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Linkage Relationships of Genes Controlling Isoleucine, Valine, and Leucine Biosynthesis in *Bacillus subtilis*

M. BARAT, C. ANAGNOSTOPOULOS, AND A.-M. SCHNEIDER

Laboratoire de Génétique Physiologique, Centre National de la Recherche Scientifique, Gif-sur-Yvette, Seine et Oise, France

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ABSTRACT

BARAT, M. (Centre National de la Recherche Scientifique, Gif-sur-Yvette, Seine et Oise, France), C. ANAGNOSTOPOULOS, AND A.-M. SCHNEIDER. Linkage relationships of genes controlling isoleucine, valine, and leucine biosynthesis in *Bacillus subtilis*. J. Bacteriol. **90**:357–369. 1965.—In *Bacillus subtilis*, the genetic loci controlling isoleucine and valine biosynthesis are not all clustered. Some of them were located on two distinct transforming deoxyribonucleic acid "molecules." One of these molecules (the "*ile-ilva*$_{2-4}$-*met* segment") carries the threonine deaminase and the dihydroxy acid dehydrase loci linked to methionine markers. The other (the "*ilva*$_{1-3}$-*leu* segment") bears the reductoisomerase locus and one or more loci involved in leucine synthesis. A phenylalanine marker was also shown to be weakly linked to this latter group. In transduction mediated by phage PBS-1, these groups are transferred jointly with other gene clusters. The phage appears to convey chromosome fragments considerably longer than the transforming "molecules." The genetic maps of both the above segments were extended by transduction. Some groups previously studied by transformation can be placed in the following linear order: the *ile-ilva*$_{2-4}$-*met* segment, the cluster of loci involved in aromatic amino acid synthesis (*try* segment), and a lysine locus. An arginine locus is cotransduced with the *phe-ilva*$_{1-3}$-*leu* segment. Recombination frequencies between linked markers are much lower in transduction by this phage than in transformation.

Transformation is the most direct approach to investigating the relationship between the chemical structure of deoxyribonucleic acid (DNA) and its role as the carrier of genetic information. Detailed knowledge of the genetic structure of transforming DNA segments and their relation to the rest of the genome would be of great help in advancing these studies.

Bacillus subtillis is a particularly suitable organism in this connection since it can be grown on simple chemically defined media, thus permitting analysis of nutritional and other biochemical markers. Furthermore, two bacteriophages mediating generalized transduction, SP-10 (Thorne, 1961, 1962) and PBS-1 (Takahashi, 1961, 1963), exist for the transformable *B. subtilis* strains, making it possible to compare recombination in the different systems and eventually map wider regions of the genome.

So far, genetic analysis in *B. subtilis* has been carried out mainly by transformation and involved mutants blocked in the biosynthesis of various amino acids. Linkage was observed for the aromatic amino acids; all loci controlling tryptophan synthesis were found to be clustered and arrayed in the same order as the biochemical sequence, with one exception (Anagnostopoulos and Crawford, 1961). The same DNA "molecule" also carries a histidine locus, loci controlling shikimic acid and tyrosine biosynthesis, and regulation genes for aromatic amino acid synthesis (Nester and Lederberg, 1961; Nester, Schafer, and Lederberg, 1963). The total number of loci on this segment (to be referred to hereafter as the "*try* segment") was estimated at 13. Histidine loci were located on two different transforming particles (Ephrati-Elizur, Srinivasan, and Zamenhof, 1961), and arginine loci on three (Mahler, Newman, and Marmur, 1963). Recombination frequencies between markers of the *try* segment were found to be identical in transformation and in transduction by phage SP-10 (Ephrati-Elizur and Fox, 1961).

While working on the production of linked mutations by in vitro treatment of DNA, we became interested in the biosynthetic pathways

of the branched amino acids isoleucine, valine, and leucine (Fig. 1; *see* Umbarger and Davis, 1962). All known loci controlling isoleucine and valine biosynthesis are linked in *Salmonella typhimurium* (Glanville and Demerec, 1960) and *Escherichia coli* (Pittard, Loutit, and Adelberg, 1963), but leucine loci are not linked to them. Moreover, the regulation of these pathways in the same organisms shows an interesting pattern ("multivalent repression"), since all four end products (isoleucine, valine, leucine, and pantothenate) are necessary for repression of the enzymes common to isoleucine and valine synthesis (Freundlich, Burns, and Umbarger, 1962; Freundlich and Umbarger, 1963).

In a preliminary note (Anagnostopoulos, Barat, and Schneider, 1964), we reported the first results on the linkage of certain markers of these pathways in *B. subtilis*. Isoleucine markers were found to be linked to one class of isoleucine-valine and to methionine markers. Another class of isoleucine-valine markers, located on a different DNA "molecule," is linked to leucine markers. Linkage of an isoleucine and a methionine marker has also been reported by Yoshikawa and Sueoka (1963). In the present paper we describe (i) details (and an extension) of the genetic analysis of isoleucine, isoleucine-valine, leucine, and methionine mutants by transformation; (ii) cases of weak linkage in the two new linkage groups; and (iii) transduction experiments with phage PBS-1 which enabled us to extend mapping outside the limits of the transforming DNA "molecules" bearing isoleucine-valine markers, and to show linkage of one of these clusters to the *try* segment.

MATERIALS AND METHODS

Strains. The original mutants bearing the markers studied (Table 1) were derived either by ultraviolet irradiation of *B. subtilis* 168 (*try*$^-$) or by treatment of DNA from wild type (WT) with nitrous acid. WT was a prototroph obtained by transformation of strain 168 with DNA from strain 23 of Burkholder and Giles (1947). Multiple marked strains were constructed from those of Table 1 by introducing the other markers through transformation. Mutants possessing the wild allele of *try*$^-$ were also obtained by transformation with DNA from WT.

Strains SB25 (*try*$^-$ *his*$^-$) and SB137 (*shik*$^-$ *his*$^-$) were kindly provided by E. W. Nester.

Media. The minimal medium was that of Spizizen (1958).

DNA preparations. The method of extraction of DNA from protoplasts was described previously (Anagnostopoulos and Spizizen, 1961). Deproteinization was carried out by use of the chloroform-octanol procedure. Treatment with the solvent mixture was repeated four to six times. Ribonucleic acid (RNA) was removed by ribonuclease action. The purified DNA was dissolved in 2 M NaCl and stored at 4 C.

For preliminary studies, crude preparations were used. The cells were completely lysed with lysozyme in the presence of 0.1 M ethylenediaminetetraacetic acid (EDTA). The lysate was added to ethyl alcohol, and the fibers obtained were dissolved in 2 M NaCl. Recombination values were identical whether the highly purified DNA or this material was used.

DNA was determined by use of the colorimetric method of Burton (1956).

Transformation procedure. For transformation procedure, the protocol of Anagnostopoulos and Spizizen (1961) was followed. The DNA concentration was 0.01 μg/ml or lower to exclude double transformants by two separate segments observed with saturating levels in this system (Anagnostopoulos and Crawford, 1961; Nester and Stocker, 1963).

Exposure of the cells to the DNA lasted either for 15 min or for the whole length of the last stage in which competence is developed (100 min). Re-

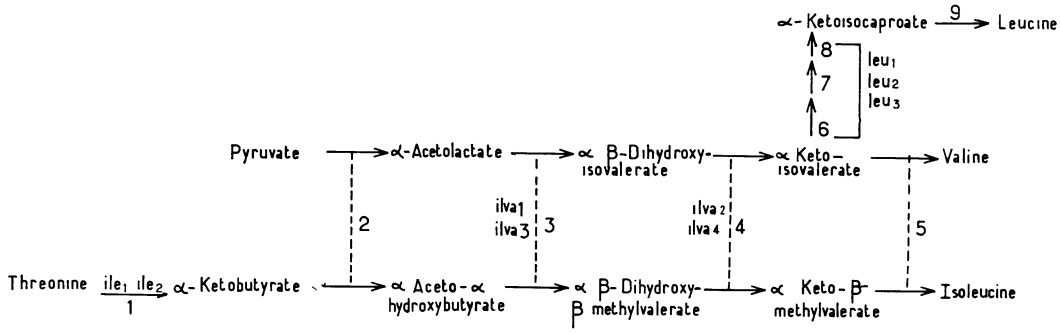

FIG. 1. *Pathway of isoleucine, valine, and leucine biosynthesis. Enzymes catalyzing the first five steps are as follows: (1) threonine deaminase, (2) condensing enzyme, (3) reductoisomerase, (4) dehydrase, (5) transaminase. The intermediates of steps 6 to 9 were omitted. The biochemical blocks corresponding to the markers of Table 1 are indicated.*

TABLE 1. *Origin of the basic strains used in this study**

Strain No.	Genotype	Origin
GSY 111	$ile_1^-try^-$	UV irradiation of strain 168
GSY 184	$ilva_1^-try^-$	UV irradiation of strain 168
GSY 186	$leu_2^-try^-$	UV irradiation of strain 168
GSY 225	$phe_1^-try^-$	HNO_2-DNA (WT)† (recipient GSY 184)
GSY 226	$met_2^-try^-$	HNO_2-DNA (WT) (recipient GSY 111)
GSY 227	$met_3^-try^-$	HNO_2-DNA (WT) (recipient GSY 111)
GSY 228	$met_4^-try^-$	HNO_2-DNA (WT) (recipient GSY 111)
GSY 231	$ilva_3^-try^-$	HNO_2-DNA (WT) (recipient GSY 225)
GSY 250	arg^-try^-	HNO_2-DNA (WT) (recipient GSY 254)
GSY 254	lys^-try^-	HNO_2-DNA (WT) (recipient GSY 225)
GSY 260	$ilva_2^-try^-$	HNO_2-DNA (WT) (recipient GSY 228)
GSY 276	$ilva_4^-try^-$	UV irradiation of strain 168
GSY 277	$ile_2^-try^-$	UV irradiation of strain 168
GSY 301	$leu_3^-try^-$	HNO_2-DNA (WT) (recipient GSY 184)

* Abbreviations: *try*, tryptophan; *ile*, isoleucine; *ilva*, isoleucine + valine; *leu*, leucine; *phe*, phenylalanine; *met*, methionine; *arg*, arginine; *lys*, lysine; UV, ultraviolet.

† Wild-type DNA treated in vitro with nitrous acid. The recipient strain used in the transformation experiment with this DNA is indicated in brackets.

combination frequencies were unaffected by the duration of contact. The efficiency of transformation was routinely about 1% for the *try*$^-$ marker of strain 168 at saturating DNA concentrations.

Transduction procedure. Phage PBS-1 was provided by courtesy of I. Takahashi. Lysates of lysogenic strains were used in the transduction experiments. These lysates were prepared as follows. Exponentially growing lysogenic cultures in Pennassay Broth (Difco) were centrifuged, and the cells were suspended in normal saline and ultraviolet-irradiated (GE Sterilamp, 30 sec at 50 cm). The suspensions were diluted with an equal volume of Pennassay Broth, shaken for 2 hr at 37 C, and further incubated overnight at 37 C without shaking. The lysates were centrifuged at low speed, treated with deoxyribonuclease (20 to 50 μg/ml), and filtered through Millipore membranes.

The transduction experiments were performed according to Takahashi (1961). The frequency of transductants was about 10^{-6} to 10^{-5} among the recipient population.

Scoring of transformants and transductants. The cultures were plated on minimal agar supplemented with the specific growth requirements for the various classes of recombinants (4 μg/ml for tryptophan, 16 μg/ml for the other amino acids). In the case of crosses involving strains of distinguishable phenotypes, the replica-plating technique was used to identify organisms arising from recombination between two markers. When no such distinction could be made, recombination was estimated by reference to a third marker lying outside the segment studied. The calculation of the recombination values is explained in Results.

Nitrous acid treatment of DNA. DNA was treated with nitrous acid by use of a modification of a previously described method (Anagnostopoulos and Crawford, 1961). A mixture of highly purified native wild-type DNA (30 μg/ml), $NaNO_2$ (0.25 M), and acetate buffer (*p*H 4.5, 0.25 M) was maintained for 90 min at 32 C, after which it was diluted fivefold in phosphate buffer (*p*H 8.5, 1.0 M) and used in transformation experiments at a final DNA concentration of 1 μg/ml. Of the initial transforming activity, 96% was lost by this treatment. The yield of mutants was 0.05 to 0.50% among the transformants for a single marker of the recipient strain. The majority of the new markers introduced in this way were found to be linked to the endogenous marker.

Shorter treatment with nitrous acid considerably reduced the yield of mutants. No difference in the yield was observed when heat-denatured, instead of native, DNA was treated and subsequently renatured. Strack, Bautz-Freese, and Freese (1964) also reported the same observation.

RESULTS

Characterization of mutants. Biochemical studies with mutants of the isoleucine and valine pathways involved growth response to intermediates, identification of accumulated products, and the measurement of enzyme activities. These studies will be reported elsewhere (Guirard and Anagnostopoulos, *unpublished data*). Mutants requiring isoleucine alone (*ile*$^-$) were found to lack threonine deaminase activity. Strains requiring both isoleucine and valine (*ilva*$^-$) are the result of single-step mutations, as judged by the simultaneous reversion for both requirements, and the absence of recombination between the two characters. These *ilva*$^-$ strains fall into two classes: (i) those blocked at the reductoisomerase step (Fig. 1, step 3; markers $ilva_1$ and $ilva_3$ of Table 1) and (ii) those blocked at the dehydrase step (Fig. 1, step 4; markers $ilva_2$ and $ilva_4$ of Table 1).

Mutants blocked in steps 2 and 5 have not as yet been isolated.

All leucine (*leu*$^-$) mutants responded to ketoleucine (α-ketoisocaproate). The exact genetic block(s) has not yet been identified.

The methionine (*met*$^-$)-requiring strains grew on homocysteine but not on cystathionine. The arginine (*arg*$^-$) mutant did not respond to ornithine or citrulline. The phenylalanine (*phe*$_1^-$) and lysine (*lys*$^-$) mutants have not been studied biochemically. The tryptophan (*try*$^-$), histidine (*his*$^-$), and shikimic acid (*shik*$^-$) markers were previously characterized (Anagnostopoulos and Crawford, 1961; Nester et al., 1963).

Two-factor transformation crosses. Linkage relationships were sought by a series of crosses between strains of different phenotypes with each marker alternatively in the recipient strain and the donor DNA. Table 2 shows the calculation of the recombination values for such crosses.

The search for linked markers was facilitated by the use of the nitrous acid-treated DNA. The nitrous acid-induced mutations were looked for among the prototrophs for the marker of the recipient strain and, in most cases, the new markers were found to lie on the same DNA piece as this marker.

In the case of crosses which involve markers displaying the same phenotype, linkage was evaluated by calculation of the "recombination index" (RI; Lacks and Hotchkiss, 1960; Table 3). The *try*$^-$ marker of strain 168 was taken as the outside reference marker. Transformations with DNA from WT and from another mutant (also bearing *try*$^+$) were always performed on the same competent culture. The relative frequency of transformation varied very little from one experiment to another for the great majority of the markers (Table 4). The values shown in Table 4 probably reflect differences in the efficiency of integration of the markers into the bacterial genome.

The two-point repulsion crosses revealed that the markers examined fall into two different groups (Tables 5 and 6): (I) *ile*$^-$ markers linked to *ilva*$_2^-$, *ilva*$_4^-$, and the *met*$^-$ markers; (II) *ilva*$_1^-$ and *ilva*$_3^-$ linked to *leu*$^-$ markers. Markers of group I are not linked to those of group II.

Recombination frequencies of reciprocal crosses were in good agreement, except in cases in which the relative frequencies of transformation of the two markers were greatly different, and the crosses were analyzed with replica plating (*see* *ilva*$_1^-$ → *leu*$_1^-$ and *leu*$_1^-$ → *ilva*$_1^-$, Table 5). The possibility that a marker may influence the integration of another marker nearby is now being investigated.

In another series of two-factor crosses, the recipient strain had two markers of the same cluster, and DNA from WT was used as donor (Table 7). Recombination values were very similar to those of the repulsion crosses. Cotransfer is therefore observed whatever the arrangement of a pair of markers may be (*cis* or

TABLE 2. *Example of analysis of reciprocal two-factor transformation crosses by replica plating*

Recipient strain (genotype)	Donor DNA	Transformants			Recombination*
		Total colonies tested	Class	Colonies of each class	
					%
ilva$_3^-$*leu*$_2^+$	*ilva*$_3^+$*leu*$_2^-$	257	*ilva*$_3^+$*leu*$_2^-$	114	55.6
			ilva$_3^+$*leu*$_2^+$	143	
ilva$_3^+$*leu*$_2^-$	*ilva*$_3^-$*leu*$_2^+$	256	*ilva*$_3^-$*leu*$_2^+$	119	53.5
			ilva$^+$*leu*$_2^+$	137	

* Per cent recombination = (wild-type recombinants/total number of transformants tested) × 100

TABLE 3. *Analysis of two-factor transformation crosses by the recombination index (RI) method*

Recipient strain (genotype)	Donor DNA	Transformants/ml		Ratio of *leu*$^+$ (or *ilva*$^+$):*try*$^+$	RI* × 100
		leu$^+$ (or *ilva*$^+$)	*try*$^+$		
leu$_2^-$*try*$^-$	Wild type	48,000	112,000	0.43 (a)	30.0
leu$_2^-$*try*$^-$	*leu*$_1^-$*try*$^+$	28,500	225,000	0.13 (b)	
ilva$_1^-$*try*$^-$	Wild type	47,700	81,000	0.59 (a)	10.5
ilva$_1^-$*try*$^-$	*ilva*$_3^-$*try*$^+$	1,760	28,400	0.062 (b)	

* RI = ratio of (b)/(a).

trans). When the doubly marked strains were exposed to a mixture of DNA preparations from the two single mutants, simultaneous transformation for the two markers at nonsaturating concentrations was extremely low.

The effect of the concentration of wild-type DNA on the transformation of a doubly marked strain is shown in Fig. 2. The constant value of recombination for concentrations corresponding to the linear part of the dose-response curve is another indication of true linkage. With saturating concentrations, cotransfer was a little more frequent, and this is probably due to the absorption by some cells of two segments carrying these markers. Similar results were obtained with several other doubly marked strains.

Three-factor transformation crosses. The recombination values obtained by the two-point crosses suggested a linear order for the linked markers. This order was further verified by a series of three-point tests (Table 8). In these experiments, strains bearing two markers were used either as recipients or donors, and the third marker was carried by the other parent. Phenotypes were distinguished by use of replica plating. Interpretation was based on the assumption that the least frequent class is that corresponding to a quadruple recombination event. In the first cross of Table 8 for instance, the proportion of *leu*$^-$ organisms was determined among the *ilva*$^+$ recombinants. Two possible configurations were considered which appear in Fig. 3.

The fact that the wild-type class was very small clearly favors configuration I, and the results of the reciprocal cross supported this conclusion. In some cases, four different crosses were carried out to determine the sequence of two markers in relation to the other group(s) located on the same DNA molecule. The order implied by the three-factor tests agreed well with the one suggested by the two-point crosses.

Linkage maps inferred from transformation results. The above results enabled us to construct partial maps for the two DNA segments carrying *ilva*$^-$ markers (Fig. 4 and 5). The relationship of *phe*$_1^-$ to the *ilva*$_{1\text{-}3}$-*leu* cluster is analyzed below.

TABLE 4. *Relative frequency of transformation of various markers by wild-type DNA**

Genotype of strains: X$^-$ try$^-$	
X	Ratio X$^+$/try$^+$
ile$_1^-$	0.33
ile$_2^-$	0.46
ilva$_1^-$	0.59
ilva$_2^-$	0.60
ilva$_3^-$	0.50
ilva$_4^-$	0.37
leu$_1^-$†	1.03
leu$_2^-$	0.43
leu$_3^-$	0.61
met$_2^-$	0.68
met$_3^-$	0.36
met$_4^-$	2.06
phe$_1^-$	0.66

* DNA concentration, 0.02μg/ml or lower.

† Considerably higher values (up to 2.4) were often found for this marker.

TABLE 6. *Recombination (per cent) in two-factor transformation crosses involving met markers*

Marker in recipient strain	Marker in donor DNA	Recombination	Marker in recipient strain	Marker in donor DNA	Recombination
		%			%
met$_2^-$	*ile*$_2^-$	89.0	*met*$_4^-$	*ile*$_2^-$	99.0
	ilva$_2^-$	49.0		*ilva*$_2^-$	79.5
	met$_3^-$	0.0		*met*$_2^-$	8.0
	met$_4^-$	11.5		*met*$_3^-$	5.5
met$_3^-$	*ilva*$_2^-$	43.0	*ilva*$_2^-$	*met*$_2^-$	48.0
	met$_2^-$	0.0		*met*$_3^-$	35.0
	met$_4^-$	7.5		*met*$_4^-$	43.0

TABLE 5. *Recombination (in per cent) in two-factor transformation crosses (ile, ilva, and leu markers)*

Marker in recipient strain	Marker in donor DNA								
	ile$_1^-$	*ile*$_2^-$	*ilva*$_1^-$	*ilva*$_2^-$	*ilva*$_3^-$	*ilva*$_4^-$	*leu*$_1^-$	*leu*$_2^-$	*leu*$_3^-$
ile$_1^-$	—	9.5	100	66.5	100	66	100	100	100
ile$_2^-$	9.5	—	100	64	100	60	100	100	100
ilva$_1^-$	100	100	—	100	10.5	100	65	59	63
ilva$_2^-$	71	68	100	—	100	21	100	100	100
ilva$_3^-$	100	100	12.5	100	—	100	60	56	56.5
ilva$_4^-$	70	66	100	13	100	—	100	100	100
leu$_1^-$	100	100	78	100	74	100	—	31	11
leu$_2^-$	100	100	60	100	53	100	29.5	—	18
leu$_3^-$	100	100	65	100	64	100	12	18.5	—

TABLE 7. *Transformation by wild-type DNA of strains bearing two linked markers*

Recipient (genotype)	Transformants		Recombination
	Class	Cells/ml	
			%
$ilva_1^-leu_1^-$	$ilva_1^+leu_1^+$	25,500	
	$ilva_1^-leu_1^+$	79,500	76.0 (among leu^+)
	$ilva_1^+leu_1^-$	45,500	64.0 (among $ilva^+$)
$ile_1^-ilva_4^-$	$ile_1^+ilva_4^+$	107,000	
	$ile_1^-ilva_4^+$	259,000	70.7

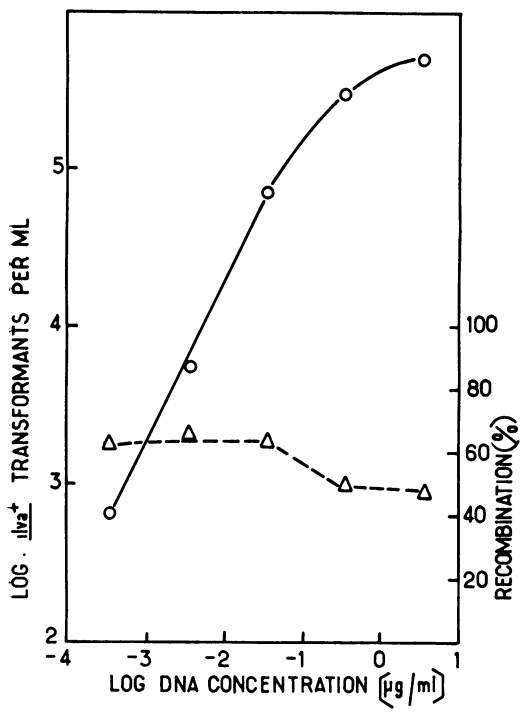

FIG. 2. *Effect of DNA concentration on the recombination between two linked markers. Recipient strain = GSY 201 ($ilva_1^-$ leu_1^-); donor DNA = wild type. Symbols:* ○, *$ilva^+$ transformants;* △, *per cent recombination.*

In their studies on the sequential replication of the *B. subtilis* chromosome, Yoshikawa and Sueoka (1963) used strains with leu^- and met^- markers. We did a certain number of transformation experiments with one of their strains, leu^- met^- ade^- (Mu8u5u6, obtained by courtesy of P. Schaeffer), and found that these leu^- and met^- markers are also located in the corresponding regions of our maps.

Cases of weak linkage in transformation. Simultaneous transformation for some pairs of the linked markers studied was particularly infrequent. This was the case for the distal markers of the segment shown in Fig. 4. Another case of weak linkage was encountered during the study of the segment $ilva_{1\text{-}3}$ *leu*: a phenylalanine (phe_1^-) marker was found in an $ilva_1^+$ transformant by use of nitrous acid-treated DNA. Subsequently, through the same procedure, marker $ilva_3^-$ was introduced when strain GSY 225 bearing phe_1^- was the recipient (Table 1). These facts suggested a linkage relationship between phe_1^- and the $ilva_{1\text{-}3}$ region. However, reciprocal crosses between strains carrying $ilva_1^-$ and phe_1^- gave very high recombination values (98 to 99%), which indicated that linkage (if any) must be very weak. The doubly marked strain GSY 230 (leu_1^- phe_1^-) was then constructed, and the kinetics of its transformation by wild-type DNA were studied (Fig. 6). The value for the joint transfer of the two markers dropped by dilution of the DNA to about 1% and remained unchanged by further dilution. Furthermore, with very low concentrations of wild-type DNA, the frequency of double transformants phe_1^+ leu_1^+ was found to be many times higher than if a mixture of two DNA's of equivalent concentrations (each bringing in one of the markers) were used instead (Table 9; 17 times higher in this experiment).

All these data suggested that joint transformation for phe_1^- and leu_1^- (or $ilva_1$) at low DNA concentrations is mediated by DNA pieces carrying both markers.

A plausible hypothesis regarding these cases of weak linkage is that preparations of DNA purified by our usual procedure contain a very small number of fragments, the length of which considerably exceeds the average length of the transforming "molecules." This hypothesis was supported by the following sets of facts: (i) crude lysates of wild type yielded higher cotransfer values (sometimes up to 5%, at concentrations for which purified preparations showed 1%); (ii) transduction by phage PBS-1 (data presented

TABLE 8. *Three-factor transformation crosses*

Recipient (genotype)	Donor (genotype)	Transformant classes			Order implied by results†
		Phenotype*	Frequency (cells/ml)	Ratio (wild/mutant)	
$ilva_1^-leu_1^-$	$ilva_3^-$	$ilva^+leu^-$	13,270	0.16	$ilva_1^-ilva_3^-leu_1^-$
		wild	2,030		
$ilva_3^-$	$ilva_1^-leu_1^-$	$ilva^+leu^-$	7,930	1.4	$ilva_1^-ilva_3^-leu_1^-$
		wild	11,110		
$ilva_3^-leu_2^-$	$ilva_1^-$	$ilva^+leu^-$	5,240	0.94	$ilva_1^-ilva_3^-leu_2^-$
		wild	4,920		
$ilva_1^-$	$ilva_3^-leu_2^-$	$ilva^+leu^-$	418	41.3	$ilva_1^-ilva_3^-leu_2^-$
		wild	17,240		
$ilva_3^-leu_2^-$	leu_3^-	$ilva^-leu^+$	2,170	0.82	$ilva_3^-leu_2^-leu_3^-$
		wild	1,785		
leu_3^-	$ilva_3^-leu_2^-$	$ilva^-leu^+$	393	45.8	$ilva_3^-leu_2^-leu_3^-$
		wild	18,010		
$ilva_1^-leu_1^-$	leu_2^-	$ilva^-leu^+$	7,730	0.048	$ilva_1^-leu_2^-leu_1^-$
		wild	370		
leu_2^-	$ilva_1^-leu_1^-$	$ilva^-leu^+$	4,490	1.07	$ilva_1^-leu_2^-leu_1^-$
		wild	4,810		
$ile_1^-ilva_2^-$	ile_2^-	ile^-ilva^+	83,380	0.002	$ile_1^-ile_2^-ilva_2^-$
		wild	170		
$ile_1^-ilva_2^-$	$ilva_4^-$	ile^-ilva^+	4,900	0.069	$ile_1^-ilva_4^-ilva_2^-$
		wild	340		
$ilva_4^-$	$ile_1^-ilva_2^-$	ile^-ilva^+	2,280	1.05	$ile_1^-ilva_4^-ilva_2^-$
		wild	2,410		
$ilva_4^-met_3^-$	$ilva_2^-$	$ilva^+met^-$	14,430	0.126	$ilva_4^-ilva_2^-met_3^-$
		wild	1,820		
$ilva_2^-$	$ilva_4^-met_3^-$	$ilva^+met^-$	746	0.54	$ilva_4^-ilva_2^-met_3^-$
		wild	404		
$ilva_4^-met_3^-$	met_4^-	$ilva^-met^+$	4,720	1.32	$ilva_4^-met_3^-met_4^-$
		wild	6,240		

* Scored by replica plating.
† See text.

below) showed close linkage of all pairs of markers for which transformation suggested weak linkage.

Transduction with PBS-1. PBS-1 is a large sized phage comparable in its dimensions to T4 (Takahashi, 1963). It was hoped, therefore, that it might transfer DNA segments longer than the transforming particles and help to elucidate the question of weak linkage (i.e., that of phe_1 to the $ilva_{1\text{-}3}$-*leu* cluster). Results in Table 10 show that phe_1^+ is very frequently cotransduced with leu_1^+ or $ilva_1^+$. On the other hand, there is no cotransduction of either phe_1^+ or $leu_1^+$with *try*$^+$. Tests with the markers in *trans* position (Table 11) confirmed these results. Strong cotransduction was observed for pairs of markers showing weak linkage in transformation (phe_1-leu_1; phe_1-$ilva_1$; ile_1-met_3; ile_2-met_3), and absence of cotransduction was observed for some pairs where transformation also had not shown indication of linkage (phe_1-ile_2; phe_1-*lys*; ile_2-*lys*).

An interesting finding was the significant cotransduction of phe_1^- with *arg*$^+$, because no linkage, however weak, was detectable for these markers by transformation with our DNA preparations.

Markers for which linkage was well established from transformation experiments were much more frequently cotransferred by transduction (Table 12).

Cotransduction was also observed for several pairs of markers other than phe_1 and *arg* which behave as unlinked in transformation (Table 13). All the markers of the *ile*-$ilva_{2\text{-}4}$-*met* segment were transduced simultaneously with markers of the region in which the *try* cluster was located (*try*$^-$ or *his*$^-$). A *lys*$^-$ marker was also transferred together with the above. Cotransduction of *arg*$^+$ and leu_1^+ was not surprising in view of the known relationship of both these markers to phe_1^-. No cotransduction was found for any marker of the phe_1^--$ilva_{1\text{-}3}$-*leu-arg* group with markers of either the *try* or the *ile*-$ilva_{2\text{-}4}$-*met* segments.

Cotransduction frequencies favored a linear sequence of the segments previously studied by

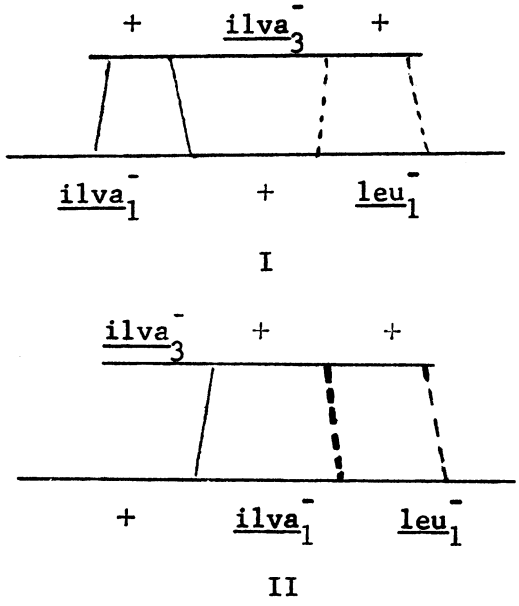

FIG. 3. *Possible configurations when the proportion of leu⁻ organisms was determined among the ilva⁺ recombinants.*

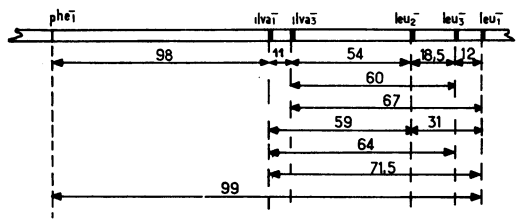

FIG. 4. *Linkage map of the segment ilva$_{1-3}$-leu constructed from transformation data. Numbers are percentages of recombination: (1-cotransfer) × 100 (mean values of the reciprocal two-factor crosses). They indicate, approximately, the relative position of the markers and are not meant as scale map distances. The order of the markers was confirmed by three-factor crosses (Table 8). For the relationship of phe1⁻ to the other markers, see text, Fig. 5, and Table 9. Three-factor crosses were not run with this marker. Its position on the left is inferred from transduction results.*

transformation which are transduced simultaneously. This order is shown in Fig. 7 and 8. Due to the very high cotransduction of markers inside each transformation segment, it has not as yet been possible to establish clearly to which side of the *try* group each one of the other groups is related (Fig. 7).

The view that the cotransduced clusters of markers may lie side by side on the bacterial

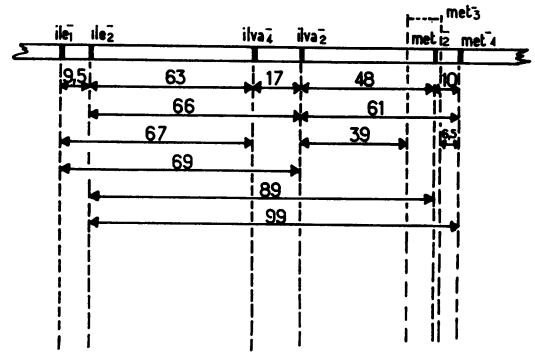

FIG. 5. *Linkage map of the segment ile-ilva$_{2-4}$-met constructed from transformation data. Recombination values calculated as for Fig. 3. The met$_3$⁻ marker is represented as affecting a larger region which contains met$_2$⁻. This is based on (i) the absence of recombination between met$_3$⁻ and met$_2$⁻, (ii) the absence of reversion of met$_3$⁻, and (iii) the lower recombination values for ilva$_2$⁻-met$_3$⁻ and met$_3$⁻-met$_4$⁻ as compared with the corresponding crosses involving met$_2$⁻.*

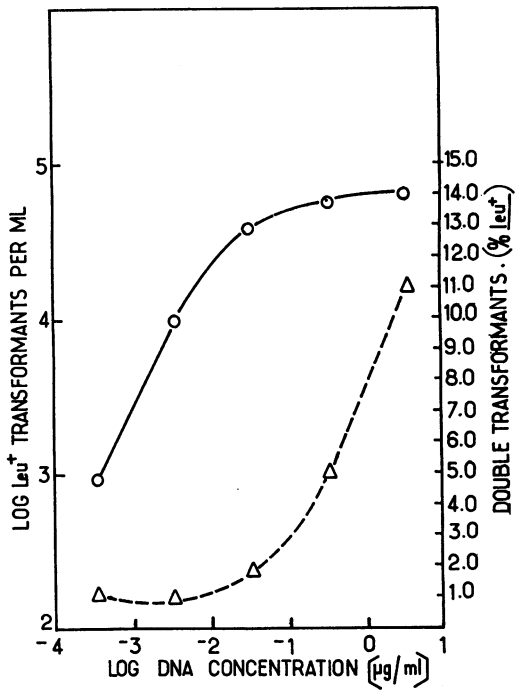

FIG. 6. *Contransfer of markers phe1⁺ and leu1⁺ in function of DNA concentration. Recipient strain = GSY 230 (phe1⁻-leu1⁻); donor DNA = wild type. Symbols: ○, ilva⁺ transformants; △, double transformants = phe⁺ ilva⁺ (in per cent of singles:leu⁺).*

TABLE 9. *Linkage of phe_1 and leu_1 in transformation, with GSY 230 (phe_1^--leu_1^-) as recipient strain*

Donor DNA		Transformants/ml			Competent cells†	phe_1^+ leu_1^+ per ml expected as random co-incidence‡
Genotype	Concn	phe_1^+	leu_1^+	phe_1^+ leu_1^+		
	μg/ml				%	
Wild type	0.0007	3,965	5,130	49.6*	—	2.9
Mixture of ($phe_1^+leu_1^-$) and ($phe_1^-leu_1^+$)	0.0007 (of each)	5,090	7,325	5.35	6.32	—

* Mean values.

† Percentage of competent cells = [(number of phe_1^+ transformants × number of leu_1^+ transformants)/(number $phe_1^+leu_1^+$ transformants × total recipient cells per milliliter)] × 100. Total recipient cells per milliliter = 1.1×10^8.

‡ Calculated with above formula.

TABLE 10. *Cotransduction of leu_1^+ (or $ilva_1^+$) and phe_1^+**

Recipient (genotype)	Transductants		Cotransduction	
	Class	Cells/ml		
			%	
$phe_1^-leu_1^-$	phe^+leu^-	458		61.5†
	phe^-leu^+	367		
	phe^+leu^+	670		
$phe_1^-leu_1^-try^-$	$phe^-leu^+try^-$	410	($phe_1^+leu_1^+$)	64.3
	$phe^+leu^+try^-$	740	($leu_1^+try^+$)	0.0
	$phe^-leu^+try^+$	0	($phe_1^+try^+$)	0.0
	$phe^+leu^+try^+$	0		
	$phe^-leu^-try^+$	843		
$phe_1^-ilva_1^-$	phe^+ilva^-	762		76.8
	phe^+ilva^+	2531		

* Donor strain = wild type.

† Frequency of double transductants among those transduced for one of the markers. Mean values.

chromosome is supported by the results of Kelly and Pritchard (1965) on transformation. With DNA prepared by mild extraction techniques and protected by the addition of carrier DNA, they were able to show linkage of *ile* and *met* markers to the *try* cluster.

DISCUSSION

Linkage of loci involved in isoleucine, valine, and leucine biosynthesis. The transformation results reported in this paper show that, in *B. subtilis*, the situation with regard to linkage is not the same as in *S. typhimurium* or *E. coli*. In *B. subtilis*, the genes controlling the enzymes threonine-deaminase and dihydroxy acid dehydrase (Fig. 1, steps 1 and 4) are linked, but the reductoisomerase gene is located in another region of the genome and carried by a DNA "molecule" which also carries one or more genes involved in leucine biosynthesis.

The location of the deaminase and the dehydrase genes next to each other seems, however, to be a more general feature. In *S. typhimurium*, the order of the genetic loci recently established (Armstrong and Wagner, 1964) is not exactly that of the biochemical sequence, but the following: reductoisomerase-threonine deaminase-dihydroxy acid dehydrase-transaminase. Moreover, results on the regulation of these pathways suggest that the genes controlling the threonine deaminase and the dehydrase form a single operon in *S. typhimurium* (Freundlich and Umbarger, 1963), and are part of an operon which also comprises the gene controlling the transaminase (Fig. 1, step 5) in *E. coli* (Ramakrishnan and Adelberg, 1964). It is not yet known whether in *B. subtilis* these two linked genes also constitute an operon. The observed linkage of a locus concerned with isoleucine and valine biosynthesis (reductoisomerase) to leucine loci may also be of some

TABLE 11. *Repulsion tests by transduction with phage PBS-1*

Recipient (genotype)	Donor (genotype)	Transductants		Cotransduction
		Class	Cells/ml	
				%
leu$_1^-$	*phe*$_1^-$	*phe*$^-$*leu*$^+$	1,303	68.0
		phe$^+$*leu*$^+$	613	
ilva$_1^-$		*phe*$^-$*leu*$^+$	894	67.5
		phe$^+$*leu*$^+$	430	
arg$^-$		*phe*$^-$*arg*$^+$	207	20.0
		phe$^+$*arg*$^+$	827	
lys$^-$		*phe*$^-$*lys*$^+$	2	0.2
		phe$^+$*lys*$^+$	830	
lys$^-$	*ile*$_2^-$	*lys*$^+$*ile*$^-$	0	0.0
		lys$^+$*ile*$^+$	2,900	
phe$_1^-$		*phe*$^+$*ile*$^-$	0	0.0
		phe$^+$*ile*$^+$	1,283	
ile$_2^-$	*met*$_3^-$	*ile*$^+$*met*$^-$	70	82.0
		ile$^+$*met*$^+$	16	
ile$_1^-$		*ile*$^+$*met*$^-$	226	78.0
		ile$^+$*met*$^+$	64	

TABLE 12. *Recombination between closely linked markers in transduction by PBS-1**

Recipient (genotype)	Recombination
	%
try$^-$*his*$^-$	2.7
shik$^-$*his*$^-$	8.5
ilva$_1^-$*leu*$_1^-$	2.8
ile$_1^-$*ilva*$_4^-$	11.5
ile$_1^-$*met*$_3^-$	10.5
ilva$_2^-$*met*$_3^-$	4.5
ilva$_4^-$*met*$_4^-$	4.5

* Donor strain = wild type.

significance regarding the regulation of these pathways in this organism.

Weak linkage in transformation. For several pairs of markers in the segments studied, the recombination frequencies were over 50%. Values exceeding 50% could be expected (i) if fragmentation of the donor DNA during extraction and purification is more or less random, (ii) if the maximal length of the integrated segment is smaller than the transforming particle, or (iii) if a region of effective pairing exists in the interval between the two markers. Experimental evidence is not yet available which would permit exclusion of any of these hypotheses. Values higher than 50% were also found in the *try* segment (Nester et al., 1963; Anagnostopoulos, *unpublished data*). In this segment, however (which has been mapped in more detail), the interval over which recombination (in two-point crosses) does not exceed 50% includes several genetic loci and corresponds to a large section (calculated at about ⅓) of the transforming "molecule."

The cases of "very weak linkage" (e.g., *phe*$_1$ to the *ilva*$_{1\text{-}3}$-*leu* segment, Fig. 3) seem to have a different basis. Fragments carrying *phe*$_1$ and *ilva*$_1$ also carry all the other markers of the *ilva*$_{1\text{-}3}$-*leu* region with high frequency. The hypothesis that they correspond to rare DNA pieces much longer than the average transforming particles seems probable, especially in view of the transduction results. "Very weak linkage" was not found in the *try* segment. Similar cases were, however, reported for pneumococcal transformation (Michel, Sicard, and Ephrussi-Taylor, 1964). The "unstable linkage" described by Kelly and Pritchard (1965) involves markers further apart than those of our "very weak linkage" cases.

Comparison of transformation and transduction data. Comparison of recombination data obtained by transformation and by transduction with phage PBS-1 reveals two striking differences between the two systems. (i) Chromosome fragments transferred in transduction by this phage seem to be much longer than the transforming DNA "molecules", and (ii) recombination frequencies are much lower in transduction than in transformation.

Markers which are only rarely carried by the same transforming particle are very frequently cotransferred in transduction. More genetic loci than those carried by two transforming particles were found to be simultaneously transduced (Fig. 8). It is believed that they are all carried by a single fragment. If this is the case, Fig. 7 and 8 represent the physical sequence of markers in two quite long sections of the *B. subtilis* chromosome. The alternative hypothesis, that a single phage particle may introduce more than one chromosome fragment, seems unlikely for the following reasons. (i) It requires the additional assumption that the phage genome pairs simultaneously at two or more specific regions of the bacterial chromosome, since the same markers always are cotransduced by a single phage particle. Since transduction with this phage is of the generalized type, the model would involve a great number of these special patterns of phage attachment. (ii) The linkage relationships and order of markers depicted in Fig. 7 for the *ile* to *lys* region coincide with the map of Kelly and Pritchard (1965), compiled from data of unstable linkage in transformation.

In contrast to the observations with the SP-10 mediated transduction (Ephrati-Elizur and Fox, 1961), recombination frequencies in our transduction system are very different from those in transformation. Compared with the maps obtained by transformation, those obtained by transduction of the same regions are shrunken.

TABLE 13. *Cotransduction of markers behaving as unlinked in transformation**

Recipient (genotype)	Transductants		Cotransduction
	Class	Cells/ml	
			%
$ile_2^-try^-$	ile^+try^-	760	49.5†
	ile^-try^+	770	
	ile^+try^+	753	
$met_3^-try^-$	met^+try^-	564	53.5
	met^-try^+	716	
	met^+try^+	732	
$met_3^-his^-$	met^+his^-	170	57.0
	met^-his^+	177	
	met^+his^+	231	
$ile_2^-lys^-$	ile^+lys^-	407	25.0
	ile^-lys^+	457	
	ile^+lys^+	143	
$ile_2^-phe_1^-$	ile^+phe^-	640	0.0
	ile^-phe^+	1,000	
	ile^+phe^+	0	
lys^-try^-	lys^+try^-	1,238	67.0
	lys^-try^+	560	
	lys^+try^+	1,775	
$leu_1^-met_4^-try^-$	$leu^-met^+try^+$	1,066	40.5 ($met^{4+}try^+$)
	$leu^-met^-try^+$	1,577	0.0 ($leu_1^+met_4^+$)
	$leu^+met^-try^+$	0	
	$leu^+met^+try^+$	0	
arg^-try^-	arg^+try^-	280	0.0
	arg^-try^+	235	
	arg^+try^+	0	
$arg^-leu_1^-$	arg^+leu^-	612	51.0
	arg^-leu^+	1,516	
	arg^+leu^+	1,007	
$ilva_1^-try^-$	$ilva^+try^-$	242	0.0
	$ilva^-try^+$	172	
	$ilva^+try^+$	0	

* Donor strain = wild type.
† Calculated as for Table 10. Mean values.

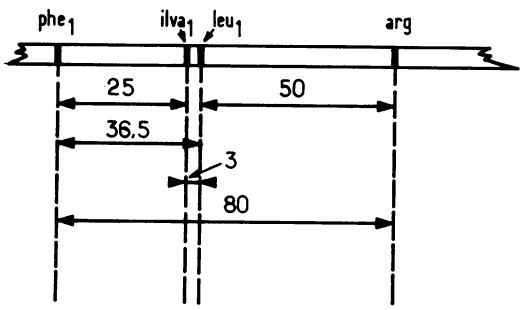

FIG. 7. *Linear sequence for phe_1, the $ilva_{1-3}$-leu segment, and arg markers, as suggested by results of transduction with phage PBS-1. Numbers are percentages of recombination.*

More or less random fragmentation of the DNA during extraction may account for a certain "magnification" of the maps obtained by transformation. On the other hand, the discrepancies in recombination values may simply reflect the difference in length of the donor segments in transformation and in transduction by PBS-1. Since the probability for effective pairing outside the interval considered is higher in the case of the (much longer) transducing fragments, the frequency of simultaneous integration of the two markers is increased. The situation seems similar to that encountered in *E. coli* K-12, when comparing recombination frequencies by transduction and conjugation. The hypothesis that the phage causes a modification in the state of the bacterial DNA that influences recombination cannot be excluded either. Linkage studies with transforming bacterial DNA extracted from phage PBS-1 might perhaps be helpful ln furthering the solution of this problem. An investigation along these lines is now in progress.

Mapping in B. subtilis. The results of the present study indicate that the combined use of the three techniques—transformation, selective

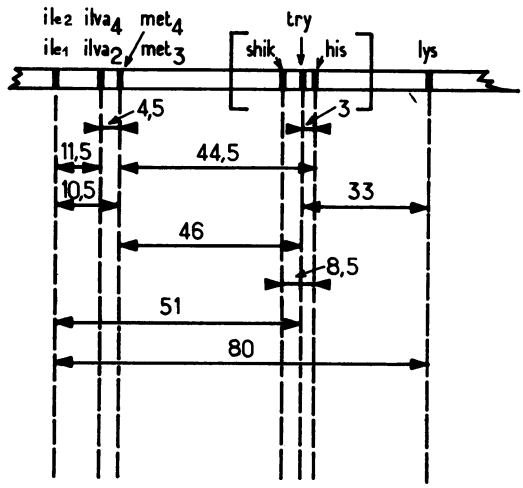

FIG. 8. *Linear sequence suggested by results of transduction with phage PBS-1 for the il-ilva$_{2-4}$-met segment, the try cluster, and lys markers. Numbers are percentages of recombination (mean values of several experiments). Brackets imply that it is not yet definitely established which end of the "try segment" is closer to the ile-met segment.*

isolation of linked markers by nitrous acid-treated DNA, and transduction with PBS-1—offers possibilities for detailed mapping of relatively long regions of the *B. subtilis* genome. If some overlapping should be found in the segments analyzed, it may then be possible to construct a map extending over the whole length of the chromosome.

Yoshikawa and Sueoka (1963) proposed a genetic map of *B. subtilis* constructed by a method based on the hypothesis of oriented replication of a single chromosome: relative distances of markers are evaluated from the ratios of transformation frequencies with DNA extracted from cultures of the donor strain in exponential and stationary growth phases. Our results, as well as those of Kelly and Pritchard (1965), are not in agreement with this map, particularly with regard to the position of the *try* segment. In the latest version of this map (Oishi, Yoshikawa, and Sueoka 1964), the *try* segment occupies a position in the middle of the chromosome very close to the region of the *leu* markers, and the *ile-met* is located at the end. Our transduction data suggest that the *try* segment lies next to the *ile-met* segment and is not linked to the *leu* region. It could be argued that the donor strains of Yoshikawa and Sueoka (1963) and ours differ in a translocation of the *try* segment. This possibility is now being investigated. Another hypothesis which would account for these discrepancies (and which is also put forward by Kelly and Pritchard) involves an alternative model considered by Yoshikawa and Sueoka (1963) for the interpretation of their results: the genome of *B. subtilis* might be composed of more than one independently replicating unit. The implications of such a model cannot at present be tested.

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