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Genetic Transfer of Large DNA Inserts to Designated Loci of the *Bacillus subtilis* 168 Genome

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It was found that contiguous DNA segments of up to 50 kb can be transferred between *Bacillus subtilis* genomes when a sufficient length of the flanking genomic region is provided for homologous recombination, although the efficiency of transfer was reduced as the insert size increased. Inserts were translocated to different loci, where appropriate integration sites were created.

A previous comparison of sequenced bacterial genomes strongly indicated a frequent exchange of genes between bacterial species in nature which is called a "horizontal transfer" (5). *Bacillus subtilis* 168, a gram-positive endospore-forming soil bacterium, has been shown to develop a competence to integrate cognate genomic regions (8) and noncognate DNA inserts into its genome (3, 4, 10–13). The *B. subtilis* 168 genes for developing competency are known, and the mechanism underlying the uptake of single-stranded DNA through the cell membrane has been elucidated (6, 7). While the average size of the DNA segments incorporated into *B. subtilis* cytoplasm was estimated to be 8.5 kb (7), the maximum genomic distance covered by contiguous DNA was estimated by the mapping of two linked genomic markers to be approximately 30 kb (8). Because these two figures are inconsistent, a systematic investigation was needed to clarify how great a length of contiguous DNA can be integrated into the *B. subtilis* genome. Previously constructed noncognate lambda DNA inserts ranging from 16.8 to 48.5 kb in the *B. subtilis* genome (11) were considered appropriate for such an investigation.

The lambda inserts constructed in the pBR322 sequence that had been integrated into the *Not*I site of the *proB* gene are described in reference 11 and in the legend to Fig. 1. The donor strains BEST2204 to BEST2207 listed in Table 1 and Fig. 1 and those constructed in this study each have two antibiotic markers at the end of the variable length of the lambda insert, i.e., the 1.0-kb chloramphenicol resistance gene cassette (Cm), the 1.2-kb erythromycin resistance gene cassette (except for the 16.8-kb insert) (Em), and the tetracycline resistance gene (Tc; 1.9 kb), integrated into the *leuB* gene. The transfer of the insert to the recipient 168 *trpC2* genome was carried out by homologous recombination of the genomic regions flanking the *proB* genes of the recipient and the donor, as shown in Fig. 1. If the entire noncognate section of donor DNA is integrated into the recipient genome according to this scheme, both the Cm and the Em markers should be simultaneously integrated. Thus, transformants selected by chloramphenicol (CM) (at 5 μg/ml) should be erythromycin (EM) resistant (at 5 μg/ml), and vice versa.

For example, in the 39.5-kb transfer, the 200 CM-resistant transformants obtained by BEST2205 × 168 *trpC2* were all EM resistant. Genomes of the six representative clones were analyzed by *Not*I, *Sfi*I, and *Hin*dIII and by Southern analyses using pBR322 and lambda DNA as probes. It was confirmed that all six clones acquired the contiguous 39.5-kb insert in the *proB* locus (data not shown). Similarly, the 200 transformants selected by EM, six of which had genome structures identical to those selected by CM, were all CM resistant (data not shown). Other inserts with variable lengths of lambda DNA were similarly examined, and it was verified that all of the CM-resistant colonies tested were also EM resistant (data not shown). *Hin*dIII was obtained from Toyobo (Tokyo, Japan), *Sfi*I was obtained from New England Biolabs (Beverly, Mass.), and *Not*I was obtained from Takara Shuzo (Kyoto, Japan). DNA manipulations in vitro were done according to the methods described in reference 20 or the manufacturers' instructions unless otherwise specified. The Southern hybridization procedure has been described previously (11). Luria Bertani broth (17) was used for the growth of *B. subtilis* at 37°C.

For the measurement of transformation efficiency, competent *B. subtilis* 168 *trpC2* was prepared as previously described (23) and stored at −70°C in the presence of dimethyl sulfoxide at 20% (vol/vol). Frozen competent cells were used within several weeks, during which the transformation efficiency remained unaltered, giving ~10^4 transformants per μg of DNA. High-molecular-weight genomic DNA was prepared by the method of Saito and Miura (19), and the concentrations of DNA solutions, as determined by their UV absorption at 260 nm measured by a spectrophotometer (DU640; Beckman Instruments, Inc., Fullerton, Calif.), were 45 μg/ml for BEST2204, 27.5 μg/ml for BEST2207, 29.9 μg/ml for BEST2042, 31.3 μg/ml for BEST2205, and 30.0 μg/ml for BEST2206. Diluted solutions were left for at least 1 day at room temperature with occasional gentle shaking before use. Each transformation experiment was performed at least three times.

The calculated numbers of CM-resistant transformants per μg of DNA are plotted against insert sizes from 0 to 48.5 kb in Fig. 2. A logarithmic decrease in CM-resistant transformants was observed with each increase in the concentration of DNA (from 0.27 to 6.7 μg/ml) in contrast to the constant number of tetracycline-resistant transformants (selected at 10 μg/ml). Transfer of the *leuB*::*tet* marker occurred independently of the lambda transfer, because the *leuB* gene is located 1,518 kb away from the *proB* gene (12, 15) and the distance is far longer than the lengths of donor DNAs, which are comprised of sheared, 200- to 400-kb segments. The donor DNA of strain BEST2206, having the longest (55 kb) insert (comprised of lambda [48.5 kb] plus pBR322 [4.3 kb] plus Em [1.2 kb] plus Cm [1.0 kb]), still gave 111 ± 8 CM-resistant transformants per μg of DNA (Fig. 2). It is likely that DNA segments longer than 55 kb are included in the DNA uptake and recombinational

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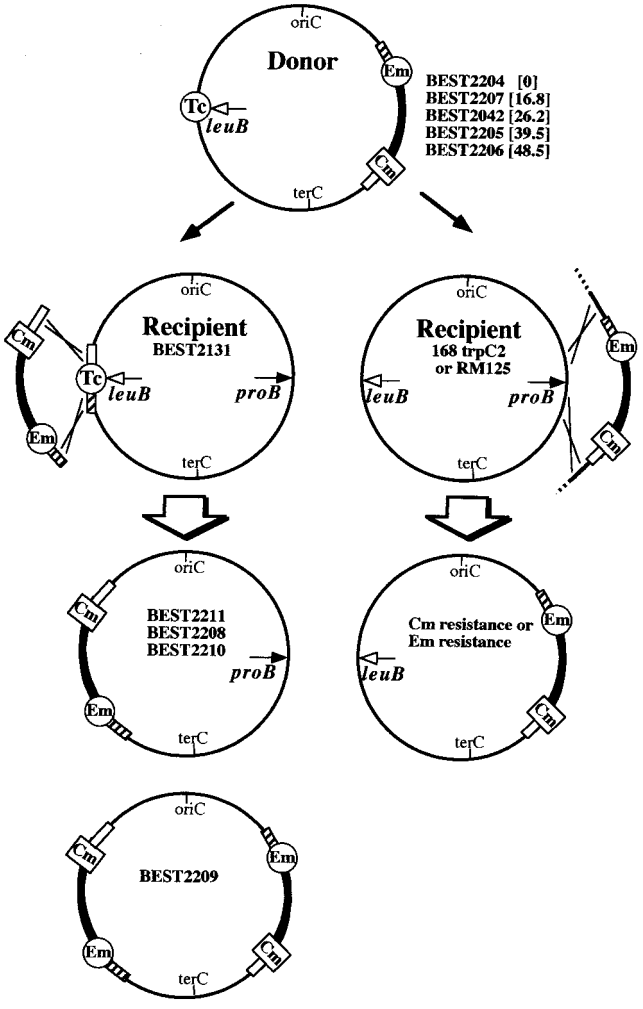

FIG. 1. Transfer of the lambda insert in the recipient *B. subtilis* genome. Large circles represent the *B. subtilis* genome with the locations of *oriC*, *proB*, *terC*, and *leuB* labeled. The donor strains had two extra DNA segments in their genomes; one is a lambda DNA sequence (heavy line) flanked by pBR322 sequences (closed and hatched boxes) integrated in the *proB* gene (11), and the other is a tetracycline resistance gene inserted in the *leuB* gene (Table 1). The transfer of the lambda DNA plus pBR322 sequences to the recipient genome proceeded via crossovers in the flanking genomic region when strain 168 *trpC2* was used as a recipient. Transfer of the lambda insert to the *leuB* locus was carried out by crossovers within the pBR322 sequence prepared in the BEST 2131 genome (10). BEST2209 had two 26.2-kb lambda DNA inserts, one in *proB* and the other in *leuB*.

processes of transformation. The almost linear logarithmic decrease in transfer efficiency at the unsaturated concentration (approximately 1/10 per 50 kb [Fig. 2]) supports the adverse affect of the insert size. A slight downshift from the linear decrease was observed at other concentrations. The rate of tetracycline resistance transformations did not change at saturated concentrations, and this lowered efficiency suggests a rate-determining step by the recombination machinery to replace the DNA segment including the long noncognate inserts.

A controversial observation with respect to the size of incorporated single-stranded DNA in cytoplasm, i.e., on average 8.5 kb, remained to be confirmed (7). One possible explanation is that higher-molecular-weight DNA is actually involved in integration and that the DNA processed during rapid recombination remained and was observed as a major population. Alternatively, perhaps long inserts with nonhomologous sequences stabilized the DNA during uptake and/or during a recombinational process. Results similar to those shown in Fig. 2 were obtained by using strain RM125 as a recipient (data not shown), indicating that the restriction modification system did not discriminate the lambda transfer.

Since the horizontal transfer from *proB* to *proB* was established, the question of whether the sequences for integration are provided in different loci of recipient genomes arose. Recipient BEST2131 has a pBR322 sequence integrated in the *leuB* gene (10), and this type of transfer was mediated by homologous recombination within pBR322, which flanks the lambda insert (as schematically illustrated in Fig. 1). Since all donors have the *leuB*::*tet* marker, only recombinants whose Tc gene is displaced by the lambda insert become tetracycline sensitive. The Cm marker was used to select the lambda transfer to BEST2131, and the DNA concentration was increased to 6.7 μg/ml to obtain more transformants. Among the 225 CM-resistant colonies in the case of the 16.8-kb transfer, only one tetracycline-sensitive strain (BEST2211) was obtained. Similarly, 2 (BEST2208 and BEST2209) of 231 CM-resistant colonies in the 26.2-kb transfer and 1 (BEST2210) of 334 CM-resistant colonies in the 39.5-kb transfer were obtained. No transfer

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	DNA insert or length (kb) at:		Reference or crossover
		proB	*leuB*	
B. subtilis				
168 *trpC2* (=1A1)	*trpC2*			9
BEST2131	*trpC2*		*leuB*::pBRTc[c]	10
RM125	*arg-15* ΔSPβ *hsdMR*		*leuB8*	13
BEST2007		*proB*::pBRCm		10
BEST2012	*trpC2*	pBREm/Cm	+	pBASE2001 × BEST2007[d]
BEST2037	*trpC2*	16.8[a]	+	11
BEST2045	*trpC2*	39.5[b]	+	11
BEST2046	*trpC2*	48.5[b]	+	11
BEST2204	*trpC2*	0[b]	*leuB*::*tet*	pBMAP105TT × BEST2012
BEST2207	*trpC2*	16.8[a]	*leuB*::*tet*	pBMAP105TT × BEST2037
BEST2042	*trpC2*	26.2[b]	*leuB*::*tet*	11
BEST2205	*trpC2*	39.5[b]	*leuB*::*tet*	pBMAP105TT × BEST2045
BEST2206	*trpC2*	48.5[b]	*leuB*::*tet*	pBMAP105TT × BEST2046
BEST2211	*trpC2*	+	16.8[a]	BEST2207 × BEST2131
BEST2208	*trpC2*	+	26.2[b]	BEST2042 × BEST2131
BEST2210	*trpC2*	+	39.5[b]	BEST2205 × BEST2131
BEST2209	*trpC2*	26.2[b]	26.2[b]	BEST2042 × BEST2131
E. coli plasmids[e]				
pBMAP105TT			*leuB*::*tet*	11
pBASE2001			pBREm	10

[a] Integrated in the 5.5 kb segment (4.3 kb pBR322 + 1.2 kb Em).
[b] Integrated in the 6.5 kb segment (4.3 kb pBR322 + 1.2 kb Em + 1.0 kb Cm).
[c] The TC-resistance gene in pBR322 did not function in *B. subtilis* (10). Other details are described in reference 10.
[d] Selected by EM.
[e] Purified by CsCl-ethidium bromide ultracentrifugation.

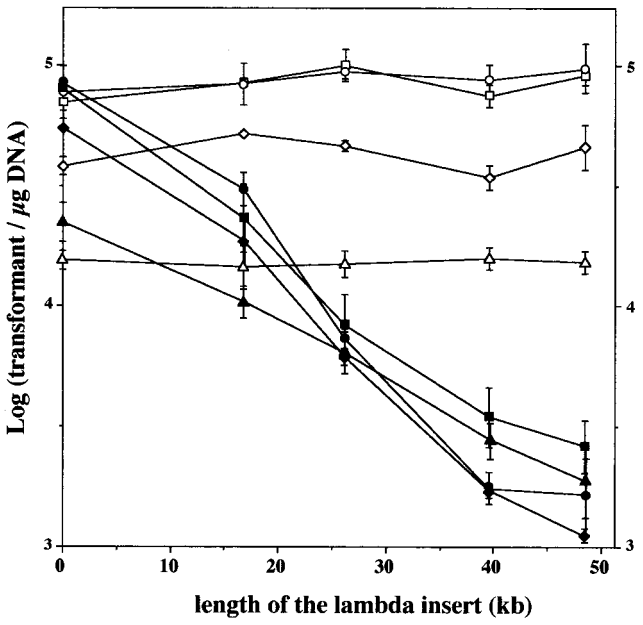

FIG. 2. Decrease of DNA transfer efficiency with increase in size of the lambda inserts. The numbers of CM-resistant (closed symbols) and tetracycline-resistant (open symbols) transformants per microgram of DNA are plotted against the lambda insert size of the donor strains. The concentrations of DNA used are 0.27 μg/ml (● and ○), 0.67 μg/ml (■ and □), 1.34 μg/ml (◆ and ◇), and 6.7 μg/ml (▲ and △). Standard deviations are indicated by vertical bars. Maximum standard deviation of the 0-kb value was 35% (◆), and others ranged between 8 and 28%.

of 48.5 kb was observed after screening 792 CM-resistant colonies.

The lambda insert in the *leuB* loci of BEST2208 through BEST2211 was verified by an increase of *Sfi*I fragments to which the *leuB* gene is attached (Fig. 3). The Southern analysis of the *Hin*dIII digests of these genomic DNAs indicated no obvious rearrangements in the newly transferred lambda DNA (data not shown). These observations are consistent with the scheme shown in Fig. 1, although the DNA transfer efficiency was significantly reduced. This low efficiency was probably due to the short sequence of pBR322 and may be improved by using a longer sequence for integration or a rapid selection method for the integrants.

It is noteworthy that strain BEST2209 was shown to have two 26.2-kb inserts, one in *leuB* (characterized above) and the other in *proB* (Fig. 1). The *Sfi*I fragments in which *leuB* and *proB* are located were increased in size by 26.2 kb and were detected by the lambda DNA probe (Fig. 3) and pBR322 (data not shown). Southern analyses of the *Hin*dIII digests indicated that lambda and adjacent genomic regions of BEST2209 were the sum of those of BEST2042 (insert only in *proB*) and BEST2208 (insert only in *leuB*) (data not shown). These two lambda structures did not segregate in colonies of BEST2209 (data not shown). Strain BEST2209 grew normally without noticeable instability of the genome structure. This strain may be useful in the study of intrachromosomal DNA rearrangements and duplications or the subgenomic formation of the approximately 1,518-kb region between *leuB* and *proB* (14, 24).

Unlike *Haemophilus influenzae*, the competent strains of *B. subtilis* did not discriminate the nucleotide sequences of the incoming DNA (16). Interchromosomal recombination efficiency did not significantly differ within the *B. subtilis* genome (1), which allows the choice of appropriate genomic loci for the

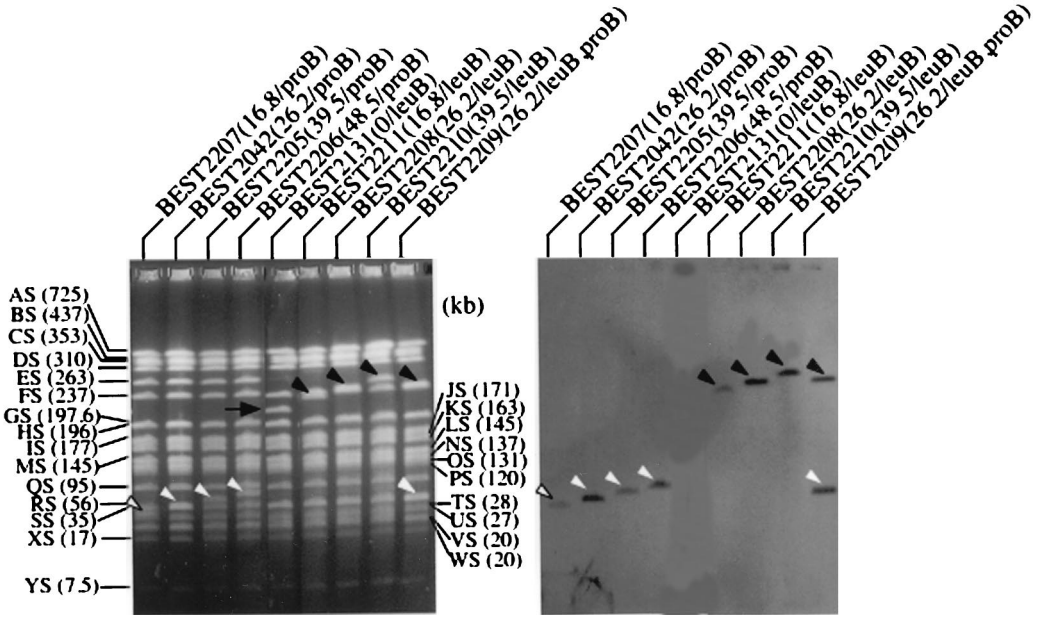

FIG. 3. Transfer of lambda insert to the *leuB* locus. *Sfi*I digests of the genome (2 to 3 μg) of the indicated *B. subtilis* strains were separated by contour-clamped homogeneous electric field gel electrophoresis, and the results are shown in the left panel. Running conditions were as follows: 3 V cm^{-1}, 24-s pulse time, and 40-h running time at 14°C. Twenty-six *Sfi*I fragments originally designated based on the physical map (9, 12) were verified by the determined nucleotide sequence (15) and are shown as AS to ZS, with predicted sizes in parentheses. The 28 kb fragments of the donor strains in which the *proB* gene is located (labeled TS) were increased by the insert size (indicated by open arrowheads) and hybridized by labeled lambda DNA (right panel). The fragment of BEST2131 in which the *leuB* gene is located (labeled ES) is shown by a horizontal arrow in the BEST2131 lane. The ES fragments of BEST2131 derivatives (closed arrowheads), hybridized by labeled lambda DNA (right panel), were increased by the size of lambda DNA integrated. BEST2209 had two lambda inserts. The shortened ES fragment (202 kb) was caused by a newly created *Sfi*I site in the *leuB* gene at 2,896 kb (10).

integration and relocation of new inserts. The present results will facilitate the manipulation of longer segments of DNA in genome engineering with *B. subtilis*, using techniques reported previously (11, 14).

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