APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2004, p. 7241–7250 Vol. 70, No. 12
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.12.7241–7250.2004

New Integrative Method To Generate *Bacillus subtilis* Recombinant Strains Free of Selection Markers

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Received 6 April 2004/Accepted 20 July 2004

The novel method described in this paper combines the use of *blaI*, which encodes a repressor involved in *Bacillus licheniformis* BlaP β-lactamase regulation, an antibiotic resistance gene, and a *B. subtilis* strain (BS1541) that is conditionally auxotrophic for lysine. We constructed a BlaI cassette containing *blaI* and the spectinomycin resistance genes and two short direct repeat DNA sequences, one at each extremity of the cassette. The BS1541 strain was obtained by replacing the *B. subtilis* P_{lysA} promoter with that of the P_{blaP} β-lactamase promoter. In the resulting strain, the cloning of the *blaI* repressor gene confers lysine auxotrophy to BS1541. After integration of the BlaI cassette into the chromosome of a conditionally *lys*-auxotrophic (BS1541) strain by homologous recombination and positive selection for spectinomycin resistance, the eviction of the BlaI cassette was achieved by single crossover between the two short direct repeat sequences. This strategy was successfully used to inactivate a single gene and to introduce a gene of interest in the *Bacillus* chromosome. In both cases the resulting strains are free of selection marker. This allows the use of the BlaI cassette to repeatedly further modify the *Bacillus* chromosome.

The completion of the sequencing and annotation of the *Bacillus subtilis* 168 genome supply a complete view of the *B. subtilis* protein machinery, and this knowledge stimulates new approaches to analyze biochemical pathways (12, 15). This postgenomic study requires genetic tools that allow the combination of several gene manipulations in the same strain. Classically, these chromosomal modifications could be achieved by a method using a positive selection marker, usually an antibiotic resistance marker generated by the insertion of a selection marker gene in the *B. subtilis* chromosome. In this strategy, the introduction of a second chromosomal modification requires a second resistance gene, or, if the same resistance gene is used, the eviction of this gene by a single crossover event prior to further genetic manipulation. In the first case, the number of chromosomal modifications is limited by the number of available resistance genes, and, moreover, the multiantibiotic pressure could modify the physiology of the manipulated strain. In the second case, selection of the strain which has lost resistance is tedious due to the relatively low frequencies and absence of positive selection. In the same way, the optimization of a recombinant *B. subtilis* strain for overproduction and secretion of a protein can require chromosomal modifications which could involve the integration of several copies of the gene of interest (7), the construction of multiprotease- and sporulation-deficient strains (18, 19, 25, 26), and/or the coexpression of chaperones to amplify the posttranscriptional maturation of the protein (2, 24). Another example of the usefulness of generating multiple mutants is for the study of complex physiological pathways, such as sporulation. Indeed, mutations in many sporulation genes regulated by sporulation-specific sigma factors or found by DNA arrays give no obvious phenotypes (4, 5). It is thought that the products of these genes could be largely redundant and that multiple mutants may be needed to unravel gene function.

In terms of routinely carrying out chromosome integrations, only one method is described for *B. subtilis* that allows the subsequent excision of the selection marker coupled with positive selection (6). This method relies on the use of an integrative cassette containing an antibiotic resistance gene and the *upp* gene, encoding uracylphosphoribosyl transferase as a counterselection marker (the upp cassette). The use of the upp cassette is linked to a *B. subtilis* strain deleted of the *upp* gene conferring resistance to 5-fluorouracyl. Here we describe a conditional auxotrophy-based method for the eviction of the selection marker. This alternative to the method of Fabret et al. (6) combines the use of *blaI*, which encodes a repressor involved in *Bacillus licheniformis* BlaP β-lactamase regulation (8, 9, 13), an antibiotic resistance gene, and a conditional lysine-auxotrophic *B. subtilis* strain. This strategy was successfully used to inactivate a single gene and to introduce a gene of interest into the *B. subtilis* chromosome. In both cases the resulting strains are free of selection marker, thus allowing the repeated use of the method for further modifications of the *Bacillus* chromosome.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. All *B. subtilis* recombinant strains are *B. subtilis* 168 derivatives. Specific primers used for PCR amplification were synthesized by Eurogentec (Table 3).

Culture and growth conditions. All organisms were grown in Luria-Bertani (LB) broth (rich medium) or LB agar supplemented, when required, with the appropriate antibiotic. Terrific Broth (TB) without glycerol was used as a rich liquid nutrient broth for β-lactamase production. Minimal medium (MM) ($Na_2HPO_4 \cdot 7H_2O$, 12.8 g/liter; KH_2PO_4, 3 g/liter; NaCl, 0.5 g/liter; NH_4Cl, 1 g/liter; $MgSO_4$, 1 mM; $CaCl_2$, 0.1 mM; glucose, 0.4%; L-tryptophan, 20 mg/liter; pH 7.4) was used for auxotrophy determination (11). In order to check lysine auxotrophy, MM was supplemented with lysine at a concentration of 50 μg/ml. The final concentrations of antibiotics were the following: 100 μg of ampicillin/

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TABLE 1. Strains used in this study

Strain	Description[a]	Source or reference
E. coli dH5α	*F*$^-$ ϕ*80dlacZ*Δ*M15* Δ(*lacZYA-argF*) *U169 endA1 recA1 hsdR17* (r_k^-, m_k^+) *DeoR thi-1 phoA supE44* λ$^-$ *gyrA96 relA1*	Life Technologies
B. subtilis		
BS168	*trpC2*	*Bacillus* Genetic Stock Center
BS1539	BS168 *spoVAF*::*Kan*^{r}::*lysA*	This work
BS1541	BS168 *lysA* controlled by P_{blaP} promoter	This work
BS1549	BS1541 *amyE*::rep Spc^r *blaI* rep	This work
BS1549S	BS1541 *amyE*::rep	This work
BS1567	BS1541 *amyE*::rep Spc^r *blaI* rep *blaP*	This work
BS1567S	BS1541 *amyE*::rep *blaP*	This work
B. licheniformis 749I		16

[a] rep, repeat unit corresponding to the last 138 bp of the green fluorescent protein gene.

ml, 100 μg of spectinomycin/ml, and 50 or 10 μg of kanamycin/ml when selecting for a recombinant in *Escherichia coli* or *Bacillus* spp., respectively.

α-Amylase expression by *Bacillus* colonies was detected by growing colonies overnight on an LB plate containing 1% starch and staining the plate with iodine as described elsewhere (3).

DNA manipulation techniques. The isolation and manipulation of recombinant DNA was performed with standard techniques. Enzymes were commercial preparations and were used as specified by the suppliers (Gibco, Promega, and Biolabs). *Bacillus* chromosomal DNA was prepared with the Wizard Genomic DNA purification kit (Promega). *E. coli* transformation was performed as described by Sambrook et al. (21). *B. subtilis* transformation was performed by the competent cell method (14). In Southern blot experiments, the AlkPhos Direct Labeling kit (Amersham Pharmacia Biotech) was used to label the DNA probe with alkaline phosphatase.

Construction of pDML1539. *spoVAF* and *lysA* genes were amplified by PCR from purified chromosomal *B. subtilis* 168 DNA with the oligonucleotide pairs spoVAFBglII/spoVAFMluI and lysABamHI/lysAEnd, respectively (Table 3). The two PCR products were subcloned into pGEM-T-Easy to generate pDML1534 and pDML1537 (Table 2). pDML1536 was constructed by cloning a 362-bp SacII-ApaI fragment from pDML1570, containing the P_{blaP} promoter, into SacII-ApaI sites of pDML1534. pDML1538 was constructed by cloning the *lysA* gene from pDML1537 (on a 1,430-bp BamHI-HincII fragment) into the BamHI-EcoRV sites of pDML1536. The 1,526-bp XbaI-BglII fragment of pDG792, containing the gene conferring kanamycin resistance, was inserted into pDML1538 digested with the same restriction enzymes to generate pDML1539.

Construction of pDML1541. The P_{blaP} promoter was amplified by PCR from pDML995 by using primers BlaIbam+ and promblaPBglII. The PCR product was cloned into pCR-Script to generate pDML1570. pDML1540 is a derivative of pDML1570 in which the 1,244-bp SalI-BglII fragment of pDML1543 (carrying a truncated 5′ end of *spoVAF*) was cloned. The 1,516-bp ApaI-BamHI fragment of pDML1540, containing the last 1,244 bp of *spoVAF* and the P_{blaP} promoter, was inserted into pDML1539 digested with the same enzymes to generate pDML1541. In this construct, the *lysA* gene is under the control of the P_{blaP} promoter.

Construction of the BlaI cassette. The two repeat units (*repfront* and *repback*) corresponding to the last 138 bp of the green fluorescent protein (GFP) gene were amplified by PCR, using pDML967 as template and two sets of primers, repGFPfrup/repGFPfrdo and repGFPbackup/repGFPbackdo, respectively. The PCR-amplified fragments of 155 and 276 bp were cloned in pGEM-T-Easy to generate pDML1543 and pDML1544, respectively. The *blaI* gene was amplified by PCR using pDML995 as template and oligonucleotides pBlaIBglII and stop-BlaIBamHI as amplimers. The 513-bp amplified fragment was cloned into pGEM-T-Easy to form pDML1545. The 208-bp EcoRV-SacI fragment of pDML1544 was inserted into pDML1543, which was digested with EcoRV and SacI to generate pDML1546. The 2,194-bp BamHI fragment of pIC333 was recircularized by ligation overnight to yield pDML1566. The 505-bp BglII-BamHI fragment of pDML1545 was inserted into pDML1566 digested with BamHI to give pDML1547. The 1,753-bp XbaI-BamHI fragment of pDML1547 was subcloned into the XbaI-BamHI sites of pDML1546. The resulting pDML1548 plasmid carries the BlaI cassette.

Construction of pDML1549. The 5.8-kb BlpI-SphI fragment of pAC7 was isolated, the cohesive ends were filled in with Klenow fragment, and the DNA was recircularized by ligation to generate pDML1542. Plasmid pDML1549 was constructed by inserting the BlaI cassette from pDML1548 (on a 2,077-bp SalI-NruI fragment) into the SalI and EcoRV sites of pDML1542.

Construction of pDML1567. The 1,282-bp MluI-BamHI fragment from pDML1515, containing the *blaP* gene and the P_{blaP} promoter, was ligated to MluI- and BglII-digested pDML1549, resulting in pDML1567.

Electrophoresis, Western blotting, and β-lactamase assay. The conditions and reagents employed for running sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, agarose gels, and Western blotting were described elsewhere (17). β-Lactamase assay was performed as previously described (8)

RESULTS

Replacement of the *B. subtilis* 168 P_{lysA} promoter with the *B. licheniformis* P_{blaP}-regulated promoter. In the *B. subtilis* 168 (BS168) chromosome, the *lysA* gene, located directly downstream of the *spoVAF* gene, encodes diaminopimelate decarboxylase, the last enzyme involved in the lysine biosynthetic pathway that catalyzes the conversion of mesodiaminopimelate into lysine. Complementation experiments showed that the *lysA* gene is essential when the mutant strain is cultivated on minimal medium (12, 20). Therefore, to generate a precursor strain that simplifies the screening for marker replacement with the P_{blaP} promoter, the expression of *lysA* was interrupted by replacing the BS168 chromosome region containing the *spoVAF* 3′ end and the intergenic region between *spoVAF* and *lysA* genes with a kanamycin resistance gene. This was accomplished by transforming BS168 with the ApaI-linearized *E. coli* plasmid pDML1539, a pGEM-T-Easy derivative, carrying the Kan^r gene of pDG792 flanked by the *spoVAF* 5′ end and the *lysA* gene, respectively (Fig. 1). The cells were plated on rich medium supplemented with 10 μg of kanamycin/ml. The selected kanamycin-resistant clones were replicated on minimal medium supplemented with 10 μg of kanamycin/ml and with or without 50 μg of lysine/ml. In 33% of the kanamycin-resistant colonies, the 752-bp *spoVAF* 3′ end, the intergenic region upstream of *lysA*, and the start codon of *lysA* were exchanged by double crossover with part of pDML1539 to generate the BS1539 strain exhibiting a Kan^r, Δ*lysA*, and Δ*spoVAF* phenotype. This strain was unable to grow on minimal medium devoid of lysine, confirming that absence of the P_{LysA} promoter can provide a reliable *lysA* mutant phenotype that can be easily screened. The high levels (67%) of kanamycin-resistant and *lysA*$^+$ colonies obtained were the result of a single crossover,

TABLE 2. Plasmids used in this study

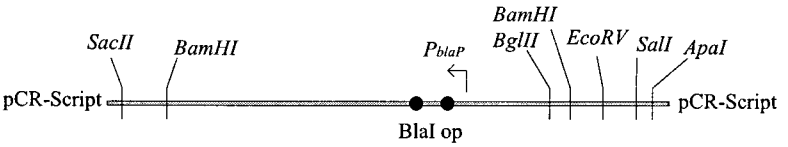
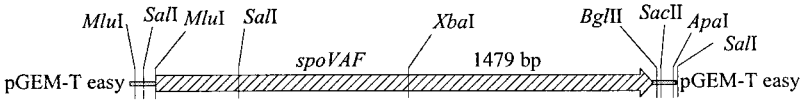
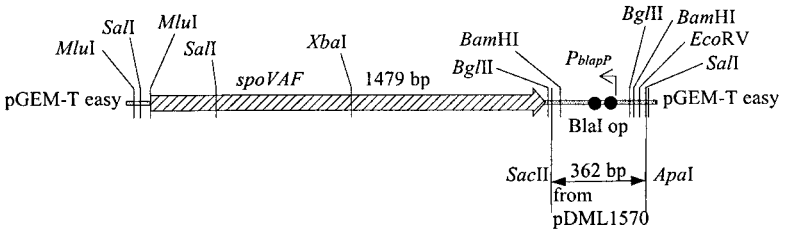
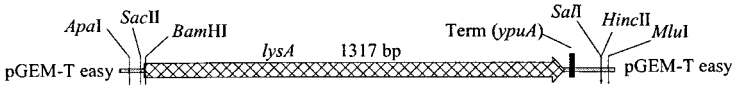
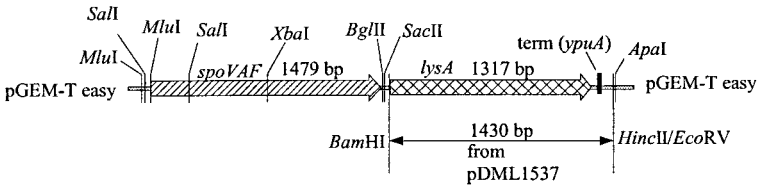
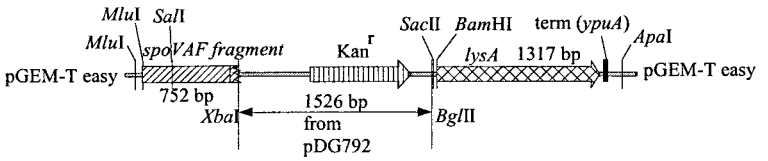
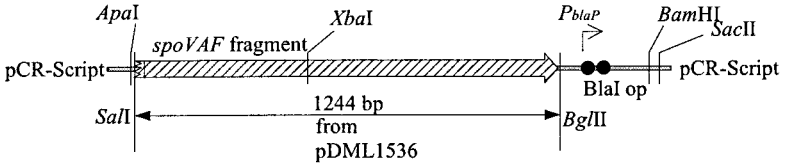
Plasmids	Description or structure	Source or reference
pGEM-T Easy	Cloning vector	Promega
pPCR-Script SK(+)	Cloning vector	Stratagene
pIC333	Mini-Tn*10* delivery vector	23
pAC7	Integrative vector at *amyE* locus from *B. subtilis* 168	22
pDML967	pSL1190 derivative with 0.7-kb PCR fragment containing *gfp*	A. Brans
pDG792	Plasmid containing a kanamycin resistance gene	10
pDML995	pMK4 derivative with *blaP* from *B. licheniformis* 749I	A. Brans
pRB373	*B. subtilis*/*E. coli* shuttle plasmid	1
pDML1515	pRB373 with 1.3-kb fragment containing *blaP* from *B. licheniformis* 749I	A. Brans
pDML1570		This work
pDML1534		This work
pDML1536		This work
pDML1537		This work
pDML1538		This work
pDML1539		This work
pDML1540		This work

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TABLE 2—*Continued*

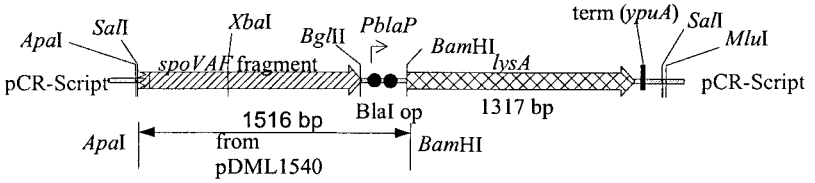
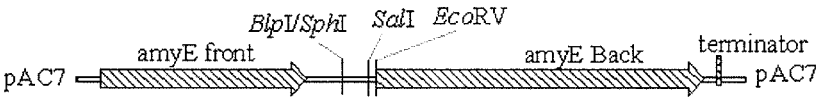
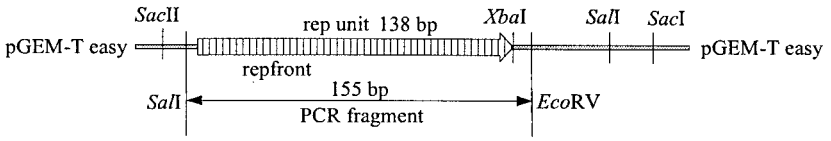
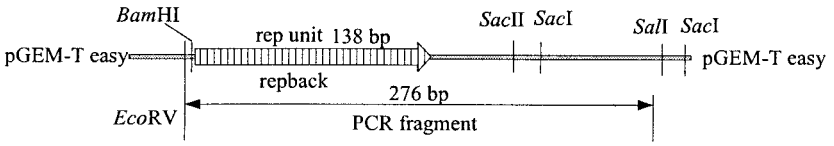
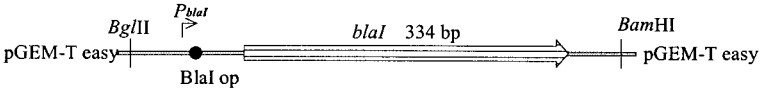
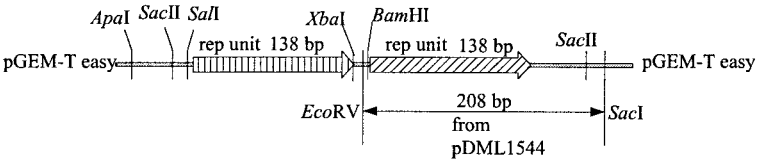
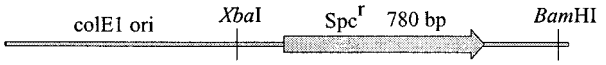
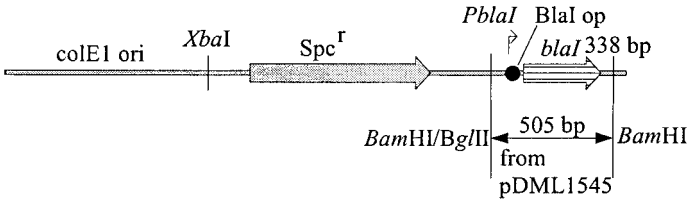
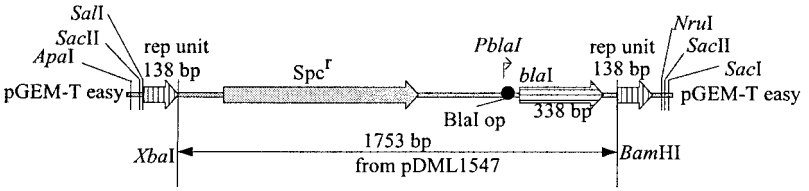
Plasmids	Description or structure	Source or reference
pDML1541		This work
pDML1542		This work
pDML1543		This work
pDML1544		This work
pDML1545		This work
pDML1546		This work
pDML1566		This work
pDML1547		This work
pDML1548		This work

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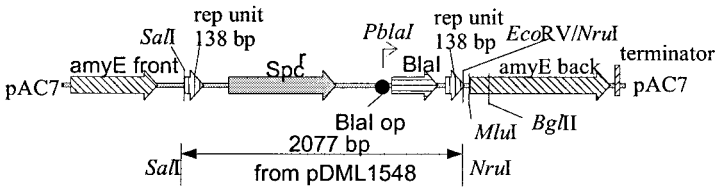
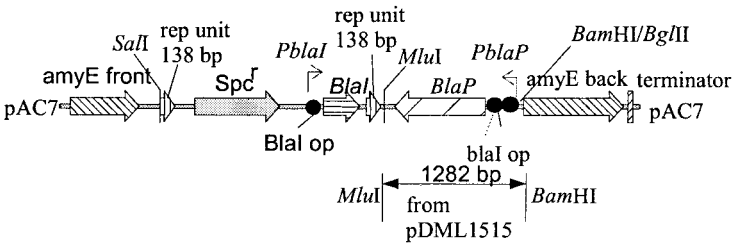
TABLE 2—*Continued*

Plasmids	Description or structure	Source or reference
pDML1549		This work
pDML1567		This work

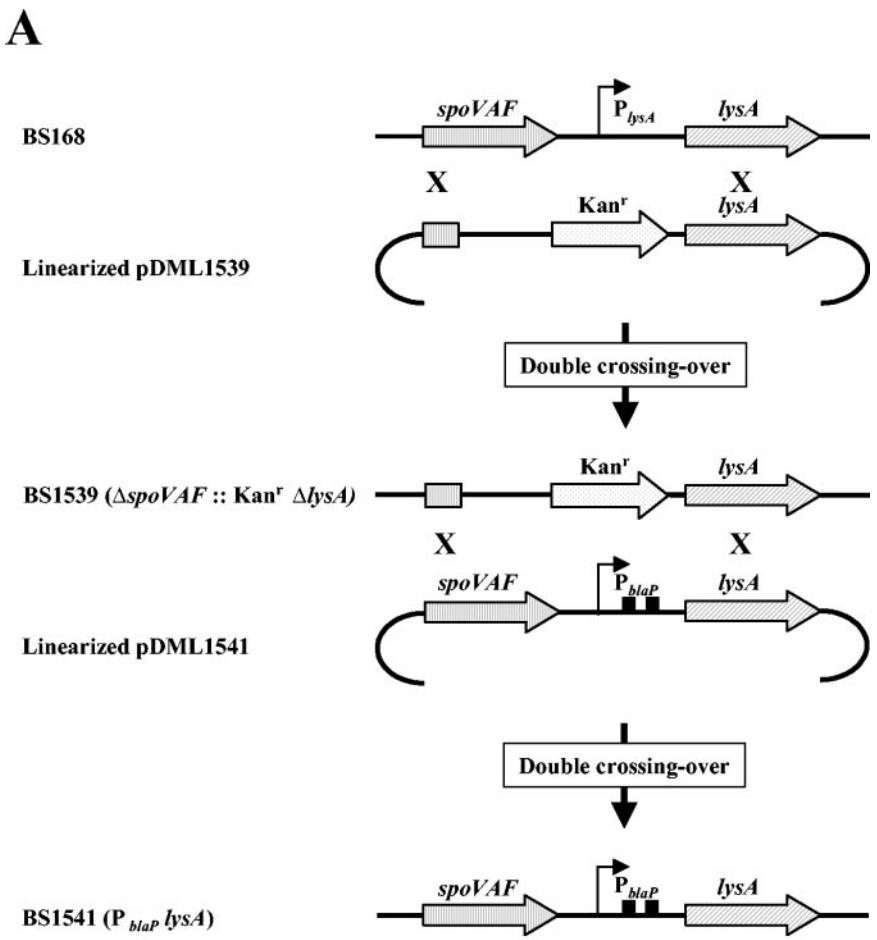
probably due to the partial cleavage of the pDML1539 plasmid with the ApaI endonuclease.

Plasmid pDML1541 is a derivative of the *E. coli* pCR-Script plasmid that contains the 1,244-bp *spoVAF* 3′ end and the *lysA* genes, between which a 362-bp fragment was inserted. This fragment contains the regulated *B. licheniformis* P_{blaP} promoter of the β-lactamase *blaP* gene (for details see Materials and Methods). This plasmid, previously linearized by the action of the ApaI restriction endonuclease, was introduced into BS1539 to achieve a double-crossover event. In the resulting BS1541 strain, selected on minimal medium (Fig. 1), the homologous recombination restores the *spoVAF* gene, the Kan^r gene is removed, and the *lysA* gene is under the control of the P_{blaP} promoter (for this experiment the yield of double crossover was around 1%). Consequently, the BS1541 strain could be switched to lysine auxotrophy if the *blaI* gene that negatively controls the P_{blaP} promoter is added in this strain. For this reason, the BS1541 strain is a conditionally auxotrophic strain for lysine. The double-crossover events in the selected BS1539 and BS1541 strains were confirmed by Southern blot analysis (Fig. 1).

Construction of the selection-eviction BlaI cassette. To achieve positive selection for genetic modification of BS1541 and the eviction of the gene encoding antibiotic resistance used as a selection marker, the 2,029-bp BlaI cassette illustrated in Fig. 2 was constructed. In this cassette, the Spc^r gene responsible for spectinomycin resistance in *Bacillus* spp. and the *blaI* gene encoding a DNA-binding protein are flanked by two

TABLE 3. Synthetic primers for PCR amplification

Name	Length (bp)	Sequence (5′ → 3′)
BlaIbam+	30	ATATGGATCCATCAAAATCGTCTCCCTCCG
promblaPBglII	32	ATCCAGATCTTCCCTCCGTTCATTTGTCCCCG
lysABamHI	34	ATGGATCCACACGGCACAAGCAGACAAAATCAAC
lysAend	25	CATTGATTTCTTCGTATCTATCTGG
repGFPbackup	33	GATATCGGATCCTTTTACCAGACAACCATTACC
repGFPbackdo	27	AGATCTATGACCATGATTACGCCAAGC
repGFPfrup	30	GTCGACTCCTTTTACCAGACAACCATTACC
repGFPfrdo	33	GATATCTCTAGATTTGTATAGTTCATCCATGCC
spoVAFBglI	31	AGATCTTGCGATTATGAATTGGTAGGCTGCC
spoVAFMluI	27	ACGCGTATGCCGGACCACAAGGAAGAG
pBlaIBglII	30	CAGATCTAAGTGATGGAATTAAAAATGCAG
stopBlaIBamHI	31	CGGATCCTCATTCCTTCTTTCTGTTCTTATG
Spc+	31	GGAAGTTCAATACTTGGAGTATATCTATTTG
Spc−	21	CTTATCATCACACTCTCCCCG
BlaP+	33	CGCTTCGATATAGTGACAATGCGGCACAGAATC
BlaP−	31	GCCGTTCATGTTTAAGGCTTTCATTACCACC
BlaINdeI	30	ATACATATGAAAAAAATACCTCAAATCTCTG
BlaIEcoRI	37	ATAGAATTCATTTCATTCCTTCTTTCTGTTCTTATG
kanaup	22	ATGGCTAAAATGAGAATATCAC
kanadown	21	CTAAAACAATTCATCCAGTAA
amyEfront	25	TTTATTGCTGTTTCATTTGGTTCTG
amyEback	24	GATGGTGTATGTTTTGCCAAATTG



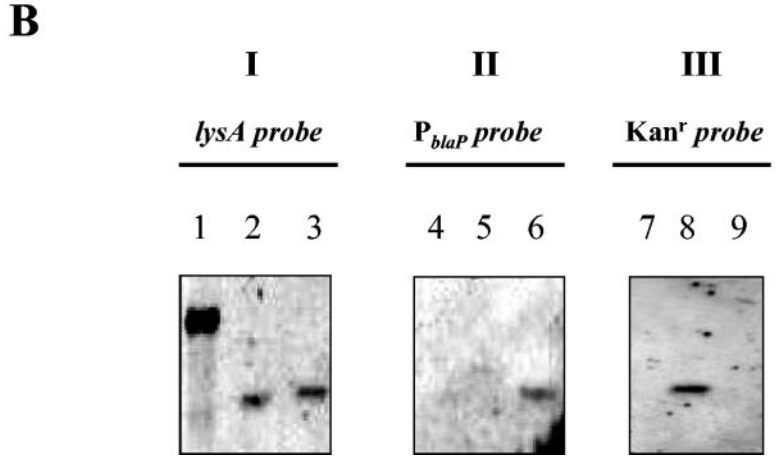


FIG. 1. (A) Construction of BS1539 (Δ*spoVAF*::Kan^r Δ*lysA*) and BS1541 (P_{blaP} *lysA*). pDML1539 and pDML1541 are pGEM-T-Easy and pCR-Script derivatives, respectively, constructed as described in Materials and Methods. P_{lysA} and P_{blaP} are *B. subtilis* and *B. licheniformis* promoters. The operator DNA sequences recognized by BlaI are indicated by shaded boxes. The Kan^r gene was used for kanamycin selection. An X indicates the recombination events leading to the chromosomal constructions of BS1539 and BS1541. In the latter strain, the *B. licheniformis* P_{blaP} promoter replaces the *B. subtilis* P_{lysA} promoter. (B) Southern Blot analyses of the BglII-digested chromosomal DNA from chromosomal BS168 (lanes 1, 4, and 7), BS1539 (lanes 2, 5, and 8), and BS1541 (lanes 3, 6, and 9) DNA. Panels I, II, and III contain the same patterns of DNA digests hybridized to *lysA* (I), P_{blap} (II), and Kan^r (III) probes. *lysA*, P_{blaP}, and Kan^r probes were generated by PCR with the following pairs of primers as amplimers: lysABamHI/lysAEnd, BlaIBam+/promblaPBglII, and kanaup/kanadown. pDML1539 or pDML1541 was the DNA template.

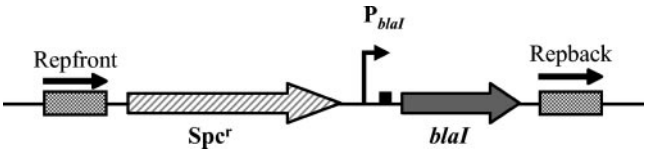


FIG. 2. Schematic representation of the BlaI cassette. The *blaI* gene is under the control of its own promoter (P_{blaI}). Spc^r indicates the spectinomycin resistance gene from pIC333. Rectangles represent the operator DNA sequence recognized by the BlaI repressor. *repfront* and *repback* contain direct repeat unit sequences (138 bp) used for the eviction of the BlaI cassette. For details see the text.

direct repeat units corresponding to the last 138 bp of the *Aequorea victoria gfp* gene (for details see Materials and Methods). The pDML1548 plasmid, a pGEM-T-Easy derivative, carries the BlaI cassette, which can be easily excised or fused with another sequence of interest by the presence of flanking unique restriction sites.

Integration and eviction of the BlaI cassette in BS1541. To probe the feasibility of the eviction of the BlaI cassette after its integration into the BS1541 chromosome, the BlaI cassette was subcloned in the *E. coli* pAC7 plasmid. In the resulting pDML1549 plasmid, the BlaI cassette is inserted between two DNA fragments corresponding to the 5′ and 3′ ends (amyEfront and amyEback) of the nonessential *B. subtilis amyE* gene, which encodes an α-amylase. Competent BS1541 cells were transformed with linearized pDML1549 and were plated on rich medium supplemented with 100 μg of spectinomycin/ml. The selected spectinomycin-resistant BS1549 strain (Fig. 3) is auxotrophic for lysine when the strain is replicated on minimal medium supplemented with spectinomycin (100 μg/ml), and the double-crossover event was confirmed by the lack of α-amylase activity visualized on starch plate assay and by Southern blot hybridization (data not shown). In BS1549, the *lysA* gene under the control of the P_{blaP} promoter is more tightly repressed by BlaI than the *blaI* gene itself, because the two BlaI operators located in the P_{blaP} promoter are recognized by BlaI with a higher affinity than the operator present in the P_{blaI} promoter (9) (Fig. 4). The auxotrophy of the BS1549 strain for lysine confirms that BlaI tightly represses the *lysA* gene. The fact that this strain is unable to grow on minimal medium without lysine suggests that it can be used as a counterselection marker during the eviction of the BlaI cassette.

The eviction of the BlaI cassette by a single crossover event (Fig. 3) was achieved by growing BS1549 for 24 h in 10 ml of antibiotic-free LB medium. After dilution of the culture, the clones isolated on minimal medium were analyzed for their ability to grow on minimal medium alone or supplemented with spectinomycin. It appeared that 50% of the isolated clones that had recovered the ability to grow on minimal medium without lysine (*lysA*$^+$) were spectinomycin sensitive. The BlaI cassette had thus been excised from these clones. To increase the yield of the *lysA*$^+$ and spectinomycin-sensitive recombinant strain, a 24-h culture of BS1549 cultivated in

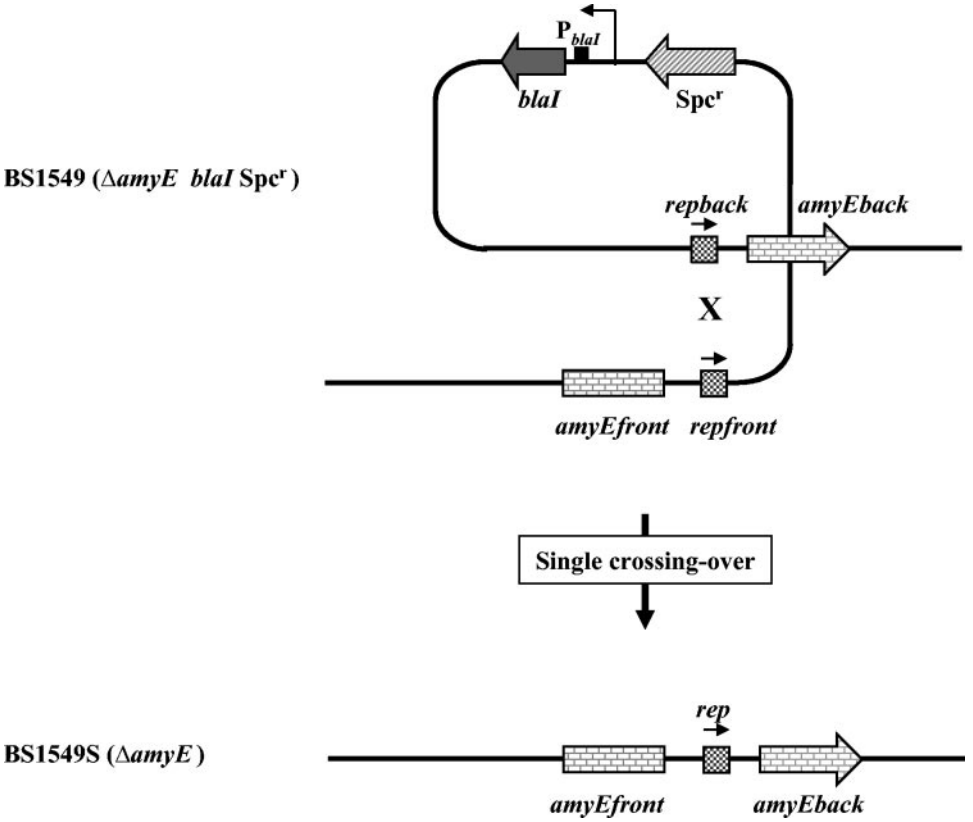


FIG. 3. Eviction of the BlaI cassette by single crossover between the two direct repeat unit sequences (*repfront* and *repback*) to generate the BS1549S (Δ*amyE*) strain. The lysine auxotrophy of the BS1549 strain, linked to the presence of the *blaI* gene (conditional auxotrophy), was used to select the recombinant strain that had excised the BlaI cassette.

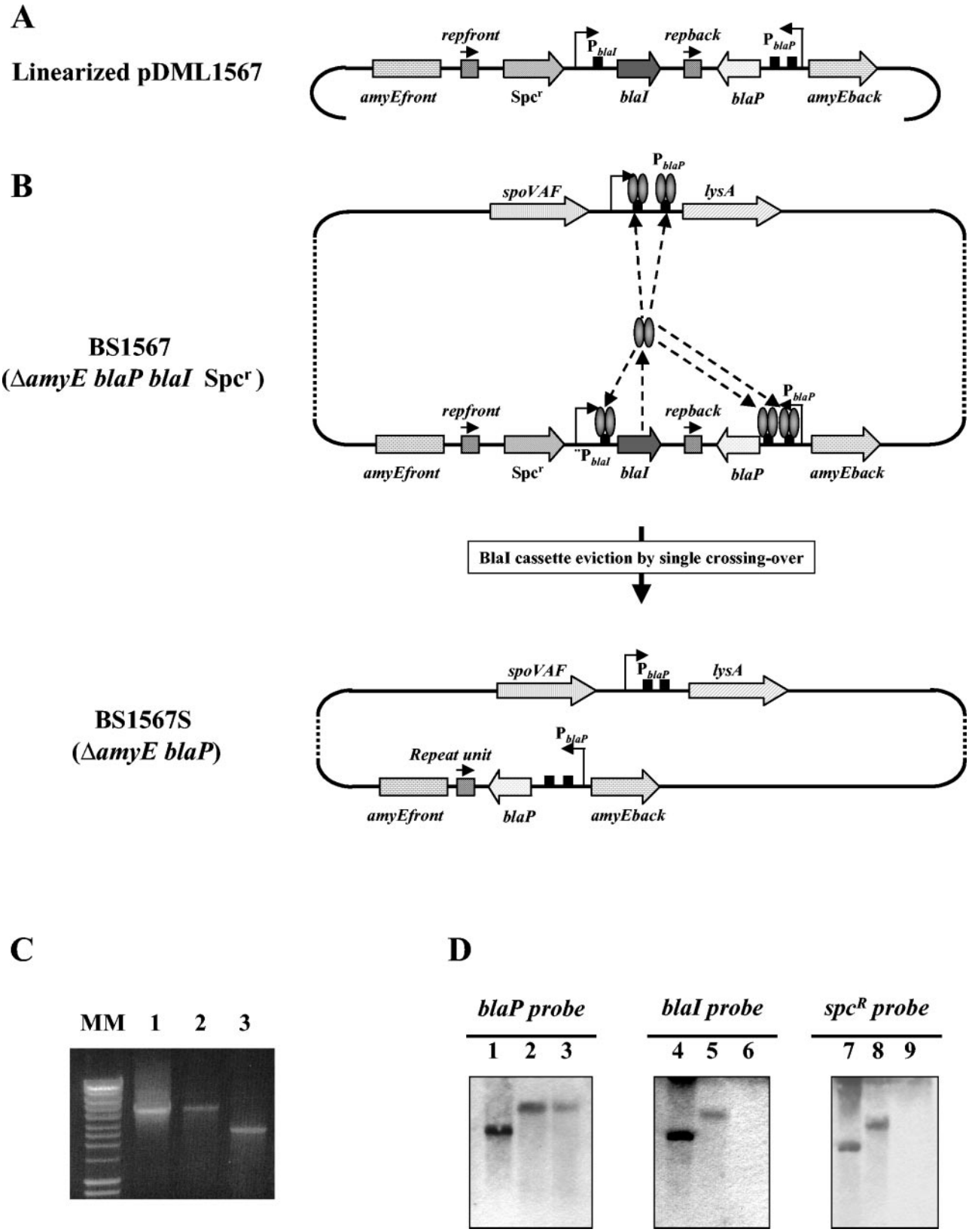


FIG. 4. Construction and characterization of BS1567 and BS1567S strains by PCR and Southern blot analyses. (A) Linearized pDML1567 plasmid carrying *blaP*, the BlaI cassette, and amyEfront and amyEback sequences. P_{blaI} and P_{blaP} are the native promoters of the *B. licheniformis* 749/I *blaI* and *blaP* genes, respectively. Rectangles correspond to the operator DNA sequences recognized by the BlaI repressor. (B) The pDML1567 insert was introduced by double crossover into the BS1541 strain to generate the BS1567 strain. This strain exhibits lysine auxotrophy

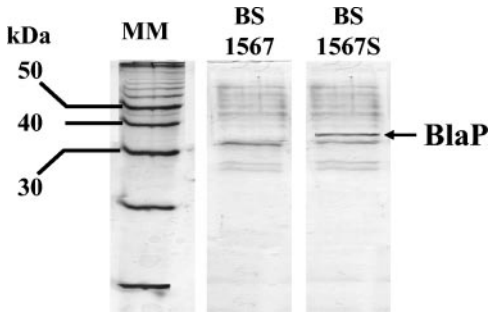


FIG. 5. β-Lactamase production in BS1567 and BS1567S. SDS-PAGE analysis of BS1567 and BS1567S culture supernatants is shown. MM, molecular mass marker.

antibiotic-free LB medium was diluted 1,000-fold into minimal medium and was further cultivated for 24 h. This latter step was repeated once, and the cells were spread on minimal agar plates. After analysis, 95% of the clones in the 72-h culture exhibited the *lysA*$^+$ and spectinomycin-sensitive phenotype. This result highlights that after 72 h of culture, the selection cassette had been excised in the majority of the *lysA*$^+$ clones. All colonies that had the *lysA*$^+$ and spectinomycin-sensitive phenotype (BS1549S) were tested for α-amylase production on a starch plate assay and were all *amyE* mutants, in agreement with the fact that, after the eviction of the counterselection and selection markers, one repeat unit remains in the chromosome and interrupts the *amyE* gene (Fig. 3). The reason why 50% of BS1549 cells have not excised the BlaI cassette after 24 h of culture in LB medium followed by selection on solid minimal medium remains unexplained. One hypothesis is that the small leakage of the P_{blaP} promoter repressed by BlaI combined with the exogenous lysine coming from the LB medium used to inoculate the minimal medium could allow *lysA* mutant cells to survive. On the other hand, a time course of the BlaI cassette eviction followed by the appearance of reporter β-lactamase activity showed that the BS1567 strain (see the next paragraph) cultivated in minimal medium grew very slowly over the first 24 h (A_{600} varied from 0.094 to 0.165) and that the enzymatic activity was only detected after 24 h of culture. Thereafter the cells grew normally and reached a cellular density similar to that of a *B. subtilis lysA*$^+$ strain.

Integration of a gene of interest in BS1541 and subsequent eviction of the BlaI cassette. The *B. licheniformis* 749/I *blaP* gene, encoding a class A β-lactamase, was used as a gene of interest and was inserted between amyEback and the second repeat of the BlaI cassette in the pDML1541 plasmid to generate pDML1567 (Fig. 4A). The chromosomal integration of the *blaP* gene and the BlaI cassette into the BS1541 *amyE* gene and the eviction of the BlaI cassette from the chromosome were carried out as described above. This generated BS1567 (Spc^r) and the desired spectinomycin-sensitive BS1567S strain (Fig. 4B). These two strains were characterized by PCR and Southern blot experiments, and their ability to express the *blaP* gene was estimated by measuring the β-lactamase activity in the culture medium. The PCR amplifications obtained by using complementary amplimers for amyEfront and amyEback and BS1567 or BS1567S chromosomal DNA as template is shown in Fig. 4C. The amplified fragment in BS1567 (4.5 kb) was the same length as that obtained when pDML1567 was used as DNA template, and it corresponds to the *amyE* gene in which *blaP* and the BlaI cassette have been inserted. By contrast, when the BS1567S chromosome was used as DNA template, the length of the amplified fragment was shorter by about 1.9 kb. This difference corresponds to the expected amplified fragment resulting from the excision of a fragment containing one repeat, *blaI* and the spectinomycin resistance gene, from the BS1567 chromosome. Southern blot analysis supported this conclusion (Fig. 4D). Indeed, BglII digestion of BS1567S chromosomal DNA yielded no detectable signal when *blaI* and Spc^r probes were used, whereas the presence of the *blaP* gene was detected (Fig. 4D). The analysis of the protein content of BS1567 and BS1567S culture media was carried out by SDS-PAGE, and, as expected, the excision of the counterselection and selection markers is correlated with overexpression of a protein that exhibits an apparent molecular size of 31 kDa, corresponding to that of the BlaP β-lactamase. The absence of this band in the culture medium of BS1567 and the determination of the β-lactamase activity in the two supernatants highlights the repression of the β-lactamase synthesis when the product of the *blaI* gene is present (Fig. 5). A 1,000-fold increase in β-lactamase activity was observed for BS1567S (BlaP specific activity per cell density, 12,000 ± 300 nmol of nitrocefin hydrolyzed/min · A_{600}) compared to that of BS1567 (BlaP specific activity per cell density, 15 ± 1 nmol of nitrocefin hydrolyzed/min · A_{600}). These experiments show that the repression mediated by BlaI is very efficient and that the basal expression of the gene of interest is maintained at a low level until excision of the repressor. This result pinpoints another interesting feature of the proposed method, i.e., the use of the BlaI repressor to control the gene of interest. In this case, the strong repression of the gene of interest until the excision of the BlaI cassette allows one to determine the permissive insertion sites in the *Bacillus* chromosome. Indeed, if no recombinant strain carrying the BlaI cassette is obtained for a specific chromosome insertion, it can be concluded that this insertion site is not permissive, because the lethal phenotype does not result in the overexpression of the gene of interest but is linked to the inactivation of the gene used for the target insertion site.

due to the presence of the BlaI repressor that negatively controls the expression of the *lysA* gene. For the same reason, the expression of the *blaP* gene is very low. The eviction of the BlaI cassette by single crossover between the two direct repeat unit sequences (*repfront* and *repback*) was achieved as described in the legend to Fig. 3 to generate the BS1567S (Δ*amyE blaP*) strain. (C) PCR amplifications of *amyE*. The amplified fragments were generated with primers amyEfront and amyEback and were analyzed by agarose gel electrophoresis. Lanes 1, 2, and 3 correspond to PCR experiments carried out with BS1567 chromosomal DNA, plasmid pDML1567, and BS1567S chromosomal DNA as template, respectively. MM, molecular size marker (Smart ladder; Eurogentec). (D) Southern Blot analysis of the BglII-digested chromosomal DNA from BS1567 (lanes 2, 5, and 8) and BS1567S (lanes 3, 6, and 9). Linearized pDML1567 (lanes 1, 4, and 7) was used as positive control. *blaP*, *blaI*, and Spc^r probes were generated by PCR with the following primers pairs as amplimers: BlaP+/BlaP−, BlaINdeI/BlaIEcoRI, and Spc+/Spc−. pDML1567 was the DNA template. For more details see Materials and Methods.

On the contrary, the absence of a recombinant that had excised the BlaI cassette during selection on minimal medium suggests that inappropriate expression of the protein of interest is lethal.

DISCUSSION

In the present study, we developed a method for directed genetic manipulation of BS168 combined with eviction of the antibiotic resistance gene used as a selection marker. This method relies on the use of the *B. licheniformis* β-lactamase P_{blaP} promoter, regulated by the BlaI repressor, to confer conditional lysine auxotrophy in a BS1541 strain in which the endogenous P_{lysA} promoter has been replaced with the *B. licheniformis* P_{blaP} promoter.

We used this method both to inactivate a specific gene and to introduce a gene of interest into the BS1541 strain. This strategy can also be used to replace one promoter with another, to generate a large chromosomal deletion, or to deliver a point mutation. In the latter case, the point mutation must be present in the two direct repeat sequences flanking the BlaI cassette. In addition, these genetic manipulations can be combined in the same strain, because the strategy can be repeated sequentially after eviction of the BlaI cassette. The direct repeat sequences flanking the BlaI cassette can be replaced by any other nucleotide sequence; for example, by a portion of the target sequence itself. This flexibility in choice of direct repeat sequences offers many potential applications, among which are (i) the selective deletion of gene(s) inserted in an operon without alteration of the gene(s) downstream of the deleted gene(s); (ii) the engineering of an artificial operon; and (iii) the replacement of a promoter or the replacement of a nonessential endogenous gene with an exogenous gene without modifying the native promoter and terminator of the replaced gene.

Compared to the delivery system described by Fabret et al. (6), based on the use of the upp cassette and a *B. subtilis upp* mutant, our approach gives similar results and offers the following two advantages. First, in our strategy the selection of the $lysA^+$ and spectinomycin-sensitive *B. subtilis* cells that have excised the BlaI cassette can be achieved by a simple transfer of the selected $lysA^+$ cells onto a spectinomycin-containing medium. That is not the case with the *upp* cassette strategy, for which PCR selection followed by strain isolation is required. Second, in our case, except for the introduced modification, the excised strain possesses the same phenotype as the wild-type *B. subtilis* isolate and is not a *upp* mutant. In conclusion, the strategy described in this paper is very efficient and can be used as a tool to manipulate the *B. subtilis* chromosome.

ACKNOWLEDGMENTS

This work was supported by the Belgian program on Interuniversity Poles of Attraction, initiated by the Federal Office for Scientific, Technical, and Cultural Affairs (PAI no. P5/33), the Fond National de la Recherche Scientifique (FNRS; crédit aux chercheurs no. 1.5201.02, credit FRFC 2.4530.03), la Région Wallonne (Bioval 3822–007), and the Communauté Française de Belgique (Actions de Recherche Concertées 2003–2008). B.J. is a FNRS associate researcher. P.F. was a fellow of the Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture (FRIA; Brussels, Belgium).

REFERENCES

1. **Bruckner, R.** 1992. A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli.* Gene **122:**187–192.
2. **Collier, D. N.** 1994. Expression of *Escherichia coli* SecB in *Bacillus subtilis* facilitates secretion of the SecB-dependent maltose-binding protein of *E. coli.* J. Bacteriol. **176:**4937–4940.
3. **Dahl, M. K., and C. G. Meinhof.** 1994. A series of integrative plasmids for *Bacillus subtilis* containing unique cloning sites in all three open reading frames for translational lacZ fusions. Gene **145:**151–152.
4. **Driks, A.** 1999. *Bacillus subtilis* spore coat. Microbiol. Mol. Biol. Rev. **63:**1–20.
5. **Eichenberger, P., S. T. Jensen, E. M. Conlon, C. van Ooij, J. Silvaggi, J. E. Gonzalez-Pastor, M. Fujita, S. Ben-Yehuda, P. Stragier, J. S. Liu, and R. Losick.** 2003. The σE regulon and the identification of additional sporulation genes in *Bacillus subtilis.* J. Mol. Biol. **327:**945–972.
6. **Fabret, C., S. D. Ehrlich, and P. Noirot.** 2002. A new mutation delivery system for genome-scale approaches in *Bacillus subtilis.* Mol. Microbiol. **46:**25–36.
7. **Fahnestock, S. R., and K. E. Fisher.** 1986. Expression of the staphylococcal protein A gene in *Bacillus subtilis* by gene fusions utilizing the promoter from a *Bacillus amyloliquefaciens* α-amylase gene. J. Bacteriol. **165:**796–804.
8. **Filée, P., K. Benlafya, M. Delmarcelle, G. Moutzourelis, J. M. Frère, and B. Joris.** 2002. The fate of BlaI repressor during the induction of the *Bacillus licheniformis* BlaP β-lactamase. Mol. Microbiol. **44:**685–694.
9. **Filée, P., C. Vreuls, R. Herman, I. Thamm, T. Aerts, P. P. De Deyn, J. M. Frère, and B. Joris.** 2003. Dimerization and DNA binding properties of the *Bacillus licheniformis* 749/I BlaI repressor. J. Biol. Chem. **278:**16482–16487.
10. **Guerout-Fleury, A. M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis.* Gene **167:**335–336.
11. **Harwood, C. R., and S. M. Cutting (ed.).** 1990. Molecular methods for *Bacillus*. John Wiley & Sons, Chichester, United Kingdom.
12. **Kobayashi, K., S. D. Ehrlich, A. Albertini, G. Amati, K. K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, P. Bessieres, F. Boland, S. C. Brignell, S. Bron, K. Bunai, J. Chapuis, L. C. Christiansen, A. Danchin, M. Debarbouille, E. Dervyn, E. Deuerling, K. Devine, S. K. Devine, O. Dreesen, et al.** 2003. Essential *Bacillus subtilis* genes. Proc. Natl. Acad. Sci. USA **100:**4678–4683.
13. **Kobayashi, T., Y. F. Zhu, N. J. Nicholls, and J. O. Lampen.** 1987. A second regulatory gene, *blaR1*, encoding a potential penicillin-binding protein required for induction of β-lactamase in *Bacillus licheniformis.* J. Bacteriol. **169:**3873–3878.
14. **Kunst, F., T. Msadek, and G. Rapoport.** 1994. Signal transduction network controlling degradative enzyme synthesis and competence in *Bacillus subtilis*, p. 1–20. *In* P. J. Piggot, C. P. Moran, and P. Youngman (ed.), Regulation of bacterial differentiation. American Society for Microbiology, Washington, D.C.
15. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis.* Nature **390:**249–256.
16. **Lampen, J. O., J. B. Nielsen, K. Izui, and M. P. Caulfield.** 1980. *Bacillus licheniformis* beta-lactamases: multiple forms and their roles. Philos. Trans. R. Soc. Lond. B Biol. Sci. **289:**345–348.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Olmos-Soto, J., and R. Contreras-Flores.** 2003. Genetic system constructed to overproduce and secrete proinsulin in *Bacillus subtilis.* Appl. Microbiol. Biotechnol. **62:**369–373.
19. **Pierce, J. A., C. R. Robertson, and T. J. Leighton.** 1992. Physiological and genetic strategies for enhanced subtilisin production by *Bacillus subtilis.* Biotechnol. Prog. **8:**211–218.
20. **Rosner, A.** 1975. Control of lysine biosynthesis in *Bacillus subtilis*: inhibition of diaminopimelate decarboxylase by lysine. J. Bacteriol. **121:**20–28.
21. **Sambrook, J. E. F. Fritsch, and T. Maniatis.**1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Shimotsu, H., and D. J. Henner.** 1986. Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis.* Gene **43:**85–94.
23. **Steinmetz, M., and R. Richter.** 1994. Easy cloning of mini-Tn*10* insertions from the *Bacillus subtilis* chromosome. J. Bacteriol. **176:**1761–1763.
24. **Williams, R. C., M. L. Rees, M. F. Jacobs, Z. Pragai, J. E. Thwaite, L. W. Baillie, P. T. Emmerson, and C. R. Harwood.** 2003. Production of *Bacillus anthracis* protective antigen is dependent on the extracellular chaperone, PrsA. J. Biol. Chem. **278:**18056–18062.
25. **Wu, S. C., M. Hassan Qureshi, and S. L. Wong.** 2002. Secretory production and purification of functional full-length streptavidin from *Bacillus subtilis.* Protein Expr. Purif. **24:**348–356.
26. **Wu, S. C., J. C. Yeung, Y. Duan, R. Ye, S. J. Szarka, H. R. Habibi, and S. L. Wong.** 2002. Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. Appl. Environ. Microbiol. **68:**3261–3269.