The Size and Continuity of DNA Segments Integrated in Bacillus Transformation

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ABSTRACT

We investigated the size and continuity of DNA segments integrated in *Bacillus subtilis* transformation. We transformed *B. subtilis* strain 1A2 toward rifampicin resistance (coded by *rpoB*) with genomic DNA and with a PCR-amplified 3.4-kb segment of the *rpoB* gene from several donors. Restriction analysis showed that smaller lengths of donor DNA integrated into the chromosome with transformation by PCR-amplified DNA than by genomic DNA. Nevertheless, integration of very short segments (<2 kb) from large, genomic donor molecules was not a rare event. With PCR-amplified segments as donor DNA, smaller fragments were integrated when there was greater sequence divergence between donor and recipient. There was a large stochastic component to the pattern of recombination. We detected discontinuity in the integration of donor segments within the *rpoB* gene, probably due to multiple integration events involving a single donor molecule. The transfer of adaptations across Bacillus species may be facilitated by the small sizes of DNA segments integrated in transformation.

GENETIC exchange in bacteria is notorious for allowing the transfer of adaptations, such as antibiotic resistance, across taxon boundaries. In some cases, whole gene operons have been transferred into new taxa by plasmid carriage (YOUNG and LEVIN 1992); in other cases, adaptive alleles have been transferred by homologous recombination (MAYNARD SMITH *et al.* 1991). Whether by plasmid carriage or by homologous recombination, the transfer of adaptations in bacteria is facilitated by the ability of bacteria to accept and express genetic material from other taxa.

The transfer of adaptations is also facilitated by the ability of bacteria to limit genetic exchange to a very small number of genes (COHAN 1994). This is because genes that can be adaptively transferred across taxa are necessarily a very limited set that confer *general* adaptations, which are not limited to the ecological and genetic context of a particular taxon (*e.g.*, genes conferring resistance to widely used antibiotics). The potential for adaptive gene transfer should be greater when a generally adaptive gene can be transferred alone without the co-transfer of other, more narrowly adaptive genes, whose adaptive value is limited to a particular taxon (MAYNARD SMITH *et al.* 1991; COHAN 1994).

Recent work suggests that the size of DNA segments transferred by homologous recombination in nature may be quite small. For example, some strains of *Neisseria meningitidis* and *N. gonorrhoeae* have acquired penicillin resistance by homologous recombination of <100 bp from *N. flavescens;* similar patterns have been found in Streptococcus and Haemophilus (MAYNARD SMITH *et al.* 1991). The present study addresses whether this potential for adaptive transfer of very short segments also exists in another bacterial genetic exchange system, that of transformation in the genus Bacillus.

In bacterial transformation there are several mechanisms by which a short segment (*e.g.*, <2 kb) might be transformed without the cotransformation of immediately flanking DNA. First, the segments taken up by the cell and then integrated into the chromosome may both be very short (<2 kb) (model 1). This mechanism is possible for the Bacillus transformation system, although the rate of transformation declines sharply for short donor molecules (*i.e.*, <6000 bp; MORRISON and GUILD 1972; ZAWADZKI *et al.* 1995). Also, an excluded length of at least 400–500 bp is removed from donor molecules before integration, establishing a lower bound for the length of donor molecules that can be at least partially integrated (DUBNAU 1993).

Alternatively, uptake of a long segment may result in integration of one or more shorter subsegments (DUBNAU 1993): the long segment may be cleaved and integrated as a single short segment (model 2), or it may be processed into multiple short segments that are integrated discontiguously (model 3). In this last mechanism, there is an opportunity for cotransfer of some linked genes. Nevertheless, both Models 2 and 3 provide a mechanism by which a single gene or domain of a gene may be transformed without the co-transfer of immediately flanking DNA.

Previous work on Bacillus transformation has shown

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that a single exogenous DNA segment may be processed and integrated as multiple discontiguous segments (consistent with model 3). Electron microscopy has shown that transformation by large, genomic donor segments results in an area of integration spanning 9 kb (DUBNAU and CIRIGLIANO 1972; FORNILLI and FOX 1977). The area of integration is not always integrated in full but may consist of multiple shorter subsegments that are integrated discontiguously (*i.e.*, with nonrecombined segments intervening). These previous studies did not address the sizes of the individual subsegments that are integrated.

The present study introduces a restriction-digest approach to investigate the likelihood of integration of very short segments <2 kb. Our approach was first to transform a recipient strain, *B. subtilis* strain 168, toward rifampicin resistance (coded by rif^R alleles of *rpoB*) using DNA from several resistant donors. We then PCR-amplified a 3.4-kb segment of the *rpoB* gene from a given transformant, as well as from the recipient and donor. We compared the restriction digests of the *rpoB* segments of transformants to those of the recipient and donor to determine the part of the *rpoB* gene that was transferred from donor to recipient.

With this approach we were able to analyze the statistical distribution of the size and continuity of integrated segments under several conditions of transformation. We addressed the following issues bearing on the probability of adaptive transfer of small segments across taxon boundaries. First, how does the length of the donor DNA presented affect the size of integrated segments? Second, how does sequence divergence between donor and recipient affect the size of transforming fragments? Third, in cases where a single donor molecule is integrated discontinuously as multiple, shorter subsegments, how large are the individual integrated units?

MATERIALS AND METHODS

Strains: *Bacillus subtilis* strain 1A2 (a derivative of strain 168) was obtained from the Bacillus Genetic Stock Center. *B. atrophaeus* type strain NRRL NRS-213 was obtained from the Agricultural Research Service Culture Collection of the National Center for Agricultural Utilization Research. *B. licheniformis* type strain ATCC 14580 was obtained from the American Type Culture Collection. Strain RO-E-2 of *B. subtilis* was isolated from a natural population in the Mojave Desert (as reported by COHAN *et al.* 1991).

Isolation of rifampicin-resistant mutants: Rifampicin-resistant (rif^R) mutants of all strains used as DNA donors were isolated as described by ROBERTS and COHAN (1993).

Purification of genomic DNA: Genomic DNA was isolated as described by ROBERTS and COHAN (1993).

PCR-amplification of *rpoB* segment: A 3367-bp region of the *rpoB* gene of the recipient strain (*B. subtilis* strain 1A2), of each donor, and of the transformants was amplified by PCR as described by ROBERTS and COHAN (1993). The amplified region extended from bp 1102 to 4468 of the unpublished sequence kindly provided by KATHRYN BOOR, MARIAN DUNCAN, and CHESTER W. PRICE.

Transformation: In each transformation experiment, *B. subtilis* strain 1A2 was transformed toward rifampicin resistance using DNA from one of three rif^R donors (rif^R mutants of *B. subtilis* strain RO-E-2, the type strain of *B. atrophaeus*, or the type strain of *B. licheniformis*). The recipient was transformed toward rifampicin resistance using either genomic DNA or a PCR-amplified 3367-bp segment of the *rpoB* gene, as described by ROBERTS and COHAN (1993). The genomic DNA preparation presented to the recipient consisted of segments of ~50 kb in length. To avoid congression (*i.e.*, transformation of a single cell by multiple fragments of donor DNA), the final DNA concentration was 0.05 μg/ml, a concentration typically used in mapping studies (CUTTING and VANDER HORN 1990). Transformation frequencies reported here take into account the appearance of rif^R colonies by spontaneous mutation.

We limited the possibility that our preparation of PCR-amplified DNA might contain enough genomic DNA (used as template in the PCR) to bring about transformation. This was accomplished by first performing PCR using genomic DNA as the template and then using 0.2 μg of DNA from the PCR reaction as template for a second round of PCR. We measured the contribution of the genomic DNA template toward the total rate of transformation by transforming the recipient with a PCR reaction mixture containing all components except the *Taq* polymerase. This mixture yielded a rate of transformation that was only 1/300 of that found for the complete PCR reaction containing PCR product.

Eleven or 12 randomly chosen transformants from each transformation treatment were isolated for further analysis.

Restriction-digest analysis of the recipient, donors, and transformants: A 3367-bp PCR-amplified segment of the *rpoB* gene from the recipient strain, from each donor, and from each transformant was digested by each of five four-cutter restriction endonucleases (*Dpn*II, *Hin*fI, *Hin*PI, *Msp*I and *Rsa*I; New England Biolabs), and restriction digests were assayed by electrophoresis, as described by ROBERTS and COHAN (1993). Fragment lengths were estimated using the DNA molecular weight marker XI of Boehringer Mannheim.

RESULTS

Frequencies of transformation: The recipient strain, *B. subtilis* strain 1A2, was transformed toward rifampicin resistance with genomic and PCR-amplified DNA from each of three donors representing different levels of sequence divergence from the recipient [*B. subtilis* strain RO-E-2 with 3.1% divergence from 1A2, the type strain of *B. atrophaeus* with 7.0% divergence, and the type strain of *B. licheniformis* with 14.2% divergence; sequence divergence data for gene *rpoB* from ROBERTS and COHAN (1993)]. Frequencies of transformation were on average, over all donors, 6.8 times higher with genomic DNA than with PCR-amplified DNA. For both genomic and PCR-amplified DNA, the frequencies of transformation decreased with increasing levels of sequence divergence (as previously shown by ROBERTS and COHAN 1993) (Table 1). Transformation frequencies were at least 22 times higher than that of mutation (mean mutation rate = 2.10×10^{-8}).

Restriction-digest analysis of donor segments integrated in transformation: We took the following approach to analyzing the segments of DNA transferred from donor to recipient in transformation. First, we

TABLE 1

Frequencies at which the recipient strain, *B. subtilis* strain 1A2, was transformed with a PCR-amplified 3.4-kb segment of *rpoB* and with genomic DNA from different donors

Donor strain	Donor-recipient sequence divergence at *rpoB*	Transformation frequency	
		Genomic DNA	PCR-amplified DNA
B. subtilis RO-E-2	3.1%	2.82×10^{-4}	6.47×10^{-5}
B. atrophaeus NRS-213	7.0%	8.10×10^{-5}	6.22×10^{-6}
B. licheniformis ATCC 14580	14.2%	1.72×10^{-6}	5.67×10^{-7}

Transformation frequencies have been corrected for mutation.

digested the 3367-bp region of the *rpoB* gene of the recipient and each donor with each of five restriction enzymes. Figure 1 shows the restriction map of the recipient and compares the restriction map of the recipient to that of each donor. For our analysis of transformants, we used only those restriction fragments that were present in the restriction digest of the recipient but were absent in the donor. We excluded from our analysis all restriction fragments that were too small to be detected on the gel (*i.e.*, <50 bp). We would also have excluded pairs of fragments that were too close in size to be distinguished, but no such pairs were found in our study.

We then PCR-amplified the *rpoB* gene of each of 11 or 12 rif^R transformants from each transformation treatment, and we digested each PCR product with the five restriction enzymes (Table 2). For each recipient-strain band that was missing in a transformant, we concluded that the donor's DNA was integrated somewhere within that restriction fragment. By comparing each transformant's restriction digest to the digest of the recipient (for each restriction enzyme), we estimated the extent of donor DNA that integrated into each transformant's *rpoB* gene, as we describe below.

Whenever possible, we have analyzed the integrated segments by assuming that a single, continuous segment transformed the recipient. The minimum possible integrated DNA fragment was determined by the following algorithm. For each restriction enzyme, the minimum possible integrated segment was taken to span the inside ends of the two most peripheral recipient-strain fragments that were missing in the transformant (see Figure 2 for rationale). For enzymes in which two or fewer recipient-strain fragments were missing, the minimum span could not be estimated. Putting the data for all enzymes together, the 5′-most of the 5′ estimates and the 3′-most of the 3′ estimates were taken as the minimum span of integration.

We used the following approach to estimate the maximum size of the integrated segment for each transformant. For each restriction enzyme that yielded at least some missing recipient-strain fragments, we first identified the following two "flanking" restriction fragments: these were the two recipient-strain restriction fragments that were *not* missing in the transformant, were missing in the donor strain, and most closely flanked each end of the string of recipient-strain restriction fragments that were missing in the transformant. For those enzymes not yielding any differences between the transformant and the recipient (*i.e., no* missing recipient-strain fragments in the transformant), the 5′ and 3′ flanking segments were determined as those recipient-strain segments most closely flanking the minimum integrated segment (as determined above).

In separate analyses for each restriction enzyme, the 5′-most possible extent of the integrated region was estimated to be at the 5′ end of the 5′ flanking segment, and likewise, the 3′-most possible extent of the integrated region was estimated to be at the 3′ end of the 3′ flanking segment (for rationale, see Figure 2). Taking the information from all restriction enzymes into account, Figure 3 shows the maximum and minimum ranges of donor DNA that integrated in each transformant.

In several transformants the pattern of missing fragments was not consistent with transformation by a single segment. In these cases, restriction analysis showed donor-derived segments to be separated by recipient-derived segments. As shown in Figure 3, transformation involved integration of discontiguous segments in transformants 24, 26, 29, 37, and 55. The discontiguously integrated segments ranged in size from at most 547 bp (transformant 29), to at least 1631 bp (transformant 24), to integrated segments that extended beyond the analyzed region (transformant 37).

Effect of the length of donor DNA on the size of the integrated segment: *B. subtilis* strain 1A2 was transformed with genomic DNA, of length ~50 kb, and with the PCR-amplified *rpoB* gene segment, of length 3.4 kb, from three donor strains. For all three donors, much longer fragments of donor DNA integrated into the genome after transformation with genomic DNA than with PCR-amplified DNA (Figure 3). In most cases transformation with genomic DNA replaced the entire *rpoB* gene segment (in 26 out of 36 cases, over all three donors), while in no case did transformation with PCR-amplified DNA replace the entire gene segment. Although transformation with genomic DNA generally resulted in integration of 3.4 kb or more, integration of segments shorter than 2 kb was not uncommon (occurring in six of the 36 transformants). With PCR-amplified DNA, donor-derived segments <2 kb were integrated in 28 out of 33 transformants. Thus, while there is a greater potential for integration of short DNA segments from short donor molecules, integration of short

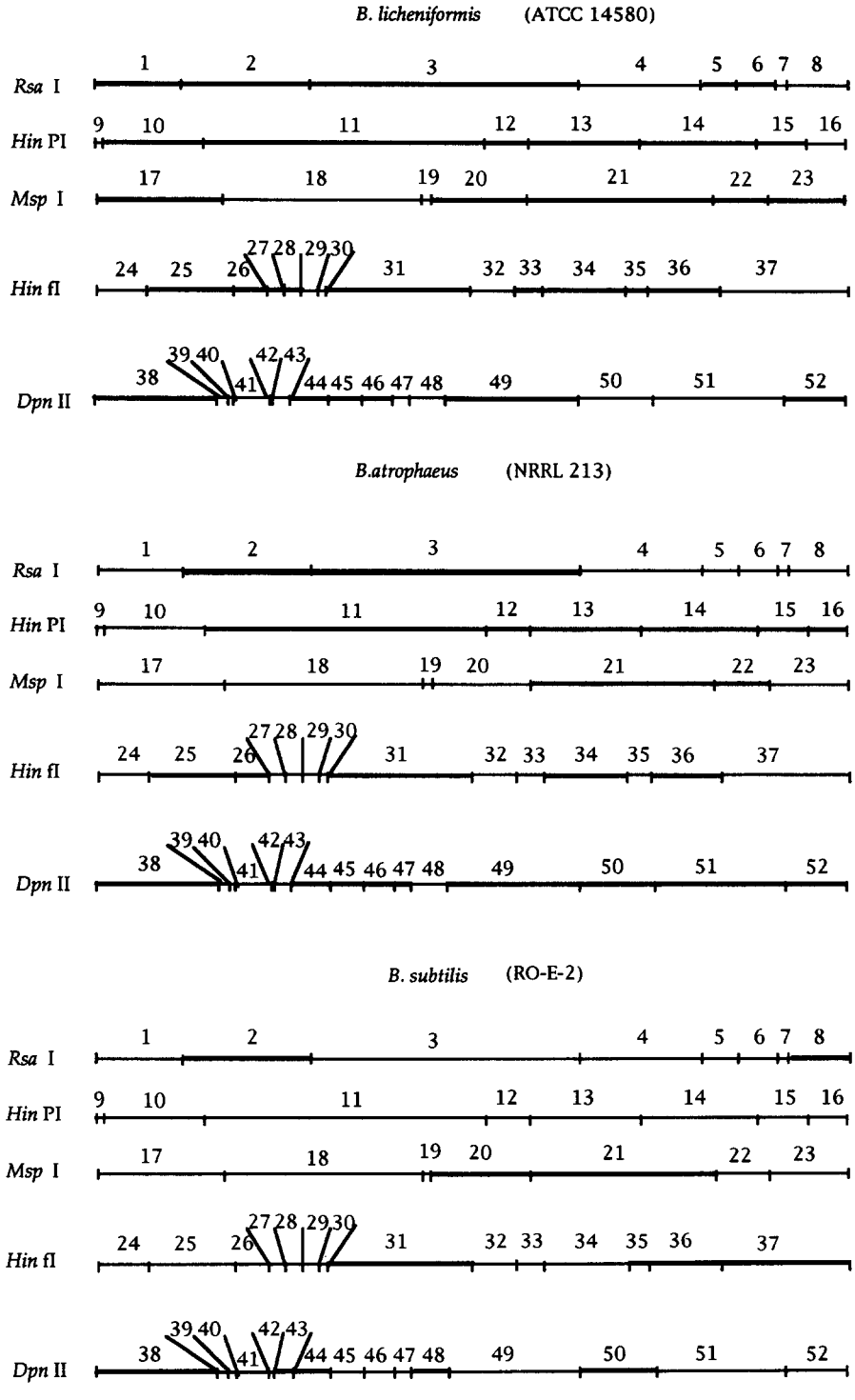

FIGURE 1.—Comparison of the restriction digests of the recipient strain, *B. subtilis* strain 1A2, with those of each donor strain. The figure shows the restriction digest of the recipient strain; fragments that are absent in the digest of each donor strain are highlighted in bold. *Rsa*I cuts the recipient at sites 1463, 2048, 3258, 3821, 3980, 4158, and 4201; *Hin*PI cuts at sites 1135, 1596, 2850, 3059, 3554, 4078, and 4298; *Msp*I cuts at sites 1667, 2567, 2592, 3053, 3873, and 4116; *Hin*fI cuts at sites 1334, 1714, 1862, 1942, 2020, 2070, 2122, 2775, 2993, 3101, 3482, 3579, and 3907; *Dpn*II cuts at 1649, 1706, 1721, 1877, 1892, 1982, 2156, 2300, 2440, 2510, 2677, 3280, 3614, and 4207.

segments from large donor molecules is not a rare event.

In one case with transformation with PCR-amplified DNA (transformant 10, transformed with PCR-amplified DNA from *B. licheniformis*), donor DNA was not detected. This could be because only a very small segment of donor DNA was integrated, or the putative transformant may actually have been a mutant of the recipient.

Effect of donor-recipient sequence divergence on the size of the recombinational replacement: In transformation with genomic DNA, the length of DNA integrated within the 3367-bp segment was similar across all three donors, with the entire segment being replaced in a majority of transformants for all donors. However, in the case of transformation with PCR-amplified DNA, much smaller segments were integrated from the most divergent donor, the type strain of *B. licheniformis*, than from the less divergent donors (Figure 3). Nine of the 11 transformants transformed by *B. licheniformis* incor-

TABLE 2

Restriction digests of the PCR-amplified 3367-bp segment of *rpoB* for each transformant

Donor DNA	Transformant number	*rpoB* gene fragment number																																						
		1	2	3	5	6	8	10	11	12	13	14	15	16	17	20	21	22	23	25	26	27	28	31	33	34	35	36	37	38	43	44	45	46	47	48	49	50	51	52
B. licheniformis ATCC 14580 (PCR-amplified)	1	+	+	+	+	+	0	+	−	+	+	+	+	0	+	+	+	+	+	+	+	+	+	−	+	+	+	+	0	+	0	+	+	+	0	0	+	0	0	+
	2	+	+	+	+	+	0	+	−	+	+	+	+	0	+	+	+	+	+	+	+	+	+	−	+	+	+	+	0	+	0	+	+	−	0	0	+	0	0	+
	3	+	+	+	+	+	0	+	−	+	+	+	+	0	+	+	+	+	+	+	+	+	+	−	+	+	+	+	0	+	0	+	+	+	0	0	+	0	0	+
	4	+	+	+	+	+	0	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	−	+	+	+	+	0	+	0	+	+	+	0	0	+	0	0	+
	5	−	−	−	+	+	0	−	−	+	+	+	+	0	−	−	+	+	+	−	−	−	−	−	+	+	+	+	0	−	0	−	−	−	0	0	+	0	0	+
	6	−	−	−	+	+	0	+	−	−	−	+	+	0	−	−	−	+	+	−	−	−	−	−	−	−	+	+	0	−	0	−	−	−	0	0	−	0	0	+
	7	+	+	+	+	+	0	+	−	+	+	+	+	0	+	+	+	+	+	+	+	+	+	−	+	+	+	+	0	+	0	+	+	+	0	0	+	0	0	+
	8	+	+	+	+	+	0	+	−	+	+	+	+	0	+	+	+	+	+	+	+	+	+	−	+	+	+	+	0	+	0	+	+	+	0	0	+	0	0	+
	9	+	+	+	+	+	0	+	−	+	+	+	+	0	+	−	+	+	+	+	+	+	+	−	+	+	+	+	0	+	0	+	+	+	0	0	+	0	0	+
	10	+	+	+	+	+	0	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	0	+	+	+	0	0	+	0	0	+
	11	+	+	+	+	+	0	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	−	+	+	+	+	0	+	0	+	+	+	0	0	+	0	0	+
B. licheniformis ATCC 14580 (genomic)	12	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0	−	0	−	−	−	0	0	−	0	0	−
	13	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0	−	0	−	−	−	0	0	−	0	0	−
	14	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0	−	0	−	−	−	0	0	−	0	0	−
	15	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0	−	0	−	−	−	0	0	−	0	0	−
	16	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0	−	0	−	−	−	0	0	−	0	0	−
	17	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0	−	0	−	−	−	0	0	−	0	0	−
	18	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0	−	0	−	−	−	0	0	−	0	0	−
	19	+	+	+	+	+	0	+	−	+	+	+	+	0	+	+	+	+	+	+	+	+	+	−	+	+	+	+	0	+	0	+	+	+	0	0	+	0	0	+
	20	−	−	−	+	+	0	−	−	−	−	−	+	0	−	−	−	+	+	−	−	−	−	−	−	−	−	−	0	+	0	−	−	−	0	0	−	0	0	+
	21	+	+	+	+	+	0	+	−	+	+	+	+	0	+	+	+	+	+	+	+	−	−	−	+	+	+	+	0	+	0	−	−	−	0	0	+	0	0	+
	22	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0	−	0	−	−	−	0	0	−	0	0	−
	23	−	−	−	−	−	0	−	−	−	−	−	−	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0	−	0	−	−	−	0	0	−	0	0	−
B. atrophaeus NRRL NRS-213 (PCR-amplified)	24	0	−	−	0	0	0	0	−	−	−	−	−	−	0	0	−	−	0	−	−	0	0	−	0	−	0	−	0	−	0	−	−	−	−	0	−	−	+	−
	25	0	+	−	0	0	0	0	−	+	+	+	+	+	0	0	−	+	0	+	+	0	0	−	0	−	0	+	0	+	0	+	+	−	−	0	−	+	+	+
	26	0	+	−	0	0	0	0	−	−	−	−	−	+	0	0	−	−	0	−	+	0	0	−	0	−	0	−	0	−	0	+	+	+	+	0	−	−	+	+
	27	0	+	−	0	0	0	0	−	+	+	+	+	+	0	0	+	+	0	+	+	0	0	+	0	−	0	+	0	+	0	+	+	+	+	0	+	+	+	+
	28	0	+	−	0	0	0	0	−	+	+	+	+	+	0	0	+	+	0	+	+	0	0	+	0	+	0	+	0	+	0	+	+	+	+	0	+	+	+	+
	29	0	+	−	0	0	0	0	−	−	−	+	+	+	0	0	−	+	0	−	+	0	0	−	0	+	0	+	0	+	0	+	+	+	+	0	−	−	+	+
	30	0	+	−	0	0	0	0	−	+	+	+	+	+	0	0	+	+	0	+	+	0	0	−	0	+	0	+	0	+	0	+	+	+	+	0	+	+	+	+
	31	0	−	−	0	0	0	0	−	+	+	+	+	+	0	0	+	+	0	+	+	0	0	−	0	−	0	+	0	+	0	−	−	−	−	0	+	+	+	+
	32	0	−	−	0	0	0	0	−	−	−	+	+	+	0	0	+	+	0	−	−	0	0	−	0	−	0	+	0	+	0	−	−	−	−	0	−	−	+	+
	33	0	−	−	0	0	0	0	−	−	+	+	+	+	0	0	+	+	0	+	+	0	0	−	0	+	0	+	0	+	0	−	−	−	−	0	−	+	+	+
	34	0	−	−	0	0	0	0	−	+	−	+	+	+	0	0	+	+	0	+	−	0	0	−	0	−	0	+	0	+	0	−	−	−	−	0	+	+	+	+

TABLE 2

Continued

Donor DNA	Transformant number	*rpoB* gene fragment number 1	2	3	5	6	8	10	11	12	13	14	15	16	17	20	21	22	23	25	26	27	28	31	33	34	35	36	37	38	43	44	45	46	47	48	49	50	51	52
B. atrophaeus NRRL NRS-213 (genomic)	35	0	–	–	0	0	0	0	–	–	–	–	–	–	0	0	–	–	0	–	–	0	0	–	0	–	0	–	0	–	0	–	–	–	–	0	–	–	–	–
	36	0	–	–	0	0	0	0	–	–	–	–	–	–	0	0	–	–	0	–	–	0	0	–	0	–	0	–	0	–	0	–	–	–	–	0	–	–	–	–
	37	0	–	–	0	0	0	0	–	–	–	–	–	–	0	0	–	–	0	–	–	0	0	–	0	–	0	–	0	–	0	–	+	+	–	0	–	–	–	–
	38	0	–	–	0	0	0	0	–	–	–	–	–	–	0	0	–	–	0	–	–	0	0	–	0	–	0	–	0	–	0	–	–	–	–	0	–	–	–	–
	39	0	–	–	0	0	0	0	–	–	+	+	+	+	0	0	+	+	0	+	–	0	0	–	0	–	0	+	0	+	0	–	–	–	–	0	+	+	+	+
	40	0	–	–	0	0	0	0	–	–	+	+	+	+	0	0	+	+	0	+	–	0	0	–	0	–	0	+	0	+	0	–	–	–	–	0	+	+	+	+
	41	0	–	–	0	0	0	0	–	–	+	+	+	+	0	0	+	+	0	+	–	0	0	–	0	–	0	+	0	+	0	–	–	–	–	0	+	+	+	+
	42	0	–	–	0	0	0	0	–	–	+	+	+	+	0	0	+	+	0	+	–	0	0	–	0	–	0	+	0	+	0	–	–	–	–	0	+	+	+	+
	43	0	–	–	0	0	0	0	–	–	–	–	–	–	0	0	–	–	0	–	–	0	0	–	0	–	0	–	0	–	0	–	–	–	–	0	–	–	–	–
	44	0	–	–	0	0	0	0	–	–	–	–	–	–	0	0	–	–	0	–	–	0	0	–	0	–	0	–	0	–	0	–	–	–	–	0	–	–	–	–
	45	0	–	–	0	0	0	0	–	–	–	–	–	–	0	0	–	–	0	–	–	0	0	–	0	–	0	–	0	–	0	–	–	–	–	0	–	–	–	–
	46	0	–	–	0	0	0	0	–	–	–	–	–	–	0	0	–	–	0	–	–	0	0	–	0	–	0	–	0	–	0	–	–	–	–	0	–	–	–	–
B. subtilis RO-E-2 (PCR-amplified)	47	0	+	0	0	0	+	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	+	+	+	+	–	–	0	0	0	–	0	+	0	0
	48	0	–	0	0	0	+	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	–	0	0	+	+	+	+	–	–	0	0	0	–	0	+	0	0
	49	0	–	0	0	0	+	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	+	+	+	+	+	0	0	0	–	0	–	0	0
	50	0	–	0	0	0	+	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	–	0	0	+	+	+	+	–	–	0	0	0	–	0	+	0	0
	51	0	–	0	0	0	+	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	–	0	0	+	+	+	+	–	–	0	0	0	–	0	+	0	0
	52	0	–	0	0	0	+	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	+	+	+	+	–	–	0	0	0	–	0	+	0	0
	53	0	–	0	0	0	+	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	+	+	+	+	–	–	0	0	0	–	0	+	0	0
	54	0	–	0	0	0	+	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	–	0	0	+	+	+	+	–	–	0	0	0	–	0	+	0	0
	55	0	+	0	0	0	+	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	+	–	–	–	0	0	0	–	0	–	0	0
	56	0	–	0	0	0	+	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	–	0	0	+	+	+	+	–	–	0	0	0	–	0	+	0	0
	57	0	–	0	0	0	+	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	–	0	0	+	+	+	+	–	–	0	0	0	–	0	+	0	0
	58	0	–	0	0	0	+	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	–	0	0	+	+	+	+	+	+	0	0	0	–	0	+	0	0
B. subtilis RO-E-2 (genomic)	59	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0
	60	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0
	61	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0
	62	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0
	63	0	–	0	0	0	+	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	+	–	–	–	0	0	0	–	0	–	0	0
	64	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	+	+	+	–	–	–	0	0	0	–	0	+	0	0
	65	0	–	0	0	0	+	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0
	66	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0
	67	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0
	68	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0
	69	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0
	70	0	–	0	0	0	–	0	0	0	0	0	0	0	0	–	–	0	0	0	0	0	0	–	0	0	–	–	–	–	–	–	0	0	0	–	0	–	0	0

The *rpoB* gene fragment numbers correspond to the numbers in Figure 1. Only those fragments that were used in the analyses are included in the table. 0, for a particular donor, means that the fragment could not be used to analyze transformants; +, means that the fragment was present in the transformant; –, means that the fragment was absent in the transformant.

FIGURE 2.—Rationale for determining the minimum and maximum size of an integrated donor segment, as illustrated by the restriction analysis of a hypothetical transformant. The numbers represent restriction fragments occurring in the recipient strain. The segments highlighted in bold (as either dashed or nondashed lines) are absent in the digest of the donor strain. The recipient-strain bands represented by dashed lines are missing from the digest of the transformant. Thus, the transformant is missing three consecutive recipient-strain segments: restriction segments 12, 13, and 14. The letters indicate restriction sites: sites A, B, C, and D are known to occur in the recipient strain; sites E and F are sites that may possibly occur in the donor but not in the recipient (see below). Determination of the minimum span of integration: The minimum possible segment integrated from the donor would contain only restriction band 13. This is because a donor fragment that spans only from restriction site A to B would cause the transformant to be missing the three recipient bands 12–14. Determination of the maximum span of integration: The maximum size of the integrated fragment would extend from the 5′ end of band 11 to the 3′ end of band 15. (These are the segments flanking the chain of recipient-strain segments missing in the transformant.) We include restriction fragments 11 and 15 in the maximum estimate because we do not know if the absence of fragments 11 and 15 in the donor strain is due to an absence of restriction sites C and D in the donor or additional restriction sites within segments 11 and 15 (*e.g.*, sites E and F) in the donor. Because the hypothesized sites E and F may be at the distal ends of segments 11 and 15, respectively, the maximum span of the integrated segment could be as 5′ as the 5′ end of segment 11 and as 3′ as the 3′ end of segment 15. The maximum span of integration is thus estimated as the region between the distal ends of the two nonmissing recipient segments that flank the chain of missing recipient segments.

porated <903 bp (from bp 2156 to 3059), while this is the case for at most six of the 11 *B. atrophaeus* transformants and for at most seven of the 12 *B. subtilis* transformants.

It is not a simple matter to quantify the differences among donors in the lengths of DNA incorporated, since we can estimate only a maximum and minimum length for each segment integrated. Our approach was to compare donors for the fraction of their transformants missing each of several recipient-strain fragments. We included in this analysis only those restriction fragments that were present in the recipient but were absent in all three donor strains.

Consider first the results of transformation with PCR-amplified DNA as donor. For all three donors, the highest rate of recombination occurred within the region coding for rifampicin resistance (between nucleotides 2478 and 2684) (C. PRICE, personal communication) (Figure 4A). The three donors were uniformly high in the fraction of transformants missing the band (band 31) spanning the rif^R region: the fraction missing this band ranged from 0.91 in *B. licheniformis* transformants to 1.00 in *B. subtilis* transformants (Figure 4A). This result was expected because we had selected for transformants on the basis of rifampicin resistance.

For bands of intermediate distance from the rif^R region (≤533 nucleotides away, including bands 2, 21, and 44, but not including the band spanning the rif^R region), the donors were heterogeneous in the fraction of transformants missing each band, with fewer *B. licheniformis* transformants missing these bands. The fraction missing these bands ranged from an average of 0.15 for *B. licheniformis* transformants to an average of 0.67 for *B. subtilis* transformants (Figure 4A). The heterogeneity among donors was significant in the case of segment 44 (after accounting for multiple comparisons). For bands with the greatest distance from the rif^R region (>533 nucleotides away, including bands 36 and 38), all donors were uniformly low in the fraction of transformants missing the recipient bands (Figure 4A).

In contrast, with transformation by genomic DNA, there was no significant heterogeneity among donors in the fraction of transformants missing any recipient band (Table 2) (analysis of data not shown).

In the three-way comparison described above, we could only compare transformants for the presence of six restriction fragments because only these six bands of the recipient were missing in all three donors. To extend our analysis to a greater number of restriction fragments, we compared transformants of each *pair* of donors for the set of bands that were present in the recipient but were missing in both donor strains (Figure 4, B–D). In the comparison of *B. atrophaeus* and *B. licheniformis*, we could use 20 such restriction fragments; we could use eight fragments in the *B. subtilis-B. licheniformis* comparison, and seven fragments in the *B. subtilis-B. atrophaeus* comparison.

The results of these pairwise comparisons are generally similar to the three-way analysis. First, transformation by genomic DNA yielded no heterogeneity between donors in the fraction of transformants missing any recipient band (Table 2) (analysis of data not shown).

Second, with transformation by PCR-amplified DNA, the frequencies of integration of donor DNA were highest for all donors for bands overlapping the rif^R region, and the frequencies were similar across all donors. However, the donors were not as uniform in this region as in the three-way comparisons. For example, in the comparison between *B. atrophaeus* and *B. licheniformis*, 93% of *B. atrophaeus* transformants were missing the recipient's bands from the rif^R region, but only 62% of these bands were missing in *B. licheniformis* transformants. For one band overlapping the rif^R region (band 3), *B. licheniformis* transformants were significantly less likely to be missing the recipient band than were *B. atrophaeus* transformants (Figure 4B). Apparently the segments integrated from *B. licheniformis* donor DNA were so small that even the segments overlapping the rif^R region frequently failed to integrate.

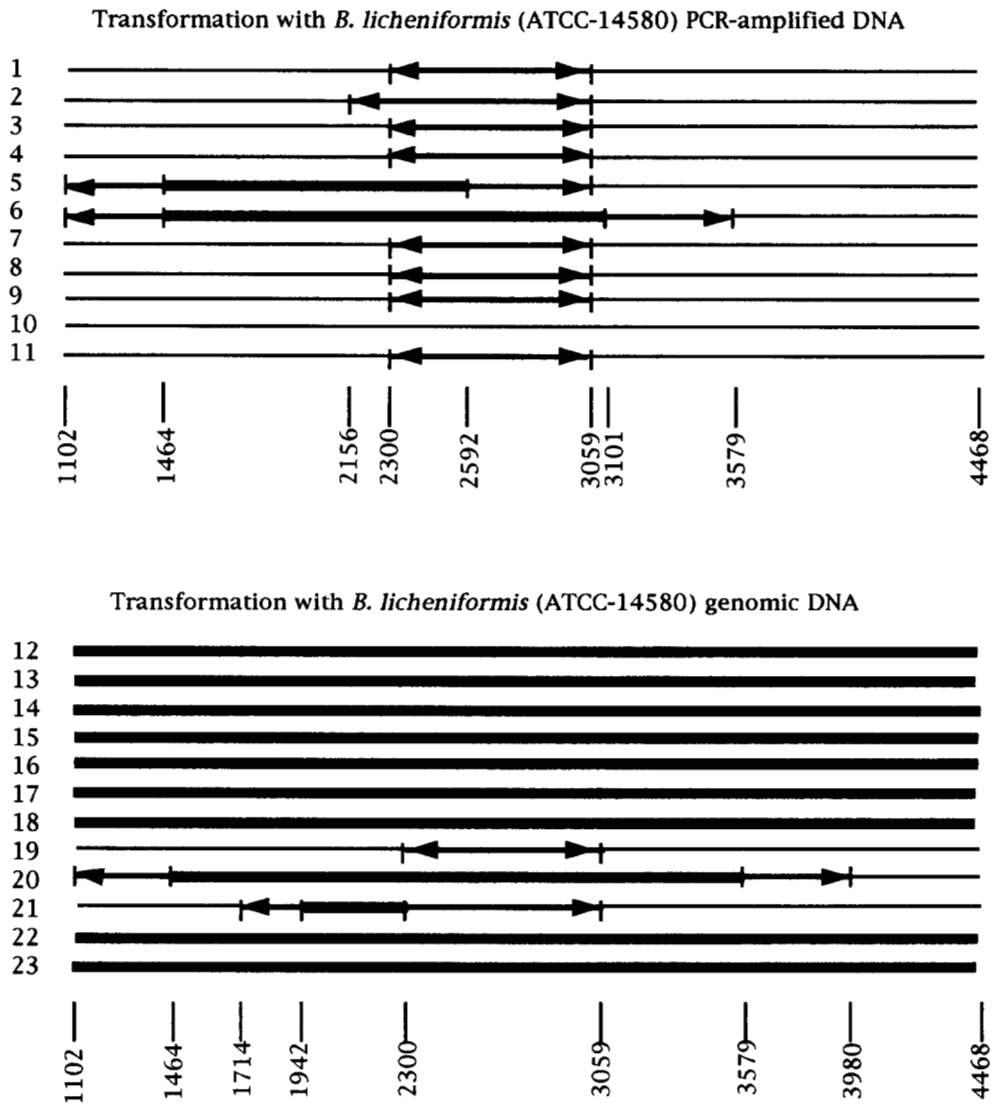

FIGURE 3.—The maximum and minimum ranges of donor DNA integrated in each transformant. Each line represents the 3367-bp segment of a single transformant, whose number is indicated at the left. Numbers at the bottom represent nucleotide sites of the *rpoB* gene. The minimum range of each transformant is spanned by the boldest bar; in cases where no bold bar is present, the restriction data provide no minimum estimate. The maximum range is spanned by arrows. The methods for determining the maximum and minimum ranges are described in the text.

Third, as in the case of the three-way comparison, the greatest differences among donors were for bands of intermediate distance from the rifampicin-resistance region. For bands in the region from 2048 to 3053, but not overlapping the rif^R region, there was a (nonsignificantly) greater fraction of transformants missing recipient bands with *B. atrophaeus* as donor than with *B. licheniformis* (Figure 4B). In this region there was a significantly greater fraction of *B. subtilis* transformants missing bands than with *B. licheniformis* transformants for two out of eight bands (bands 2 and 44) (Figure 4C).

Finally, as in the three-way comparisons, all donors were uniformly low in the frequency of bands missing at both ends of the *rpoB* gene.

Pairwise comparisons between the *B. subtilis* and *B. atrophaeus* donors revealed no significant differences in the fraction of transformants missing recipient bands (Figure 4D).

We conclude that in transformation with PCR-amplified DNA, the most divergent donor yielded shorter integrated segments than did the less divergent donors. In transformation with genomic donor DNA, the donors did not differ in the length of DNA integrated within the *rpoB* gene.

DISCUSSION

The restriction-digest approach to determining the pattern of integration of donor DNA: The restriction-digest approach presented in this study was designed to determine the size and continuity of transformed

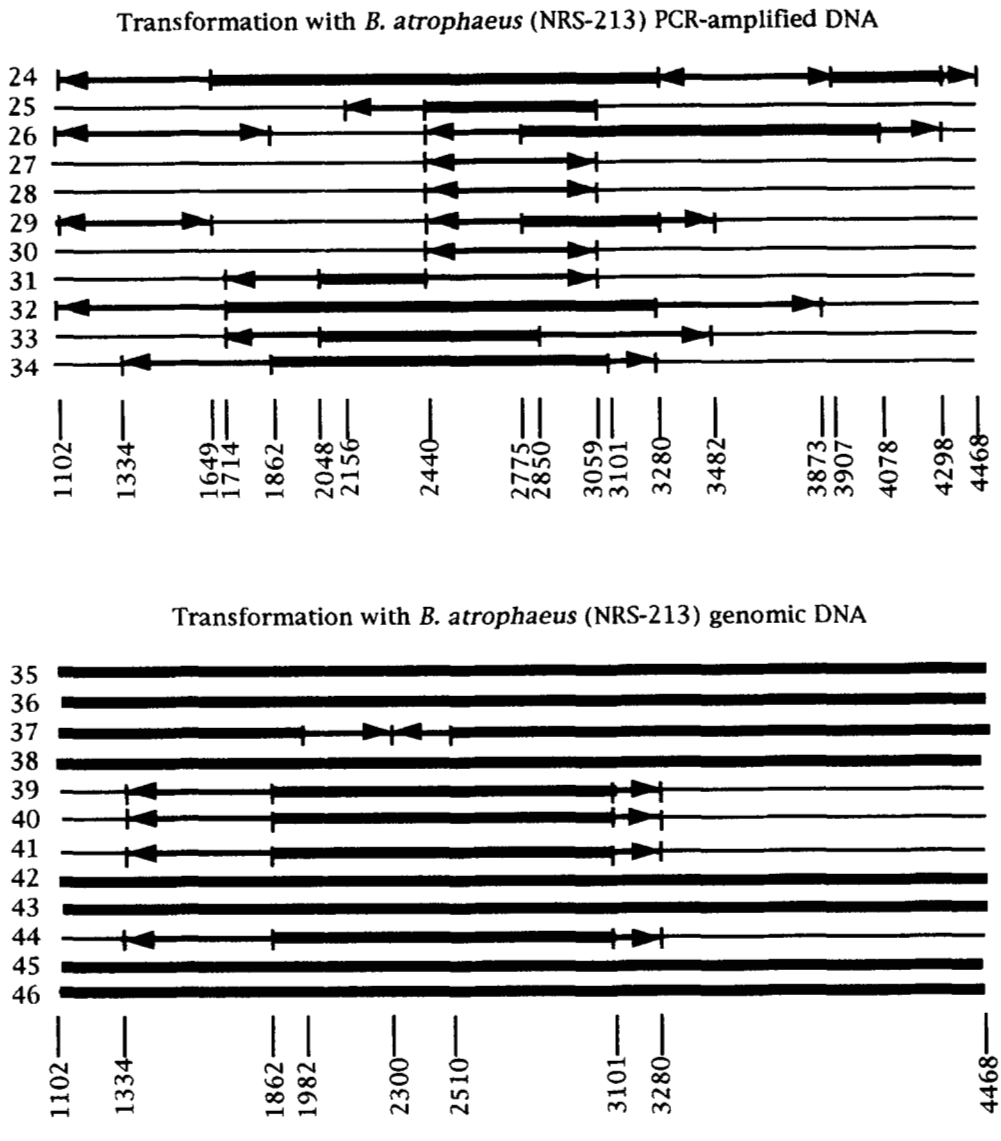

FIGURE 3.—*Continued*

segments within a stretch of DNA short enough to be PCR-amplified. The method is easy to apply because it requires sequence data from the recipient strain only and then only restriction-digest data from donor strains and transformants. This method has detected transformed segments ranging from one that is at most 547 bp long (transformant 29) to those that spanned the entire 3.4-kb region of *rpoB* under study. The method is simple enough so that one may perform many replicates of a given transformation experiment, thus allowing investigation of the intrinsic stochastic variation in the outcome of transformation events.

While the restriction-digest method cannot give precise breakpoints for the segments integrated in transformation, the method has yielded maximum and minimum bounds for these segments. The confidence interval (quantified as the difference between the maximum and minimum estimates) was shortest for the more divergent donors: *B. licheniformis* as donor, with 14.2% divergence from the recipient, yielded a mean confidence interval of 789 ± 16 bp (SE), and *B. atrophaeus* (with 7.0% divergence) yielded a mean confidence interval of 883 ± 102 bp. With the least divergent donor, *B. subtilis* strain RO-E-2 (with 3.1% divergence), the confidence interval was greater, at 1319 ± 87 bp.

Because it is now possible to use PCR to amplify regions of DNA as long as 35 kb (BARNES 1994), one may in principle determine the fate in transformation of an entire large donor segment (*e.g.*, a segment nearly as large as the genomic segments used as donor in this study). However, one cannot yet apply this approach for analyzing transformants of *rpoB*, since the requisite sequence data are not yet available in the *rpoB* region (C. W. PRICE, personal communication).

Effect of the length of donor DNA on the length of the integrated segment: Most transformants treated

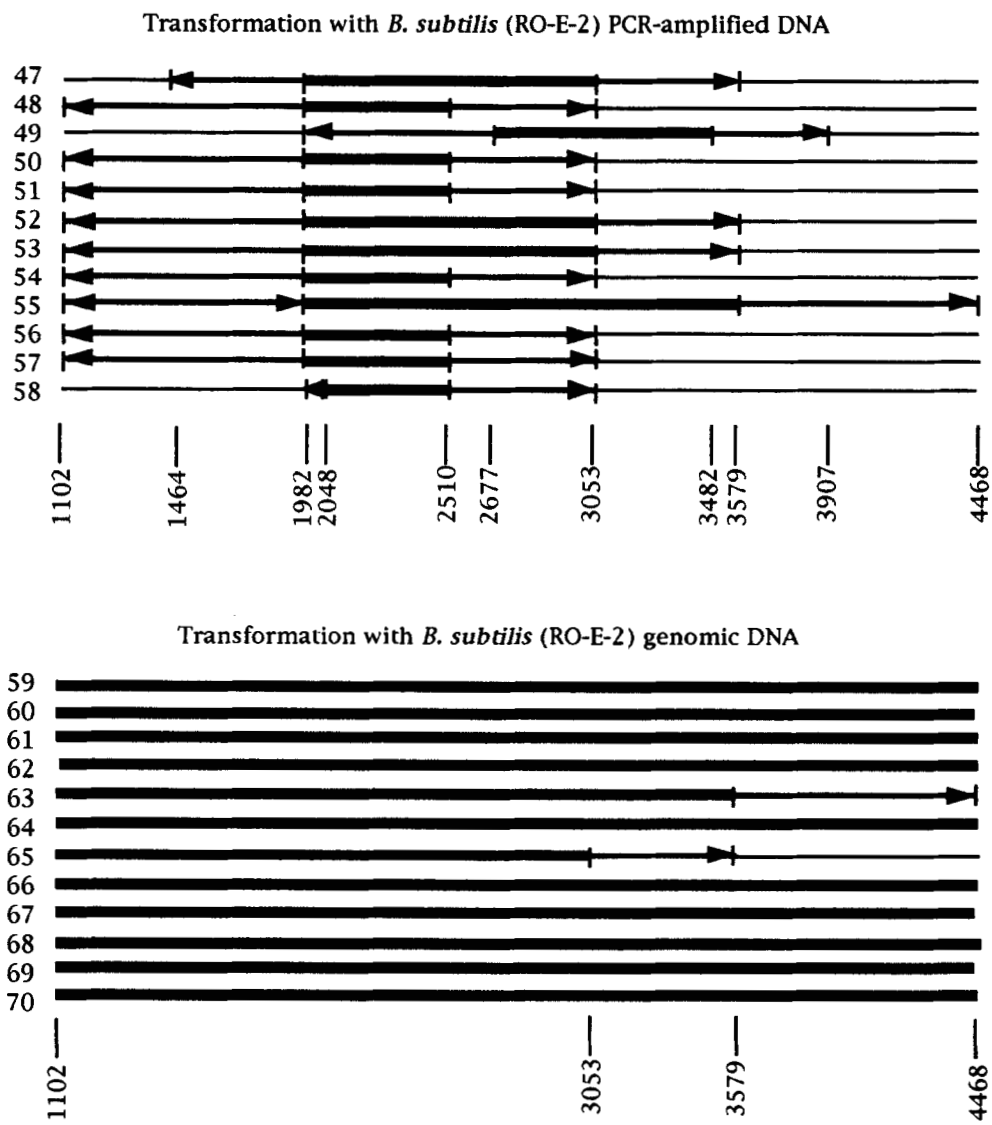

FIGURE 3.—*Continued*

with genomic DNA (of length ~50 kb) integrated the entire 3.4-kb segment under study, while none of the transformants treated with the 3.4-kb segment integrated the entire segment. In many cases, the DNA integrated from PCR-amplified donor DNA was less than half of the 3.4-kb segment. These results suggest that when larger donor segments are presented to a competent cell, longer donor fragments are integrated. Nevertheless, even with large genomic donor segments there was a relatively high frequency of integration of fragments shorter than 2 kb (occurring in 17% of transformants with genomic DNA).

Why did transformation with large genomic molecules tend to yield longer integrated segments? One explanation is that, regardless of the length of donor DNA molecules presented the cell, each donor molecule entering the cell was cleaved at its ends, perhaps by an exonuclease. This explanation is supported by the results of CONTENTE and DUBNAU (1979), who concluded that at least 400–500 bp at the ends of a donor molecule are excluded from integration. This cleavage would usually have little consequence on the outcome of transformation of a small region by a large genomic segment. One way to test this model further would be to present competent cells with PCR-amplified DNA in which the rifampicin resistance region is located near the end of the segment. If this donor molecule failed to transform cells toward rifampicin resistance, this would suggest that the end regions of donor segments are always cleaved. This approach could yield a statistical distribution of the length of a segment's end that is excluded from transformation.

The stochastic nature of transformation: This study has shown a strong stochastic component in the outcome of transformation. Transformation with each of the PCR-amplified donor molecules has resulted in

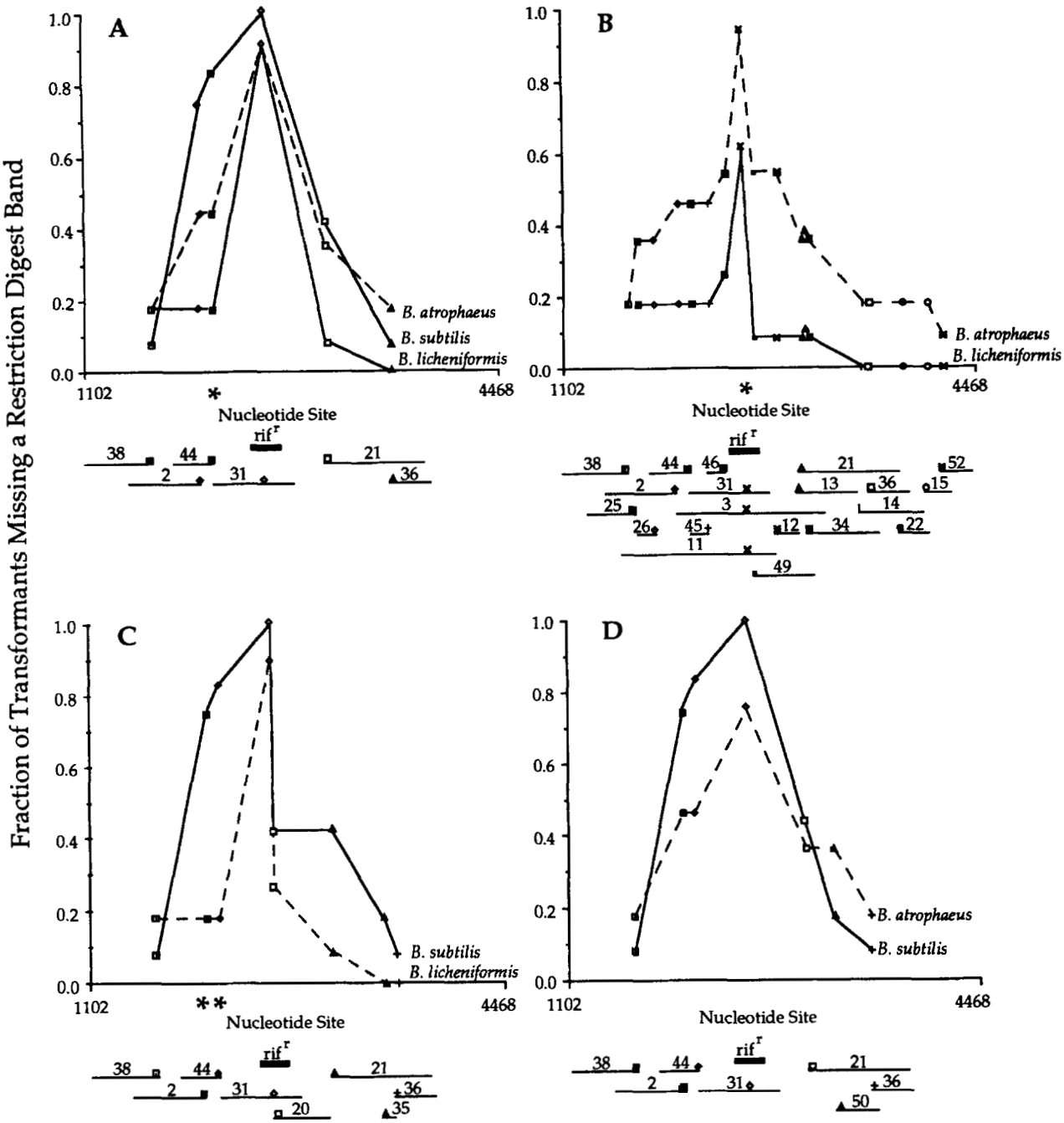

FIGURE 4.—For each donor strain (using PCR-amplified DNA), the fraction of transformants missing restriction-digest bands present in the recipient strain. (A) Comparison among all three donors. (B) Comparison between *B. licheniformis* and *B. atrophaeus* as donors. (C) Comparison between *B. licheniformis* and *B. subtilis* as donors. (D) Comparison between *B. atrophaeus* and *B. subtilis* as donors. Each comparison involves only those recipient-strain bands that are missing in all (or both) donors being compared. The span of each band is indicated below the graph. The bands overlapping the center of the rif^R region are pooled in the graph; all other bands are positioned on the graph by their closest point to the center of the rif^R region. The asterisks indicate significant heterogeneity among donors for a given band ($P < 0.05$ in G test, accounting for multiple comparisons by the sequential Bonferroni method). In the case of the comparison between *B. atrophaeus* and *B. licheniformis*, the asterisk refers to significant heterogeneity for band 3.

outcomes that are almost all distinguishable from one another. Even the large degree of variation in the pattern of recombination illustrated in Figure 3 has underestimated the true diversity of integration patterns. This is because different combinations of missing restriction fragments sometimes yielded the same interpretation for the range of DNA integration (Table 3).

The outcome of transformation with the larger genomic donor molecules was less variable within the 3.4-kb segment assayed, as most transformants integrated the entire 3.4 kb. It is possible that the outcome of transfor-

TABLE 3

The number of distinguishable recombination patterns found in each transformation treatment

Donor DNA	No. of different recombination patterns detected	Total no. of analyzed transformants
B. licheniformis		
PCR	7	11
Genomic	4	12
B. atrophaeus		
PCR	10	11
Genomic	3	12
B. subtilis		
PCR	6	12
Genomic	3	12

Data from Table 2.

mation with genomic DNA may be just as variable on a larger scale, outside the 3.4-kb region, as with the shorter PCR-amplified DNA.

Previous work has shown that replicate substrains transformed toward rifampicin resistance at *rpoB* are much more variable in the fitness effects of rifampicin resistance when transformed with PCR-amplified DNA than with genomic DNA (COHAN *et al.* 1994). The results of the present study suggest that the greater variation in fitness caused by transformation with PCR-amplified DNA may be due to the greater variability in the size and location of integrated segments within the *rpoB* gene.

The discontinuity of integrated segments: This study has shown that transformation may yield a discontiguous pattern of integration within the 3.4-kb segment of *rpoB*. Five transformants yielded restriction-digest patterns inconsistent with transformation by a single continuous molecule, where a recipient-derived segment was flanked by two donor-derived segments within the gene. It is possible that there is more discontinuity of integration than is apparent because our method of analysis assumed integrated segments to be continuous unless the restriction-digest pattern was inconsistent with continuous integration. The discontinuity detected in our experiments was probably caused by separate integration of parts of the same donor molecule, rather than integration of two or more donor molecules taken up independently into the cell (congression), owing to the low concentration of DNA presented the cells.

Our results corroborate earlier work using physical measurement and electron microscopy, which showed that a given donor molecule was likely to be integrated as a set of several discontiguous fragments (DUBNAU and CIRIGILIANO 1972; FORNILLI and FOX 1977). In these previous studies, the set of fragments stemming from a single molecule spanned an average of 9 kb, but no estimates were given for the size of the separately integrated donor subsegments. Our results suggest that these separately integrated segments range in size from ~500 bp to segments exceeding the range of the analyzed region.

The effect of donor-recipient sequence divergence on the length of the segment integrated: When genomic DNA was used as donor, the three donors yielded rif^R transformants with very similar lengths of donor DNA integrated (within the *rpoB* gene). However, when PCR-amplified DNA was used as donor, the most divergent strain (the type strain of *B. licheniformis*) yielded transformants with much shorter stretches of donor DNA than was the case for the less divergent donors (Figure 4). One possible explanation is that the greater sequence divergence between *B. licheniformis* and the recipient was much more likely to bring about cleavage of the heteroduplex by mismatch repair enzymes. It is not clear, however, why this greater cleavage of the *B. licheniformis*-recipient heteroduplex would not also occur in cells transformed by genomic DNA.

The reduced length of DNA transferred from *B. licheniformis* to *B. subtilis* may contribute to the sexual isolation observed between these species (ROBERTS and COHAN 1993), at least when donor DNA molecules are short (*e.g.*, 3.4 kb). It is interesting that the degree of sexual isolation between *B. licheniformis* and *B. subtilis* is about sevenfold higher with PCR-amplified donor DNA than with genomic DNA (ROBERTS and COHAN 1993). This corresponds approximately to the ratio of the average maximum estimate of the length of DNA transferred in *B. subtilis-B. subtilis* transformation to that transferred in *B. licheniformis-B. subtilis* transformation, when PCR-amplified DNA was used as donor (Figure 3). Thus, the greater degree of sexual isolation with transformation by PCR-amplified DNA may be due to the smaller segments transferred from *B. licheniformis* to *B. subtilis* (compared to intraspecific transformation) under this condition of transformation.

Evolutionary consequences of transfer of short segments of donor DNA: This study has shown that very short segments of donor DNA (~500 bp) may be integrated in Bacillus transformation. The integration of such short segments may be most likely when the donor DNA presented the recipient cells is short (*e.g.*, as short as the 3.4-kb segment presented in this study), and when there is greater sequence divergence between donor and recipient. Transformation may even bring about the discontiguous integration of two or more short segments within a single gene.

Our results show that Bacillus is like Neisseria, Streptococcus, and Haemophilus (MAYNARD SMITH *et al.* 1991) in being able to transfer very short segments of DNA across taxa by homologous transformation. This ability may facilitate the transfer of general adaptations across taxa, since the transfer of a very short segment of DNA is less likely to involve the cotransfer of closely

linked DNA whose cotransfer would be harmful. The cotransfer of DNA flanking a general adaptation, whether from the same gene or from other closely linked genes, may be harmful if the flanking DNA codes for adaptations that are only of value in the ecological or genetic context of the donor organism. It will be interesting to determine how harmful the cotransfer of flanking gene regions might be.

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