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Genetics

Molecular cloning of genetically active fragments of *Bacillus* DNA in *Bacillus subtilis* and properties of the vector plasmid pUB110

(genetic complementation/tryptophan/*Eco*R1 endonuclease)

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ABSTRACT Plasmid pUB110 (~2.8×10^6 daltons), originally detected in *Staphylococcus aureus*, specifies resistance to neomycin and has been transformed into *Bacillus subtilis* 168. In *B. subtilis*, pUB110 is stably maintained at about 50 copies per chromosome and renders the host resistant to neomycin sulfate at 5 μg/ml. pUB110 isolated from *B. subtilis* transforms Rec$^+$ and *recE4*-containing strains of *B. subtilis* at frequencies $\geq 10^3$ transformants per μg of plasmid. pUB110 was transferred by PBS1 or SP10 transduction from *B. subtilis* to strains of *B. pumilus* and *B. licheniformis*. pUB110 is compatible with each of four previously described *Bacillus* plasmids, including pPL576, pPL10, pPL7065, and pPL2. pUB110 contains a single *Eco*R1-sensitive site and was used as vector to clone DNA fragments that complement the *trpC2* mutation in *B. subtilis* 168 from *Eco*R1 digests of the chromosome DNA isolated from *B. pumilus* strains NRRL B-3275 and NRS576, *B. licheniformis* strains 9945A and 749C, and *B. subtilis* 168. Genetic and physical properties of each of the constructed Trp derivatives of pUB110 are described.

The technique of molecular cloning involves the *in vitro* insertion of fragments of DNA into small replicons, plasmids, or phage genomes, followed by selection of chosen recombinant molecules by transformation of appropriate recipient cells (1, 2). Direct application of recombinant DNA technology to the study of *Bacillus subtilis* will ultimately provide a general method for constructing partial diploid strains which will, in turn, permit genetic complementation analyses of specific mutations and provide a source of easily obtainable DNA highly enriched for genes of chromosomal origin whose *in vitro* expression may be of special interest such as sporulation genes.

Among those *Bacillus* plasmids currently available, three determine host functions that do not permit direct selection of plasmid-containing transformants of *B. subtilis* (3–5), or the plasmids govern no known host function [i.e., they are cryptic plasmids (6–8)]. In contrast, certain antibiotic resistance plasmids originally detected in *Staphylococcus aureus* have recently been transformed into *B. subtilis* where they are stably maintained and express the appropriate antibiotic resistance trait (ref. 9). In the present report we describe the properties of one such antibiotic resistance plasmid, pUB110, and the use of this plasmid for cloning *Eco*R1 endonuclease-generated DNA fragments in *B. subtilis* 168.

MATERIALS AND METHODS

Bacteria, Media, and Growth Conditions. The strains used are listed in Table 1. Media used included tryptose blood agar base (TBAB; Difco), Spizizen minimal medium (14), and antibiotic medium no. 3 [Penassay broth (PB) Difco]. When neomycin sulfate was added to these media, the final antibiotic concentration was 5 μg/ml. Incubation was at 37°; liquid cultures were grown with rotary shaking.

Gradient Centrifugation of DNA. Isolation of covalently closed circular duplex (CCC) DNA by CsCl/ethidium bromide gradient centrifugation and sedimentation of DNA through 5–20% neutral sucrose gradients were as described (4). In sucrose gradients the reference was [^{14}C]thymidine-labeled T7 DNA, assigned an S value of 32 (15).

Agarose Gel Electrophoresis. Conditions for electrophoresis of DNA fragments through horizontal slab gels of 0.7% agarose were those described in detail by Dean *et al.* (13). *Eco*R1 digested λ DNA was reference for molecular weight estimations (16).

Procedure for Cloning DNA Fragments. Twice the activity of *Eco*R1 endonuclease (Miles Laboratories) required to produce limit digests of a given concentration of plasmid pUB110 (e.g., 1 μg), as monitored by agarose gel electrophoresis, was routinely used to digest the plasmid. The same ratio of enzyme activity to DNA concentration was used to digest phenol-purified cell DNA extracted from *B. licheniformis* strains 9945A and 749C, *B. pumilus* NRRL B-3275, and *B. pumilus* NRS 576 (Table 1). After digestion, enzyme activity was terminated by incubating the DNA-containing solutions at 65° for 15 min. Annealing of the cohesive ends was achieved by combining digested cellular DNA (3 μg) with *Eco*R1-cleaved pUB110 (0.5 μg), both in *Eco*R1 digestion buffer (13), and holding the mixture at 2° for 18 hr. The solution (generally 100–200 μl) was adjusted by adding dithiothreitol to 10 mM, ATP to 50 mM, and 1 unit of T4-induced DNA ligase (Miles Laboratories). Incubation was then continued for 8 hr at 10° and then 8 hr at 15°. The resulting DNA preparations were dialyzed against 2-{[Tris(hydroxymethyl)methyl]amino}ethanesulfonate (TES) buffer (3) prior to use.

Ligated DNA preparations (2 μg) were added to 5×10^8 competent (17) BR151 cells (*trpC2 metB10 lys-3*). Cells and DNA were shaken for 1 hr at 37° and the entire 1-ml transformation mixture was diluted into 20 ml of PB containing neomycin sulfate (5 μg/ml). Controls, including cells not exposed to DNA and DNA incubated without cells, were similarly treated. After overnight incubation at 37°, only the culture containing the transformed cells had grown to saturation. Portions of this culture were washed with Spizizen minimal medium and diluted 1:10 into several 10-ml portions of Spizizen minimal medium supplemented with 0.05% acid-hydrolyzed casein and neomycin sulfate. Each of these cultures was then shaken at 37° overnight, at which time each had grown to saturation. A loopful of each culture was streaked to minimal agar containing 0.5% casein hydrolysate. After overnight incubation,

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Abbreviations: PB, Penassay broth; CCC, covalently closed circular duplex; NeoR, neomycin resistance; PFU, plaque-forming unit.

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Table 1. Strains of *Bacillus subtilis, pumilus,* and *licheniformis*

	Relevant properties*	Source or ref.
B. subtilis†		
BD366	*trpC2 thr-5* pUB110^{+}	D. Dubnau
BD224	*trpC2 thr-5 recE4*	D. Dubnau; 10
BR151	*trpC2 metB10 lys-3*	4
T24	*trpE*	S. Hoch; 11, 12
T22	*trpD*	S. Hoch; 11, 12
T5	*trpC*	S. Hoch; 11, 12
T12	*trpF*	S. Hoch; 11, 12
T20	*trpB*	S. Hoch; 11, 12
T4	*trpA*	S. Hoch; 11, 12
SB38	*aroB2 his-2*	J. Hoch
BR144	*tyrA1 his-2 trpC2*	J. Hoch
JH86T	*phe-1 spoB*	4
strain W23	Streptomycin-resistant	R. Gordon
strain ATCC7003	Wild type; pPL2^{+}	6
B. licheniformis		
9945A	Wild type	C. Thorne
9945A	Mutant FD052 *trp-1 pur-1 tyr-1*	C. Thorne
749C	Prototroph	W. Brammar
B. *pumilus*		
NRS576	Wild type; pPL576^{+}	3
NRS576(pPL7065)	pPL576^{+}, pPL7065^{+}	5
NRS576(pPL10)	pPL576^{+}, pPL10^{+}	4
NRRLB-3275	Wild type	13

* pUB and pPL refer to the presence of specific plasmids.

† *B. subtilis* strains are derivatives of strain 168, except for W23 and ATCC7003.

one or two colonies were selected and subjected to two successive single-colony isolations on TBAB containing neomycin. Each was checked to ensure that the cells were sensitive to ϕ105 and exhibited a requirement for lysine and methionine. A single colony was grown for plasmid isolation (4). The resulting purified plasmid DNA was used to transform BR151 to neomycin resistance.

Several of the transformants (50 or more) were tested for the tryptophan requirement. In every case, the neomycin-resistant transformants were Trp^{+} but remained Lys^{-} and Met^{-}. One transformant was selected for further study and served as a source of the specific constructed Trp plasmid (Table 2). Plasmid pSL106 contains a Trp fragment cloned from *B. subtilis* 168 DNA. The construction of pSL106 followed a slight variation of the method used to construct the other Trp plasmids. Construction and manipulation of recombinant DNA molecules were performed at the P1 level of containment. The cloning system has been approved by the Recombinant DNA Offices of the National Science Foundation and the National Institutes of Health.

Transduction and Transformation. PBS1 and SP10 transduction and DNA-mediated transformation were as described (18, 19) with the exception that phage and cells or DNA and cells were incubated for 1 hr at 37° prior to plating on selective media.

RESULTS

Properties of Plasmid pUB110. Approximately 7.6 ± 0.6% of the total cell DNA extracted from strain BD366 (pUB110) was isolated as CCC DNA molecules that sedimented at 21 ± 0.5 S in neutral sucrose gradients. An S value of 21 corresponds to a CCC DNA molecule with a molecular weight of approximately 2.9×10^{6} (20). The molecular weight of the *B. subtilis* chromosome has been estimated as 2×10^{9} (21). pUB110 was therefore present in *B. subtilis* at about 50 copies per chromosome.

pUB110 purified from BD366 (pUB110) [or from BR151 (pUB110)] by CsCl/ethidium bromide density centrifugation transformed strain BR151 to neomycin resistance (NeoR) at a frequency of about 10^{3}–10^{4} transformants per 1 μg of DNA. pUB110 contains a single *Eco*R1-sensitive site. Complete digestion of pUB110 (2.0 μg/ml) with *Eco*R1 (monitored by agarose gel electrophoresis) decreased the transformation frequency by more than 4 orders of magnitude. A portion of the digested plasmid was diluted to 0.05 μg/ml, allowed to anneal for 12 hr at 6°, treated with ligase, dialyzed against TES buffer, and used to transform BR151 to NeoR at a DNA concentration of 0.02 μg/ml. This procedure regenerated 63% of the original transforming efficiency.

We previously demonstrated that bacteriophage PBS1 inefficiently mediated transduction of the plasmids pPL10 and pPL7065 in strains of *B. pumilus* (5, 22). By contrast, PBS1-mediated transduction of pUB110 between derivatives of *B. subtilis* 168 occurred relatively efficiently at frequencies on the order of 1 transductant per 10^{5} plaque forming unit (PFU). PBS1-mediated transduction of any of several chromosome markers in *B. subtilis* was less efficient, occurring at frequencies on the order of 0.1–1 transductant per 10^{6} PFU. PBS1 propagated on BR151 (pUB110) generated NeoR transductants of *B. pumilus* strains NRS576 and W20 at an efficiency of 1

Table 2. Properties of pUB110 and derivative plasmids

		$M_r \times 10^{6}$		Complementing activity*							
					trp						
Plasmid	DNA source of cloned fragment	Intact†	*Eco*R1-digested	*aroB*	*E*	*D*	*C*	*F*	*B*	*A*	*his-2*
pUB110	—	2.9	2.8	0	0	0	0	0	0	0	0
pSL101	*B. licheniformis* 749C	5.4	5.4	0	0	0	+	+	+	+	0
pSL103	*B. pumilus* NRRLB-3275	5.0	2.8, 2.3	0	+	+	+	+	0	0	0
pSL104	*B. pumilus* NRS576	5.0	2.8, 2.3	0	0	±	+	+	0	0	0
pSL105	*B. licheniformis* 9945A	5.4	2.8, 2.6	0	0	±	+	+	0	0	0
pSL106‡	*B. subtilis* 168	4.5	4.5	0	0	0	+	+	0	0	0

* Complementing activity of each plasmid was determined in *B. subtilis*. +, mutation was complemented by the plasmid; 0, mutation was not complemented; ±, mutation was complemented but cells grew only poorly.

† Intact molecular weights were based on S values for each plasmid: pUB110, 21S; pSL101, 27S; pSL103, 26S; pSL104, 26S; pSL105, 27S; pSL106, 25S.

‡ pSL106 complements the *trpC2* mutation in a *recE4*-containing strain of *B. subtilis*. The ability of pSL106 to complement other mutations in *B. subtilis* under conditions that eliminate recombination has not been tested because all hosts tested were *Rec*$^{+}$.

transductant per 10^6 PFU. By comparison, transfer of chromosome markers between *B. subtilis* and *B. pumilus* by transduction has never been demonstrated.

pUB110 was transferred from BR151 (pUB110) to *B. subtilis* W23 by PBS1-mediated transduction. A single NeoR transductant was cloned and an SP10 lysate was prepared on these cells by the plate-lysis technique. The SP10 lysate was then used to generate NeoR transductants of an auxotrophic derivative of *B. licheniformis* 9945A (strain FDO52). The transduction frequency was approximately 1 transductant per 10^7 PFU. A single transductant was repeatedly cloned on TBAB containing neomycin and then grown for plasmid isolation. Approximately 8% of the total DNA extracted from the transductant was isolated as CCC DNA molecules that cosedimented through neutral sucrose gradients with pUB110 from BD366.

PBS1 propagated on BR151 (pUB110) was used to transfer pUB110 to *B. pumilus* and *B. subtilis* strains harboring other plasmids to test plasmid compatibility. Transduction of pUB110 into a derivative of *B. pumilus* NRS576 which harbored both pPL576 and pPL7065 (5) generated NeoR transductants at a frequency of 1 per 10^7 PFU. Each of two of the NeoR transductants contained 11 ± 1% of the total DNA as CCC molecules. Sucrose gradient centrifugation of the CCC DNA demonstrated the presence of the three plasmid species, pPL576, pPL7065, and pUB110. Similarly, two pUB110-containing NeoR transductants of a derivative of *B. pumilus* NRS576 that harbored pPL576 and pPL10 (4) contained approximately 10% of the total cell DNA as CCC molecules, and sedimentation of the CCC DNA through neutral sucrose gradients resolved the three plasmid species. pUB110 was also inserted by PBS1 transduction into *B. subtilis* ATCC7003 where it is stably maintained and compatible with the resident 46×10^6 dalton cryptic plasmid pPL2 (6).

Cloning *Eco*R1 Generated Fragments of *B. licheniformis* and *B. pumilus* DNA that Complement the *trpC2* Mutation in *B. subtilis*. *B. licheniformis* and *B. pumilus* are species related to *B. subtilis* (23). Specific chromosomal mutations in *B. licheniformis* and *B. pumilus* have been transferred to *B. subtilis* by transformation [e.g., streptomycin resistance, rifampin resistance (24–26)]. However, transformation of many nutritional markers in *B. subtilis* to prototrophy by using DNA from *B. pumilus* or *B. licheniformis* either does not occur or does so at a frequency approaching the reversion rate of the mutations tested. Hybridization between the DNA extracted from *B. pumilus*, *B. subtilis*, and *B. licheniformis* detects only about 20% base sequence homology (27). Thus, the limited transformation of *B. subtilis* with the heterologous DNA is due partly, if not exclusively, to extensive regions of nonhomology among the chromosomes of the three species. In contrast, genetic mapping studies in *B. pumilus* and *B. licheniformis* suggest that the arrangement of genes on the chromosomes of these species may be similar to that in *B. subtilis* (13, 28, 29).

We chose initially to clone fragments from *B. pumilus* and *B. licheniformis* DNA that complemented the *trpC2* mutant allele in *B. subtilis* 168 for two reasons. First, the *trpC2* mutation in *B. subtilis* is not detectably transformed to Trp$^+$ by using DNA from strains of *B. pumilus* and *B. licheniformis*. Second, the gene order within the *trp* gene cluster is well established and several genetic markers that flank the cluster have been identified (30).

trpC2-Complementing fragments were cloned from *Eco*R1-digested *B. licheniformis* and *B. pumilus* DNA onto plasmid pUB110 by using *B. subtilis* strain BR151 as transformation recipient. BR151 harboring each of the constructed Trp derivatives of pUB110 contained 8 ± 1% of the total cell DNA as CCC molecules. *Eco*R1 digestion of the constructed Trp plasmids pSL103, pSL104, and pSL105 generated two DNA fragments, one of which comigrated with the *Eco*R1-generated linear form of pUB110 during electrophoresis in agarose gels (Fig. 1; Table 2). The second *Eco*R1-generated fragment from each of these Trp plasmids had a molecular weight less than that of pUB110 (Table 2). The sum of the molecular weights of the two *Eco*R1-generated fragments from each of these Trp plasmids approximated the molecular weight of the intact plasmid (Table 2).

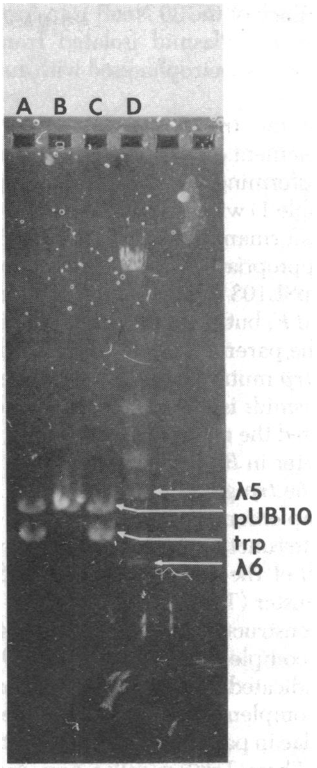

FIG. 1. Electrophoresis of *Eco*R1 fragments of pSL103 (A), pUB110 (B), pSL104 (C), and λ DNA (D) through a horizontal slab gel of 0.7% agarose. Each DNA sample (1–2 μg in 50 μl of digestion buffer) was incubated with 2 μl of *Eco*R1 at 37° for 30 min and then at 65° for 15 min before loading. Conditions for electrophoresis were as described (13). Migration was from top to bottom.

Plasmid pSL101 was constructed by cloning a *trpC2* complementing fragment from *Eco*R1-digested *B. licheniformis* 749C DNA. Intact pSL101 had a molecular weight of approximately 5.4×10^6 (Table 2). Unexpectedly, complete *Eco*R1 digestion of pSL101 generated only a single linear fragment with a molecular weight of approximately 5.4×10^6. It seems evident that, during or after the construction of pSL101, an *Eco*R1-sensitive site was deleted.

pSL101, pSL103, pSL104, and pSL105 were used in transformations of BR151. NeoR transformants were selected and each one tested (200/200 for each plasmid) was also Trp$^+$. By contrast, NeoR transformants of BR151 generated by using pUB110 remained tryptophan-requiring (200/200). Moreover, approximately 0.1% of the cells in logarithmic-phase PB cultures of BR151 harboring the above Trp plasmids were sensitive to neomycin. In each case (76 tested), the neomycin-sensitive cells were tryptophan-requiring. Thus, there was complete linkage of NeoR and the ability to complement the *trpC2* mutation in BR151.

pSL103 (Table 2) was digested with *Eco*R1, diluted to about 0.05 μg/ml, annealed, ligated, and then used to transform

BR151 to NeoR. Each of the 30 NeoR transformants remained tryptophan-requiring. Plasmid isolated from one of these transformant clones coelectrophoresed with authentic pUB110 in agarose gels.

The ability of the *trpC2*-complementing derivatives of pUB110 to complement other mutations in *B. subtilis* was determined by transforming appropriate auxotrophic derivatives of *B. subtilis* (Table 1) with pUB110 and each of the Trp plasmids. NeoR transformants were selected and 50–200 colonies were picked to appropriate media to determine the phenotype. By this method, pSL103 was found to complement mutations in *trpE, D, C,* and *F*, but not mutations in *trpA* or *trpB* (Table 2). In contrast, the parent plasmid, pUB110, did not complement any of the *trp* mutations. The complementing ability of the other Trp plasmids is shown in Table 2. None of the plasmids complemented the mutations *tyrA*, *aroB2*, or *his-2*, which flank the *trp* cluster in *B. subtilis* (30).

The order of the *trp* genes in the cluster is *EDCFBA* (30). Based on the complementation analyses, none of the plasmids carried a complete functioning *trp* cluster. However, the data suggest that each of the Trp plasmids carried a functioning segment of the cluster (Table 2).

Two of the constructed plasmids, pSL104 and pSL105, showed "spotty" complementation of the *trpD* mutation in *B. subtilis*. This is indicated as ± complementation in Table 2. In practice, spotty complementation refers to the appearance of many Trp^+ papillae in patches of *B. subtilis* T22 harboring the above plasmids. These Trp^+ papillae remained spotty upon repeated cloning, suggesting the plasmid-linked *trpD* gene product functions poorly in *B. subtilis*. This result may reflect decreased expression of the plasmid-linked *trpD* gene at the level of transcription/translation or decreased ability of the product of the "foreign" *trpD* gene to associate stably with the host-specified *trp* enzymes.

Insertion of the Trp Plasmids into a *recE4*-Containing Strain of *B. subtilis*. Dubnau and Cirigliano (10) described the properties of a recombination-deficient mutant of *B. subtilis* harboring the mutation designated *recE4*. A strain of *B. subtilis* carrying this mutation, BD224 (*recE4 trpC2 thr-5*), was transformed to NeoR at a frequency of approximately 10^3 transformants per μg of pUB110 DNA, but we were not able to transform the strain to threonine-independence or tryptophan-independence by using *B. subtilis* chromosome DNA at 1–5 μg/ml. Transformation of BD224 with each of the Trp plasmids generated NeoR transformants at a frequency of about 10^3 transformants per μg of DNA. The NeoR transformants tested (50 for each plasmid) simultaneously became Trp^+. Plasmid DNA was isolated from individual transformant clones of BD224 carrying pUB110 and pSL103. Sedimentation of this DNA through neutral sucrose gradients demonstrated homogeneously sedimenting species having the S value predicted for each plasmid: 21S and 26S for pUB110 and pSL103, respectively.

Cloning a Trp Fragment from *B. subtilis* DNA. *B. subtilis* BD224 (*recE4 trpC2 thr-5*) can be transformed with plasmid DNA but not with chromosome DNA. BD224 was therefore an ideal recipient for the cloning of a *B. subtilis trp* fragment onto pUB110. The cloning procedure followed that outlined in *Materials and Methods*, with the following exceptions: the donor chromosome DNA was purified from JH86T, a Trp^+ derivative of *B. subtilis* 168 (Table 1), and the transformation recipient was BD224. The resulting constructed plasmid, pSL106, sedimented at 25 ± 1 S in neutral sucrose gradients, indicating a molecular weight of approximately 4.5×10^6 (4). *Eco*R1 digestion of pSL106 generated a single linear molecule with a molecular weight of approximately 4.5×10^6 as determined by electrophoresis in agarose gels relative to *Eco*R1-digested λ DNA (16). Thus, pSL106 appeared to contain a single *Eco*R1-sensitive site as did the constructed plasmid pSL101.

Purified pSL106 DNA was used to generate NeoR transformants of each of the tryptophan-requiring mutants of *B. subtilis*, and 100–200 of the resulting transformants were tested for the tryptophan requirement. Insertion of pSL106 into strains T12 (*trpF*) and T5 (*trpC*) resulted in a Trp^+ phenotype. Strains T22 (*trpD*), T24 (*trpE*), T20 (*trpB*), and T4 (*trpA*) remained Trp^- when carrying pSL106. Insertion of pSL106 into SB38 and BR144 was also accomplished by selecting NeoR transformants. Each of 200 of the NeoR transformants of SB38 remained Aro^- and His^-. Similarly the NeoR transformants of BR144 (200 tested) were Tyr^- and His^- but Trp^+.

pSL106 was transformed into a Rec^+ recipient (BR151). Each of 50 transformants selected for NeoR were also Trp^+. One transformant clone was subjected to several single-colony isolations on TBAB containing neomycin and was then grown for approximately 100 generations in neomycin-free PB. Approximately 0.1% of the cells in the culture were sensitive to neomycin (27 were detected) and each of these was Trp^+. By contrast, when BD244 (pSL106) was used in this type of experiment, each of 16 neomycin-sensitive clones was Trp^-. These data suggest that pSL106 is capable of recombining with the *B. subtilis* chromosome in a Rec^+ host, but not in a Rec^- host.

DISCUSSION

Several of the properties of pUB110 demonstrate the general usefulness of the plasmid as a vector for molecular cloning in *B. subtilis*. Small *Eco*R1-generated DNA fragments can be inserted into the single *Eco*R1 site present in pUB110 without altering the ability of the plasmid to replicate in *B. subtilis* or the plasmid-specified NeoR trait. pUB110 and each of the derivative plasmids were maintained by recipient cells in a relatively stable state at high copy number (~50 per chromosome). Moreover, pUB110 has been transferred by PBS1 or SP10 transduction and by transformation among strains of three *Bacillus* species, *pumilus*, *subtilis*, and *licheniformis*.

pUB110 and each of the Trp derivatives of this plasmid were directly transformed into a *recE4*-containing strain of *B. subtilis* at frequencies on the order of 10^3 transformants per μg of plasmid. *recE4*-containing strains appear incapable of recombining homologous DNA fragments into the host chromosome (10). As predicted by these observations, it has been possible to clone a *trpC2*-complementing DNA fragment from *Eco*R1 digests of *B. subtilis* DNA onto pUB110 by using a *B. subtilis recE4*-containing recipient. The constructed plasmid pSL106 is stably maintained by the Rec^- strain in an extrachromosomal state. It seems likely that many fragments of the *B. subtilis* chromosome can be joined to pUB110 by the general method used for the construction of pSL106. The cloning of specific *Bacillus* chromosome fragments in *E. coli* by using phage or plasmid vectors has been demonstrated by several investigators (e.g., ref. 31). However, the *B. subtilis* system offers the potential for identifying and isolating specific DNA fragments on the basis of their biological effect on the process of sporulation.

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