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHSG575</td>
<td>CmR, lacZα, derivative of pSC101</td>
<td></td>
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<tr>
<td>pKBU4</td>
<td>CmR, derivative of pHSG575 carrying a 7.5-kb B. subtilis chromosomal fragment</td>
<td></td>
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<tr>
<td>pKBU5</td>
<td>CmR, KmR, lacZΔM15, derivative of pKBU4</td>
<td></td>
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<tr>
<td>pKBU5M</td>
<td>CmR, KmR, derivative of pKBU5 carrying a modified -20 to +33 lacZΔM15 region</td>
<td></td>
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<tr>
<td>pKBU6</td>
<td>CmR, KmR, derivative of pKBU5M carrying the modified lacZΔM15 gene preceded by the lactococcal promoter P23</td>
<td></td>
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<tr>
<td>pKBU7</td>
<td>CmR, KmR, derivative of pKBU5M carrying the modified lacZΔM15 gene preceded by the B. subtilis phage SPO2 promoter</td>
<td></td>
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<tr>
<td>pGHS1</td>
<td>KmR, lacZΔM15, derivative of pWVO1</td>
<td></td>
</tr>
<tr>
<td>pLSG1</td>
<td>KmR, lacZΔM15, pSC101 derivative</td>
<td></td>
</tr>
<tr>
<td>pHPl3-2</td>
<td>CmR, EρR, pTA1060-pUC9 derivative</td>
<td></td>
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<tr>
<td>pHPS9</td>
<td>CmR, EρR, cat-86::lacZα, pTA1060-pUC9 derivative</td>
<td></td>
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<tr>
<td>pHPS10</td>
<td>ErR, cat-86::lacZα, derivative of pHPl3-2</td>
<td></td>
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<tr>
<td>pGKH1</td>
<td>ErR, cat-86::lacZα, pWVO1 derivative</td>
<td></td>
</tr>
<tr>
<td>pGKH2</td>
<td>ErR, cat-86::lacZα, derivative of pGKH1</td>
<td></td>
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<tr>
<td>pGKV259</td>
<td>ErR, cat-86, pWVO1 derivative</td>
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<tr>
<td>pGKV259-2</td>
<td>ErR, cat-86, derivative of pGKV259 lacking the PstI and SalI sites</td>
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<tr>
<td>pGKV223</td>
<td>ErR, cat-86, pWVO1 derivative</td>
<td></td>
</tr>
<tr>
<td>pGKV11</td>
<td>ErR, cat-86, pWVO1 derivative</td>
<td></td>
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<tr>
<td>pPCT2</td>
<td>ErR, cat-86, PUB110 derivative containing the E. coli r Tes T5, T2 terminators on an EcoRI fragment</td>
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<tr>
<td>pUC9</td>
<td>ApR, pBR322 derivative</td>
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This medium was as described by Biswal et al. (1967). For E. coli, Ap plus Km and Km plus Er were used at 50 μg/ml each. For B. subtilis, Km was used at 10 μg/ml, Cm at 5 μg/ml and Er at 1 μg/ml. XGal was used at 40 μg/ml for E. coli and at 80 μg/ml for B. subtilis. The B. subtilis cells were grown to competence as described by Bron and Venema (1972) and, unless stated otherwise, transformed as described before (Haima et al., 1987). The E. coli cells were made competent and transformed as described before (Haima et al., 1987). When βGal α-complementation in B. subtilis was assayed, transformants were selected at 30°C for 40 h.
of maximal competence. We tried to stabilize the lacZAM15 gene by integrating it, together with a KmR marker gene, via a double cross-over event into the B. subtilis chromosome.

The plasmid used for the integration, pKBU4 (Fig. 1, Table I), which is nonreplicative in B. subtilis, contains a 7.5-kb PstI fragment derived from the B. subtilis chromosome. Sequence analysis of this fragment (J.A.K.W. Kiel, J.M. Boels, G. Baldman, G. and G.V., unpublished) revealed that the BgII site in this fragment starts approximately 2 kb downstream from the rnmB gene cluster (Green et al., 1985), approximately at position 275° on the genetic linkage map of B. subtilis (Piggot and Hoch, 1985). Since it appeared that integration of DNA fragments in this region did not affect essential functions, we decided to target the lacZAM15 gene to this part of the B. subtilis chromosome.

Plasmid pKBU5 (Fig. 1) was constructed by inserting the NheI-HindIII fragment from pGHS1, containing the lacZAM15 and KmR genes, into the BgII site of the pSC101-based plasmid pKBU4. The pGHS1 moiety of pKBU5 was flanked with large nonessential regions of homology with the B. subtilis chromosome (2.5 kb to the left and 5 kb to the right of the BgII site), thus allowing homologous recombination to occur. To obtain the desired double crossover integration event, B. subtilis 6GM competent cells containing the lacZa-complementing plasmid pHPS9 (Haima et al., 1990), were transformed with PstI-cleaved pKBU5 DNA. This can only result in single-copy integration of the fragment of interest. All transformants selected on TY plates containing Km, Cm and XGal, showed ßGal activity, suggesting that the lacZAM15 gene had been integrated into the chromosome. As expected, Southern hybridization of restriction digests of chromosomal DNA from the transformants with appropriate probes, revealed that all integration events had taken place by double crossover recombination (results not shown), resulting in a stable copy number of one lacZAM15 gene per chromosome equivalent.

(b) Optimizing the expression of the integrated lacZAM15 gene

B. subtilis cells containing the integrated lacZAM15 gene and the lacZa-complementing plasmid pHPS9, produced faint-blue colonies on media containing XGal. Since deep-blue colonies were obtained in controls when the lacZAM15 gene was present on plasmid pGHS1, this indicates that the level of expression of the integrated gene was reduced compared to the plasmid-borne gene. Most likely this was due to a gene dose effect: pGHS1 was present at approximately five copies per chromosome equivalent. To quantitate the level of reduction of expression of the lacZAM15 gene due to integration, we measured the ßGal activity in exponentially growing cultures. The results (Table II) show that integration resulted in about a fourfold reduction of the ßGal activity (pKBU5-derived integrants).

### Table II

<table>
<thead>
<tr>
<th>Strain</th>
<th>ßGal activity (u/ml)*</th>
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<tbody>
<tr>
<td>6GM[pGHS1 + pHPS9]</td>
<td>11.1</td>
</tr>
<tr>
<td>6GM[pKBU5][pHPS9]*</td>
<td>2.7</td>
</tr>
<tr>
<td>6GM[pKBU6][pHPS9]*</td>
<td>9.9</td>
</tr>
<tr>
<td>6GM[pKBU7][pHPS9]*</td>
<td>5.4</td>
</tr>
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</table>

* In these strains the lacZAM15 gene was integrated into the chromosome.

ßGal activity was assayed as described by Miller (1982) and expressed as u/ml culture (normalized for cell density).

Since the lacZAM15 gene was under control of the strong lactococcal promoter P23, which is also highly active in B. subtilis (Van der Vossen et al., 1987), we reasoned that transcription would not be a limiting factor for expression of the gene. Therefore, we tried to increase the level of expression by improving the efficiency of translation. This was done by modifying the −20 to +33 region of the gene (Fig. 1), using synthetic oligos. Three kinds of modifications were introduced. First, the region upstream of the lacZAM15 gene, which as a consequence of the construction is not the regular upstream sequence of the gene (Haima et al., 1989), was replaced by the −20 to −1 region of the B. subtilis spoOF gene, which was shown to be efficiently translated in B. subtilis (Shimotsu et al., 1983; Hager and Rabinowitz, 1985). Second, we adapted several third nt of the codons in the 0 to +32 region of the lacZAM15 gene, such that these corresponded to the average nt distribution in this region of Gram+ genes, as deduced from the work of Hager and Rabinowitz (1985). Third, the 2nd (ACC), 7th (CTG) and 8th (GCC) codon of the lacZAM15 gene, which are rare in highly expressed B. subtilis genes (Sharp et al., 1988), were substituted with frequently used codons. None of these changes resulted in aa substitutions in the protein.

The modified lacZAM15 gene was placed under control of either the lactococcal promoter P23 (Van der Vossen et al., 1987), resulting in plasmid pKBU6 (Fig. 1), or the B. subtilis phage SPO2 promoter (Williams et al., 1981), resulting in plasmid pKBU7 (Fig. 1), and stably integrated into the B. subtilis chromosome in one copy by a double cross-over event (as described for pKBU5). These modifications of the gene resulted in about a fourfold increase of the ßGal activity (Table II; pKBU5 vs. pKBU6). Furthermore, promoter P23 appeared to result in about twofold higher levels of ßGal activity than the SPO2 promoter (pKBU6 vs. pKBU7). One of the pKBU6-derived integrants, denoted B. subtilis strain 6GM15, was selected for further use.
(c) Molecular cloning with the host-vector system 6GM15-pHPS9

To test the cloning properties of the novel host-vector system 6GM15-pHPS9, BamHI-cleaved bacteriophage λ DNA was ligated to BamHI-cleaved pHPS9 DNA at a mass ratio of 25:1 and a final DNA concentration of 500 μg/ml. The ligation mixture was used to transform 6GM15 competent cells and transformants were selected on Cm- and XGal-containing plates.

Approximately 10^4 transformants per ml competent cells were obtained, among which 75% were potential clones since these produced white colonies. Restriction analysis of plasmid DNA extracted from 24 randomly chosen potential clones revealed that all contained a recombinant pHPS9 plasmid, carrying inserts ranging from 5.6 to 29 kb (the average insert size amounted to 13.5 kb). From these results it can be concluded that the host-vector system 6GM15-pHPS9 was reliable (white colony producing cells contained a recombinant plasmid), and enabled the cloning of large heterologous inserts (up to at least 29 kb) with high efficiency.

(d) Structural stability of recombinant plasmids

Plasmids from Staphylococcus aureus are rather inefficient cloning vehicles (Gryczan et al., 1982; Jannière et al., 1989; 1990; Michel et al., 1980), and inserts carried on these plasmids often suffer deletions (Ehrlich et al., 1986). Moreover, cloning in such vectors has been shown to result in high levels of size-dependent segregational instability (Bron et al., 1985; 1988). It has been postulated that the inferior cloning properties of staphylococcal plasmids are a consequence of their mode of replication (Jannière et al., 1990); all these plasmids appear to replicate via ssDNA intermediates (termed ss+ plasmids), probably by rolling-circle replication (for a review, see Gruss and Ehrlich, 1989). One observation supporting this hypothesis was that plasmids

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Fig. 3. Construction of plasmid pHPS10. The PstI and SalI sites on the pWVO1-based plasmid pGKV259 were removed with T4 DNA polymerase and PolIk, respectively, resulting in plasmid pGKV259-2 (filling-in of the SalI site resulted in the generation of a PstI site). The SalI-SphI fragment of pGKV259-2, containing promoter P59 and part of the cat-86 gene, was used to replace the corresponding SalI-SphI fragment of plasmid pGKH1, resulting in plasmid pGKH2. Plasmid pHPS10 was constructed by ligating the 1200-bp SalI-BamHI fragment from pGKH2 to plasmid pHPI3-2 digested with HindIII (filled-in) and BamHI. The E. coli MC1000[pLGS1] transformants carrying pHPS10 were selected on Km + Er + XGal plates. Open bars, lacZα sequences; closed bars, cat-86 sequences; thin arrows in the plasmid, direction of transcription; bold arrows, promoter P59; ori pUC, ori of replication derived from plasmid pUC9; rep, primary replication functions of pTA1060. The following restriction sites (only those relevant for the construction) are indicated: B, BamHI; E, EcoRI; H, HindIII; P, PstI; Pv, PvuI; S, SalI; Sa, Sau96I; Sc, ScaI; Sm, Smal; Sp, SphI. EmR, gene encoding Er resistance.
like pAMβ4, which replicate without generating ssDNA (termed ss− plasmids), appeared to be structurally more stable than plasmids based on the ssDNA generating replicon pC194, at least for certain types of deletions. Further support came from the observation that large DNA segments could be cloned on ss− plasmids, while only small fragments could be cloned on the ss+ plasmid (Jannière et al., 1990).

Several observations in our laboratory indicate that the B. subtilis plasmid pTA1060 also replicates via a rolling-circle mechanism (S.B. and S. Holsappel, unpublished). A region of pTA1060 was identified which is required for optimal segregational stability (Bron et al., 1987), and which appeared to contain the origin of minus strand synthesis (ori−; S.B. and S. Holsappel, unpublished). Because plasmid pHPS9 lacks the functional ori− and therefore accumulates ss DNA, it might be structurally unstable. To examine this possibility, we analyzed the structural stability of four recombinant pHPS9 plasmids, carrying bacteriophage λ DNA fragments of 7.2, 16.8, 24 and 29.1 kb, respectively. All transformed colonies (12 of each type were examined) appeared to contain only the original plasmid, indicating that the inserts carried by pHPS9 were stable during transformation of competent cells. To examine the long-term stability of the inserts, transformants containing the four recombinant pHPS9 plasmids were grown in batch culture under Cm selective pressure for 100 generations. All cultures appeared to contain the original plasmid (results are shown for the 7.2-, 16.8- and 29-kb inserts in Fig. 2), indicating that the inserts carried by pHPS9 were also stable during long-term vegetative growth. It is therefore concluded that, although pTA1060-based plasmids replicate via a ssDNA-generating rolling-circle mechanism, they are far superior to the commonly used S. aureus vectors with regard to cloning efficiencies and structural stability.

At present we can only speculate about the reasons why in B. subtilis pTA1060-derived plasmids are superior to the commonly used S. aureus plasmids. Since both groups of plasmids replicate in a similar way (via a rolling-circle mechanism), it is unlikely that the observed differences with respect to cloning efficiencies and stability are a direct consequence of their mode of replication. One possible explanation is that the types of structural instability shown to be stimulated by ssDNA (Jannière and Ehrlich, 1987; Jannière et al., 1990), do not occur frequently enough to interfere seriously with molecular cloning of foreign DNA in pTA1060-based plasmids. A significant difference between the two groups of plasmids is their copy number: five per chromosome equivalent for pTA1060, and from 20 to 50 for most of the S. aureus plasmids. It has been shown that the S. aureus plasmids generate, in addition to ssDNA replication intermediates, aberrant replication intermediates of high-molecular-weight (HMW) DNA (Gruss and Ehrlich, 1988), if they carry foreign DNA inserts. HMW DNA was also observed in certain derivatives of pUB110 (Viret and Alonso, 1987). Both ss and HMW DNA have been conceived to cause plasmid instability (Alonso et al., 1987; Gruss and Ehrlich, 1988; Viret and Alonso, 1987), possibly in an indirect way by interfering with cell growth. It is attractive to speculate that pTA1060-based plasmids generate only small amounts of ss and HMW plasmid DNA. Preliminary experiments suggest that this is indeed the case (S.B. and S. Holsappel, unpublished). The production of ss DNA can even be further reduced by the introduction of the ori− in plasmids like pHPS9, which we expect to result in an even further improvement of the stability properties, also segregationally, of these plasmids.

(e) Construction of improved cloning vectors

To extend the number of unique target sites in the MCS of the lacZα-complementing plasmid pHPS9, one of each of the duplicate SalI and PstI sites was removed, resulting in plasmid pHPS10. The details of pHPS10, along with an outline of its construction, are shown in Fig. 3.

Unexpectedly, plasmid pHPS10 failed to express Cm resistance in B. subtilis. Furthermore, the βGal activity in 6GM15[pHPS10] was considerably higher (60.6 u/ml) than in 6GM15[pHPS9] (9.9 u/ml). Since the copy numbers of pHPS9 and pHPS10 were similar (five copies per chromosome equivalent, results not shown), the most likely explanation for the decreased expression of the CmR gene is strong read-through transcription from promoter P59 in pHPS10 into the divergently transcribed Cm gene. This was concluded from the fact that the insertion of the E. coli rnmB T1T2 terminators (Brosius et al., 1981) into the EcoRI site of the cat−: lacZα fusion on pHPS10 restored the CmR phenotype.

Although plasmid pHPS10 does not express CmR in B. subtilis, the vector is very useful for molecular cloning in this organism. It confers ErR to B. subtilis and contains eight unique restriction sites suitable for the direct selection of cloned heterologous DNA fragments. Furthermore, the plasmid might be useful for isolating transcription terminators. We have shown that by screening for CmR transformants, recombinant pHPS10 plasmids carrying the T1T2 terminator fragment were selected.

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