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Integration of Linear, Heterologous DNA Molecules into the *Bacillus subtilis* Chromosome: Mechanism and Use in Induction of Predictable Rearrangements

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Linear DNA molecules composed of a central region nonhomologous with the *Bacillus subtilis* chromosome and two flanking regions homologous with the chromosome can integrate into the chromosome, provided that the homologous regions have the same relative orientation. The resulting chromosome can be maintained in a haploid or in a merodiploid cell together with a parental chromosome. This can most easily be explained by supposing that the integration occurs by crossing over at each homologous region and that a part of the chromosome between these regions is deleted and replaced by the central nonhomologous region of the integrating molecule. If no essential genes were replaced during that process a haploid cell would be obtained; if essential genes were replaced a merodiploid cell would be obtained. The use of appropriate linear molecules therefore should allow the induction of deletions, extending from a given chromosomal site in a predetermined direction, and defined duplications in the *B. subtilis* chromosome.

Circular transforming plasmid molecules are probably cleaved on contact with competent *Bacillus subtilis* cells in a manner analogous to that described previously for the transforming chromosomal DNA (10, 21). Linearized plasmids are not viable (12) and must circularize on uptake to become established in the transformed cell. The plasmids which are internally repeated because of oligomerization in vivo or construction in vitro may circularize by an intramolecular process (5, 9, 11, 25). Two types of intramolecular circularization have been suggested to occur (25). The first corresponds to recombination between internal repeats and produces a circular molecule that is smaller than the parental transforming plasmid. The second corresponds to the repair of a break introduced in one repeat, using the homologous intact repeat as a template, and produces a circular molecule of the parental size. Gene conversion can occur during this process (25). Transforming plasmid molecules may also circularize by an intermolecular process, provided that they are homologous with genomes (plasmids, chromosomes) that are resident in the competent cells (3, 4, 7, 26). Circularization has been postulated to occur by repair of the break, using homologous resident sequences as templates, which leads to gene conversion when the interacting homologous sequences are not identical (20).

Circular DNA molecules unable to replicate in *B. subtilis* (e.g., an *Escherichia coli* plasmid carrying a gene well expressed in *B. subtilis* [13, 15, 28]) are likely to undergo similar processing during transformation. To become established in the recipient cell, however, such molecules must integrate into a resident genome (plasmid, chromosome), which can be accomplished only if they carry a segment homologous with the resident genome (15, 26, 30, 36). Integration may occur after intra- or intermolecular circularization by a single crossing over recombination event of the Campbell type.

Linear DNA molecules partially homologous with the *B. subtilis* chromosome, but unable to replicate in this bacterium, can integrate into the chromosome, provided that the region of nonhomology they contain is flanked on both sides by regions homologous with the chromosome (17, 29, 30, 34). Two crossing over events, one within each homologous region, have been suggested to be necessary for integration. As a result of this process, chromosomal sequences were replaced by heterologous sequences and were maintained stably in the cell if no gene essential for cell survival was carried within the replaced region (19, 29, 30).

We report here results of additional studies of the integration of linear, partially homologous DNA molecules in the *B. subtilis* chromosome which indicate that (i) heterologous parts of such molecules may be inserted into the chromosome only if the two homologous flanking regions have the same relative orientation; (ii) replacement of an essential gene by heterologous sequences leads to a merodiploid cell in which these sequences are unstable; and (iii) deletions starting from a given site and extending for various distances in a predetermined direction may be induced in the chromosome.

MATERIALS AND METHODS

Bacterial strains and plasmids used in this study are listed in Table 1. Enzymes were commercial preparations used as specified by the suppliers. Chromosomal DNA was prepared by centrifugation of lysed bacteria in CsCl (16), and plasmid DNA was prepared by centrifugation in CsCl (6) or hydroxyapatite chromatography of cleared cell lysates prepared as described previously (28). Competent *E. coli* and *B. subtilis* cells were prepared and transformed as described previously (8, 28). DNA samples were labeled by nick translation (33) and used for hybridization as described by Southern (35).

Hybrid plasmids were constructed by cloning in *E. coli* HVC45 (Fig. 1 and 2).

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TABLE 1. Bacterial strains

Strains	Genetic markers or other identifying characteristics	Source or reference
E. coli		
HVC45	*thrA1 leu-6 thi-1 lacY1 tonA21 supE44 hsdR rpsL*	R. Davis
B. subtilis		
GSY649	*ilvD2 thyA1 thyB1*	C. Anagnostopoulos
GSY914	*ilvA5 trpC2*	C. Anagnostopoulos
HVS49	*trpC2 tyrA1 aroB2 hisH2*	P. Schaeffer
HVS260	SPβCII	S. Zahler
c26	*trpC2 tyrA1 aroB2 hisH2* ins(pHV438) del(*ilvA2*)[a]	This laboratory
GSY914 Km^r	ins(pHV453) dup(*thyB ilvA5* X)[a]	This study
HVS49 Km^r	*tyrA1 aroB2 hisH2* ins(pHV453) dup(*thyB* X)[a]	This study
c26 Km^r	*tyrA1 aroB2 hisH2* ins(pHV438) del(*ilvA2*) ins (pHV453) dup(*thyB* X)[a]	This study
Plasmid[b]		
pUB110	Natural isolate	T. Gryczan
pHV438	Hybrid between pHV32 *thyB* and X segments of *B. subtilis* DNA	29
pHV445	Hybrid between pHV32 A and K segments of *B. subtilis* DNA	This study
pHV453	*Sau*3A segments I and IV of pUB110 joined to *Bgl*II-cleaved pHV438	This study
pHV459	*Bam*HI-*Bgl*II segments of pHV438 joined in the opposite orientation	This study
pHV460	*Bam*HI-*Bgl*II segments of pHV445 joined in the opposite orientation	This study
pHV463	*Bam*HI-*Bgl*II segments of pHV438 containing X joined to *Bam*HI-*Bgl*II segment of pHV445 containing A	This study
pHV465	*Bam*HI-*Bgl*II segment of pHV438 containing *thyB* joined to the *Bam*HI-*Bgl*II segment of pHV445 containing K	This study
pHV469	*Eco*RI segments of pHV438 joined in the opposite orientation	This study
pHV470	*Bam*HI-*Bgl*II segments of pHV469 joined in the opposite orientation	This study

[a] Genetic modifications because of the insertion of plasmids into the chromosome have been described previously as follows (30): (i) ins followed by the plasmid name denotes the inserted plasmid; (ii) del or dup followed by a gene or sequence name or number in parentheses denotes deletions or duplications, respectively, induced by the insertion of plasmids.

[b] Relevant details for plasmids are given in the text and Fig. 1 and 2.

pHV438. We have previously described *B. subtilis* c26 (Fig. 3), in which plasmid pHV32 (a hybrid between pBR322 and the Cm^r gene of pC194 [32]) replaced the chromosomal *ilvA* gene (30). Plasmid pHV438 was obtained by cleaving c26 DNA with *Bgl*II and transforming *E. coli* with ligated DNA segments to ampicillin resistance (30). It confers on *E. coli* resistance to ampicillin (Ap^r), tetracycline (Tc^r), and chloramphenicol (Cm^r), as well as independence from thymine, and consists of pHV32 (5.8 kilobases) [kb]), the *B. subtilis thyB* gene (2 kb), and a genetically uncharacterized segment of the *B. subtilis* chromosome labeled X (3.3 kb; Fig. 1).

pHV445. Plasmid pHV445 was obtained as follows. *Eco*RI-cleaved pHV32 joined in a shotgun experiment to *Eco*RI-cleaved *B. subtilis* DNA was used to transform *B. subtilis* HVS49-competent cells to chloramphenicol resistance. A Cm^r clone with a rough colonial morphology (the parental strain is smooth) which segregated smooth Cm^s progeny was found among the transformants. Its DNA was purified, cleaved with *Bgl*II, ligated, and used to transform *E. coli* to ampicillin resistance. A plasmid isolated from a representative Ap^r clone was named pHV445. It conferred the Ap^r Tc^r Cm^r phenotype on *E. coli* and was composed of pHV32 and two *B. subtilis* chromosomal segments of unknown genetic content named A (2.9 kb) and K (3.0 kb; Fig. 2).

pHV453. Plasmid pHV453 was constructed by inserting the Km^r gene of plasmid pUB110 (carried on *Sau*3A segments I and IV, totaling 2 kb) into the *Bgl*II site of pHV438 (Fig. 1).

pHV459. Plasmid pHV459 was constructed by cleaving pHV438 with *Bam*HI and *Bgl*II and ligating the resulting segments in the presence of the two enzymes to favor the creation of hybrid *Bam*HI/*Bgl*II sites (Fig. 1).

pHV460. Plasmid pHV460 was constructed by cleaving pHV445 with *Bam*HI and *Bgl*II and ligating the resulting segments in the presence of the two enzymes to favor the creation of *Bam*HI/*Bgl*II hybrid sites (Fig. 2).

pHV461. Plasmid pHV461 was constructed by replacing the pHV438 *Bgl*II-*Bam*HI segment carrying the Cm^r gene with the equivalent pHV445 segment (Fig. 2).

pHV462. Plasmid pHV462 was constructed by replacing the pHV438 *Bgl*II-*Bam*HI segment carrying the Ap^r gene with the equivalent pHV445 segment (Fig. 2).

pHV463 and pHV465. Plasmids pHV463 and pHV465 were constructed from pHV461 and pHV462, respectively, as described above for pHV459 and pHV460 (Fig. 2).

pHV469. Plasmid pHV469 was constructed by cleaving pHV438 with *Eco*RI and joining the released segments. pHV32 and the *thyB*-X region of pHV469 had the orientation opposite to that found in pHV438 (Fig. 1).

pHV470. Plasmid pHV470 was constructed from pHV469 as described above for pHV459 and pHV460 (Fig. 1).

RESULTS

Four classes of linear DNA molecules, consisting of a central region nonhomologous with the *B. subtilis* chromosome and two homologous flanking regions, can be distinguished if the orientation of the central region is disregarded (Fig. 4A). These molecules differ in the relative orientation of the flanking regions and, as a consequence, have on one of their strands sequences that are derived from only one (type 1) or from both (type 2) strands of the chromosome (Fig. 4A). Type 1 molecules should be able to integrate into the chromosome (Fig. 4B), and type 2 should not (Fig. 4C). During integration a part of the recipient chromosome, delimited by the regions of homology, may be replaced by the heterologous region of the transforming molecule, if the homologous regions are not contiguous in the chromosome (Fig. 4B) (30). The orientation of the homologous regions flanking the central nonhomologous region of a linear transforming molecule should determine which part of the recipient chromosome will be replaced upon integration. This is illustrated in Fig. 5, in which the chromosome consists of two parts, α and ω, separated by regions abc and klm that are

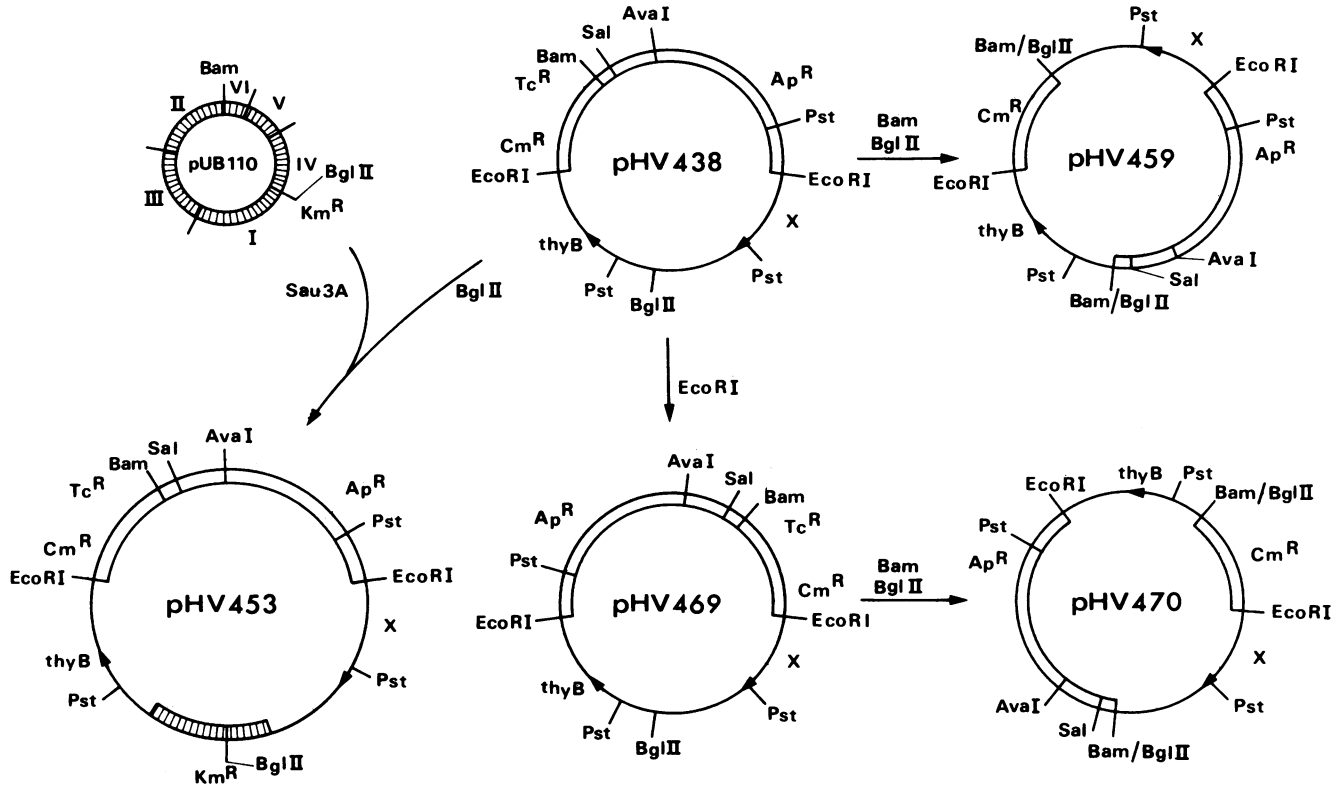

FIG. 1. Structure and construction of plasmids partially homologous with the *B. subtilis* chromosome derived from pHV438. Chromosomal, pHV32, and pUB110 sequences are shown as single, double, and hatched lines, respectively. Arrows refer to the orientation of *B. subtilis* segments (see text). Only the restriction sites referred to in this work are indicated. Roman numerals denote *Sau*3A segments of pUB110 in order of decreasing size.

homologous with the transforming molecule. One of the orientations of the flanking regions should lead to the replacement of part α; the other should lead to the replacement of part ω with the heterologous segment M. Because the two parts represent (together with regions abc and klm) the entire chromosome, if one part carried all essential genes the other would carry none. Bacteria containing the part α of the chromosome were viable (30). Those containing part ω should not be viable, unless they also possess a chromosome that carries the missing essential genes and are therefore merodiploid for the part of the chromosome not replaced by heterologous sequences (Fig. 5). We constructed the four classes of linear DNA molecules shown in Fig. 4A and used them to transform *B. subtilis*-competent cells to test whether they integrate by inducing deletions or duplications or whether they are unable to integrate, as predicted by the schemes shown in Fig. 4 and 5.

Transforming DNA molecules. The two segments of *B. subtilis* DNA, *thyB* and X, which are present on a linear molecule produced by cleaving pHV438 (Fig. 1) with *Bgl*II are oriented relative to each other in the same way as in the chromosome of strain c26 (see above; Fig. 3). We compared the *thyB* and X regions of the c26 and the wild-type chromosomes and found identical restriction maps (Fig. 3). The two *B. subtilis* DNA segments carried in pHV438 therefore have the same relative orientation as in the wild type chromosome. The appropriate cleavage of the plasmid thus should produce type 1 molecules capable of integrating in that chromosome.

Two different type 1 molecules can be generated from pHV453, a plasmid which is derived from pHV438 (Fig.1). The first molecule, obtained by cleavage within the Km^r region, resembles that obtained from pHV438 and can be schematically represented by *thy* Cm^r X. The second molecule, obtained by cleavage within the pHV32 region, has a different order of homologous segments flanking the heterologous part of the molecule and can be represented by X Km^r *thy*. To determine whether both molecules can be integrated into the chromosome, we performed the following experiments.

pHV453 transformed *B. subtilis* efficiently to chloramphenicol resistance (10^6 transformants per μg were obtained). A total of 27% of the transformants were Cm^r Km^r Ile$^+$, and 73% were Cm^r Km^s Ile$^-$. The first were probably due to the integration of a circular plasmid molecule into the chromosome by a single crossing over within *thyB* or X; the second were due to integration of a linear molecule (generated presumably by cleavage within or close to the Km^r gene during transformation) by a double crossing over at *thyB* and X. As expected, only the latter class was observed if the plasmid was cleaved with *Bgl*II before transformation. These results indicate that the type 1 molecules analogous to those obtained from pHV438 can be integrated into the chromosome.

pHV453 gave no Km^r transformants by direct selection, presumably because of rather inefficient expression of the gene integrated into the chromosome (31; unpublished data). However, such transformants could be identified in a congression experiment (Table 2). Three phenotypic classes were obtained with intact DNA. Two of them, Km^r Cm^r and

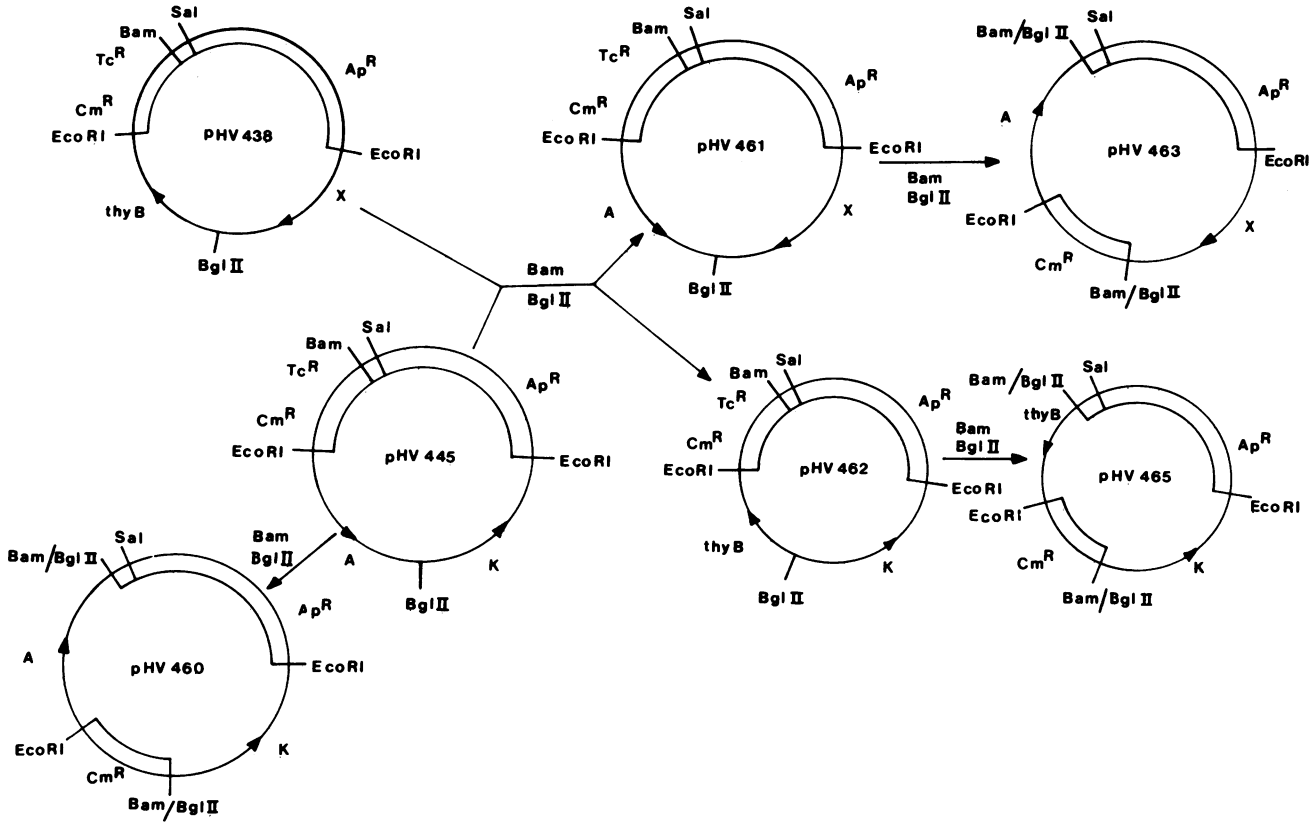

FIG. 2. Structure and construction of plasmids partially homologous to *B. subtilis* chromosome derived from pHV445. Symbols and conventions used in the figure are described in the legend to Fig. 1.

Cm^r, which appeared at a frequency similar to that observed on selection for chloramphenicol resistance (4 of 20 and 27%) were due to integration of circular molecules and linear molecules cleaved within the Km^r region of pHV453, respectively (see above). The third class, Km^r, was presumably due to a double crossing over recombination between the recipient chromosome and the pHV453 linearized during transformation by cleavage within the pHV32 region. This was the only observed class when pHV453 was cleaved before transformation with *Bam*HI, which cuts within pHV32 (Fig. 1). Type 1 molecules of the structure X Km^r *thy* therefore can be integrated into the chromosome.

Integration may be seen as occurring in two steps (Fig. 5; see also below). First, recombination of the linear molecule with the chromosome could give a circular molecule consisting of *thyB ilvA* X and Km^r (cf. Fig. 5, replacing abc, ω, klm, and M with *thyB*, *ilvA*, X and Km^r, respectively). Recombination of this circular molecule, which is unable to propagate autonomously, with the chromosome would generate a duplication with the structure *thyB ilvA* X Km^r *thyB ilvA* X (Fig. 5). Several experiments were performed to demonstrate the existence of this structure.

In the first experiment, *B. subtilis* HVS49-competent cells were transformed to kanamycin resistance with pHV453 DNA cleaved with *Bam*HI. All the transformants were Ile^+, which indicates that the integration of this linear molecule does not lead to replacement of the *ilvA* gene. A representative Km^r transformant and the parental HVS49 cells were transformed to chloramphenicol resistance with *Bgl*II-cleaved pHV438 DNA. Half of the transformants obtained with the former cells were Km^r Ile^+; the other half were Km^s Ile^-, whereas all transformants obtained with the latter cells were Ile^-. The structure of the transforming molecule can be schematically represented as *thyB* Cm^r X. Integration into the chromosome by double crossing over at *thyB* and X must lead to replacement of the *ilvA* gene, which maps between these two loci, with the Cm^r gene. The parental HVS49 chromosome carries a unique copy of the *ilvA* gene; therefore, all of the Cm^r transformants were Ile^-. The Km^r clone must have carried two copies of the *ilvA* gene, both flanked by *thyB* and X, because the integration of the Cm^r gene gave transformants which were Ile^+ (one copy replaced) and Ile^- (both copies replaced). Replacement of one copy of the *ilvA* gene was never accompanied by the loss of the Km^r gene, whereas replacement of both copies always was accompanied by the loss of the Km^r gene, which indicates that the Km^r gene was located between the two *ilvA* genes. These results support the existence of the duplicated structure postulated for the Km^r transformant.

In the second experiment, we used a Km^r clone obtained by transforming the *B. subtilis ilvA5* GSY914 cells with *Bam*HI-cleaved pHV453 (Table 2) to demonstrate that *ilvA* is duplicated. For that purpose we isolated an Ile^+ mutant and used its DNA to transform *B. subtilis* GSY649 *ilvD thyA thyB* cells to thymine independence. Phenotypes of transformants, as determined by replicating several hundred colonies, are given in Table 3.

A majority of clones were Ilv^- Val^- and therefore carried the *ilvD* gene which is closely linked to *thyB* (1) and were probably transformed at the *thyA* locus. The remaining clones were Val^+ and therefore were $ilvD^+$. They were also $thyB^+$, as shown by their thermosensitive Thy^+ phenotype

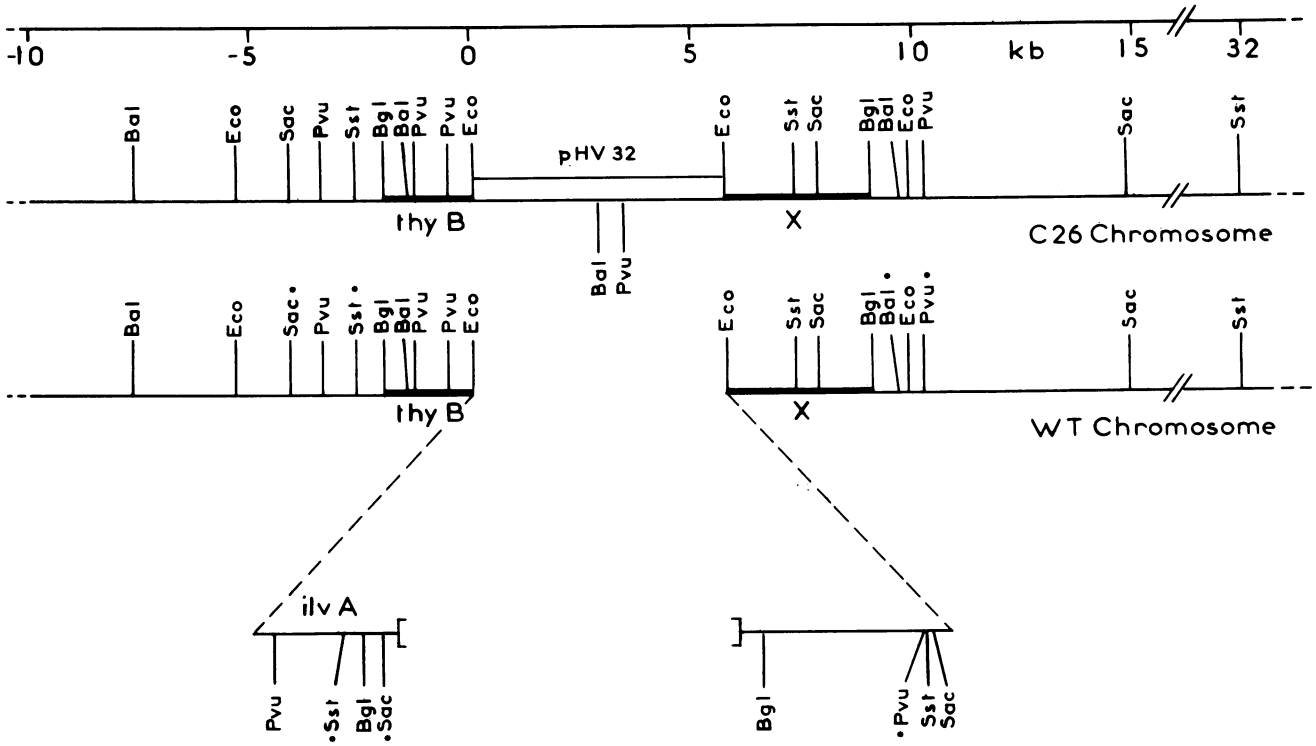

FIG. 3. Restriction enzyme map of the *thyB*-X region of the c26 and the wild-type *B. subtilis* chromosomes. *Bal*I, *Bgl*II, *Eco*RI, *Pvu*II, *Sac*I, and *Sst*II were used. The position of sites within the *thyB* or X segment (thick line) or pHV32 (double line) was determined by analyzing plasmid pHV438; the position of the other sites in the chromosome (thin line) was determined by Southern analysis of the two chromosomal DNAs with pHV438 used as a probe. Of 10 and 9 sites in the *thyB* and X regions of the chromosome, respectively, 2 could not be assigned unambiguously on the wild-type chromosome in our analysis and are indicated by an asterisk. The parentheses indicate part of the missing region that is devoid of known sites, which is probably not larger than 7 kb (28).

(27). Of these, 62% were Ile$^-$ and 38% were Ile$^+$, which shows that they harbored the *ilvA* and *ilvA*$^+$ alleles respectively. This demonstrates that the donor DNA was diploid for the *ilvA* gene.

GSY914 DNA was used to transform GSY649 cells to thymine independence in a control experiment (Table 3). The proportion of Ile$^-$ Val$^-$ transformants was similar to that observed with the DNA from the diploid Km^r clone. Ile$^+$ Val$^-$ clones were somewhat more abundant, which suggests that the structure of duplication present in the Km^r mutant was *ilvD*$^+$ *thyB*$^+$ *ilvA* X Km^r *thyB*$^+$ *ilvA*$^+$ X. Integration of the *ilvD*$^+$ *thyB*$^+$ *ilvA* mutant region could then sometimes be accompanied by integration of the linked *ilvA*$^+$ allele, which is absent in the GSY914 DNA. This is supported by the fact that the Km^r gene was more frequent among Ile$^+$ than among Ile$^-$ transformants (Table 3).

In the third experiment, the Km^r transformants obtained with pHV453 DNA cleaved with *Bam*HI were grown for 20 generations in the absence of kanamycin. 4% of the cells became sensitive to the antibiotic. This indicates that the Km^r mutant was flanked by duplicated sequences. No sensitive cells were obtained in a control experiment with Cm^r transformants obtained with pHV453 DNA cleaved with *Bgl*II. These transformants carry no duplication flanking the resistance gene.

From the results of the experiments described above we conclude that the analyzed Km^r transformants, obtained with pHV453 DNA cleaved within the pHV32 region, carry two copies of *thyB*, X, and *ilvA* and that their Km^r gene is flanked on both sides by one of the copies.

Nontransforming DNA molecules. Results of the experiments described above indicate that heterologous regions of the type 1 linear molecules can be integrated into the chromosome. To examine type 2 molecules we used plasmids pHV459 and pHV470 (Fig. 1). Linearization of the two plasmids with enzymes that cut within their pBR322 region, such as *Sal*I or *Ava*I, produces molecules in which the Cm^r gene is flanked by *thyB* and X. The relative orientation of these (Fig. 1, arrows) is such that both point toward the Cm^r gene in pHV459 and point away from that gene in pHV470 (*thyB* and X point toward and away from the Cm^r gene, respectively, in the linear type 1 molecules obtained by *Bgl*II cleavage of pHV438 or pHV469; Fig. 1). Intact pHV459 and pHV470 DNAs transformed *B. subtilis*-competent cells with an efficiency of 10^5 to 10^6 transformants per μg. *Sal*I cleavage reduced that efficiency by four orders of magnitude (10 to 100 transformants per μg). The remaining activity was probably due to incomplete *Sal*I cleavage, because additional treatment with *Ava*I, which cleaves close to *Sal*I (Fig. 1), decreased the transforming activity even further (<10 transformants per μg were obtained). The decrease upon *Sal*I treatment was not due to spurious degradation of DNA, because ligation of the cleaved samples restored about 10% of the original transforming activity. These results are in sharp contrast with those obtained with pHV438 and pHV469, in which *Bgl*II cleavage never caused a decrease exceeding 10 times. The results indicate that a heterologous region of the linear type 2 DNA molecules cannot be integrated into the *B. subtilis* chromosome.

To confirm this conclusion we made use of several other plasmids. First, we used pHV445, which resembles pHV438 because it is composed of pHV32 and two *B. subtilis*

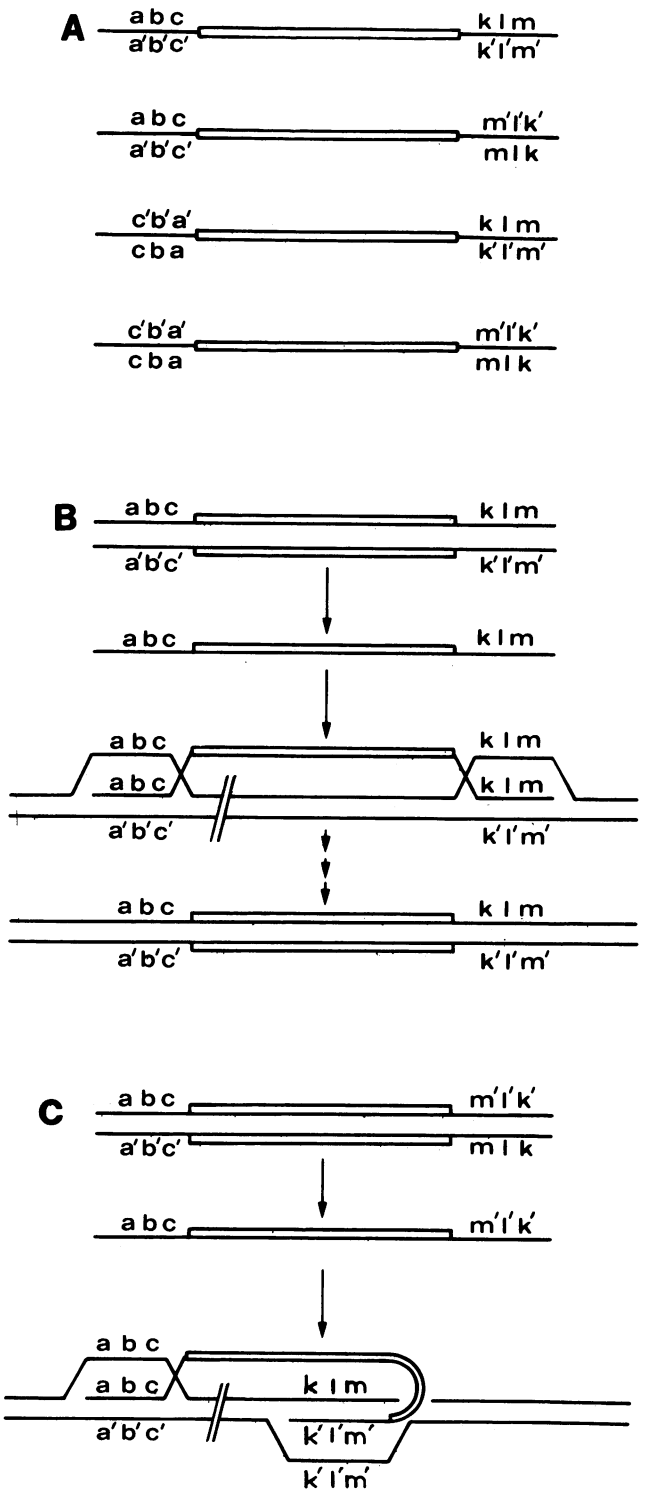

FIG. 4. Linear DNA molecules partially homologous with the *B. subtilis* chromosome and their interaction with the chromosome. Chromosomal sequences are represented as a single line, heterologous sequences are represented as a double line, letters denote sequences, and primed letters denote their complements. (A) Four orientations of two DNA segments homologous with the chromosome flanking a heterologous DNA segment. (B) A model of the integration into the chromosome of a heterologous DNA segment flanked by appropriately oriented homologous segments. (C) A model of abortive integration of a heterologous DNA segment flanked by inappropriately oriented homologous segments. The transforming molecule is shown converted into the single-stranded form, as was expected from previously described models of *B. subtilis* transformation.

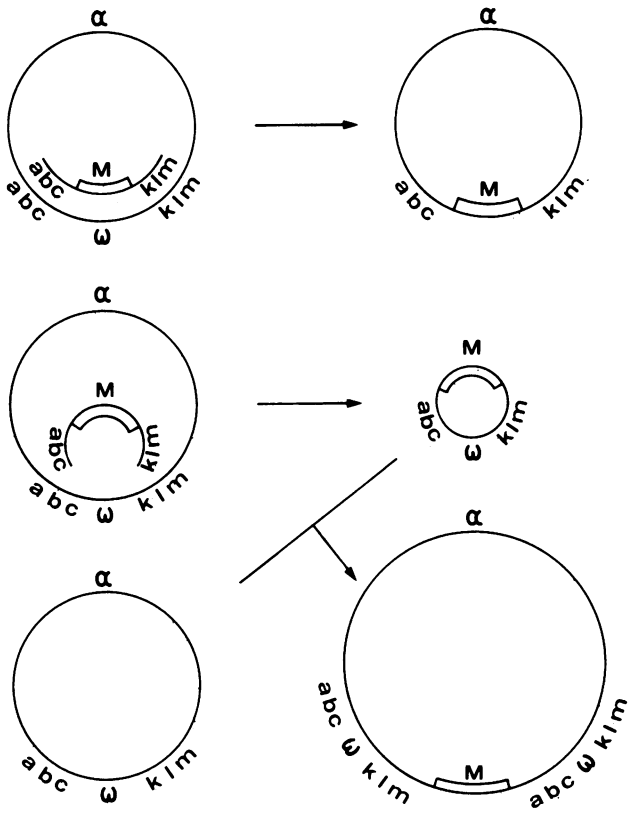

FIG. 5. Deletions induced in *B. subtilis* chromosome by the integration of a heterologous DNA segment. Single and double lines refer to chromosomal and heterologous DNA, respectively; Latin letters denote chromosomal sequences; Greek letters indicate parts of the chromosome; arrows indicate the orientation of segments carried by the integrating molecules. The process represented in this figure is discussed in the text.

chromosomal segments joined at the *Bgl*II site (Fig. 2). The genetic content of the segments, named A and K, is not known. pHV445 cleaved by *Bgl*II transformed *B. subtilis* to chloramphenicol resistance. We inverted the relative orientation of A and K and labeled the resulting plasmid pHV460 (Fig. 2). The transforming efficiency of *Sal*I-cleaved pHV460 was 10^4 times lower than that of the intact plasmid.

Next we constructed from pHV438 and pHV445 the plasmids pHV461 and pHV462 (Fig. 2). In pHV461, pHV32 was flanked by A and X; in pHV462 it was flanked by *thyB* and K. The two transformed *B. subtilis* 10^4 times more efficiently when they were intact than when they were cleaved. We then inverted the relative orientations of the flanking homologous segments. The two plasmids, pHV463 and pHV465 (Fig. 2), transformed *B. subtilis* when they were both intact or cleaved. These results indicate that a heterologous region of a linear DNA molecule integrates in a *B. subtilis* chromosome only if the flanking regions have one of the two appropriate orientations (cf. Fig. 4). They indicate that type 2 molecules that were unable to integrate were generated from pHV460, pHV461, and pHV462, whereas

type 1 molecules that are able to integrate were generated from pHV445, pHV463, and pHV465. Segments A and K therefore must have the same orientation. Their orientation must be opposite to that of *thyB* and X in the chromosome of *B. subtilis* HVS49.

It is interesting that the Cm^r transformants obtained with cleaved pHV445, pHV463, and pHV465 were all merodiploid, as judged by the fact that they had no auxotrophic requirements, except for those of the parental strain, and that they segregated chloramphenicol-sensitive progeny. Integration of these molecules may have occurred by the mechanism depicted in Fig. 5.

Deletions starting at a defined chromosomal site. Integration of linear DNA molecules, composed of a central heterologous and two flanking homologous regions, into a *B. subtilis* chromosome may result in deletion of the part of the chromosome delimited by the two regions of homology (Fig. 4 and 5). If one of the homologous regions is kept the same and the other is varied, a series of deletions should be obtained, with all deletions starting adjacent to the constant region. This was verified in the following experiment.

pHV438 was cleaved with *Pst*I and *Bam*HI which yielded a DNA segment composed of *thyB* and the Cm^r gene (Fig. 1). It was ligated in the presence of *Bgl*II with *B. subtilis* DNA cleaved with *Bgl*II. Linear molecules of the form T Cm^r B should be produced in this way, where T and B stand for *thyB* and *B. subtilis* random chromosomal sequences, respectively. These molecules cannot circularize because the T and B ends are produced by *Pst*I and *Bgl*II, respectively, and therefore are not complementary. They were used to transform *B. subtilis* HVS49-competent cells to chloramphenicol resistance. Transformants (10^3) were obtained with 0.1 μg of pHV438 DNA, whereas in control experiments less than 10 and 40 transformants were obtained if ligase and cleaved *B. subtilis* DNA, respectively, were omitted.

The phenotype of 440 Cm^r transformants was determined. Of these 413 had the same requirements as HVS49, 24 also required isoleucine, and 3 had more complex requirements which were not determined in detail. Chromosomal DNA was extracted from 12 randomly chosen clones of the first class and all the clones of the last two classes and was used to transform HVS49 to chloramphenicol resistance. The transformants obtained with the DNA extracted from Ile^+ and Ile^- clones had a phenotype identical to that of the donor bacteria. The transformants obtained with the DNA from the three remaining clones had the phenotype corresponding to that of HVS49, which indicates that the complex requirements of these clones were not a consequence of the insertion of the Cm^r gene. These were not studied further.

The *ilvA* gene, which maps next to the *thyB* gene, was expected to be deleted in all Cm^r transformants. Ile^+ clones therefore were likely to be merodiploid because of the deletion of an essential gene (cf. Fig. 5). To test this hypothesis, DNA from two representative clones was extracted, cleaved with *Bgl*II, and analyzed by hybridization with radiolabeled *thyB*-Cm^r sequences used as probes (Fig. 5). In both DNAs two *Bgl*II segments were revealed, with one corresponding to the wild-type *thyB* segments (by comparison with *Bgl*II-cleaved HVS49 DNA) and the other presumably corresponding to the *thyB* segment joined to the Cm^r gene and some other *B. subtilis* sequences. As expected, the latter segment was not the same in the two DNA preparations. This indicates the presence of two *thyB* genes in the Ile^+ Cm^r clones and suggests that these clones were merodiploid.

Stability of the Cm^r phenotype of 12 Ile^+ clones was determined. All but one segregated Cm^s cells with frequencies varying from 1 to 50% per 20 generations. This indicates that they were merodiploid. The remaining clone segregated less than 1% of Cm^s clones during 20 generations. This may be due to the fact that the duplicated region was rather short. Previously we have observed that duplications in the *B. subtilis* chromosome of about 4 kb recombine with a frequency of 10^{-4} per cell generation (31). The clone carrying such a duplication would appear to be stable in the present experiment.

Ile^- clones should result from an event during which no essential genes were deleted and should therefore be haploid. This was confirmed by Southern analysis of the DNA extracted from two such clones. In both cases a single *Bgl*II segment different from the one present in the wild-type chromosome hybridized with the *thyB*-Cm^r probe (Fig. 6). Furthermore, in all 24 clones the Cm^r character segregated less than 1% within 20 generations.

TABLE 2. Transformation of GSY914-competent cells with pHV453 and HVS260 DNAs

DNA	Plasmid treatment	No. of transformants			
		Trp+	Km^r Cm^r	Km^r	Cm^r
pHV453	None	0			
pHV453 + HVS260	None	1,410	4	7	20
pHV453 + HVS260	*Bam*HI	1,609	0	19	0
HVS260	None	2,434	0	0	0

TABLE 3. Phenotypes of Thy^+ clones obtained by transforming GSY649 *ilvD thyA thyB* cells

Donor DNA	Percentage of the following phenotypes:				
	Ile^- Val^-	Ile^- Km^r	Ile^- Km^s	Ile^+ Km^r	Ile^+ Km^s
GSY914 Km^r	71	0.5	17.5	3	8
GSY914	70		24		6

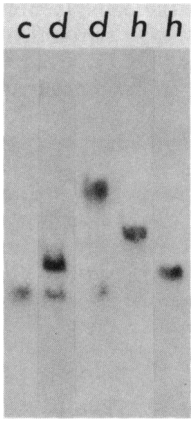

FIG. 6. Hybridization analysis of the *B. subtilis* chromosome. DNA extracted from the control HVS49 parental strain (lane c) diploid Cm^r Ile^+ (lanes d), and haploid Cm^r Ile^- (lanes h) transformants was cleaved with *Bgl*II and hybridized with a labeled probe containing the *thyB* and pHV32 sequences.

Deletions introduced into the recipient chromosome in this experiment were expected to start at *thyB* and progress toward *ilvA*. The fact that all clones that appeared to be haploid were *ilvA* (as judged by their isoleucine requirement), whereas no mutations were observed in the *ilvD* gene (these mutants would require isoleucine and valine) which is closely linked to *thyB* but on the side opposite from *ilvA*, supports this prediction. The size of deletions was analyzed by examining those genes other than *ilvA* that were missing from the Cm^r transformants. The two closest mapped loci are in prophage SPβ and code for the production of and resistance to betacine (37). Of 24 Ile^- clones, 5 were both sensitive to betacine and deficient in its synthesis. The deletions starting at *thyB* and entering into prophage SPβ therefore were induced in these clones. This indicates that there are no essential genes between *thyB* and SPβ. The next convenient genetic marker in the HVS49 chromosome is *citK* (18). None of the clones lacked this gene, as judged by their ability to sporulate.

It is interesting that the region proximal to *thyB* which is devoid of essential genes represented about 4% of the chromosome (SPβ is 128 kb; the *B. subtilis* chromosome is 3400 kb [14, 18]) and that 5% of the Cm^r clones were haploid. This suggests that the variable end of the linear molecules used may have integrated with nearly equal probability anywhere along the *B. subtilis* chromosome, which is expected, because that end was added in a shotgun experiment. The randomness of integration was also indicated by the Southern analysis (Fig. 6; unpublished data).

DISCUSSION

Integration of a linear DNA molecule, composed of a central region nonhomologous with the chromosome and two flanking homologous regions, into the *B. subtilis* chromosome has been found previously to be deletogenic (30). The induced deletion was delimited by regions of homology between the integrating molecule and the chromosome, with the deleted portion of the chromosome being replaced by the heterologous part of the molecule. No essential gene was deleted during that process, and the resulting chromosome was maintained in a haploid cell.

In the present study we examined a situation in which essential genes would be deleted during integration. We constructed a molecule which should have induced deletion of all the essential genes (pHV453 cleaved by *Bam*HI). Its integration yielded viable cells. Genetic analysis has shown that a part of the resulting chromosome, corresponding to sequences which would not be deleted during integration, was duplicated. Therefore, merodiploid cells were generated.

In two other experiments, integration was expected to delete parts of the chromosome delimited by (i) two unmapped regions of the chromosome, named A and X (experiments with pHV445 cleaved with *Bgl*II), and (ii) *thyB* and K or a region close to *thyB* and A (experiments with pHV463 and pHV465 cleaved with *Sal*I). Merodiploid cells were obtained upon integration in all cases. It is likely that essential genes were present within the deleted portions of the chromosome.

Finally, an experiment was performed in which the deletions were expected to begin at *thyB* and end at random sites around the chromosome. Haploid and merodiploid cells were obtained. Deletions eliminated no essential genes from the chromosome in haploid cells and presumably one or more such genes in merodiploid cells.

Integration of the linear molecules examined in this study may be visualized as follows. The interaction between homologous regions of a single-stranded transforming DNA molecule and a double-stranded recipient chromosome may initiate integration (Fig. 4B). The process may then continue in several ways. First, the resident displaced strand may be degraded, as postulated in the recombination model proposed by Meselson and Radding (24). The incoming strand could then be joined to the neighboring resident sequences, and the resulting recombination intermediate could be converted into a mature recombinant, possibly during chromosomal replication. A chromosome in which sequences between regions homologous with the integrating molecule are deleted and replaced by the heterologous part of that molecule thus would be generated. This chromosome could be maintained in a haploid cell if it missed no essential genes, or it could be maintained in a merodiploid cell, together with an intact chromosome (either in a single replication unit [Fig. 5] or replicating independently), if it missed one or several essential genes.

The second way that the process could continue would be for DNA replication to be initiated at the 3′ end of the integrating molecule (at m in Fig. 4B). A part of the chromosome delimited by the regions homologous with the integrating molecule (α in Fig. 5) could be replicated, and a circular genome could be generated by joining the newly synthesized and the invading DNA strand. A chromosome identical to that described above would be obtained. It has been suggested that circularization of transforming plasmid molecules partially homologous with the chromosome occurs by a similar process (4, 20, 22).

The third way that the process could occur would be for the 3′ end of the integrating DNA molecule (m in Fig. 4B) first to be joined to the corresponding strand of the chromosome. This would create a free 3′ end in the chromosomal strand, at which DNA replication could be initiated. A circular genome could then be obtained by replicating a part of the chromosome delimited by regions of homology (α in Fig. 5) and by joining the newly synthesized strand to the invading DNA strand. This genome would be composed of one intact chromosome and one chromosome identical to that described in the two preceeding paragraphs. The latter chromosome could separate from the former by recombination and could be maintained in a haploid cell if it missed no essential genes, or it could propagate in association with the former if it missed some essential genes. Such a process has been proposed to explain the integration of linear molecules similar to those used here into *Streptococcus pneumoniae* chromosome (22, 23).

A common feature of the last two alternatives is that a part of the chromosome delimited by regions of homology with the integrating molecule needs to be replicated before the invading and resident DNA strands can be joined. It could be expected then that the frequency of integration depends on the length of the replicated part. This is not borne out in the experiments with pHV453, in which the Km^r and the Cm^r genes integrated into the chromosome with a similar efficiency (Table 2). The first event would require replication of the chromosomal region delimited by *thyB* and X, which was estimated to be 10 to 15 kb (30), whereas the second event would require replication of almost the entire *B. subtilis* chromosome (except for the region described above) which is 3,400 kb. For this reason we consider the first alternative, which requires little DNA synthesis for the joining of the integrating molecule to the chromosome, to be more likely. The primary event during integration would then be the deletion of some chromosomal sequences and

their replacement with sequences carried by the integrating molecule, whereas duplications would be generated later (Fig. 5).

Molecules that are integrated could recombine not only with a single chromosome, as described above, but with two chromosomes present in a recipient cell: the region abc of the molecule that recombines with the homologous region of one chromosome and the region klm of the molecule that recombines with the homologous region of the other chromosome. Subsequent recombination between the two chromosomes could give rise to a merodiploid genome (Fig. 5). Such a process has been postulated to take place during the transformation of *B. subtilis* with DNA molecules homologous to nonadjacent regions of the recipient chromosome (these molecules were issued from *B. subtilis* strains that had undergone large chromosomal rearrangements [2]). The results of this study do not allow us to distinguish between recombination with one or two recipient chromosomes.

We have shown that two types of integrating linear DNA molecules can be distinguished. Type 1 molecules, such as those derived from the appropriate cleavage of pHV438, pHV453, pHV469 (and probably pHV445, pHV463, and pHV465 [Fig. 1]) carry on their C and W single strands sequences derived from their chromosomal C and W strands, respectively (C and W refer to cDNA strands). The central nonhomologous region of such molecules can be integrated into the chromosome. Type 2 molecules (derived in our experiments from plasmids such as pHV459 or pHV470 and probably pHV460, pHV461, and pHV462 [Fig. 1 and 2]) carry on their C (and W) strand sequences from both C and W strands of the chromosome. The central region of these molecules could not be integrated into the chromosome. The scheme presented in Fig. 4 accounts for this finding.

Finally, it is worth mentioning that the integrating molecules may have many uses, such as the following: (i) Deletions can be introduced into the chromosome next to any cloned chromosomal segment, as shown in this study with the *thyB* gene. (ii) Essential genes can be localized by determining which regions of the chromosome cannot be deleted without the formation of a merodiploid clone. (iii) Haploid cells can be produced that harbor incomplete chromosomes, which could be used as recipients to clone the missing genes (e.g., c26 cells for cloning the *ilvA* gene). Interaction of the cloned with the resident homologous sequences thus would be avoided without having to use *rec* mutants, which often have lower viability and transformability than wild-type cells. (iv) Merodiploidy can be created in a chromosome. Any chromosomal gene may be rendered merodiploid by almost any pair of cloned DNA segments, because, in general, between the two there will always be an essential gene. If one orientation of these segments in the integrating molecule leads to merodiploidy in one part of the chromosome (for example, α in Fig. 5) the opposite orientation will lead to merodiploidy in the remainder of the chromosome (ω in Fig. 5). Dominance and recessivity relationships can be studied in merodiploid cells, as shown by the example of $ilvA^+$ and *ilvA* described in this study. The switching off of genes carried on one of the two chromosomes harbored in diploid cells obtained by protoplast fusion (19) can also be studied possibly in clones with an intact and an incomplete, autonomously replicating chromosome. (v) The relative orientation of cloned segments of the *B. subtilis* chromosome can be determined by constructing appropriate integrating molecules, as shown with plasmids pHV438 and pHV445.

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