GENETIC STRUCTURE AND INTERNAL REARRANGEMENTS OF STABLE MERODIPLOIDS FROM *BACILLUS SUBTILIS* STRAINS CARRYING THE *trpE26* MUTATION

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ABSTRACT

Transformation and transduction to tryptophan independence of strains of *Bacillus subtilis* carrying the "*trpE26*" chromosomal aberrations (a translocation and an inversion) with a "normal" 168 type strain as donor induce a tandem duplication of the *thrA-ilvA* region of the chromosome. The clones possessing this unstable duplication segregate besides the Trp$^-$ some stable Trp+ cells which retain only part of the duplication (the *trpE-ilvA* region) in nontandem configuration. Such clones may also be produced directly during the crosses. The genetic map of these clones (designated as class I stable merodiploids) was constructed: they possess the tranlocation and the inversion of the *trpE26* parental strain. Another type of stable Trp+ clones (class II) also appears, although more rarely, in similar crosses. Studies on their genetic structure revealed that they are haploid for the *trpE-ilvA* region and carry a nontandem duplication of the *thrA-trpE* region. In these clones the *cysB-tre* region has the orientation of the 168 type strain. The duplications in both classes are stable, that of class I being more stable than that of class II where loss of one copy of the *thrA-trpE* region leads to about 1% haploid cells. Detailed genetic studies on heterozygous clones from both classes have shown exchange of alleles between copies of the nontandem duplications. Models are proposed for the formation of each class of merodiploids and for recombination events taking place in them. These models imply recombination at sequences of intrachromosomal homology and (or) introduction of heterologous junctions ("novel joints") by transformation or transduction.

IN the system of the "*trpE26* strains" of *Bacillus subtilis* studied in this laboratory dramatic changes occur regarding the duplication, position, orientation and excision of long chromosome segments. These changes take place during transformation or transduction crosses between strains carrying the "*trpE26* mutation" and the "normal" 168 strains, as well as later in the progeny of these crosses (AUDIT and ANAGNOSTOPOULOS 1972, 1973, 1975; TROWSDALE and ANAGNOSTOPOULOS 1975, 1976).

Strains carrying *trpE26* differ from 168-type strains by two chromosomal rearrangements: the translocation of the *trpE-ilvA* segment (about 4% of the

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genome, as calculated from the *B. subtilis* chromosome map of HENNER and HOCH 1980), and the inversion of the upper part of the chromosome including the origin of replication (*cysB*-O-*tre*). The inverted region is very long (about 36% of the chromosome). One endpoint of the translocated segment is inside the *trpE* locus (locus of the anthranilate synthetase) which is therefore split in two. (TROWSDALE and ANAGNOSTOPOULOS 1975, 1976). Abridged maps of 168- and *trpE26*-type strains are shown in Figure 1. The chromosome segments (or regions) that will be mentioned in this paper are defined in this figure. Segment I is divided into segments Ia and Ib.

In reciprocal transformation or transduction crosses between the two types of strains long tandem duplications are induced involving segments B and C, when selection is for specific markers. These markers are strongly linked in the donor and widely separated in the recipient chromosome. When the recipient is a "*trpE26* strain" and the donor is of the 168 type, selection for tryptophan independence (Trp$^+$) yields merodiploid clones possessing a C-B-C-B duplication (C-B is about 29% of the chromosome). These clones are unstable and readily segregate tryptophan auxotrophs of "*trpE26*" genetic structure. The model proposed for interpreting the induction of the duplication implies that the donor DNA segment that carries the intact *trpE*$^+$ locus, pairs and recombines with

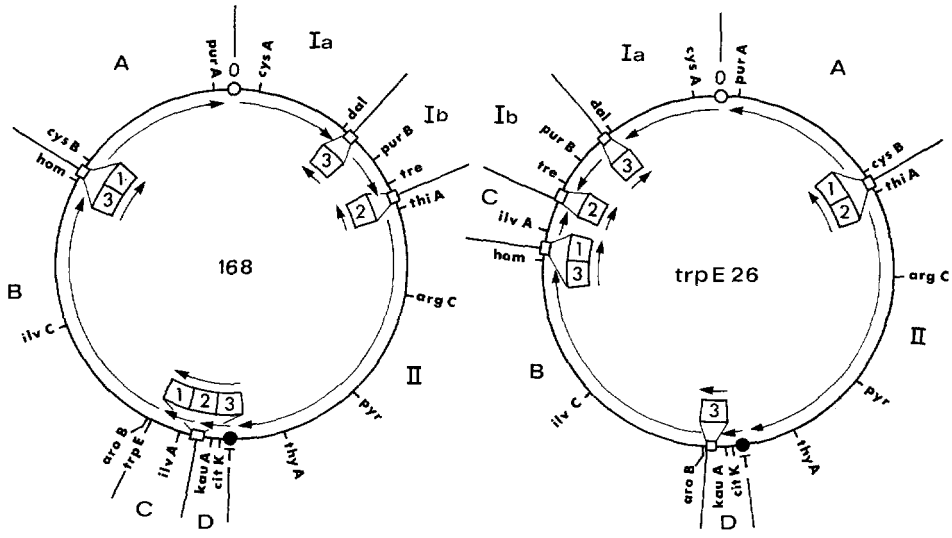

FIGURE 1.—Abridged genetic maps of 168 and *trpE26* type strains of *Bacillus subtilis*. Only landmark loci are indicated. The capitals (A-D) and roman numerals (Ia, Ib and II) designate regions of the chromosome inside which no rearrangements have so far been observed. O and T are the origin and terminus of replication. The arrows indicate orientation of the regions based on the convention of reading the 168 strain map clockwise from the origin. The open rectangles at the junctions of most of the regions represent postulated sequences of intrachromosomal homology of three different kinds (1, 2 and 3). These are enlarged and numbered in the inner part of the map. More details about their number, distribution and orientation will be provided in the DISCUSSION.

the two regions B and C (which contain the split parts of the locus) of two *trpE26* chromosomes. The chromosomes become linked to each other and a third recombination event between them (at any region except segments B and C) results in one complete circular chromosome bearing the C.B.C.B tandem duplication. A map of an unstable merodiploid will be shown later in this paper (Figure 8a). (TROWSDALE and ANAGNOSTOPOULOS 1975, 1976; ANAGNOSTOPOULOS and TROWSDALE 1976; ANAGNOSTOPOULOS 1977). Analogous situations have been encountered in other bacteria and in particular *Escherichia coli* and *Salmonella typhimurium* (for reviews see ANDERSON and ROTH 1977; STARLINGER 1977).

Among the progeny of the unstable Trp^+ merodiploids some stable clones also appear, with a frequency of about 1%, which do not segregate Trp^- cells (AUDIT and ANAGNOSTOPOULOS 1972, 1973). Growth of the unstable diploids is inhibited by L-lysine while the stable ones are resistant to this amino acid. This feature makes it easy to screen for the stable clones. The biochemical basis of the lysine effect is not as yet understood. From preliminary results it appears to be related to the biosynthesis of threonine and methionine, probably to the inhibition of aspartokinase II (E. TURPIN and C. ANAGNOSTOPOULOS, unpublished data). The stable clones were found to possess a redundancy of segment C alone (*trpE-ilvA*) which is only a part of the original C-B duplication of the unstable merodiploids (AUDIT and ANAGNOSTOPOULOS 1973, 1975). Density transfer experiments suggested that the two copies of segment C flank a copy of segment B (TROWSDALE and ANAGNOSTOPOULOS 1975). These stable merodiploids therefore derive from the unstable ones by loss of the second copy of segment B of the original C-B-C-B tandem duplication leaving C-B-C.

Since clones possessing a nontandem duplication of segment C were isolated, it was thought that it might be possible to obtain organisms which would possess a nontandem duplication of only segment B. Such organisms were sought and, so far, two clones corresponding to the prediction have been obtained.

The two categories of stable Trp^+ merodiploids derived from the *trpE26* strains were designated class I (duplication of segment C) and class II (duplication of segment B, about 25% of the chromosome).

The present paper reports detailed data on the genetic analysis of clones from each of these classes. The results made it possible to establish, unequivocally, the sequence of the chromosome segments in these strains. Data are also presented on the rare phenomena of "segregation" in the heterozygous stable merodiploids. Models are proposed regarding their formation and segregation. Many of these models call for recombination events at specific sequences of intrachromosomal homology. These sequences are shown in Figure 1.

A few preliminary results from these studies have been mentioned in two review articles (ANAGNOSTOPOULOS and TROWSDALE 1976; ANAGNOSTOPOULOS 1977) where tentative maps of these stable diploids were proposed.

MATERIALS AND METHODS

Strains: The *B. subtilis* strains used are listed in Table 1.

Media and procedures: Media, DNA preparation, procedures for transformation, PBS-1 mediated transduction and scoring of transformants and transductants were described in previous

TABLE 1

Strains of B. subtilis *used*

No.	Genotype	Origin
168 M	*trpC2*	J. SPIZIZEN
H25	*hisH2*	Laboratory stock
T7	*trpF7*	Laboratory stock
T8	*trpE8*	Laboratory stock
T16	*trpA16*	Laboratory stock
Mu8u5u5	*leu-8 thrA5 metB5*	N. SUEOKA
BD111	*trpC2 cysB3 thrA5*	D. DUBNAU
QB830	*tre-12 thiA78 purB33 sacA321*	J. A. LEPESANT
GSY110	*tyrA1*	Laboratory stock
GSY112	*aroB2*	Laboratory stock
GSY123	*trpB3*	Laboratory stock
GSY125	*trpD15*	Laboratory stock
GSY199	*leu-2*	Laboratory stock
GSY447	*trpE26 hisH2*	Laboratory stock
GSY1126	*hisH2 ilvC1/ilvC+* (Lys^{sens})*	This paper
GSY1127	*hisH2 ilvC1/ilvC+* (Lys^{res})*	This paper
GSY1269	*trpE26 ilvC1*	Laboratory stock
GSY1800	*trpE26 hisH2 tyrA+/trpE26+ hisH+ tyrA1*	This paper
GSY1801	*trpE26 hisH2 tyrA+/trpE26+ hisH+ tyrA+*†	This paper
GSY1803(a)	*trpE26 hisH2 tyrA+/trpE26+ hisH2 tyrA+*	This paper
GSY1803(b)	*trpE26 hisH2 tyrA+/trpE26+ hisH2 tyrA1*	This paper
GSY1804	*trpE26 hisH2 tyrA1/trpE26+ hisH+ tyrA1*	This paper
GSY1817	*lys-1 metB3*	Laboratory stock
GSY1897	*trpE26 ilvA2 hom-1*	Laboratory stock
GSY1992	*trpE26 tre-12*	Laboratory stock
GSY2170	*trpE26 hisH2 tyrA+/trpE26+ hisH+ tyrA+*†	This paper
GSY2171	*trpE26 hisH+ tyrA+/trpE26+ hisH2 tyrA1*	This paper
GSY2172	*trpE26 hisH2 tyrA1/trpE26+ hisH+ tyrA+*	This paper
GSY2205	*trpE26 thiA86*	Laboratory stock
GSY2390	*cysB3 ilvC1/ilvC+*‡	BD111 $\xrightarrow{TF}$ GSY1127
GSY2504	*cysB3 hisH2/hisH+*‡	BD111 $\xrightarrow{TF}$ GSY1803(a)

* Lys^{sens} and Lys^{res} designate respectively the phenotypic trait of sensitivity or resistance (wild type) to L-lysine.

† GSY1801 is a lysine resistant Trp+ transformant of GSY447 by H25 DNA and GSY2170 is a spontaneous revertant of GSY1803(a).

‡ These strains were obtained by transformation (TF). The arrow points to the recipient.

papers (ANAGNOSTOPOULOS and SPIZIZEN 1961; AUDIT and ANAGNOSTOPOULOS 1972; JAMET and ANAGNOSTOPOULOS 1969; SCHNEIDER and ANAGNOSTOPOULOS 1981). For lysine sensitivity tests the concentration of L-lysine in the plates was 5.5 μM.

Segregation: The clones to be studied were streaked on solid minimal medium plus glucose (MG) containing the growth requirements of the strain as well as those of the segregants expected. After growth the cells were suspended in liquid MG, suitably diluted and plated on the same medium as above. The phenotype of the segregants and their proportions were determined by replica plating.

RESULTS

Class I stable merodiploids

Isolation of strains GSY1800 and GSY1801: These clones were isolated directly as Trp$^+$Lys^{res} transformants from a *trpE26* strain. Strain GSY447 (*trpE26 his H2*) was transformed by DNA from a 168-type strain, GSY110 (*tyrA1*). Both markers *hisH2* and *tyrA1* are located on segment C. Among the Trp$^+$ His$^+$ Tyr$^+$ transformants 1.5% were resistant to L-lysine. They should be heterozygous for the *hisH* locus (*hisH2/hisH*$^+$). The *tyrA* locus could be either heterozygous (*tyrA1/tyrA*$^+$) or homozygous (*tyrA*$^+$/*tyrA*$^+$) according to whether *tyrA1* was or was not integrated into the recipient genome. To test this, two Trp$^+$His$^+$ Tyr$^+$Lys^{res} clones (GSY1800 and GSY1801) were used as donors to transform strain 168M (*trpC2*). Table 2 shows that GSY1800 is heterozygous for both *hisH* and *tyrA* while GSY1801 is heterozygous for *hisH* only. The data also show that in GSY1800 *hisH2* is linked to *tyrA*$^+$ and *hisH*$^+$ to *tyrA1*. The genotypes of the two strains can therefore be represented as follows:

GSY1800 : *trpE26 hisH2 tyrA*$^+$/*trpE26*$^+$ *hisH*$^+$ *tyrA1*

GSY1801 : *trpE26 hisH2 tyrA*$^+$/*trpE26*$^+$ *hisH*$^+$ *tyrA*$^+$

The frequencies of the different classes of *trpC*$^+$ transformants in Table 2 agree well with those calculated on the basis of the two sets of linked markers being brought by two independent DNA segments of the donor merodiploid. Integration of the *trpC*$^+$ from the translocated *trpE26* segment requires recombination between *trpC2*$^+$ and *trpE26*. The reduced efficiency of this segment as donor is due to lack of homology between the two types of strains beyond the "*trpE26* marker".

The genetic map of class I stable merodiploids: The linkage relationships of markers flanking the junctions of chromosome segments linked in the *trpE26* strains were investigated in strain GSY1800. The pairs of markers tested were the following: *tre-12, hisH2* (junction I-C); *ilvA2, hom-1* (C-B) and *cysB3, thiA86* (A-II) (see Figure 1). Table 3 reports transduction results which show that in GSY1800, and in one of its derivatives, GSY2504, the cotransduction fre-

TABLE 2

Evidence for the duplication of hisH *and* tyrA *loci in Trp*$^+$ *stable merodiploids of class I*

Recipient No. and genotype	Donor No. and phenotype	Trp+ transformants			
		Sample	Class	No.	%
168 M *trpC2*	GSY1800 Trp$^+$ His$^+$ Tyr$^+$	553	His$^+$ Tyr$^+$	238	44.6
			His$^+$ Tyr$^-$	237	44.5
			His$^-$ Tyr$^+$	57	10.7
			His$^-$ Tyr$^-$	1	0.2
168 M *trpC2*	GSY1801 Trp$^+$ His$^+$ Tyr$^+$	216	His$^+$ Tyr$^+$	191	88.5
			His$^+$ Tyr$^-$	0	0.0
			His$^-$ Tyr$^+$	25	11.5
			His$^-$ Tyr$^-$	0	0.0

TABLE 3

Linkage relationships in PBS-1 mediated transduction of three pairs of markers (tre-12, hisH2; ilvA2, hom-1 *and* cys83, thiA86) *in a class I stable merodiploid*

Recipient No. and genotype	Donor No. and genotype	Transductants				Cotransduction %
		Selection	Sample	Class	No.	
GSY1992 *trpE26 tre-12*	GSY1800 *hisH2 tyrA+/ hisH+ tyrA1*	Tre+	275	His+	158	42.5
				His-	117	
GSY1897 *trpE26 ilvA2 hom-1*	GSY1800 *hisH2 tyrA+/ hisH+ tyrA1*	Ile+	1355	Hom+	497	36.7
				Hom-	858	
		Hom+	843	Ile+	441	52.3
				Ile-	402	
GSY2504 *cycB3 hisH2/hisH+*	GSY2205 *trpE26 thiA86*	Cys+	140	Thi+	50	64
				Thi-	90	

GSY2504 is a derivative of GSY1800.

quency of each pair of markers is the same as in *trpE26* strains (TROWSDALE and ANAGNOSTOPOULOS 1976). The presence of the B-C junction was ascertained in a transduction experiment where GSY1800 was the donor with a recipient of 168 type, GSY1817. The markers of this strain, *lys-1* and *metB3*, are located on segments B and C respectively. In a control experiment this recipient was transduced with a lysate of a homologous strain of 168 type, GSY110 (*tyrA1*). The cotransfer frequency of *lys*$^+$ and *met*$^+$ is the same, about 25%, in both crosses (Table 4). The similarity in the relative frequencies of the various classes of transductants in the two experiments suggests that only one of the copies of segment C (*trpE26*$^+$ *hisH*$^+$ *tyrA1*) of GSY1800 was active in the transduction. If the other copy (*trpE26 hisH2 tyrA*$^+$) had transmitted *metB*$^+$ with an appreciable frequency, one would expect a decrease in the cotransfer of *metB*$^+$ and *lys*$^+$. The appearance of rare His$^-$ transductants seems not to be due to the contribution of this copy. Among seven His$^-$ clones found, five are His$^-$ Tyr$^-$. This linkage could have already been established in the donor GSY1800 resulting in the introduction of *hisH2* in the effective segment. The excess of His$^+$ Tyr$^+$ transductants in the first cross may also be due to a similar reassociation of markers. This phenomenon is further analyzed below. The absence of effect of the second copy of C is probably due to the fact that its markers *hisH2* and *metB*$^+$ lie close to regions not homologous to those of the recipient. However this second copy of C was active in transformation (Table 2). This can be explained by the much higher recombination frequencies in transformation as compared to PBS-1 transduction.

The above results have shown that GSY1800 possesses two copies of segment C in nontandem configuration and that most of the junctions of segments are as in the *trpE26* strains. Class I stable clones do therefore possess the A-I inversion. Considering also the well established fact that they are haploid for segment B

TABLE 4

Linkage in transduction of lys *and* metB *loci in a class I stable merodiploid and ineffectiveness as donor of the other copy of the segment C duplication*

Recipient No. and genotype	Donor No. and genotype	Transductants				
		Selection	Sample	Class	No.	%
GSY1817 *lys-1 metB3*	GSY1800 *tyrpE26+ hisH+ tyrA1/ trpE26 hisH2 tyrA+*	Lys+	2480	His+ Tyr+ Met+	75	3.0
				His+ Tyr+ Met-	1166	47.0
				His- Tyr+ Met+	1	0.04
				His- Tyr+ Met-	0	0.0
				His+ Tyr- Met+	565	22.8
				His+ Tyr- Met-	671	27.1
				His- Tyr- Met+	1	0.04
				His- Tyr- Met-	1	0.04
		Met+	1580	Lys+ His+ Tyr+	43	2.7
				Lys+ His- Tyr+	0	0.0
				Lys- His+ Tyr+	845	53.5
				Lys- His- Tyr+	1	0.06
				Lys+ His+ Tyr-	336	21.3
				Lys- His+ Tyr-	352	22.3
				Lys+ His- Tyr-	1	0.06
				Lys- His- Tyr-	2	0.13
GSY1817 *lys-1 metB3*	GSY110* *tyrA1*	Lys+	727	His+ Tyr+ Met+	7	1.0
				His+ Tyr+ Met-	312	42.9
				His+ Tyr- Met+	188	25.9
				His+ Tyr- Met-	220	30.2
		Met+	667	Lys+ His+ Tyr+	4	0.6
				Lys- His+ Tyr+	313	46.9
				Lys+ His+ Tyr-	164	24.6
				Lys- His+ Tyr-	186	27.9

* Control experiment (168 type donor and recipient).

(AUDIT and ANAGNOSTOPOULOS 1975), it is possible to propose a linkage map of their chromosome (Figure 2).

The stability of the duplication: AUDIT and ANAGNOSTOPOULOS (1972, 1973, 1975) have demonstrated that these Trp$^+$ Lys^{res} merodiploids do not segregate Trp$^-$ cells. The possibility that they may become Trp$^+$ haploid was considered. Such a haploidization was investigated in the heterozygous strain GSY1800 (*hisH2 tyrA$^+$/hisH$^+$ tyrA1*). It was found that this strain segregates His$^-$ and Tyr$^-$ bacteria with a frequency of about 0.15%. The haploid or diploid state of the two markers was tested in three such segregants (2 His$^-$ and 1 Tyr$^-$) and in their prototrophic revertants (one from each). The results of the transformation of strain 168 (*trpC2*) by the DNA of these six clones are presented in Table 5. They indicate that (a) the three auxotrophic segregants are diploid for the *hisH-tyrA* region since their revertants transmit both the + and − alleles of the markers concerned (b) the two His$^-$ clones are not identical since one retains the *tyrA1* allele of GSY1800 while the other does not.

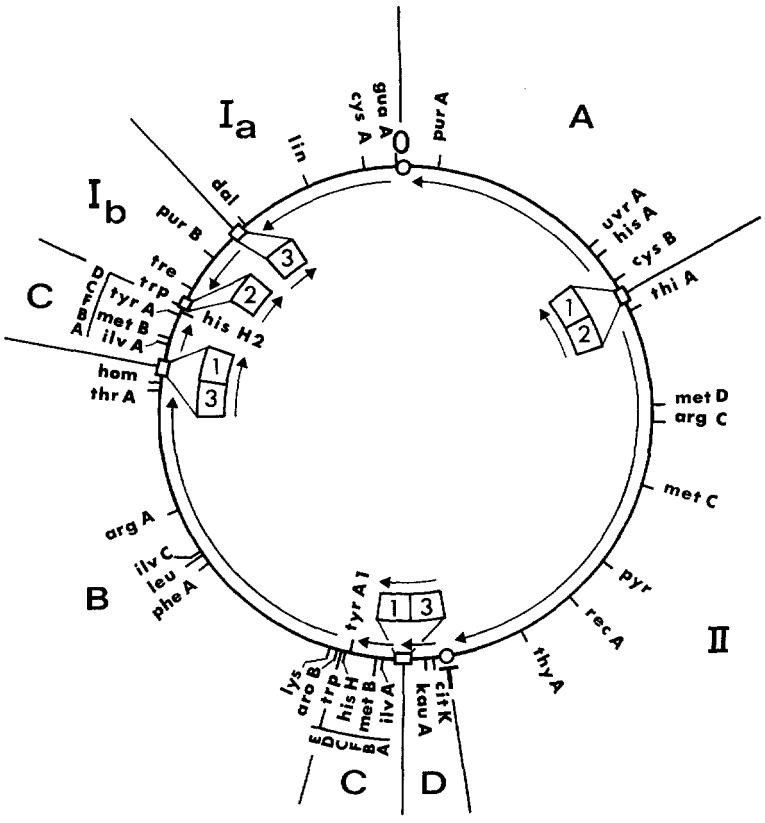

FIGURE 2.—Genetic map of strain 1800, a class I stable merodiploid, carrying a nontandem duplication of segment C (*trpE-ilvA*). The strain is heterozygous for *hisH2* and *tyrA1*. For all other details see Figure 1.

The genotypes of these clones can therefore be represented as follows:

GSY1803(a) : *trpE26 hisH2 tyrA*$^+$*/trpE26*$^+$ *hisH2 tyrA*$^+$ (His$^-$)

GSY1803(b) : *trpE26 hisH2 tyrA*$^+$*/trpE26*$^+$ *hisH2 tyrA1* (His$^-$)

GSY1804 : *trpE26 hisH2 tyrA1/trpE26*$^+$ *hisH*$^+$ *tyrA1* (Tyr$^-$)

These three clones are segregants of GSY1800.

GSY2170 : *trpE26 hisH2 tyrA*$^+$*/trpE26*$^+$ *hisH*$^+$ *tyrA*$^+$, His$^+$ revertant of GSY1803(a)

GSY2171 : *trpE26 hisH*$^+$ *tyrA*$^+$*/trpE26*$^+$ *hisH2 tyrA1*, His$^+$ revertant of GSY1803(b)

GSY2172 : *trpE26 hisH2 tyrA1/trpE26*$^+$ *hisH*$^+$ *tyrA*$^+$, Tyr$^+$ revertant of GSY1804.

As in the crosses of Table 2 the relative frequencies of the various classes of Trp$^+$ transformants are in agreement with the hypothesis that these clones were derived by transformation of 168 M with either of two independent transforming segments. In the case of the *trpE26* segment, a recombination event is necessary between this marker and *trpC2*.

TABLE 5

Evidence for the duplication of hisH *and* tyrA *loci in auxotrophic segregants of a class I stable merodiploid and in their revertants*

Recipient No. and genotype	Donor No. and phenotype	Trp+ transformants			
		Sample	Class	No.	%
168 M *trpC2*	GSY1803a Trp+His- Tyr+	381	His+ Tyr+	150	39.4
			His+ Tyr-	0	0.0
			His- Tyr+	231	60.6
			His- Tyr-	0	0.0
168 M *trpC2*	GSY1803b Trp+ His- Tyr+	437	His+ Tyr+	143	32.7
			His+ Tyr-	4	0.9
			His- Tyr+	77	17.6
			His- Tyr-	213	48.7
168 M *trpC2*	GSY1804 Trp+ His+ Tyr-	456	His+ Tyr+	217	47.6
			His+ Tyr-	190	41.7
			His- Tyr+	5	1.1
			His- Tyr-	44	9.6
168 M *trpC2*	GSY2170 Trp+ His+ Tyr+	733	His+ Tyr+	682	93.0
			His+ Tyr-	0	0.0
			His- Tyr+	51	7.0
			His- Tyr-	0	0.0
168 M *trpC2*	GSY2171 Trp+ His+ Tyr+	577	His+ Tyr+	380	65.9
			His+ Tyr-	7	1.2
			His- Tyr+	43	7.5
			His- Tyr-	147	25.5
168 M *trpC2*	GSY2172 Trp+ His+ Tyr+	702	His+ Tyr+	673	95.9
			His+ Tyr-	0	0.0
			His- Tyr+	5	0.7
			His- Tyr-	24	3.4

GSY1803a, GSY1803b and GSY1804 are auxotrophic clones which appeared in a culture of GSY1800.
GSY2170 and GSY2171 are His+ revertants of GSY1803a and GSY1803b, respectively.
GSY2172 is a Tyr+ revertant of GSY1804.

The above studies did not reveal any haploidization in strain GSY1800. The duplication of segment C in these merodiploid clones may therefore be considered very stable. The observed "segregation" must be the result of the introduction of the mutant allele into the second copy of the duplication. The mechanism of such reassociation of alleles will be considered in the DISCUSSION.

Class II stable merodiploids

Isolation of strains GSY1126 and GSY1127: These strains were obtained as follows: strain GSY1269 (*trpE26 ilvC1*) was transformed by DNA from strain H25 (*hisH2*) at saturating concentration and Trp+ Ilv+ His- transformants were sought. Six such clones were isolated among 140 Trp+ Ilv+ double transformants examined. Two of them (GSY1126 and GSY1127) did not segregate Trp- cells.

Transformation studies with the DNA of His$^+$ revertants showed that they were haploid for *hisH2*. The two strains however are not physiologically identical. GSY1126 is lysine sensitive, does not sporulate and is very poorly transformable and transducible. GSY1127 is lysine resistant, it sporulates although at low rate and is much more transformable and transducible.

The genetic map of class II merodiploids: These studies were performed with strain GSY1127 but several experiments with GSY1126 gave the same results. A first hint of the heterozygous state of the *ilvC* locus in these strains was the segregation of Ilv$^-$ bacteria. Its frequency varied from 0.1% to 2% in different experiments. The heterozygous state *ilvC1/ilvC*$^+$ was confirmed by using DNA from GSY1127 and from one of its derivatives, GSY2390 (*cysB3 ilvC1/ilvC*$^+$), to transform GSY199 (*leu-2*). Cotransformation of *leu-2* and *ilvC1* is 59% in haploid strains (see Table 9, 1st cross). Table 6 shows that GSY1127 and GSY2390 do possess *ilvC1*. The two-fold reduced frequency of its cotransfer with *leu*$^+$ (about 28%) was in agreement with the hypothesis of two independent segments, *leu*$^+$ *ilvC1* and *leu*$^+$ *ilvC*$^+$, in the donor DNA, both taking part in the transformation with equal probability.

It was necessary to assess whether the duplication covered the whole of segment B. ANAGNOSTOPOULOS and TROWSDALE (1976) have shown that the *thrA* locus (situated near one of the endpoints of B) was linked to *cysB* (on segment A) in GSY1126 and GSY1127. This was the first indication that in these strains the inverted region A-I had returned to its original orientation (that of the 168 type strains). This result was confirmed (Table 7, 1st cross). The decrease of the *cysB*$^+$-*thrA*$^+$ cotransduction frequency among the Thr$^+$ clones selected, as compared to this frequency in the Cys$^+$ selection, suggests the contribution of a second B segment carrying *thrA*$^+$ but not *cysB*$^+$. In order to ascertain the duplication of *thrA*, the same two markers were used in a repulsion cross (Table 7, 2nd cross). The recipient was GSY2390 (*cysB3*) and the donor, of 168 type, carried *thrA5*. No Thr$^-$ transductants were recovered. This apparent absence of linkage between *cysB*$^+$ and *thrA5* supported the evidence of the duplication of *thrA*$^+$ in GSY2390. Clones that have acquired *thrA5* must be heterozygous *thrA5/thrA*$^+$. It was reasonable to assume that the second B segment would be linked to the *ilvA* endpoint of segment C, as in the *trpE26* strains. Linkage of *ilvA*$^+$ to *hom*$^+$ is clearly shown in cross 3 of Table 7 (*hom-1* is most probably

TABLE 6

Evidence for the duplication of the ilvC *locus in a class II stable merodiploid*

Recipient No. and genotype	Donor No. and phenotype	Leu+ transformants			Cotransformation %
		Sample	Class	No.	
GSY199	GSY1127	482	Ilv+	358	25.7
leu-2	Ilv+		Ilv$^-$	124	
GSY199	GSY2390	367	Ilv+	256	30.2
leu-2	Ilv+		Ilv$^-$	111	

GSY2390 (*cysB3 ilvC1/ilvC*$^+$) is a derivative of GSY1127.

TABLE 7

Evidence for the duplication of the thrA *region in class II stable merodiploids. Linkage relationships in transduction of markers at the A-B* (cysB-thrA), *C-B* (ilvA-hom-1) *and I–II* (tre-thiA) *junctions*

Cross No.	Recipient No. and relevant genotype	Donor No. and relevant genotype	Transductants				Cotransduction %
			Selection	Sample	Class	No.	
1	BD111 *cysB3 thrA5*	GSY1127	Thr+	1054	Cys+	333	31.6
					Cys-	721	
			Cys+	674	Thr+	287	42.6
					Thr-	387	
2	GSY2390 *cysB3*	Mu8u5u5 *thrA5*	Cys+	377	Thr+	377	0.0
					Thr-	0	
3	GSY1897 *trpE26 ilvA2 hom-1*	GSY1127	Ile+	189	Hom+	60	31.7
					Hom-	129	
			Hom+	522	Ile+	72	13.8
					Ile-	420	
4	QB830 *tre-12 thiA78*	GSY1127	Tre+	81	Thi+	24	30
					Thi-	57	

GSY2390 is a derivative of GSY1127.

a deletion that covers *thrA5*). Here again the decrease in cotransfer, among Hom+ transductants, indicates duplication of the *thrA* region. The above results show that the duplication of segment B in GSY1127 extends as far as *thrA*. One of the copies is linked to *cysB* (junction A-B) and the other to *ilvA* (junction C-B). The last cross of Table 7 demonstrates linkage of *tre-12* to *thiA78*, markers of segments I and II respectively. This linkage was first observed by TROWSDALE and ANAGNOSTOPOULOS (1976) (cited by ANAGNOSTOPOULOS 1977). Segments I and II are therefore linked in GSY1127 as in 168 type strains.

The next step was to verify the duplication of the other endpoint of segment B. Linkage relationships were first determined between the *hisH2* marker of GSY1127 and markers lying on both sides of the B-C junction. DNA from 168 type strains carrying these markers was used to transform GSY1127 (Table 8). Among the donor markers of these experiments, *aroB2* and also *trpE8* are on segment B, since the splitting of the *trpE* locus, which created the *trpE26* marker, is between *trpE8* and *trpD15* (AUDIT and ANAGNOSTOPOULOS 1973). The other markers lie on segment C. *HisH2* is between *trpA16* and *tyrA1* (ANAGNOSTOPOULOS and CRAWFORD 1967). The results of Table 8 show that all these auxotrophic markers, with the exception of *aroB2*, are cotransfered with *hisH*+ into GSY1127, and the frequencies are similar to those obtained in crosses between 168-type strains. The *trpD-tyrA* region is therefore haploid in GSY1127. The very low frequency of introduction of *aroB2* can be explained by the duplication of the *aroB* locus in GSY1127, as we have shown above for *thrA*. This was confirmed after isolating four His+ Aro+ transformants from cross 8 and using their DNA to transform 168 M (*trpC2*). One of the four clones cotransmitted

TABLE 8

Evidence for the duplication of the aroB *locus and for the haploid state of most part of the* trp *gene cluster* (trpD-tyrA) *in strain GSY1127*

Cross No.	Donor No. and genotype	His+ transformants			Cotransformation %
		Sample	Class	No.	
1	GSY110 *tyrA1*	745	Tyr+	138	81.5
			Tyr-	607	
2	T16 *trpA16*	909	Trp+	122	86.6
			Trp-	787	
3	GSY123 *trpB3*	725	Trp+	113	84.4
			Trp-	612	
4	T7 *trpF7*	356	Trp+	108	69.7
			Trp-	248	
5	168M *trpC2*	740	Trp+	152	79.5
			Trp-	588	
6	GSY125 *trpD15*	687	Trp+	212	69.1
			Trp-	475	
7	T8 *trpE8*	581	Trp+	245	57.8
			Trp-	336	
8	GSY112 *aroB2*	666	Aro+	663	0.5
			Aro-	3	

Transformation crosses with GSY1127 (relevant genotype: *hisH2*) as the recipient.

aroB2 with *trpC*+. Cotransformation frequency (75%) was similar to normal 168-type strains (data not shown). The isolation of the heterozygous clone *aroB2/aroB*+, apart from proving the existence of the B-C junction in GSY1127, clearly demonstrates duplication of the *aroB* side endpoint of segment B. As regards the three Aro- clones of cross 8, it will be shown later in this paper that they were haploid.

The case of the *trpE8* marker is of particular interest. Since this marker is easily integrated (Table 8, cross 7), only part of the *trpE* locus must be duplicated in the merodiploid recipient. The *trpE26* strains carry this part (*trpE*(*a*)) which includes *trpE8*, on the B segment at the B-D junction. In GSY1127, *trpE*(*a*) must be duplicated, one copy lying at the B-D junction and the other being part of the intact *trpE* locus at the B-C junction (Figure 3).

Integration of *trpE8* in the intact locus results in anthranilate requirement since there is no complementation with the curtailed *trpE* part of the second B segment. This was confirmed. The Trp+ and Trp- transformants of cross 7 were found to be heterozygous *ilvC1/ilvC*+ like the recipient: 9 Trp+ and 7 Trp- clones segregated Ilv- cells with a mean frequency of 0.8%. Furthermore the reversion rate of the Trp- clones was found to be about 3×10^{-4}, while in the parental donor T8 (*trpE8*) it was less than 7×10^{-9}. DNA from Trp+ revertants of four Trp- transformants contained both *ilvC*+ and *ilvC1* markers. Since the

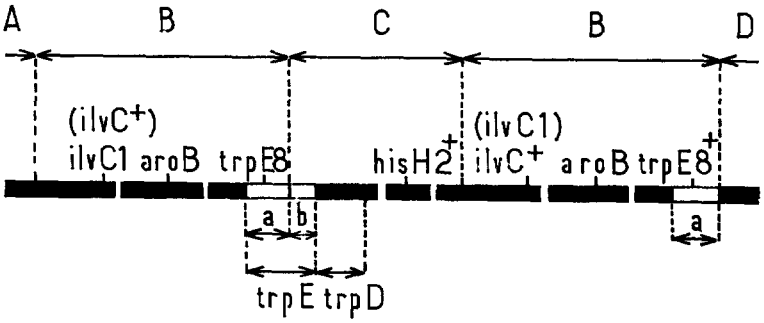

FIGURE 3.—Genetic map of the duplication of the His+Trp- clones obtained by transformation of GSY1127 with DNA from strain T8 (*trpE8*). For more details, see text.

duplication of segment B is retained, the "reversion" of *trpE8* must be due to the exchange of the alleles *trpE8* and *trpE8*+ of the two copies of *trpE*(*a*). When *trpE8*+ is introduced into the complete *trpE* locus at the B-C junction, the organism has a Trp+ phenotype.

Having shown duplication of the two endpoint regions (*hom-thrA5* and *aroB-trpE*(*a*)) and of two loci (*ilvC, leu*) near the middle, we can conclude that the whole B segment is duplicated in GSY1127. The presence of the A-B, B-C and I-II junctions confirmed in this work makes it possible to construct a map of the class II merodiploids (Figure 4). These strains possess a nontandem duplication of segment B and show a return of the A-I inversion to its original orientation.

It is of interest to mention the isolation of a particularly unstable clone from GSY1127. It was obtained during the transformation of this strain by DNA from T8 (*trpE8*) (Table 8, cross 7). While studying segregation in ten Trp+ His+ transformants, one of them was found to segregate auxotrophs with an unusually high frequency: Trp- His+ Ilv+ (38%), Trp- His+ Ilv- (32%) and Trp+ His- Ilv- (26%). This can be explained by assuming that the clone possesses a triplication of segment B and a duplication of segment C. The left side of its map would be: O.A.B.C.B.C.B.D. This could happen by interaction of the donor DNA segment carrying *trpE8* with two chromosomes of the recipient, recombining at the second copy of segment B of the first chromosome and at the unique copy of segment C of the second. This resembles the induction of the tandem duplication during transformation and transduction crosses between *trpE26* and 168 type strains and suggests again that fusion of two chromosomes through the exogenous DNA segment is not infrequent in *B. subtilis*.

Stability of the duplication in GSY1127: The low segregation of Ilv- bacteria from strain GSY1127 may be due to two different causes (a) haploidization of the parent cell by loss of one copy of segment B (b) introduction of a second *ilvC1* marker into the genome. Phenomena similar to the latter have already been observed with the class I stable merodiploid GSY1800.

Six Ilv- segregants of GSY1127 and the three Aro- derivatives of this strain obtained by transformation with GSY112 (*aroB2*) DNA (Table 8, cross 8) were studied. All the Ilv- clones transmit *ilvC1* to GSY199 (*leu-2*) with a cotransfer

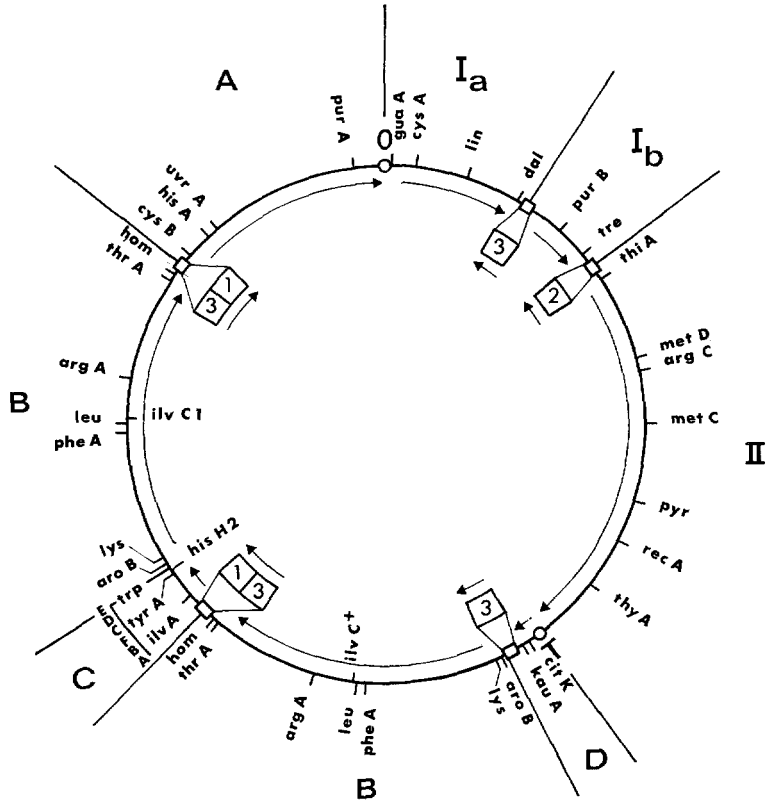

FIGURE 4.—Genetic map of strains GSY1126 and GSY1127, class II stable merodiploids, carrying a nontandem duplication of segment B (*hom-aroB*). The strains are heterozygous for *ilvC1* and haploid for *hisH2*. For other details, see Figure 1.

frequency of *leu*$^+$ *ilvC1* close to that observed with haploid strains. An example is given in Table 9, cross 1. The haploid or diploid state of the *ilvC* locus was tested by using revertants of those segregants as donors. When Ilv$^+$ clones do not transmit *ilvC1* it may be concluded that they are haploid (Table 9, cross 2). The three Aro$^-$ Ilv$^+$ used as donors gave the same result. Duplication of *ilvC* involves the transmission of *ilvC1* by the revertants (Table 9, cross 3). Haploidy of the *aroB* locus was shown in this second group of Ilv$^-$ segregants after transformation to His$^+$ by donor DNA of GSY112 (*aroB2*). The cotransfer frequency observed was high (Table 9, cross 4) and contrasted with the very low introduction of *aroB2* into GSY1127 (Table 8, cross 8), or into the *Ilv*$^-$ clones diploid for *ilvC*. The same nine clones were also tested for the haploid or diploid state of *trpE*(*a*). In their *trpE8* transformants a high reversion rate indicated the diploid state. The results of these tests were in agreement and showed that among six Ilv$^-$ segregants, four were haploid and two were diploid for *ilvC*, *aroB* and *trpE*(a). The three Aro$^-$ transformants were haploid for *ilvC* and *trpE*(*a*). It is reasonable to believe that these conclusions apply to the whole of segment B. The existence of

TABLE 9

Analysis of Ilv⁻ segregants from strain GSY1127 and their revertants

Cross No.	Recipient No. and genotype	Donor and phenotype	Transformants				Cotransformation
			Selection	Sample	Class	No.	
1	GSY199 *leu-2*	clone *a* Ilv⁻	Leu+	402	Ilv+ Ilv⁻	164 238	59.2
2	GSY199 *leu-2*	clone *a'* Ilv+	Leu+	570	Ilv+ Ilv⁻	570 0	0.0
3	GSY199 *leu-2*	clone *c'* Ilv+	Leu+	274	Ilv+ Ilv⁻	208 66	24.1
4	clone *a* *hisH2* *ilvC1*	GSY112 *aroB2*	His+	843	Aro+ Aro⁻	293 550	65.2

Clone *a* is an Ilv⁻ segregant of GSY1127. Clones *a'* and *c'* are Ilv+ revertants of clone *a* and of another Ilv⁻ segregant of GCY1127 respectively.

Ilv⁻ merodiploid segregants suggests recombination between two different copies of segment B belonging to two chromosomes which leads to homodiploidization (*ilvC1/ilvC1*). In the case of the haploid clones one copy of this segment was lost. It should be noted that the haploid cells have a great selective advantage during sporulation of GSY1127 since it forms spores at very low frequency.

Another case of haploidization was observed in GSY1127. The same cross as cross 8 in Table 8, (recipient: GSY1127; donor: GSY112 (*aroB2*)) was performed by transduction instead of transformation. The different classes of His+ transductants were: Aro+ Ilv+ (52.4%), Aro+ Ilv⁻ (5.9%) Aro⁻ Ilv+ (4.8%) and Aro⁻ Ilv⁻ (36.9%). The control culture (not infected) contained 1.4% of His⁻ Ilv⁻ cells. In this transduction experiment the donor segments being much longer than the DNA segments in transformation, they could bridge the C and D regions of the recipient with a high frequency. Consequently, its second B segment loops out and is excised (Figure 5). Since all the Aro⁻ transductants must be haploid, it can be calculated that in the culture used, 89% of the GSY1127 cells carried *ilvC1* on the first copy of B (as shown in Fig. 5) and 11% had the inverse configuration of the two alleles. The mechanisms for the production of haploid and diploid segregants as well as the exchange of alleles in heterozygous clones will be presented in the DISCUSSION.

DISCUSSION

The genetic structure of class I and II stable merodiploids from *trpE26* strains (Figures 2 and 4) are two examples of duplication of long chromosomal segments where the two copies are not contiguous (nontandem duplications). Such duplications are rare in bacteria and those described are rather short (JACKSON and YANOFSKY 1973; BEEFTINK, CUNIN and GLANSDORFF 1974; FAELEN, TOUSSAINT and DE LAFONTEYNE 1975). Almost all of them are the direct outcome of transpositions.

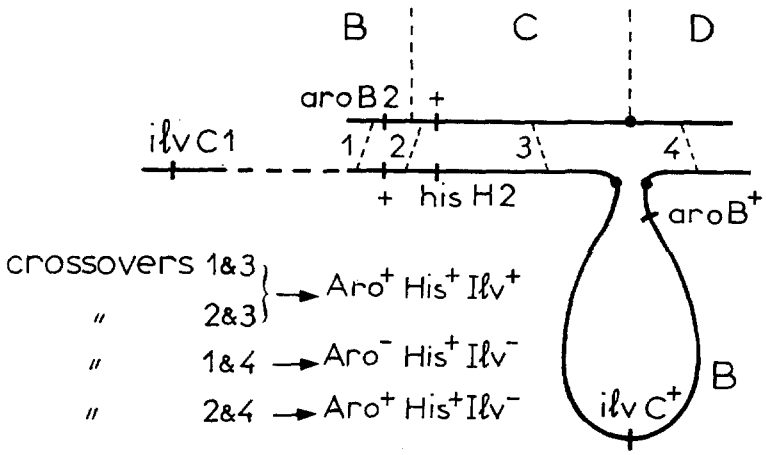

FIGURE 5.—Haploidization of strain GSY1127 during PBS-1 mediated transduction with strain GSY112 (*aroB2*) as donor.

The formation of the class I clones can be explained as being the result of the exact excision of the second copy of segment B from the C.B.C.B. tandem duplication of the unstable merodiploids. To account for its excision it was postulated that recombination takes place between sequences of intrachromosomal homology (direct repeats) at the endpoints of this copy of B (ANAGNOSTOPOULOS and TROWSDALE 1976; ANAGNOSTOPOULOS 1977). The existence of zones of internal homology on the chromosome of *B. subtilis* was assumed in order to interpret the following phenomena: (a) the simultaneous transmission of both the rearrangements of the *trpE26* strains (translocation and inversion), by transformation, to the 168 type strains (b) the production of the class I and class II stable merodiploids. This hypothesis admits the presence of three different kinds of such homologous sequences: 1, 2 and 3. These are presented on the maps of 168- and *trpE26*-type strains shown in Figure 1. Sequences of each kind should be present in at least two copies and they should be distributed along the chromosome according to a certain pattern so that direct and inverted repeats exist (ANAGNOSTOPOULOS 1977). The hypothesis fits all the facts observed. In the case of the excision of a copy of segment B, recombination would take place between the two direct repeats, No. 3 located at the C-B and B-D junctions (Figure 6a). Similarly the excision of this copy of segment B makes the class II stable clones haploid with a 168 type structure (Figure 6b). From the frequency of the appearance of class I stable clones from the unstable ones (AUDIT and ANAGNOSTOPOULOS 1972), it can be deduced that this excision of one copy of segment B occurs in about 1% of the population.

Class I stable merodiploids can however also be selected directly after transformation or transduction of a *trpE26* strain. This was the case for GSY1800 and GSY1801. It is possible that they have arisen without the intermediate formation of an unstable diploid. An alternative mechanism is proposed in Figure 7 to account for such a direct formation. In this case, too, recombination between two sequences of No. 3 kind is necessary.

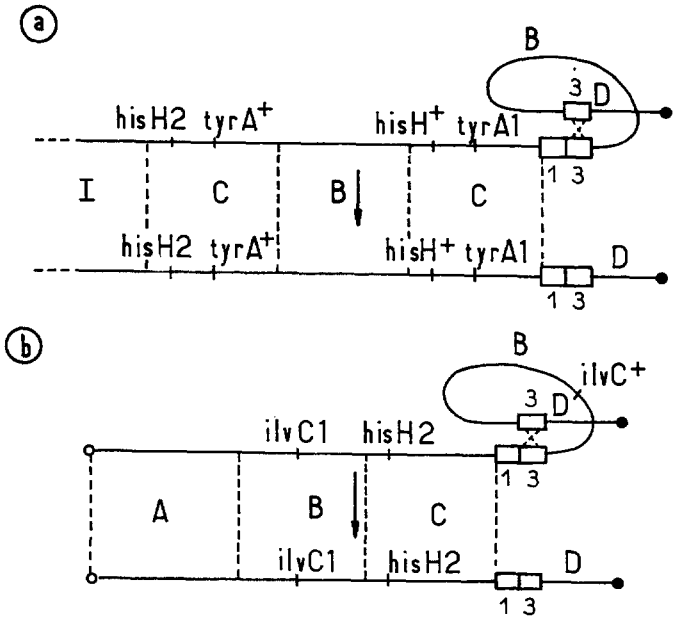

FIGURE 6.—Excision of segment B, when located between C and D, as a result of recombination inside a pair of zones of intrachromosomal homology (a) Model for the formation of strain GSY1800 (class I) from an unstable merodiploid transformant of GSY447 (*trpE26 hisH2*) by DNA of GSY110 (*tyrA1*). (b) Model for the formation of a haploid Ilv⁻ segregant of GSY1127 (class II).

Formation of class II merodiploids can also be explained by two models. The first assumes the excision of the first C segment of the tandem duplication of an unstable clone (Figure 8a). This excision re-establishes the A-B and I-II junctions of the 168-type strains and thus the A-I inversion of the *trpE26* strains recovers its original orientation. According to this model, it should be possible to obtain haploid clones of 168 structure from the class I stable merodiploids. Such clones have so far not been isolated. The second model implies direct formation of class II clones during the transformation of the *trpE26* recipient (Figure 8b). The donor DNA segment (*trpE*$^+$ *his H2*) links two chromosomes; at the same time there is recombination at the homologous sequences as in the first model. These events result in the insertion of the whole of segment B of one chromosome into the other while re-establishing also the 168-type orientation of the A-I region. However in the case of the second model, one can also imagine, instead of recombination at sequences 1 and 2, transformation by one or two additional DNA segments bringing the A-B and (or) the I-II junctions of the 168 donor. In this case a class II stable merodiploid would be created by congression of three transforming DNA segments and recombination at one zone of homology or four segments without any implication of internal homology sequences. Such congression seems rather unlikely, however.

Looking at the variety of chromosomal rearrangements encountered with the *trpE26* strains it may be concluded that they occur by two mechanisms: (a) by

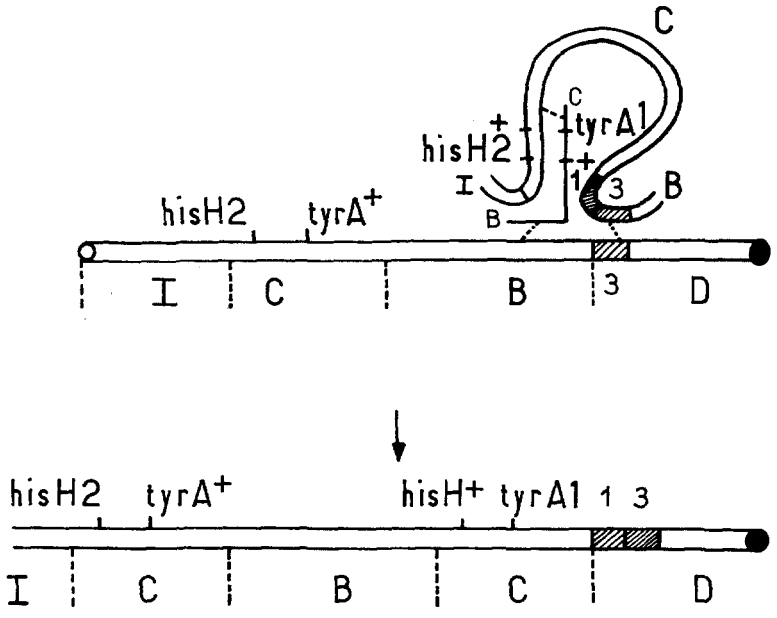

FIGURE 7.—Alternative model for the formation of GSY1800 (class I) during the transformation of GSY447 (*trpE26 hisH2*) by DNA from GSY110 (*tyrA1*) without the intermediate production of an unstable merodiploid. There is interaction of two chromosomes. The donor DNA segment brings the B-C junction of 168 strains and there is pairing of two copies of a zone of internal homology. Segment C of one *trpE26* chromosome is inserted into the other chromosome. The thin line represents the donor DNA segment and the dotted lines recombination events.

introduction of "heterologous junctions" ("novel joints"), (b) by recombination at zones of intrachromosomal homology. The first mechanism prevails when the two parent strains differ in the linkage of two chromosome regions. The donor DNA bringing the joint point pairs with two homologous regions widely separated in the recipient chromosome. Examples of this mechanism are the induction of the C.B.C.B. and B.C.B.C. tandem duplications in reciprocal crosses between *trpE26* and 168-type strains (TROWSDALE and ANAGNOSTOPOULOS 1976) as well as the cases illustrated by Figures 5, 7 and 8b of the present paper. The second mechanism is exemplified by Figures 6, 7 and 8a. In certain cases, it is difficult to ascertain the mode of induction of the aberrations observed. In the case of the formation of the class II stable clones both kinds of interaction might take place, even simultaneously (Figures 7 and 8b). In a recent paper we described a strain carrying a nontandem duplication of segment Ib (*purB-tre*) which, according to the proposed models, was created by introduction of a heterologous junction and recombination at a zone of internal homology No. 3 (SCHNEIDER and ANAGNOSTOPOULOS 1981).

Regarding the nature of the sequences of intrachromosomal homology it was speculated that they may correspond (a) to multiple copies of a prophage (or parts of it), (b) to insertion sequences (IS elements) or (c) to particular chromosomal sequences (ANAGNOSTOPOULOS and TROWSDALE 1976). One possibility

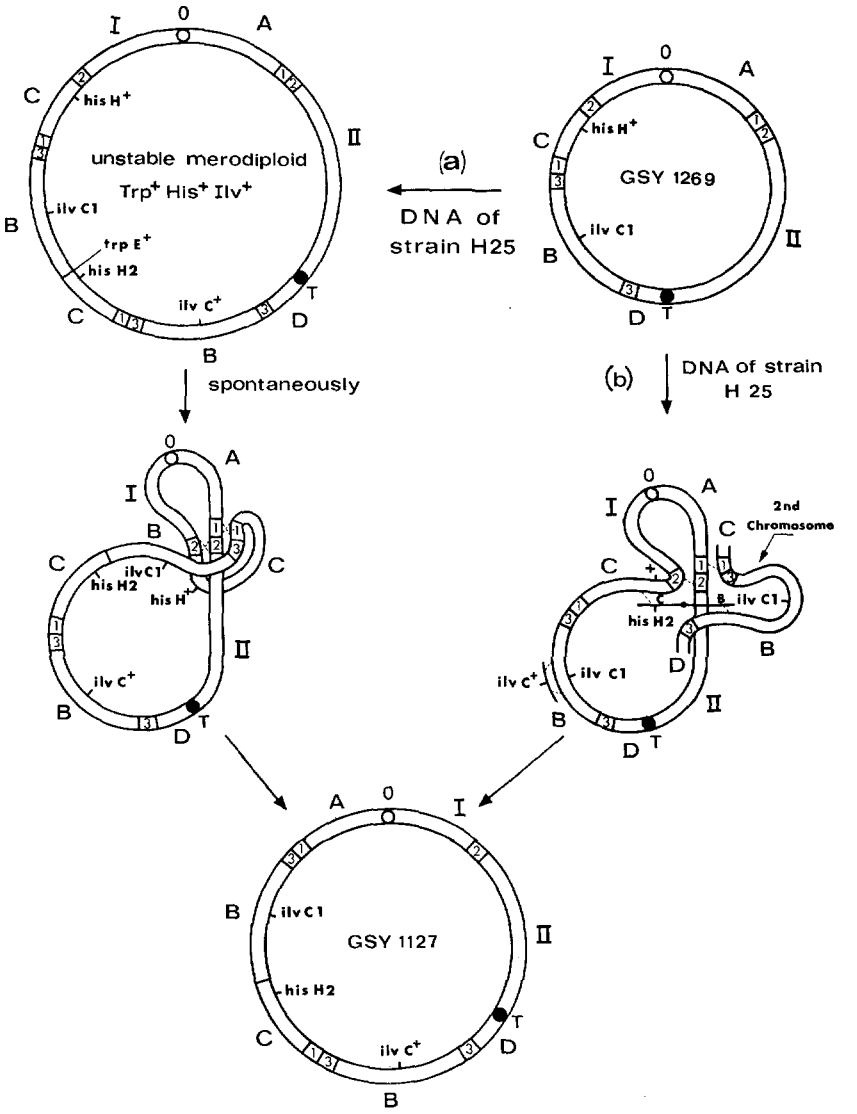

FIGURE 8.—Models for the formation of GSY1127 (class II). Strain GSY1269 (*trpE26 ilvC1*) is transformed by two DNA segments (*trpE+ hisH2* and *ilvC+*). Two possibilities are considered. (a) An unstable merodiploid is formed, carrying the C-B tandem duplication by interaction of two chromosomes with the donor DNA segment *trpE+ hisH2* (see INTRODUCTION). From this strain the first copy of segment C is excised by recombination at pairs of the homologous zones 1 and 2. (b) GSY1127 is directly formed during the above transformation. Most of the recombination events are the same as in (a) but there is no intermediate formation of an unstable merodiploid clone. The B segment of one chromosome (of which only the relevant part is drawn) is integrated into the other one, thus creating the nontandem duplication. For more details, see Figure 7.

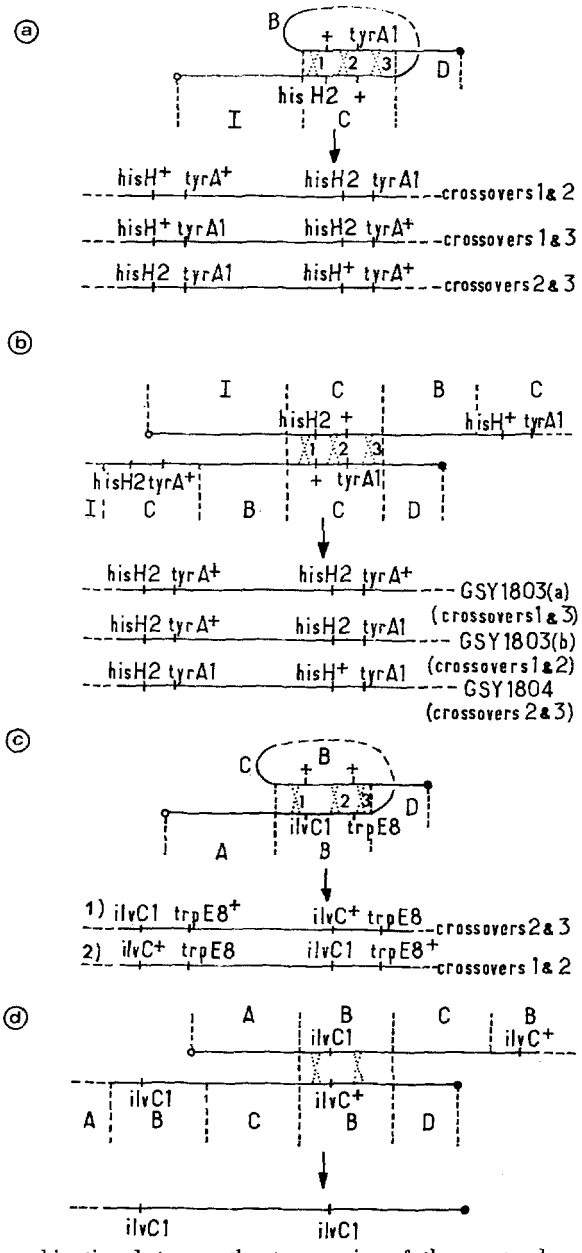

FIGURE 9.—Recombination between the two copies of the nontandem duplication in the class I and II stable merodiploids. These events may involve either one or two chromosomes and lead to reciprocal exchange of alleles. The duplication is maintained. (a) Reassociations of markers of segment C in strain GSY1800 (class I) shown by the data of Table 3. (b) "Segregation" of His$^-$ and Tyr$^-$ clones from strain GSY1800. (c) Reassociations in a Trp$^-$ (*trpE8*) transformant of strain GSY1127 (see Figure 2) giving (1) a Trp$^+$ "revertant" and (2) an exchange of the *ilvC1* and *ilvC*$^+$ alleles. (d) Segregation of as Ilv$^-$ homodiploid clone from strain GSY1127.

is phage SP β discovered by WARNER *et al.* 1977, and extensively studied by ZAHLER and coworkers (ZAHLER *et al.* 1977, ROSENTHAL *et al.* 1979). SP β exists as prophage in all 168-type strains. Its normal attachment site lies between *ilvA* and *kauA*. It might correspond to the zone at the C-D junction in 168-type strains. Part of this prophage may have been translocated with the C segment in *trpE26* strains. According to ZAHLER and KORMAN (Abstracts of the Eighth International Spore Conference, October 9–12, 1980, No. 121), SP β is able to attach to various sites on the *B. subtilis* chromosome. One of these sites lies between *dal* and *purB* (LIPSKY, ROSENTHAL and ZAHLER, Abstracts of the Annual Meeting of the American Society for Microbiology 1981, H 92). This position seems to coincide with that of one copy of our postulated sequence No. 3, which contributes to the induction of the *purB-tre* nontandem duplication of strain GSY1835 (SCHNEIDER and ANAGNOSTOPOULOS 1981). The phage sequence involved must belong to the distal part of SP β (near *kauA*), which remains at the B-D junction in the *trpE26* strains. This part of the phage therefore is either present in several copies on the *B. subtilis* chromosome or possesses a sequence of good homology with at least two other sites of this chromosome (near *hom-thr* and near *purB*). This hypothesis explains several facts reported in the present paper. We are at present investigating the possible relationships of this phage to the junctions of the different chromosome segments involved in the rearrangements of the *trpE26* strains.

Recombination events between the two copies of the nontandem duplication of class I and class II stable merodiploids seem not to be infrequent. Figure 9 represents the mechanisms proposed. If recombination takes place between the two copies of the same chromosome there is reassociation of the + and − alleles of two heterozygous loci (Figure 9a and c). Appearance of diploid auxotrophic "segregants" calls for recombination between two different duplicate segment copies of two chromosomes (Figure 9b and d). One might also interpret this as a case of gene conversion. However, since reciprocal exchange of alleles does occur in these strains, it is more reasonable to attribute "segregation" to the same mechanism: that of general recombination.

The class I and and class II stable merodiploids are good material for dominance and complementation studies in the *hom* to *ilvA* region of the *B. subtilis* chromosome (about 29% of the genome). The recombination phenomena between copies of the nontandem duplications occur with low enough frequency not to influence such studies.

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