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Recombination Between Compatible Plasmids Containing Homologous Segments Requires the *Bacillus subtilis recE* Gene Product

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Plasmid pSL103 was previously constructed by cloning a Trp fragment (~2.3 × 10^6 daltons) from restriction endonuclease *Eco*RI-digested chromosome DNA of *Bacillus pumilus* using the neomycin-resistance plasmid pUB110 (~2.8 × 10^6 daltons) as vector and *B. subtilis* as transformation recipient. In the present study the *Eco*RI Trp fragment from pSL103 was transferred in vitro to *Eco*RI fragments of the *Bacillus* plasmid pPL576 to determine the ability of the plasmid fragments to replicate in *B. subtilis*. Endonuclease *Eco*RI digestion of pPL576 (~28 × 10^6 daltons) generated three fragments having molecular weights of about 13 × 10^6 (the A fragment), 9.5 × 10^6 (B fragment), and 6.5 × 10^6 (C fragment). Trp derivatives of pPL576 fragments capable of autonomous replication in *B. subtilis* contained the B fragment (e.g., pSL107) or both the B and C fragments (e.g., pSL108). Accordingly, the B fragment of pPL576 contains information essential for autonomous replication. pSL107 and pSL108 are compatible with pUB110. Constructed derivatives of the compatible plasmids pPL576 and pUB110, harboring genetically distinguishable *Eco*RI-generated Trp fragments cloned from the DNA of a *B. pumilus* strain, exhibited relatively high-frequency recombination for a *trpC* marker when the plasmid pairs were present in a recombination-proficient strain of *B. subtilis*. No recombination was detected when the host carried the chromosome mutation *recE4*. Therefore, the *recE4* mutation suppresses recombination between compatible plasmids that contain homologous segments.

Specific plasmids detected in naturally occurring *Bacillus* strains have thus far been shown to determine only three host functions: resistance to tetracycline, the production of, and immunity or resistance to, killing activities, and the reduced ability of the host to form spores (1, 12, 13, 17, 18). Recently, several small antibiotic resistance plasmids initially detected in *Staphylococcus aureus* were transformed directly into *Bacillus subtilis* 168, where the plasmids were stably maintained and expressed the appropriate antibiotic resistance trait (6). In addition to plasmids that determine known host functions in *Bacillus*, several strains of *B. subtilis* and *B. pumilus* have been shown to harbor cryptic plasmids (15, 16, 21, 22). Studies from two laboratories suggest that the frequency of occurrence of *B. pumilus* and *B. subtilis* strains that harbor cryptic plasmids is within the range of 10 to 40% (15, 22, 23).

The recent development of convenient molecular cloning systems for *B. subtilis* has made use of vector plasmid pUB110, which was initially detected in *S. aureus* (9a). pUB110 transforms *B. subtilis* efficiently, the plasmid is maintained at high copy number, and the neomycin resistance trait specified by the plasmid provides an easily selectable host characteristic. We have used plasmid pUB110 to clone restriction endonuclease *Eco*RI fragments of the chromosome of *B. pumilus*, *B. licheniformis*, and *B. subtilis* strains that contain segments of the tryptophan gene cluster (9a). The Trp fragments cloned from *B. pumilus* and *B. licheniformis* appear incapable of recombining with the *B. subtilis* chromosome (9a). Moreover, these Trp fragments are not capable of autonomous replication in *B. subtilis* unless joined to a replicon such as a plasmid. Therefore, these Trp fragments can be used to identify *Eco*RI fragments of plasmids essential for autonomous replication in *B. subtilis*, essentially in the manner that was used to identify the "replication fragment" of the F factor (10, 24).

In the present report we demonstrate transfer of a *B. pumilus* Trp fragment from a pUB110-Trp derivative to *Eco*RI fragments of *Bacillus* plasmid pPL576, with the resulting generation

of new Trp derivative plasmids that are stably maintained by *B. subtilis* and are compatible with pUB110. That the compatible plasmids pPL576 and pUB110 can be modified through recombinant DNA technology to harbor homologous Trp fragments has permitted us to study recombination between the plasmid pairs in recombination-proficient and recombination-deficient strains of *B. subtilis*.

MATERIALS AND METHODS

Bacteria, media, and growth conditions. Strains used are listed in Table 1. Media used include tryptose blood agar base (Difco), Spizizen minimal medium (19), antibiotic medium no. 3 (Penassay broth, Difco), and SG sporulation agar (3). When neomycin sulfate was added to these media, the final drug concentration was 5 μg/ml. Incubation was at 37°C; liquid cultures were grown with rotary shaking.

Gradient centrifugation of DNA. Isolation of covalently closed circular (CCC) duplex DNA by cesium chloride-ethidium bromide gradient centrifugation and sedimentation of DNA through 5 to 20% neutral sucrose gradients were as previously described (18). In sucrose gradients, [^{14}C]thymidine (New England Nuclear Corp.) -labeled T7 DNA was the reference. T7 DNA was assigned an S value (sedimentation coefficient) of 32 (21).

Agarose-gel electrophoresis. Electrophoresis of DNA fragments through horizontal slab gels of 0.7% agarose followed previously described methods (9a). *Eco*RI-digested lambda phage DNA was the reference for molecular-weight estimations. Electrophoresis of DNA fragments through tube gels of 0.7% agarose was according to Helling et al. (7).

In vitro joining of DNA fragments. The methods for cloning *Eco*RI-generated fragments of chromosome DNA in *B. subtilis*, using plasmid pUB110 as vector, including procedures for digestion with endonuclease *Eco*RI, annealing, ligation, and transformation, were as previously detailed (9a). Containment was at the P1 level.

Transformation of *B. subtilis*. The method for generating competent cultures of *B. subtilis* was that described by Bott and Wilson (2). Cells and DNA (2 μg/ml or less) were incubated for 1 h at 37°C prior to plating on selective media.

RESULTS

Identification of an EcoRI fragment of pPL576 essential for autonomous replication in *B. subtilis*. Plasmid pPL576 (~28 × 10^6 daltons) was initially detected in *B. pumilus* NRS576, where its presence correlates with the reduced ability of the host to form spores (12, 13). Complete digestion of pPL576 with *Eco*RI endonuclease generates three fragments having molecular weights of approximately 13.0 × 10^6 (A fragment), 9.5 × 10^6 (B fragment), and 6.5 × 10^6 (C fragment) (14). To identify an *Eco*RI fragment of pPL576 essential for autonomous replication in *B. subtilis*, we transferred the Trp fragment from pSL103 to one of the *Eco*RI fragments of pPL576. pSL103 (~5 × 10^6 daltons) was originally produced by cloning an *Eco*RI-generated Trp fragment from *B. pumilus* NRRL B-3275 chromosome DNA onto plasmid pUB110 (~2.8 × 10^6 daltons) using a *B. subtilis trpC* mutant as the transformation recipient (9a). The Trp fragment present on pSL103 has a molecular weight of about 2.3 × 10^6, complements mutations in *trpE, trpD, trpC,* and *trpF,* and shows no ability to recombine with the *B. subtilis* 168 chromosome in a recombination-proficient host (9a).

TABLE 1. *Strains of B. subtilis and B. pumilus*

Strains	Relevant properties	Source/ reference
B. subtilis[a]		
BR151	*trpC2 lys-3 metB10*	9a
BD224	*trpC2 thr-5 recE4*	5, 9a
BR151R	*trpC2 lys-3 recE4*	[b]
T24	*trpE*	8, 9a
T22	*trpD*	8, 9a
T5	*trpC*	8, 9a
T12	*trpF*	8, 9a
T20	*trpB*	8, 9a
T4	*trpA*	8, 9a
JH86T	*phe-1 spoB*	18
BR151(pUB110)	*trpC2 lys-3 metB10-* (pUB110$^+$)	9a
BR151(pSL103)	*trpC2 lys-3 metB10-* (pSL103$^+$)	9a
B. pumilus		
NRS576	pPL576$^+$	12
BpB503[c]	*trpC*	9

[a] All *B. subtilis* mutants are derivatives of strain 168. pUB110$^+$, pSL103$^+$, pPL576$^+$ refer to the presence of specific plasmids. pUB110 confers neomycin resistance, and pSL103 both confers neomycin resistance and complements mutations in *trpE* → *trpF*.

[b] BR151R was constructed by congression (5) using BD224 DNA as donor and BR151 as recipient. BR151R is sensitive to methyl methane sulfonate and cannot be transformed to Trp$^+$ using *B. subtilis* JH86 DNA; it can be transformed with pUB110.

[c] BpB503 is a *trpC* mutant of *B. pumilus* NRRL B-3275.

Transfer of the Trp fragment from pSL103 to an *Eco*RI fragment of pPL576 was accomplished by combining 1 μg of pSL103 and pPL576 in 50 μl of *Eco*RI digestion buffer, followed by *Eco*RI digestion, annealing, and ligation as described (9a). The resulting DNA preparation was used to transform BR151 (*trpC2 lys-3 metB10*) to tryptophan independence at a DNA level of 2 μg/ml. Approximately 20% of the Trp$^+$ transformants tested (63) were neomycin-sensitive (Neo^s). Plasmid DNA isolated from each of seven Neo^s Trp$^+$ transformants yielded, upon *Eco*RI cleavage, fragments corresponding to the B fragment of pPL576 and the Trp fragment from pSL103, or both the B and C fragments of pPL576 and the Trp fragment. Two Trp$^+$ clones

carrying representatives of both plasmid types were analyzed in detail.

Plasmid pSL107 sedimented at 37(±1)S in neutral sucrose gradients, indicating an approximate molecular weight of 11.2×10^6 (4). CCC DNA isolated from BR151 (pSL107) accounted for about 4% of the total cell DNA, suggesting that pSL107 was present in about seven copies per chromosome. pSL107 DNA in the CCC form transformed the recombination-deficient (Rec^-) mutant BD224 (*trpC2 thr-5 recE4*) to Trp^+ at a frequency of approximately 500 transformants per μg of plasmid. In addition, purified pSL107 was capable of generating Trp^+ transformants of *trpE, trpD, trpC,* and *trpF* mutants of *B. subtilis,* but the plasmid did not generate Trp^+ transformants of *trpB* and *trpA* mutants (Table 1). The Trp-complementing activity of pSL107 was therefore identical to that of pSL103, the source of the Trp fragment.

*Eco*RI cleavage of pSL107 generated two fragments, one of which comigrated in agarose-gel electrophoresis with the B fragment of pPL576 (Fig. 1A). The other fragment seen in Fig. 1A comigrated with the Trp fragment of pSL103 (data not shown).

Plasmid pSL108 sedimented at 46.7(±1)S in neutral sucrose gradients, suggesting an approximate molecular weight of 18.0×10^6 (4). pSL108 was present in BR151 at about seven copies per chromosome, and purified pSL108 behaved similarly to pSL107 with respect to complementation of Trp mutants and ability to transform the Rec^- mutant BD224. Agarose-gel electrophoresis of *Eco*RI-digested pSL108 demonstrated that the plasmid was composed of both the B and C fragments of pPL576 in addition to the Trp fragment (Fig. 1B).

The above results demonstrate that the B fragment of pPL576 carries the necessary information for autonomous replication in *B. subtilis.* One would therefore expect that pSL107 and pSL108 would exhibit the compatibility properties of pPL576. Although this has not been extensively tested, we have found that pSL107 is compatible with pPL10, and both pSL107 and pSL108 are compatible with pUB110. These are consistent with results previously obtained with pPL576 (9a, 18).

Cloning of the B fragment onto pUB110. pS107 (~3 μg) was digested to completion with *Eco*RI, and the two resulting linear fragments

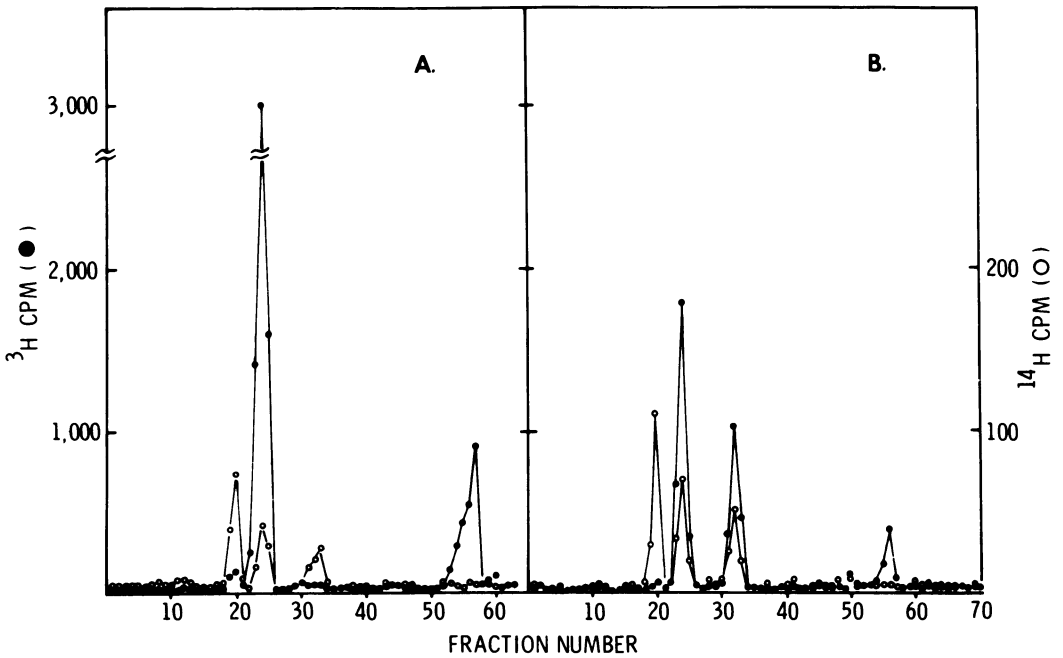

FIG. 1. *Electrophoresis of mixtures of EcoRI-cleaved pSL107 plus pPL576 and pSL108 plus pPL576 through 0.7% agarose gels. Approximately 0.5 μg of EcoRI-digested, [^{14}C]thymidine-labeled pPL576* (○) *was combined with approximately 0.5 μg of EcoRI-cleaved, [^{3}H]thymidine-labeled* (●) *pSL107 (gel A) or pSL108 (gel B). The 50-μl samples were applied to tube gels of 0.7% agarose (7). Electrophoresis proceeded at room temperature for 18 h at 34 V per gel. Gels were sliced into 2-mm sections, and each section was dissolved in concentrated ammonium hydroxide and counted in Hydromix (Yorktown Research). Migration proceeded from left to right.*

were resolved by sedimentation through a neutral sucrose gradient (Fig. 2). The fractions comprising the peak corresponding to the B fragment were pooled and dialyzed for 4 h against *Eco*RI digestion buffer. The DNA-containing solution (1 ml at ~1 μg/ml) was concentrated to 200 μl by evaporation. A 100-μl volume digestion buffer containing about 0.5 μg of the *Eco*RI-generated linear form of pUB110 was added, and the resulting solution was taken through the annealing and ligation procedure (9a). This DNA was then used to transform BR151 to Neo^r at a DNA concentration of about 0.5 μg/ml. Among five transformants tested, one harbored CCC DNA that migrated more slowly than the CCC form of pUB110 during electrophoresis through an agarose gel. This plasmid, designated pSL109, sedimented at 38.6(±1)S, suggesting a molecular weight of 12.1×10^6. Agarose-gel electrophoresis of a mixture of *Eco*RI-digested, differentially labeled pPL576 and pSL109 demonstrated the presence of the B fragment of pPL576 in pSL109 (Fig. 3). Thus, pSL109 is composed of two fused compatible replicons. Approximately 13% of the cell DNA isolated from BR151(pSL109) is in the CCC form, indicating that pSL109 is present in about 25 copies per chromosome.

The *recE4* mutation in *B. subtilis* suppresses recombination between compatible plasmids carrying homologous segments. The chromosomal mutation designated *recE4* prevents recombination of homologous DNA fragments with the *B. subtilis* chromosome regardless of whether the fragments are introduced by transformation or transduction (5). As previously shown (9a), a plasmid harboring a Trp fragment cloned from *B. subtilis* DNA (pSL106) was incapable of recombining with the host chromosome if the host carried the *recE4* mutation. The availability of compatible plasmids (e.g., pPL576 and pUB110), which through recombinant DNA technology can be used to construct derivative plasmids carrying homologous segments, permits a test of the ability of the *recE4* mutation to suppress recombination between plasmids.

pUB110 was used to clone a "*trpF*-complementing" *Eco*RI-generated fragment of the chromosome DNA isolated from BpB503, a *trpC* mutant of *B. pumilus* NRRL B-3275 (9). The initial transformation recipient for cloning was T12, a *trpF* point mutant of *B. subtilis* (Table 1). The resulting plasmid, pSL102, comigrated with pSL103 during electrophoresis in agarose gels, suggesting that pSL102 has an approximate molecular weight of 5×10^6. Agarose-gel electrophoresis of an *Eco*RI-digested mixture of differentially labeled pSL102 and pSL103 demonstrated comigration of the two *Eco*RI fragments of both plasmids. Accordingly, pSL102 and pSL103 were physically indistinguishable. This result was expected, since pSL103 and pSL102 are both pUB110 derivatives that we assumed to harbor a similar *Eco*RI fragment of *B. pumilus* NRRL B-3275 DNA (9a).

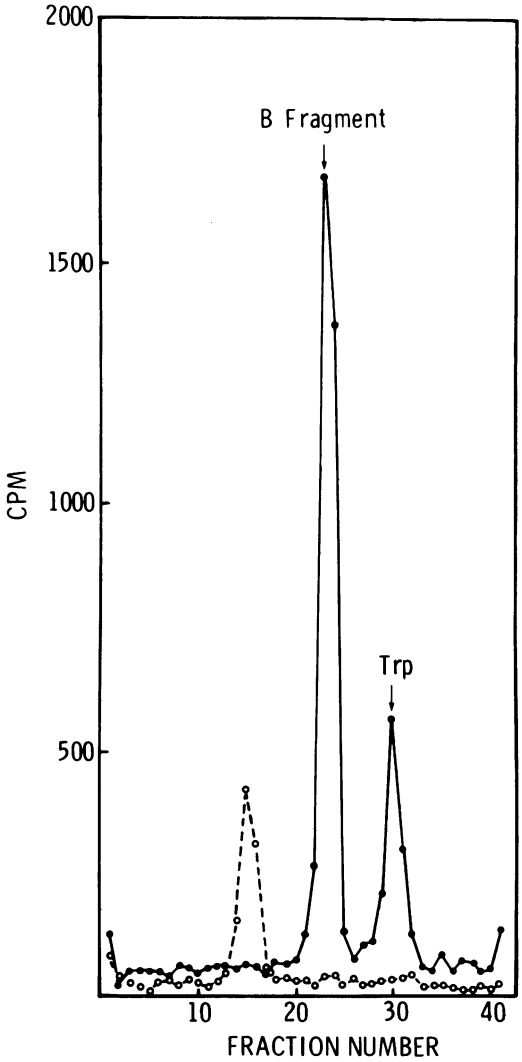

FIG. 2. *Sedimentation of T7 DNA and EcoRI-digested pSL107 through a neutral, 5 to 20% sucrose gradient. [^{3}H]thymidine-labeled (●) pSL107 (about 1 μg) was digested to completion with a twofold excess of EcoRI endonuclease (the amount of enzyme was empirically determined), held at 65°C for 10 min, and mixed with about 0.2 μg of ^{14}C-labeled (○) T7 DNA. The mixture was applied to a 5-ml, 5 to 20% linear, neutral sucrose gradient, which was then centrifuged in an SW50.1 rotor at 40,000 rpm for 3.5 h at 5°C. Fractions were collected, precipitated with 5% trichloroacetic acid, and counted as before (18).*

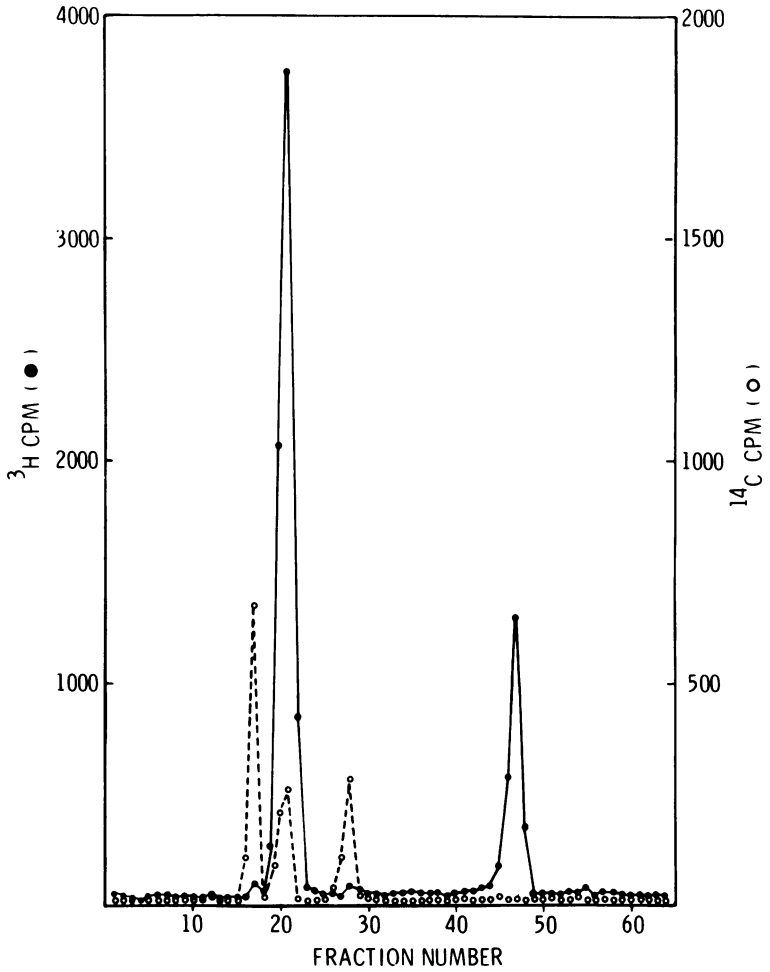

FIG. 3. *Coelectrophoresis of EcoRI-digested pSL109 and pPL576 through 0.7% agarose tube gels. Conditions were as described in Fig. 1. pSL109 was labeled with [^{3}H]thymidine* (●). *pPL576 was labeled with [^{14}C]thymidine* (○).

PSL102 complemented *trpF* and *trpD* mutations in *B. subtilis,* but not mutations in *trpE, trpC, trpB,* or *trpA.* This result was unexpected, since we anticipated that pSL102 would show the same complementation pattern as pSL103 (pSL103 complements *trpE* → *trpF*), with the exception that we expected pSL102 would not complement *trpC.* This discrepancy could reflect a difference between the Trp fragments in pSL103 and those in pSL102 in terms of the polarity of insertion into pUB110, or a minor physical difference between the fragments. However, it seems unlikely that the difference is due to the polarity of insertion of the fragment into pUB110, because cleavage of pSL102 with *Eco*RI followed by annealing and ligation did not generate plasmid molecules capable of transforming a *trpE B. subtilis* mutant to Trp$^+$. The source of the DNA from which both Trp fragments were cloned, strain NRRL B-3275, was previously shown to exhibit a very high level of spontaneous mutation (11). It is possible that the difference between pSL102 and pSL103 with respect to complementation of *trpE* results from a minor base-sequence difference in the chromosomes which were the sources of the two fragments.

DNA from strains of *B. pumilus* appears incapable of generating Trp$^+$ transformants of *B. subtilis,* presumably due primarily to extensive regions of nonhomology between the chromosomes of the two species (19). However, when *B. subtilis* BR151 (*trpC2 metB10 lys-3*) harbored plasmid pSL102 (Neo^r *trpD*$^+$ *trpF*$^+$ *trpC*), the strain could be transformed to Trp$^+$ using donor DNA extracted from *B. pumilus* NRRL

B-3275 ($\sim 10^4$ transformants per μg of DNA) or *B. subtilis* JH86T ($\sim 5 \times 10^4$ transformants per μg of DNA). We presumed that the *trpC*$^+$ transformants of BR151(pSL102) generated using *B. pumilus* donor DNA result from a recombinational event with the plasmid but not the chromosome. Conversely, *trpC*$^+$ transformants of BR151(pSL102) generated using *B. subtilis* donor DNA were presumed to result from recombination with the chromosome. Consistent with this view is the observation that when plasmid DNA, isolated from three *trpC*$^+$ transformants of BR151(pSL102) generated using donor *B. pumilus* DNA, was used to transform BR151 to Neo^r, each of 50 transformants was *trpC*$^+$. In contrast, when plasmid was isolated from three *trpC*$^+$ transformants of BR151(pSL102), generated using donor *B. subtilis* DNA, and then used to generate Neo^r transformants of BR151, each of 200 transformants tested remained *trpC*.

pSL102 was transformed into BR151R (*recE4 trpC2 lys-3*) by selecting for Neo^r. Each of two cloned transformants was incapable of being transformed to Trp$^+$ using donor *B. pumilus* or *B. subtilis* chromosome DNA. Both Neo^r clones were capable of taking up DNA during transformation, as evidenced by the fact that both were successfully transformed to Trp$^+$ using plasmid pSL108. The transformation efficiency in these experiments was on the order of 400 transformants per μg of pSL108. It therefore appears that the *recE4* mutation suppresses integration of transforming donor DNA into the resident chromosome and plasmid.

To test the ability of *recE4* to suppress recombination between plasmids, derivatives of BR151 and BR151R were constructed harboring both pSL102 and pSL108. CCC DNA purified from BR151(pSL102, pSL108) and BR151R(pSL102, pSL108) was then used to transform (at DNA levels of approximately 0.3 μg/ml) BR151 and BR151R to Neo^r. Resulting Neo^r transformants were tested for their Trp phenotype. Neo^r *trpC*$^+$ transformants were only detected when the plasmid pairs, pSL102 and pSL108, had been present in a Rec$^+$ cell (BR151) regardless of the recombination phenotype of the recipient (Table 2). Plasmid DNA isolated from each of four Neo^r *trpC*$^+$ transformants sedimented at 26(±1)S in neutral sucrose gradients, and each was capable of transforming BR151 to Neo^r and *trpC*$^+$. These results indicate that the presence of the *recE4* mutation in *B. subtilis* inhibited recombination between the plasmid pairs.

TABLE 2. *TrpC phenotype of Neo^r transformants of BR151 (Rec$^+$) and BR151R (Rec$^-$) transformed with CCC DNA extracted from BR151 (Rec$^+$) and BR151R (Rec$^-$) harboring pSL102 or pSL102 and pSL108*[a]

Source of CCC DNA	Recipient	
	BR151 (TrpC$^+$/ Neo^r)	BR151R (TrpC$^+$/ Neo^r)
BR151(pSL102)	0/400	0/382
BR151R(pSL102)	0/198	0/206
BR151(pSL102, pSL108)	289/400	315/400
BR151R(pSL102, pSL108)	0/460	0/460

[a] The presence of pSL102 or pSL102 and pSL108 in strains used as source of donor CCC DNA was confirmed by sedimentation of the CCC DNA from each strain through sucrose gradients. Transformation was performed using DNA levels of about 0.3 μg/ml.

DISCUSSION

Plasmids are CCC DNA molecules harboring genetic information essential for autonomous replication (i.e., replicons), generally linked to some additional genes. Plasmid pPL576 is cleaved by *Eco*RI into three fragments, A, B, and C, of which only the B fragment is essential for autonomous replication in *B. subtilis*. The Trp-containing derivatives of pPL576 are compatible with pUB110, and therefore both of these plasmids can be used for the construction of individual strains of *B. subtilis* which are simultaneously diploid from more than one region of the chromosome.

The *recE4* mutation was previously shown to inhibit the integration of a homologous DNA segment into the chromosome of *B. subtilis* (9a). Consistent with this result is the present observation that pairs of compatible plasmids carrying a common base sequence show no detectable recombination when the host strain carries the *recE4* mutation, but relatively high recombination when the plasmids are present in a wild-type (Rec$^+$) host. Clearly, the present data suggest that it will be possible to eliminate recombination between compatible plasmids harboring overlapping segments of the host chromosome by utilizing the *recE4* mutation in the recipient.

The *recE4* mutation has not yet been assigned to a location on the *B. subtilis* genetic map. However, the present study suggests that the *recE* gene codes for a product that is capable of acting on replicons in transconfiguration to the chromosome.

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