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New shuttle vectors for ectopic insertion of genes into *Bacillus subtilis*

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Abstract

We have constructed shuttle vectors for integration of genes via double homologous recombination into three ectopic sites on the chromosome of *Bacillus subtilis*. The sites of integration are the *pyrD*, *gltA*, and *sacA* genes located at 139°, 172°, and 333°, respectively, on the chromosome. Integration of the vectors into the target genes leads to antibiotic resistance as well as different metabolic phenotypes. *B. subtilis* strains with integrations of the empty vectors were able to sporulate at rates comparable to wild type cells. Similar levels of expression were obtained from constitutive *lacZ* fusions integrated at the different sites.

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Keywords: Double homologous recombination; *pyrD* locus; *gltA* locus; *sacA* locus

1. Introduction

The gram-positive, sporulating bacterium *Bacillus subtilis* is a popular model system (Harwood, 1992). Many tools and techniques have been developed for understanding its genetics (Cutting and Vander Horn, 1990; reviewed in the context of sporulation by Stragier and Losick, 1996). Of these, integration vectors have been one of the most important (see, for example, Guérot-Fleury et al., 1996). These plasmids allow for the ectopic insertion of cloned genes into sites on the chromosome. Integration of genes onto the chromo-

some has several advantages over maintenance of genes on plasmids. First, integration ensures that a single copy of the transgene is present, which can be important for gene regulation (Shimotsu and Henner, 1984, 1986). Second, genes integrated into the chromosome are very stable, and are maintained in the same copy number even in the absence of selection (Vazquez-Cruz et al., 1996). Another advantage of integration into the chromosome is that the transgene is present in the same physical location as other chromosomal DNA. Genes on plasmids are often not present in the same region of the bacterial cell as chromosomal genes (Gordon et al., 1997; Pogliano, 2002).

Integration of genes into ectopic loci also provides several advantages over insertion of genes by single homologous recombination. First, no

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homology to *Bacillus* genes is required, so integration vectors can be used to insert foreign genes into *B. subtilis* or to perform complementation studies in strains with deletions of chromosomal genes. Second, genes integrated by double homologous recombination are expected to be more stable than those integrated by a single crossover event.

Currently, few vectors exist for the ectopic integration of genes into the chromosome of *B. subtilis*. The most commonly used vectors are those for integration of genes into the *amyE* locus (Karmazyn-Campelli et al., 1989; Shimotsu and Henner, 1986). The inserted genes disrupt the *amyE* gene, preventing the production of α -amylase. Other integration vectors allow for the insertion of genes at the *thrC* locus, resulting in threonine auxotrophy (Guerot-Fleury et al., 1996) and at the *lacA* locus (Hartl et al., 2001).

In this work we created new shuttle vectors for stable gene integration by double homologous recombination at the *pyrD*, *gltA*, and *sacA* loci. These new vectors will allow for construction of strains with more transgenes than previously possible because genes can be integrated at these loci as well as at the *amyE* and *thrC* loci. Another positive aspect of the new vectors is that they allow for integration at different chromosomal positions. Several studies have indicated that the position of genes on the *B. subtilis* chromosome can regulate the timing and level of their expression (Dworkin and Losick, 2001; Frandsen et al., 1999; Khvorova et al., 2000; Zupancic et al., 2001). In addition, the integration vectors may be useful in marking specific locations on the chromosome for visual detection (Gordon et al., 1997; Straight et al., 1996; Teleman et al., 1998; Webb et al., 1997).

2. Materials and methods

2.1. Vector construction

For the backbone of the vectors, pUC18 plasmid DNA (Messing, 1983; Norrander et al., 1983) from Invitrogen was digested with the *PvuII* restriction enzyme, and the resulting 2.364 kb fragment was ligated to itself.

The homologous DNA for integration of constructs into the target genes was synthesized by PCR (using *Pfu* polymerase) in two steps. In the first step, 5' and 3' ends of the target genes were amplified from *B. subtilis* chromosomal DNA (strain PY79, prototroph, from P. Youngman, University of Georgia, Athens, GA), with *AscI* restriction sites incorporated into the primers nearest the middle of the target genes. The oligonucleotide primers used were as follows: *pyrD*-front5' gctagaggtgaaattgccgg, *pyrD*-frontAsc ggggcgcgcaaaagcgattccgcc, *pyrD*-backAsc ttggcgcgccccgaacgtgggctaata, *pyrD*-back3' aagcgcgatgatggcagg, *gltA*-front5' ctcaaggtctctaccgtcc, *gltA*-frontAsc ggggcgcgcaaaatagcggtaggatg, *gltA*-backAsc ttggcgcgccccaaacgctttgttcag, *gltA*-back3' gctgctgtattctgttggc, *sacA*-front5' gacagcacatgaccaggagc, *sacA*-frontAsc ggggcgcgcaaaatcgccagcccg, *sacA*-backAsc ttggcgcgccccatccgaccattgact, and *sacA*-back3' gatttccgggtcgactggc. The PCR products were gel-purified. Short flanking homology PCR (Wach et al., 1994) was then used to fuse the two portions of the genes. The resulting products contained DNA homologous to both ends of the target gene with a single *AscI* restriction site in the middle.

The target gene chimeras produced in the second round of PCR were phosphorylated using phosphonucleotide kinase, and subsequently blunt-end ligated into the *PvuII* site of the modified pUC18 vector backbone.

The antibiotic resistance genes and multiple cloning sites were amplified from pDG364 and pER82 (Cutting and Vander Horn, 1990) plasmid DNA using *Pfu* polymerase and oligonucleotides containing *AscI* restriction sites (*amyE*-frontAsc ggcgcgcaaaactggacacatgg and *amyE*-backAsc ggcgcgctaaacggatatcatc). The PCR products were then digested and cloned into the *AscI* sites in the middle of the target gene chimeras.

2.2. Construction of *B. subtilis* strains

Transformation of *B. subtilis* was carried out by the standard 2-step transformation procedure (Cutting and Vander Horn, 1990), except that when making competent cells, nutrient supplements were added to the media for *pyrD*⁻ strains: uracil was added to SpC and SpII media at a final

concentration of 40 µg/mL. Vectors were digested with the *ScaI* restriction enzyme to linearize them prior to transformation into *B. subtilis*.

2.3. Verification of integration position

Tris Spizizen salts minimal agar (TSSA) plates (Cutting and Vander Horn, 1990) with and without nutrient supplements were used to determine the phenotypes of transformants. For *pyrD* integration, uracil was added to a final concentration of 40 µg/mL. Strains with a *gltA* gene disruption required addition of glutamic acid to a final concentration of 500 µg/mL. Strains with a *sacA* gene disruption could grow on TSSA, but could not grow on a variation of this medium containing 0.1% sucrose and no glucose.

PCR was used as an additional method for verifying gene insertion. Oligonucleotide primers complementary to the ends of the target gene were used for amplification. For example, the *pyrD*-front5' and *pyrD*-back3' primers were used for the *pyrD* locus. The PCR products were analyzed by electrophoresis on a 1% agarose gel.

2.4. Measurement of growth rates

Cells were grown in TSS minimal medium with the appropriate supplements (uracil at a concentration of 40 µg/mL for *pyrD*[−] strains and glutamate at a concentration of 500 µg/mL for *gltA*[−] strains). Samples of the cultures were taken every

45 min, and their optical density (OD₆₀₀) was measured using a spectrophotometer.

2.5. Sporulation assays

Sporulation was initiated by the resuspension method (Nicholson and Setlow, 1990). Nutrient supplements were added as follows: for *gltA*[−] strains, glutamic acid was added to the resuspension medium at a final concentration of 500 µg/mL; for *pyrD*[−] strains, uracil was added to both the growth medium and the resuspension medium at a final concentration of 40 µg/mL. Heat kill assays were performed by making serial dilutions of cultures 24 h after resuspension. Aliquots of the dilutions were plated on LB medium, and the remaining portion was heated at 80 °C for 15 min, and then plated on LB medium.

2.6. Measurement of reporter gene expression

A fusion of a constitutive variant of the *spac* promoter to *lacZ* (*Phyperspac-lacZ*) was used as a reporter for gene expression at each of the different chromosomal positions. The fusion was a gift of Masaya Fujita (Fujita and Losick, 2002). It was amplified by PCR using Dynazyme polymerase (Finnzymes) from MF337-2 plasmid DNA using the oligonucleotides *spacCpr5*(*ecoRI*) *cgaattctaca* *cagccagctccagac* and *LacZbackEcoRI* *ggaattcctt* *acgcgaataacgg*. The PCR products obtained were cloned using the TOPO TA cloning kit (Invitrogen). The *EcoRI* fragment containing the *Phyper-*

Table 1
Reference numbers for sequences and strains

Construct	GenBank Accession number	BGSC number for <i>E. coli</i> strain	BGSC number for <i>B. subtilis</i> strain with empty vector integration
pPyr-Cm	AY464558	ECE170	1A806
pPyr-Kan	AY464559	ECE171	1A807
pGlt-Cm	AY464560	ECE172	1A808
pGlt-Kan	AY464561	ECE173	1A809
pSac-Cm	AY464562	ECE174	1A810
pSac-Kan	AY464563	ECE175	1A811

The sequences of the vectors described in this paper have been deposited in GenBank under the Accession numbers shown in the second column. *E. coli* strains containing the vectors and *B. subtilis* strains containing integrations of the empty vectors have been catalogued in the *Bacillus* Genetic Stock Center with the reference numbers listed in the third and fourth columns.

spac-lacZ fusion was then subcloned into the *EcoRI* sites of *amyE*, *pyrD*, *gltA*, and *sacA* vectors containing chloramphenicol resistance genes. DNA from these constructs was transformed into competent PY79 cells containing kanamycin resistance genes integrated at *amyE*, *pyrD*, *gltA*, and *sacA* respectively.

Cells were sporulated by the resuspension method and aliquots were obtained when the optical density (OD₆₀₀) of the culture reached 0.2 and 0.6 and also at 1 and 3 h after resuspension. The cells contained in these samples were frozen at -80 °C. β -Galactosidase activity was measured using an *o*-nitrophenyl- β -D-galactoside (ONPG) assay (Nicholson and Setlow, 1990).

2.7. Sequences and strains

Sequences of all six vectors have been deposited in the GenBank database. *Escherichia coli* strains containing the shuttle vectors and *B. subtilis* strains containing integrations of the empty vectors have been deposited in the *Bacillus* Genetic Stock Center. The reference numbers for them are shown in Table 1.

3. Results and discussion

3.1. Choice of new loci for ectopic gene integration

Our goal was to design vectors for the integration of genes at different loci on the *B. subtilis* chromosome. Specifically, we wanted to be able to integrate genes near the origin and near the terminus of the chromosome. Also, we wished to have a simple phenotypic screen for disruption of the target gene. Accordingly, we investigated loci of the *B. subtilis* mapping strains (Cutting and Vander Horn, 1990). Strains with disruptions in the *sacA*, *pyrD*, and *gltA* genes were available from the *Bacillus* Genetic Stock Center (Deshpande and Kane, 1980; Lepesant et al., 1972; Potvin et al., 1975). The *pyrD* and *gltA* genes are located near the terminus of the chromosome, at 139° and 172°, respectively. The *sacA* gene is near the origin, at 333°. Strains with mutations in these genes had discernable phenotypes on minimal media, grew at

normal rates in rich medium, and sporulated at frequencies similar to wild type (data not shown). Based on these results, we designed plasmids for integration at each of the three loci.

3.2. Vector design

The new plasmids were designed to be similar to the *amyE* and *thrC* integration vectors already available (Guérot-Fleury et al., 1996; Karmazyn-Campelli et al., 1989; Shimotsu and Henner, 1986). Because the new vectors share a common design, just one is diagrammed in Fig. 1. Each contains the *E. coli* origin of replication and β -lactamase gene from pUC18 (Messing, 1983; Norrander et al., 1983), allowing replication of the plasmid and selection of plasmid-containing cells in *E. coli*. The vectors also all contain two regions of homology to a *B. subtilis* gene. Depending on the gene, the regions of homology vary from 418 to 1828 bp in length. One region is homologous to the 5' end of the gene, while the other is homologous to the 3' end. There is no homology to the middle portion of the gene. Between the two regions of homology, the vectors contain an antibiotic resistance deter-

Fig. 1. A schematic of the *glt*-kan integration vector. This vector is representative of all the vectors discussed in the paper. Each contains a multiple cloning site and an antibiotic resistance gene (kanamycin resistance in this case). Flanking this are two regions of homology to the target gene (*gltA* in this case). Finally, all of the vectors have a pUC18 origin of replication and a gene encoding ampicillin resistance.

minant (either kanamycin or chloramphenicol resistance) and a multiple cloning site. The multiple cloning site includes unique *Bam*HI, *Eco*RI, and *Hind*III restriction sites in the plasmids conferring chloramphenicol resistance. In the plasmids conferring kanamycin resistance, the multiple cloning site includes unique *Bam*HI, *Eco*RI, *Kpn*I, *Spe*I, and *Xba*I restriction sites.

3.3. Integration of vectors into the chromosome

The plasmids were linearized and transformed into *B. subtilis*. Integration of the vectors into their target genes was initially screened for by phenotypic examination of colonies. Strains with *pyrD* and *gltA* gene disruptions were unable to grow on minimal medium unless uracil or glutamate, respectively, was added. Strains with a *sacA* gene disruption could grow on medium with glucose as a carbon source, but not with sucrose as the sole carbon source.

PCR was used as an additional method for verifying gene insertion. Oligonucleotide primers complementary to the 5' and 3' ends of the target gene were used for amplification. The PCR products were examined by gel electrophoresis. Differences in the sizes of the products obtained from wild type strains and strains with vector integrations indicated that the target genes were indeed disrupted by the gene insertions (Fig. 2).

Marker replacement was subsequently used to verify the position of gene integration. Antibiotic resistance genes were integrated into *B. subtilis*, and their positions were verified by phenotypic analysis and PCR. These strains were then transformed with constructs containing a gene of interest as well as a gene for resistance to a different antibiotic. The transformants could then be selected with the new antibiotic and screened for susceptibility to the old antibiotic. In this way, an additional phenotypic trait could be used to verify insertion of the gene of interest at the correct po-

Fig. 2. PCR was used to verify vector integration into the desired loci. Oligonucleotide primers complementary to the 5' and 3' ends of the target gene were used. The PCR products were run out on a 1% agarose gel along with a DNA ladder to determine their sizes. Predicted (and observed) band sizes are as follows: for the *pyrD* locus, the uninterrupted gene yields a product of 0.93 kb; the integrated chloramphenicol and kanamycin vectors change the size to 2.4 and 3.1 kb, respectively. The wild type *gltA* locus PCR product is 4.6 kb; with integrated vectors the size changes to 4.1 kb (chloramphenicol resistance) and 4.8 kb (kanamycin resistance). The *sacA* PCR product is 1.4 kb; this is increased to 2.7 and 3.4 kb upon integration of the vectors.

sition. During the course of these strain constructions, it was observed that cells with disruptions in the target genes were not noticeably different in competence compared to the wild type (data not shown).

3.4. Phenotypes of strains with empty vector integrations

Strains with empty vectors inserted into the *pyrD*, *gltA*, and *sacA* genes were assayed for growth and sporulation. Growth of strains with vector integrations in supplemented minimal me-

dium occurred at rates similar to the wild type (data not shown). However, *gltA*⁻ cultures grew to a higher cell density than wild type cultures before reaching stationary phase. This was likely due to the amount of supplemental glutamate added to the medium. When lower concentrations of glutamate were used, cultures reached stationary phase at lower cell densities. Also, when a wild type culture was grown in the presence of 500 µg/mL supplemental glutamate, it reached higher cell densities before cessation of growth (data not shown).

Sporulation was induced in strains containing empty vector integrations. Comparisons of the

Table 2
Sporulation efficiency

	Total (Cfu/mL)	Spores/mL	Sporulation efficiency
PY79 wild type	2.93×10^8	2.56×10^8	87% ± 8
PY79 <i>pyrD</i> :: <i>CmR</i>	2.88×10^8	2.04×10^8	71% ± 7
PY79 <i>pyrD</i> :: <i>KanR</i>	3.24×10^8	3.19×10^8	98% ± 10
PY79 <i>gltA</i> :: <i>CmR</i>	2.28×10^8	2.23×10^8	98% ± 6
PY79 <i>gltA</i> :: <i>KanR</i>	4.67×10^8	3.12×10^8	67% ± 5
PY79 <i>sacA</i> :: <i>CmR</i>	3.19×10^8	3.04×10^8	95% ± 7
PY79 <i>sacA</i> :: <i>KanR</i>	2.56×10^8	2.61×10^8	102% ± 7

The sporulation efficiency percentage listed was obtained by dividing the number of heat-resistant cfu/mL by the total number of cfu/mL. All strains tested sporulated with efficiencies similar to that of the wild type strain.

Beta-galactosidase Activity of *PspacC-lacZ* fusions

Fig. 3. β -Galactosidase activity was measured in strains containing the *PspacC-lacZ* fusion integrated at the various positions. Samples were taken when the cultures reached OD₆₀₀ of 0.2 (black) and 0.6 (dark grey) and at 1 (light grey) and 3 (white) h after the induction of sporulation. Note that the levels of β -galactosidase expression were nearly identical in strains where the *lacZ* fusion was inserted in opposite orientations (compare the two *amyE* strains and the two *sacA* strains).

number of cfu/mL before and after incubation at 80 °C show that there was no significant difference in the numbers of heat-resistant spores for strains with gene disruptions at any of the three positions versus the wild type strain (Table 2).

To determine whether genes integrated into the chromosome using the new vectors would be expressed at similar levels, constitutively active reporter gene fusions were cloned into the vectors and integrated into the chromosome of *B. subtilis*. The reporter gene fusion used contained a constitutive variant of the *spac* promoter driving expression of the *lacZ* gene and will be referred to as *PspacC-lacZ* (Fujita and Losick, 2002). Cells containing the reporter were grown and induced to sporulate by the resuspension method. Samples taken during early exponential phase, mid-exponential phase, and 1 and 3 h after entry into sporulation were assayed for β -galactosidase activity (Fig. 3). We found that the *lacZ* expression was similar at all positions examined including the *amyE* integration locus. The orientation of the *lacZ* fusion construct at both the *sacA* and *amyE* loci had no significant effect on its expression, indicating that *lacZ* expression was driven by the *PspacC* promoter, rather than by read-through from the flanking gene.

The three new shuttle vectors described should prove to be valuable tools for *B. subtilis* molecular genetics. They allow for the ectopic insertion of genes at different positions on the chromosome, opening up new possibilities for studying the effects of gene position or constructing strains with multiple transgenes.

4. Uncited references

Antonieuski et al. (1990), Wu and Errington (1998).

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