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Direct Selection of Cloned DNA in *Bacillus subtilis* Based on Sucrose-Induced Lethality

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Expression of the *Bacillus subtilis* or *Bacillus amyloliquefaciens sacB* gene in the presence of sucrose is lethal for a variety of bacteria. Sucrose-induced lethality can be used to select for inactivation of *sacB* by insertion of heterologous DNA in sensitive bacteria. This procedure has not been applicable to *B. subtilis* heretofore because expression of wild-type *sacB* is not detrimental to *B. subtilis*. The W29 mutation in the *B. amyloliquefaciens sacB* gene interferes with processing of the levansucrase signal peptide. The W29 mutation does not affect growth of *B. subtilis* in media lacking sucrose. However, this mutation inhibited growth of *B. subtilis* in media containing sucrose. Inactivation of the fructose polymerase activity encoded by *sacB* indicated that levan production was essential for sucrose-induced lethality. As a result, it was possible to select for cloned DNA in *B. subtilis* by insertional inactivation of the mutant *sacB* gene located on a multicopy plasmid vector in medium containing sucrose.

Bacillus subtilis has several significant advantages as a tool for biotechnology. Proteins secreted from *B. subtilis* are released directly into the culture medium rather than being trapped in a periplasmic space, as is frequently the case for *Escherichia coli* and other gram-negative bacteria (12). Furthermore, *B. subtilis* does not have lipopolysaccharide as a cell wall component so extracellular products are not contaminated by endotoxin (12). Although systems for genetic manipulation of *B. subtilis* are well developed, utilization of *B. subtilis* has lagged behind use of *E. coli* for cloning purposes. This problem is caused in part by the low efficiency of transformation of *B. subtilis* with plasmid DNA (3). As a result, it is difficult to clone DNA in *B. subtilis* that does not encode a function for which there is an efficient selection or screening procedure. There are a variety of shuttle vectors for *E. coli* and *B. subtilis* so that DNA can be initially cloned in *E. coli* and then transferred to *B. subtilis* for subsequent manipulation (3). However, in some cases, expression of a heterologous gene is toxic for *E. coli* (8, 13, 26, 29, 33). The number of systems which enrich or select for cloned DNA in *B. subtilis* is limited. One system that involves insertional inactivation of *lacZ* has been developed to screen for cloned DNA in *B. subtilis* (3). Another system is based upon insertional inactivation of a thymidylate synthetase gene to confer trimethoprim resistance on transformants with recombinant plasmids (11). Thus, there is a need for additional methods and vectors that can be used to efficiently clone DNA in *B. subtilis* without recourse to *E. coli*-based systems. A general-purpose vector for direct selection of cloned DNA would contribute to satisfying the deficiency and would greatly enhance the utility of *B. subtilis.*

Expression of *B. subtilis sacB* or *Bacillus amyloliquefaciens sacB* in the presence of sucrose is lethal to *E. coli* and a variety of other gram-negative and gram-positive bacteria (4, 9, 14–17, 20, 22, 28). The *sacB* gene encodes levansucrase (10, 18). Levansucrase catalyzes both hydrolysis of sucrose to glucose and fructose and polymerization of fructosyl groups to form levan. The basis for the lethality of levansucrase in the presence of sucrose is not fully understood. However, the inability of *E. coli* and certain other bacteria to grow if *sacB* is expressed in the presence of sucrose means that inactivation of *sacB* can be used to select directly for bacteria that contain DNA inserted into *sacB*. Several groups of workers have used *sacB* to trap insertion sequences and for marker exchange procedures (4, 9, 14, 16, 17, 22, 28). Insertional inactivation of the *B. amyloliquefaciens sacB* gene has been used to select for cloned DNA in *E. coli* (21).

Use of insertional inactivation of *sacB* has not previously been possible for *B. subtilis* because expression of wild-type *B. subtilis sacB* and *B. amyloliquefaciens sacB* in the presence of sucrose is not inhibitory for *B. subtilis* (19). However, the data in the present report indicate that a previously described mutation that alters the signal peptide of *B. amyloliquefaciens* levansucrase also causes sucrose to inhibit growth of *B. subtilis*. This observation was exploited to develop a general method for direct selection of cloned DNA in *B. subtilis.*

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. *B. subtilis* BE1510 (*trpC2 metB10 lys-3* Δ*aprE66* Δ*npr-82* Δ*sacA*::*phleo* Δ*sacB*::*ermC*) is a derivative of BE1500 (32) and was constructed by Joachim Ribbe in our laboratory by insertional inactivation of *sacA* with the phleomycin resistance gene from pUB110.

Plasmids pBE20, pBE504, and pBE517 have been described previously (2). pBE20 is an *E. coli-B. subtilis* shuttle vector. pBE504 is a derivative of pBE20 that encodes the wild-type amino acid sequence of *B. amyloliquefaciens* levansucrase. pBE517 is a derivative of pBE504 that contains the W29 mutation. Plasmid pUS19 was the source of the spectinomycin resistance gene used in construction of cloning vectors (1).

BE1510 and its derivatives were made competent and transformed by a two-step procedure (6). After incubation with DNA, the transformation cultures were diluted with 1 ml of brain heart infusion (Difco, Detroit, Mich.) and incubated on a roller drum at 37°C for 1 h prior to plating on selective medium.

LB medium and LB agar were prepared by using standard formulas (25). For some experiments, these media were supplemented with spectinomycin (100 μg/ml) or chloramphenicol (5 μg/ml).

Molecular techniques. PCR were performed by using AmpliTaq DNA polymerase (Perkin-Elmer) and a GeneAmp PCR reagent kit (Perkin-Elmer) according to the manufacturer's directions. The reactions were performed in a model 9600 GeneAmp PCR System thermocycler (Perkin-Elmer) by using 40

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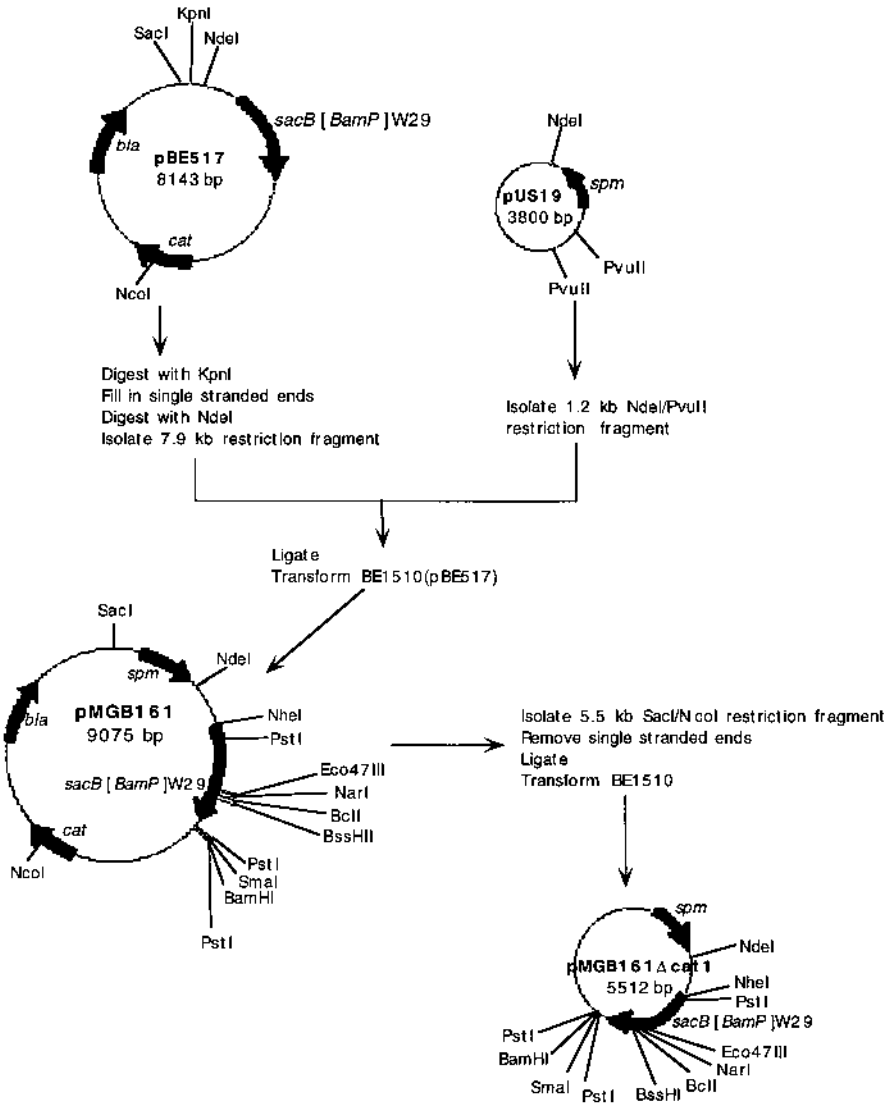

FIG. 1. Construction of pMGB161 and pMGB161Δcat1. *bla*, ampicillin resistance gene; *cat*, chloramphenicol resistance gene; *sacB*[*BamP*]W29, *B. amyloliquefaciens* levansucrase gene with W29 mutation (2); *spm*, spectinomycin resistance gene. NheI, Eco47III, NarI, BclI, and BssHI indicate unique restriction sites in pMGB161 and pMGB161Δcat1 that can be used to clone DNA by insertional inactivation of *sacB*[*BamP*]W29.

cycles consisting of a melting step (94°C for 1 min), a primer annealing step (45°C for 1 min), and a primer extension step (72°C for 2 min). After completion of the last cycle, the amplified DNA was separated from unincorporated primers by using the Wizard PCR Preps DNA purification system (Promega Corporation, Madison, Wis.).

All restriction enzymes were used according to the directions of the manufacturer (Promega). When necessary, restriction fragments were made blunt ended by treatment with T4 DNA polymerase (Promega) in 100 μl of reaction buffer (33 mM Tris-acetate [pH 7.9], 66 mM potassium acetate, 100 mM magnesium acetate, 5 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml, each deoxynucleoside triphosphate at a concentration of 0.1 mM) at 37°C for 5 min, followed by addition of 10 μl of 0.5 M EDTA and heating of the reaction mixture at 70°C for 10 min. Restriction fragments were analyzed by electrophoresis in 0.8% agarose minigels (23). Restriction fragments were removed from reaction buffers and agarose gels by using a Geneclean kit (Bio 101, Inc., La Jolla, Calif.) according to manufacturer's instructions. Ligation reactions were performed with T4 DNA ligase according to the guidelines of the manufacturer (New England Biolabs, Inc., Beverly, Mass.).

Construction of cloning vectors containing *sacB*[*BamP*]W29. Construction of cloning vectors pMGB161 and pMGB161Δcat1 is summarized in Fig. 1. Since pBE517 has origins for replication in *E. coli* and *B. subtilis*, pMGB161 is a shuttle vector for these bacteria. The *E. coli* origin of replication and a portion of a chloramphenicol acetyltransferase (*cat*) gene were deleted from pMGB161 to yield pMGB161Δcat1.

Plasmid DNA was extracted from *E. coli* DH5α(pBE517) by using the Wizard Minipreps DNA purification system (Promega). Alkaline lysis and polyethylene glycol precipitation were used to extract plasmid DNA from *E. coli* RR1(pUS19) and DH5α(pMGB161) that were grown in 500 ml of LB containing 50 μg of ampicillin per ml and were chloramphenicol amplified by standard methods (24). After BE1510 or BE1510(pBE517) was transformed with ligated DNA, samples of the transformation cultures were plated onto tryptose blood agar base (Difco) containing spectinomycin. The petri plates were incubated at 37°C for 18 h. Several spectinomycin-resistant transformants were inoculated into 3 ml of brain heart infusion containing spectinomycin and incubated on a roller drum at 37°C for 5 h. Plasmid DNA was extracted from each culture by using the Wizard Minipreps DNA purification system according to the manufacturer's directions except that lysozyme (5 mg/ml; Sigma Chemical Co., St. Louis, Mo.) was added to the resuspension buffer and the resuspended cells were incubated at 37°C for 15 min before the lysis buffer was added. The plasmid DNA was digested with *Eco*RI, and the resulting restriction fragments were analyzed by agarose electrophoresis for restriction fragments of the expected sizes (5.7, 1.7, and 1.5 kb for pMGB161; 4.0 and 1.5 kb for pMGB161Δcat1). Detailed restriction maps of pMGB161 and pMGB161Δcat1 are shown in Fig. 1.

Modification of *sacB*[*BamP*]W29 by site-directed mutagenesis. Two PCR primers were designed on the basis of the reported nucleotide sequences of *sacB*[*BamP*] (31) and the polylinker region of pBE504 (2). The primers were used to amplify a region of pMGB161Δcat1 that included the 473 bp between the *Bss*HII restriction site in *sacB*[*BamP*]W29 and the *Bam*HI restriction site in the

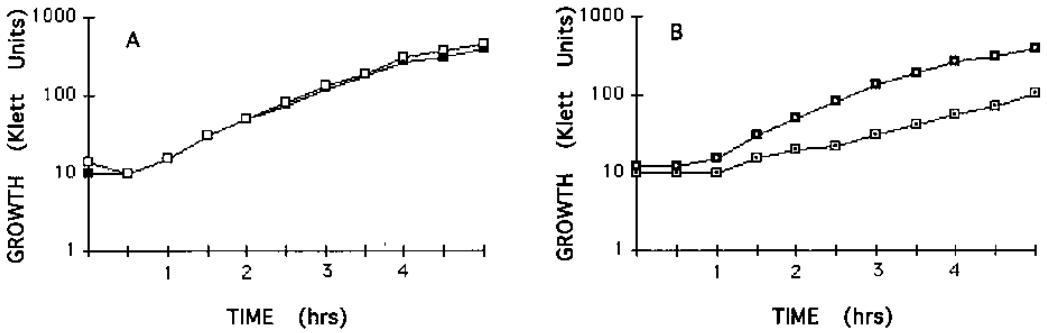

FIG. 2. Growth of BE1510(pBE504) and BE1510(pBE517). (A) BE1510(pBE504) grown with sucrose (□) and without sucrose (■). (B) BE1510(pBE517) grown with sucrose (⊡) and without sucrose (◘).

polylinker region (Fig. 1). One primer overlapped codon 331 of *sacB*[*BamP*]W29 and was designed to convert the Arg codon at position 331 into a Leu codon in the amplified DNA. The primer sequences were as follows: AGTGGATCCCC CGGGCTGCAGGAATTCACC and GAGCGCGCGAATGTTTTCAAAATG AACGGCAAATGGTACTTGTTCACTGATTCACTCGGTTC. The amplified DNA and plasmid pMGB161 were digested with *Bss*HII and *Bam*HI. The *Bss*HII-*Bam*HI-digested PCR-amplified DNA was ligated to the 8.6-kb *Bss*HII-*Bam*HI pMGB161 restriction fragment. The ligation mixture was used to transform commercially prepared *E. coli* RR1 (BRL/Life Technologies, Inc., Gaithersburg, Md.) by a standard procedure (27), with selection for ampicillin resistance. One typical transformant was designated RR1(pMGB165).

Cloning of DNA by insertional inactivation of *sacB*[*BamP*]W29. A *Bacillus* Genetic Stock Center strain, *Bacillus stearothermophilus* 9A2, was grown in 25 ml of brain heart infusion for 6.5 h at 60°C with shaking. *B. amyloliquefaciens* was grown in 25 ml of brain heart infusion for 5 h at 37°C with shaking. The bacteria were collected by centrifugation and resuspended in 2.5 ml of buffer (50 mM Tris, 10 mM EDTA; pH 8.0). Lysozyme was added to a final concentration of 200 μg/ml, and the resulting mixture was incubated at 37°C for 15 min. Proteinase K and sodium dodecyl sulfate were added (final concentrations, 50 μg/ml and 0.5%, respectively), and the resulting mixture was incubated at 55°C for 2 h. The DNA was precipitated by adding 0.5 volume of 7.5 M ammonium acetate and then 2 volumes of absolute ethanol. The DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and incubated with DNase-free RNase (final concentration, 150 μg/ml) for 30 min at room temperature. The DNA was extracted once with an equal volume of phenol-chloroform and once with chloroform and precipitated again with 7.5 M ammonium acetate and absolute ethanol. The isolated DNA was rinsed with 70% ethanol, air dried, and dissolved in TE buffer.

B. stearothermophilus DNA (60 mg) was partially digested with *Sau*3A under conditions (0.1 U of *Sau*3A at 23°C for 20 min in 50 ml of reaction buffer) that yielded predominately restriction fragments that were 0.5 to 20 kb long. The reaction mixture was extracted once with phenol-chloroform and once with chloroform. The DNA was precipitated with 7.5 M ammonium acetate and absolute ethanol and was dissolved in 10 μl of TE buffer. pMGB161Δcat1 (8 μg) was digested with *Bcl*I. The reaction mixture was extracted once with phenol-chloroform and once with chloroform, and the DNA was precipitated with 7.5 M ammonium acetate and absolute ethanol and dissolved in 10 μl of TE buffer. The *B. stearothermophilus* DNA was inserted into pMGB161Δcat1 by incubating a ligation reaction mixture (4.5 μl of a *Sau*3A partial digest of *B. stearothermophilus* DNA, 4.5 μl of *Bcl*I-digested pMGB161Δcat1, 1 μl of commercially prepared 10× ligation buffer, 200 U of T4 DNA ligase) at 23°C for 2.5 h. BE1510 was transformed with the entire volume of ligated DNA. The transformed cells were spread onto LB agar containing spectinomycin or LB agar containing spectinomycin and 5% sucrose. The petri plates were incubated at 30°C for 20 h.

The promoter and coding sequence of *B. amyloliquefaciens apr* were amplified by PCR. The sequences of the oligonucleotide primers were based on the reported nucleotide sequence of the *B. amyloliquefaciens apr* gene (32). The primers were designed to flank the target sequence with *Bam*HI restriction sites. The sequences of the primers were as follows: CCCCCAAAAATGGATCCAAAC CGTTCGACC and CGATTATGGAGCGGATTGAGGATCCGGAGG. The amplified DNA (total weight, 8 μg) was digested with *Bam*HI. The *Bam*HI-digested DNA was analyzed by electrophoresis, and a 1.5-kb fragment was extracted from the agarose gel. The DNA was resuspended in 10 μl of TE buffer. pMGB161Δcat1 (10 μg) was digested with *Bcl*I. The *Bcl*I-digested pMGB161Δcat1 and *Bam*HI-digested 1.5-kb fragment were combined, precipitated with 7.5 M ammonium acetate and ethanol, and resuspended in 10 μl of ligation buffer containing 200 U of T4 DNA ligase (New England Biolabs, Inc.). The ligation reaction mixture was incubated overnight at room temperature. BE1510 was transformed with the entire volume of ligated DNA. Transformed cells were spread onto LB agar containing spectinomycin or LB agar containing spectinomycin and 5% sucrose and incubated overnight at 30°C. Several transformants were patched from LB agar containing spectinomycin or LB agar containing spectinomycin and 5% sucrose to tryptose blood agar base containing spectinomycin and 1% skim milk. After 18 h at 37°C, the transformants were screened for overproduction of extracellular protease by examining the patches for large clear haloes.

RESULTS

Inhibition of bacterial growth by sucrose in strains with *sacB*[*BamP*]W29. The W29 mutation changes alanine into tryptophan at the −1 position of the signal peptide encoded by the *B. amyloliquefaciens* levansucrase gene (*sacB*[*BamP*]) (2). This mutation interferes with processing of the levansucrase signal peptide by *B. subtilis* and reduces the extracellular levansucrase activity to 10% of the wild-type activity (2). In these experiments, the W29 mutation did not interfere with the growth or viability of bacteria during the short exposures to sucrose used to induce levansucrase synthesis. As described below, we found that the mutant gene (*sacB*[*BamP*]W29) inhibited growth of *B. subtilis* BE1510 during prolonged exposure to sucrose. BE1510 is unable to metabolize sucrose because *sacA* and *sacB* have been inactivated in this organism. Introduction of *sacB*[*BamP*]W29 into *B. subtilis* BR151, a strain that is ancestral to BE1510 and has intact copies of *sacA* and *sacB*, did not result in growth being inhibited by sucrose (data not shown). Therefore, all subsequent experiments were performed with BE1510.

The growth curves in Fig. 2 indicate that plasmid pBE504, which encodes the wild-type amino acid sequence of *B. amyloliquefaciens* levansucrase, had no noticeable effect on the growth of *B. subtilis* BE1510 in LB or LB supplemented with 5% sucrose (Fig. 2A). Plasmid pBE517 contains *sacB*[*BamP*]W29. Growth of BE1510(pBE517) was indistinguishable from growth of BE1510(pBE504) in LB, and the doubling time was 40 min in this medium. However, BE1510(pBE517) required 75 min to double in LB containing 5% sucrose, indicating that the growth rate of BE1510(pBE517) was significantly depressed in the presence of sucrose (Fig. 2B). Likewise, the plating efficiency of BE1510(pBE517) was reduced more than 2,000-fold by the presence of 5% sucrose in LB agar, whereas the plating efficiency of BE1510(pBE504) on LB agar was unaffected by sucrose (Table 1). The results of similar experiments with *sacB*[*BamP*]W29 cloned into a single-copy bacteriophage ϕ105 vector (7) indicated that sucrose was not inhibitory for BE1510 when *sacB*[*BamP*]W29 was present in a single copy (data not shown).

Converting the arginine codon at position 331 of *sacB* (R-331) to a leucine codon does not affect the ability of levansucrase to hydrolyze sucrose but does prevent levansucrase from polymerizing the fructosyl groups of sucrose into levan (5). The

TABLE 1. Inhibition of BE1510(pBE517) growth by sucrose[a]

Strain	Presence of sucrose	CFU/ml
BE1510(pBE504)	−	5.1×10^6
	+	5.7×10^6
BE1510(pBE517)	−	3.0×10^6
	+	1.2×10^3

[a] Bacteria were inoculated into LB containing chloramphenicol and incubated at 37°C with shaking until the cultures attained an optical density of approximately 85 Klett units. Samples of serial dilutions of the cultures were plated onto LB agar containing chloramphenicol or LB agar containing chloramphenicol and 5% sucrose to determine the number of CFU per milliliter of liquid culture for each agar medium.

sacB[*BamP*]W29 gene on pMGB161 was modified by converting R-331 to a leucine codon (L-331) to form *sacB*[*BamP*]W29L331 on pMGB165. The results of a pulse-chase labeling experiment previously indicated that the W29 mutation blocked removal of the signal peptide from the precursor protein (2). A similar pulse-chase experiment demonstrated that the kinetics for processing the signal peptide for the *sacB*[*BamP*]W29 gene product in BE1510 were indistinguishable from the processing kinetics for the *sacB*[*BamP*]W29L331 gene product (data not shown). Hence, changing R-331 to L-331 did not alter the effect of the W29 mutation on removal of the signal peptide from the levansucrase precursor protein. As shown in Table 2, the plating efficiency of BE1510 (pMGB161) was reduced 1,000-fold by sucrose in LB agar. In contrast, the plating efficiency of BE1510(pMGB165) on LB agar was unaffected by sucrose. These data indicated that the levansucrase polymerase activity of the *sacB*[*BamP*]W29 gene product was required for sucrose to inhibit growth. Thus, synthesis of levan by the *sacB*[*BamP*]W29 gene product was detrimental to BE1510.

Direct selection in the presence of sucrose for the cloning of PCR-amplified DNA fragments. The observation that *sacB*[*BamP*]W29 interfered with growth in the presence of sucrose suggested that insertional inactivation of *sacB*[*BamP*]W29 could be used to select for plasmids with cloned DNA. Since the *B. amyloliquefaciens apr* gene was previously cloned, sequenced, and shown to be expressed in *B. subtilis* (31), *apr* was used as a model gene to test the effectiveness of sucrose selection and insertional inactivation of *sacB*[*BamP*]W29 for cloning DNA in *B. subtilis*. The PCR product containing *apr* was ligated into the unique *Bcl*I restriction site of pMGB161Δcat1 that is located within *sacB*[*BamP*]W29. BE1510 was transformed with the ligated DNA. Plating samples of the transformation culture onto LB agar containing spectinomycin or LB agar containing spectinomycin and sucrose revealed that fewer transformants grew on medium with sucrose (6.0×10^2 transformants per ml) than on medium without sucrose (3.4×10^4 transformants per ml). Several transformants were patched from the medium containing spectinomycin and the medium containing spectinomycin and sucrose to tryptose blood agar base containing spectinomycin and 1% skim milk to identify transformants that produced high amounts of extracellular protease. None of the 50 transformants taken from medium lacking sucrose was protease positive, whereas almost one-half of the transformants taken from medium containing sucrose (23 of 50 transformants) were protease positive. Plasmid DNA was extracted from four representative protease-positive transformants. Samples of each plasmid preparation were digested with *Eco*RI, and the resulting fragments were analyzed by agarose gel electrophoresis. An *Eco*RI digest of pMGB161Δcat1 yielded two restriction fragments that were 3.7 and 1.4 kb long. The 1.4-kb fragment contained the *sacB*[*BamP*]W29 sequence and would have been altered in size if DNA had been cloned into the *Bcl*I restriction site. The *Eco*RI digests of the plasmids from the four protease-positive transformants were similar to each other in that the 3.7-kb fragment was present in each digest but the 1.4-kb fragment was missing. In each case, the 1.4-kb fragment was replaced by one fragment that was 2.9 kb long. Hence, the plasmid from each of the four protease-positive transformants contained a cloned insertion of the size expected for the PCR-amplified *apr* gene (1.5 kb). These results indicated that sucrose selection for insertional inactivation of *sacB*[*BamP*]W29 could be used to select directly for transformants containing cloned DNA in *B. subtilis*.

Construction of a library in pMGB161Δcat1. A partial *Sau*3A digest of *B. stearothermophilus* DNA was ligated into the unique *Bcl*I restriction site within *sacB*[*BamP*]W29 on pMGB161Δcat1. Conditions of the digest were such that the restriction fragments ranged from less than 1 kb long to more than 20 kb long. Plating samples of the transformation culture onto LB agar supplemented with spectinomycin or LB agar supplemented with spectinomycin and sucrose again indicated that fewer transformants grew on the medium with sucrose (6.9×10^3 transformants per ml) than on the medium without sucrose (2.6×10^4 transformants per ml). Plasmid DNA was extracted from 10 representative transformants from medium containing spectinomycin and 10 representative transformants from medium containing spectinomycin and sucrose. Each plasmid was digested with *Eco*RI. The resulting restriction fragments were analyzed by agarose gel electrophoresis. *Eco*RI digests of the plasmids from all 10 transformants from medium lacking sucrose yielded two restriction fragments that were indistinguishable in size from pMGB161Δcat1 *Eco*RI restriction fragments. Thus, none of the transformants taken from medium lacking sucrose contained cloned *B. stearothermophilus* DNA. The *Eco*RI digests of the plasmids from the 10 transformants taken from medium containing sucrose were similar to each other in that the 3.7-kb fragment was present in each digest but the 1.4-kb fragment was missing. In each case, the 1.4-kb fragment was replaced by one fragment larger than 1.4 kb or two fragments with a combined size greater than 1.4 kb. Thus, each of the 10 transformants that had been subjected to selection on medium with sucrose contained plasmids with cloned insertions. One of the insertions was 6.3 kb long, and a second insertion was 1.1 kb long. The remaining transformants had insertions that ranged from 0.3 to 0.6 kb long.

DISCUSSION

Many of the current methods that are used to enrich or select for cloned DNA in *E. coli* have not been developed or do not work for *B. subtilis*. For example, dephosphorylated vectors cannot be used to enrich for recombinant plasmids with cloned insertions because nicked plasmids are inactive in transforma-

TABLE 2. Requirement for levansucrase polymerase activity for sucrose to inhibit growth of strains with *sacB*[*BamP*]W29[a]

Strain	Presence of sucrose	CFU/ml
BE1510(pMGB161)	−	3.0×10^6
	+	3.1×10^3
BE1510(pMGB165)	−	5.1×10^6
	+	2.7×10^6

[a] See Table 1, footnote *a*.

tion of competent *B. subtilis* cells (3). Accordingly, there is a need for efficient selection and screening procedures for cloned DNA in *B. subtilis*. This need was addressed by constructing plasmid vectors that enable direct, positive selection for cloned DNA in *B. subtilis*, even when the cloned DNA does not encode a selectable function.

Selection for cloned DNA in *B. subtilis* was based on the observation that sucrose was toxic for strain BE1510 when *sacB*[*BamP*]W29 was expressed from a multicopy plasmid. It has been suggested that sucrose is toxic when *E. coli* expresses *B. subtilis sacB* because levansucrase is trapped in the periplasm (30). Similarly, it has been suggested that sucrose is toxic for *Corynebacterium*, *Mycobacterium*, and *Nocardia* strains that express *B. subtilis sacB* because levansucrase is trapped in a pseudoperiplasmic space between the cytoplasmic membrane and an outer layer of hydrophobic mycolic acids (20). Entrapment of levansucrase could result in detrimental accumulation of levans between the inner and outer membranes and/or transfer of fructose residues to inappropriate receptors (30). The ability of the *sacB*[*BamP*]W29 gene product to synthesize levan was essential for sucrose to inhibit growth of BE1510. *B. subtilis* lacks an outer membrane to trap levansucrase. However, the W29 mutation blocks secretion of the *sacB*[*BamP*] W29 gene product by interfering with removal of the signal peptide from the levansucrase precursor protein (2). Accordingly, the *sacB*[*BamP*]W29 gene product may be tethered to the cytoplasmic membrane by its signal peptide rather than completely secreted into the culture medium, as is the case for wild-type levansucrase. Tethering of levansucrase to the cytoplasmic membrane would result in levan being synthesized and concentrated next to the cytoplasmic membrane and the peptidoglycan in a manner analogous to a cell with an outer membrane.

The cloning experiments described in this report involved transforming plasmid-negative BE1510 with a ligated mixture of vector and insertion DNA. Although no precautions were taken to prevent self-ligation of the vectors and no selection was exerted for any functions encoded by insertion DNA, cloned *B. stearothermophilus* DNA was identified in all of the transformants examined that were selected on medium containing sucrose. Transformants with cloned DNA grew on agar medium containing sucrose because insertional inactivation of *sacB*[*BamP*]W29 by the cloned DNA prevented synthesis of toxic levans. In contrast, cloned DNA was not detected in any of the transformants taken from medium lacking sucrose. Hence, insertional inactivation of *sacB*[*BamP*]W29 was highly effective in selecting transformants with cloned DNA. Any selection method or screening procedure for identifying cloned DNA can be expected to yield some false-positive colonies. In the case of sucrose selection for inactivation of *sacB*[*BamP*] W29, low-level reversion of the W29 mutation seemed to be the main source of sucrose-resistant transformants that did not have cloned DNA. However, such revertants were readily recognized because of excess production of levan, which resulted in a highly viscous colony morphology that was easily distinguished from the rougher colony morphology of transformants containing insertions.

The efficiency of insertional inactivation of *sacB*[*BamP*]W29 for selecting transformants with cloned DNA enables procedures that were not previously feasible with *B. subtilis* to be used. It was necessary to screen only 50 sucrose-resistant colonies to identify nearly two dozen transformants that expressed the *B. amyloliquefaciens apr* gene. This result indicated that *B. subtilis* can now be considered for cloning a gene that can only be identified by a difficult assay procedure because insertional inactivation of *sacB*[*BamP*]W29 could be expected to significantly reduce the number of transformants that would have to be assayed. Furthermore, plasmid vectors with *sacB* [*BamP*]W29 could be used to construct DNA libraries in *B. subtilis*. Construction of libraries in *B. subtilis* could be advantageous because expression of certain heterologous genes is toxic for *E. coli* (8, 13, 26, 29, 33). Selection for insertional inactivation of *sacB*[*BamP*]W29 would assure that nearly all transformants contained cloned DNA. Small fragments that were a few hundred base pairs long apparently predominated in the *B. stearothermophilus* DNA library described in this report. However, it was evident that pMGB161Δcat1 could accommodate insertions as large as 6 kb. Accordingly, it should be possible to construct a library that is enriched for large insertions by size selecting the restricted DNA prior to ligation with the vector.

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