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Unstable Linkage Between Genetic Markers in Transformation

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ABSTRACT

KELLY, M. (Hammersmith Hospital, London, England), AND R. H. PRITCHARD. Unstable linkage between genetic markers in transformation. J. Bacteriol. **89**:1314–1321. 1965.—A new type of association of genetic markers in a transformation system has been discovered. Linkage between certain markers is detectable only under particular conditions of deoxyribonucleic acid (DNA) extraction and is sensitive to dilution of the DNA. The data suggest that this unstable association occurs between markers which are on the same molecule but separated by a molecular distance sufficiently great for linkage to be preserved only by mild DNA extraction procedures and at high concentrations of DNA. Linkage can be stabilized by dilution in the presence of a carrier DNA. By use of these unstable linkages, the genetic map of *Bacillus subtilis* has been extended in both directions from the aromatic gene cluster.

Transformation as a method of genetic analysis does not readily permit the construction of linkage maps encompassing the whole genome of transformable bacteria, owing to the small fraction of the total genetic material contained in single molecules of deoxyribonucleic acid (DNA) isolated by orthodox procedures. The occurrence of in vitro mutation of DNA (Litman and Ephrussi-Taylor, 1959) suggested a method for the selective isolation of new mutations linked to a chosen one, and was used successfully for this purpose by Anagnostopoulos and Crawford (1961) to isolate new tryptophan-requiring mutants linked to the *his* locus in *Bacillus subtilis*. In principle, this method could be used as a means for progressively extending the known linkage map of *B. subtilis* by successive isolation of new linked mutants, thereby overcoming the major limitation of transformation in mapping. It might also provide a means for determining whether discontinuities exist in the bacterial chromosome at which fragmentation occurs preferentially during isolation and purification of DNA.

Initial attempts with this approach (Kelly and Pritchard, 1963) were not encouraging, owing to the poor yield of linked mutation found after in vitro treatment of purified DNA under a variety of conditions and with a number of mutagens. When crude lysates were treated with nitrous acid, however, a new class of linked mutation was found. Linkage was detectable when DNA was prepared by some methods but not by others, and was sensitive to dilution of the DNA. The data presented in this paper suggest that these unstable linkages occur only in preparations containing DNA with a higher molecular weight than is usually found in purified preparations. They have been used to extend the linkage map of *B. subtilis* in both directions from the aromatic gene cluster (Nester, Schaffer, and Lederberg, 1963).

Comparison of the linkage data presented here and the chromosome map drawn by Yoshikawa and Sueoka (1963*a*, *b*) suggests that the genome of *B. subtilis* may consist of more than one unit of replication.

MATERIALS AND METHODS

Strains. All strains used in this laboratory were derived from *B. subtilis* 168 (*ind*$^-$), which was kindly provided by C. Anagnostopoulos. Mutants were isolated either by transformation with nitrous acid-treated DNA according to the method of Anagnostopoulos and Crawford (1961), or after treatment of cells with ethyl methane sulfonate (Loveless and Haworth, 1959). Multiple auxotrophs *met*$^-$ *ileu*$^-$ *ind*$^-$ *tyr*$^-$ *arg*$^-$ (strain I) and *met*$^-$ *ileu*$^-$ *ind*$^-$ *tyr*$^-$ *arg*$^-$ *rib*$^-$ (strain II) were synthesized in stages by transforming an auxotroph with DNA isolated from a second auxotroph, and isolating multiple auxotrophic transformants by penicillin enrichment (*see* Nester, 1964).

Media and transformation procedure. Media and transformation procedures were based on those of Spizizen (1958), Young and Spizizen (1961), and Anagnostopoulos and Spizizen (1961). Occasional failure of cultures to grow to competence was found to be due to insufficient manganese in

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Spizizen's medium. $MnSO_4$ (10^{-7} M) was therefore added to medium of the first growth cycle. When growing strains I and II to competence, each of the required amino acids (20 μg/ml) was added to the growth medium in addition to the supplementation with casein hydrolysate (0.02%). If only the required amino acids were added, growth rate was reduced by competitive interactions, and the level of competence achieved was reduced. Competent cultures were normally transformed by incubation with DNA at 37 C for 45 min without aeration before plating.

Preparation of DNA. DNA was prepared according to the methods of Marmur (1961) and Kirby (1957, 1958). Kirby's procedure was modified as follows: (i) *p*-aminosalicylate (PAS)-phenol method. Washed cells (1.0 g, wet weight) in standard saline-citrate (40 ml) were lysed with lysozyme (0.2 mg/ml) at 37 C for 5 to 10 min. Sodium PAS (final concentration, 6%) was added to the lysate, followed by an equal volume of washed phenol, and the mixture was shaken mechanically for 30 min. The emulsion was centrifuged, and the aqueous layer was removed and shaken for 3 min with 0.1 volume of redistilled ether. The ether layer and white precipitate were discarded, and the DNA was collected by precipitation with an equal volume of 2-ethoxyethanol. The DNA was redissolved in 0.2 volume of dilute saline-citrate (0.015 M NaCl; 0.0015 M sodium citrate), and the solution was made up to standard strength (0.15 M NaCl; 0.015 M sodium citrate) with concentrated saline citrate. Ribonuclease (pretreated at 80 C for 10 min at *p*H 5 to inactivate any contaminating deoxyribonuclease) was then added (50 μg/ml), and the mixture was incubated (*p*H 7.0) at 37 C for 30 min. The DNA was reprecipitated with 2-ethoxyethanol, redissolved at a concentration of 1 to 2 mg/ml in 1.0 M NaCl (*p*H 7.0), and stored at 4 C. (ii) benzoate-phenol method. Washed cells (1.0 g, wet weight) were lysed in saline-citrate (10 or 20 ml) as described. Sodium-benzoate (0.15 to 0.3 M) was added to the lysate, followed by an equal volume of phenol. The mixture was shaken and centrifuged, and the aqueous layer was collected as before. DNA was precipitated and redissolved as before, omitting the treatment with ribonuclease. Such preparations were stored at −20 C.

In some experiments, crude cell lysates prepared by treatment of washed cells with lysozyme, as described, were used for transformation. DNA was estimated in all cases according to Burton (1956). Phenol was prepared by washing with saturated sodium metaborate, and the latter was removed by further washing with distilled water. 8-Hydroxyquinoline (0.2%) was added to the washed phenol. Enzymes were from the following sources: trypsin, ribonuclease, and deoxyribonuclease, California Foundation for Biochemical Research, Los Angeles, Calif.; chymotrypsin, lipase, and lysozyme, Koch-Light Laboratories Ltd., Colnbrook, England; phospholipase, British Drug Houses Ltd., Poole, England.

RESULTS

Mutants linked to the *ind*⁻ locus were looked for by exposing a competent *ind*⁻ culture to a crude lysate from a prototroph treated with nitrous acid and plating the mixture on medium supplemented with a purine and pyrimidine pool, a vitamin pool, and all amino acids except tryptophan. Among the new mutants isolated from the *ind*⁺ transformants was one requiring methionine for growth. When DNA from this mutant, extracted and purified by treatment with phenol, was used at saturating concentrations to transform an *ind*⁻ culture, 20% of the *ind*⁺ transformants were found to be *met*⁻. Since the frequency of cotransfer observed for unlinked pairs under these conditions was normally less than 10%, the question arose as to whether the higher value observed between this pair of markers was due to linkage. To investigate this possibility in more detail, the multiple auxotroph strain I was synthesized. With this strain it was possible to compare under identical conditions the behavior of *ind*⁻ and *met*⁻ with *ind*⁻ and *tyr*⁻ (closely linked), *ind*⁻ and *arg*⁻ (unlinked), and *met*⁻ and *ileu*⁻. The last pair was found to be loosely linked, giving 25% cotransfer with DNA prepared by Marmur's method, and 45% by use of phenol extraction.

Effect of DNA concentration on frequency of cotransfer. DNA from a prototroph, prepared by different isolation procedures, was used to transform strain I at different concentrations, and classes of single and double transformants were selected on appropriate media. The number of double transformants with respect to a given pair of markers was plotted as a percentage of the number of transformants for one member of the pair alone at each concentration of DNA. Under these conditions, the percentage of double transformants should be independent of DNA concentration in the case of linked markers, and proportional to DNA concentration in the case of unlinked markers (Goodgal, 1961).

Both *ind*⁻ and *arg*⁻, and also *ind*⁻ and *met*⁻, behaved as if they were unlinked by this criterion when DNA prepared by Marmur's method was used (Fig. 1). With DNA extracted by the benzoate-phenol method, the two pairs behaved differently (Fig. 2). *Ind*⁻ and *arg*⁻ again behaved as if they were unlinked, whereas the frequency of cotransfer of *ind*⁺ and *met*⁺ declined at a lower rate which was no longer directly proportional to DNA concentration; *ind*⁺ *met*⁺ transformants were four times as frequent as *ind*⁺ *arg*⁺ transformants at saturating DNA concentrations, but 20 times as frequent at the lowest DNA concentration used.

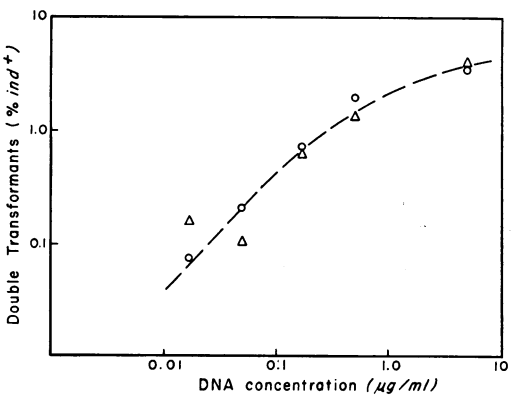

FIG. 1. *Effect of concentration of DNA prepared by Marmur's method on frequency of cotransfer. DNA from a prototroph was diluted in saline-citrate. Competent cells of strain I were added, and the number of ind+, ind+ met+, and ind+ arg+ transformants were assayed on appropriate media after incubation of the mixture for 45 min at 37 C. Double transformants are expressed as a percentage of total ind+ transformants. Symbols: △, ind+ met+; ○, ind+ arg+.*

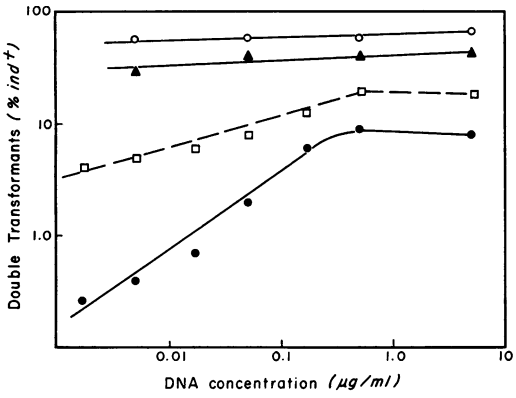

FIG. 2. *Effect of concentration of DNA prepared by the benzoate-phenol method on frequency of cotransfer. Method of transformation and presentation of data are as described in Fig. 1. Symbols: □, ind+ met+; ●, ind+ arg+; ▲, met+ ileu+; ○, ind+ tyr+.*

Although *ind*⁻ and *met*⁻ clearly do not behave as though they are unlinked, neither do they behave as orthodox linked markers, as can be seen from the behavior of two linked pairs, *ind*⁻ *tyr*⁻ and *met*⁻ *ileu*⁻ (Fig. 2).

Method of DNA preparation and ind+ met+ cotransfer. What is known about the effect of the method of DNA extraction on *ind+ met+* cotransfer can be summarized as follows. (i) The first shaking with chloroform and octanol in Marmur's procedure destroys the association of *ind+* and *met+* (Table 1). (ii) The association does not survive the published extraction procedure of Kirby (1957). (iii) The association is conserved after one extraction with phenol followed by ethanol precipitation, and it is more resistant to benzoate treatment than to treatment with PAS.

It is conceivable that the association of these markers might be an artifact of phenol extraction. Such an artificial association would have to be specific, however, since no association between *arg+* and any other locus marked in strain I was found. Moreover, the following experiment indi-

TABLE 1. *Effect of different DNA extraction procedures on ind-met linkage**

Method of extraction	Wet wt (g/10 ml) of cells of lysate	*ind+ met+* transformants (per cent *ind+*)	Ratio of *ind+ met+* to *ind+ arg+*
Benzoate-phenol.....	1.0	17	11
Benzoate-phenol.....	0.5	13	40
PAS-phenol.....	0.5	8	20
Chloroform-octanol..	1.0	2	4

* Viscosity of lysates was varied by resuspending weighed cells in appropriate volumes of saline-citrate. DNA was extracted from the lysate in 0.3 M benzoate or 6% PAS with an equal volume of phenol. In the last method, the lysate was shaken with chloroform-octanol for 30 min as in Marmur's (1961) procedure. The DNA was diluted to 0.05 μg/ml in saline citrate plus *Escherichia coli* DNA (100 μg/ml) and assayed for survival of linkage on competent cells of strain I. *Ind+ met+*, *ind+ arg+*, and *ind+* transformants were selected by plating on appropriate media.

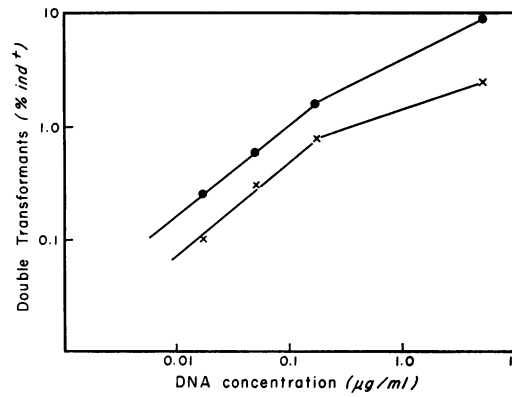

FIG. 3. *Effect of concentration of DNA isolated from a mixture of ind⁻ met+ and ind+ met⁻ cells by the benzoate-phenol method. DNA was extracted from a mixture containing equal numbers of cells of the two strains. Transformation procedure and presentation of data are as described in Fig. 1. Symbols: ×, ind+ met+; ●, ind+ arg+.*

cates that the association occurs only if the two markers are derived from the same cell, as would be predicted if it were a property of the chromosomal organization in the cell. Cultures of *ind*$^-$ and *met*$^-$ were mixed in equal proportions, and the DNA was extracted from the mixture by the benzoate-phenol method. This DNA was used to transform strain I, and the number of *ind*$^+$ *met*$^+$ and *ind*$^+$ *arg*$^+$ transformants, as a percentage of the number of *ind*$^+$ transformants, was determined at different DNA concentrations. No evidence for linkage of *ind* and *met* was found in this preparation (Fig. 3).

Stabilization of ind-met linkage by carrier DNA. The simplest interpretation of the data presented so far would be that the association between *ind*$^+$ and *met*$^+$ is due to linkage, the two markers being taken up by a recipient cell together on the same DNA molecule. It would follow that methods of DNA preparation which leave these markers unlinked do not yield material of sufficiently high molecular weight for the *ind*$^+$ and *met*$^+$ loci to occur on a single DNA fragment with measurable frequency. Furthermore, the progressive loss of linkage with decreasing DNA concentration would be attributable to loss of self-protection against hydrodynamic shear (Hershey and Burgi, 1960). If this were the case, it should be prevented by dilution of the DNA in the presence of carrier DNA. The experimental data presented in Fig. 4 show that this is, indeed, the case. In the experiment shown, DNA (100 μg/ml) isolated from a prototroph by the benzoate-phenol method was diluted serially in buffer containing 100 μg/ml of DNA isolated from strain I. The total DNA concentration was thus maintained constant at all times. The mixtures, containing different concentrations of prototrophic DNA, were used to transform strain I, and the frequency of cotransfer of different pairs of markers was determined at each concentration. Since the carrier DNA is homologous with the DNA of the recipient, it can make no contribution to transformants obtained. The *ind*$^+$ *met*$^+$ linkage was completely stable under these conditions (Fig. 4). At saturating concentrations of prototrophic DNA, the frequency of *ind*$^+$ *met*$^+$ double transformants was 20%. At the same concentration, there were 10% *ind*$^+$ *arg*$^+$ doubles (presumably due to coincident uptake of more than one DNA molecule by a single recipient cell). Dilution of the DNA resulted in a fall in the number of *ind*$^+$ *met*$^+$ doubles from 20 to 10%, at which level it remained constant. The initial drop in the frequency of cotransfer is presumably due to loss of double transformants due to coincident uptake of more than one molecule by a single cell.

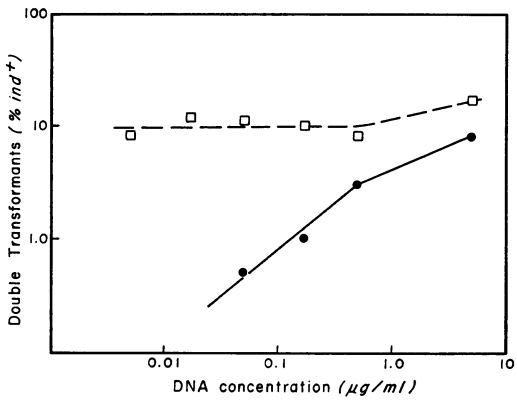

FIG. 4. *Effect of carrier DNA on frequency of cotransfer at different concentrations of DNA. DNA extracted from a prototroph by the benzoate-phenol method was diluted in DNA (100 μg/ml in saline-citrate) prepared from strain I. Transformation procedure and presentation of data are as described in Fig. 1. Symbols:* □, *ind*$^+$ *met*$^+$; ●, *ind*$^+$ *arg*$^+$.

TABLE 2. *Mode of protection of ind-met linkage by carrier DNA*

Dilution fluid*	*ind*$^+$ *met*$^+$ as a percentage of *ind*$^+$ transformants	
	Strain I carrier DNA	*Escherichia coli* carrier DNA
No carrier DNA.....	3.5	3.5
Carrier DNA added after dilution.....	6.0	6.0
Carrier DNA.....	13.0	11.0

* Prototrophic DNA extracted by benzoate-phenol method was diluted to 0.1 μg/ml (i) in presence of carrier DNA (100 μg/ml) and (ii) without carrier DNA. The mixture was diluted 20-fold into a competent culture of strain I. When carrier DNA was added after dilution, DNA was diluted without carrier DNA, this being added at time of exposure to strain I.

The loss of linkage after dilution in the absence of carrier DNA occurs mainly during the process of dilution itself (Table 2). Some protection is evident, however, even if carrier DNA is added after dilution. This residual protection must take place between the time of exposure of competent cells to the diluted DNA and DNA uptake by these cells.

Protection of linkage between *ind* and *met* also occurs in the presence of *Escherichia coli* DNA (Table 2), and this was used in all subsequent experiments.

Destruction of linkage by deoxyribonuclease and hydrodynamic shear. The sensitivity of the *ind met* linkage to DNA concentration and the method of purification suggest that the molecular distance between these markers is greater than that between markers showing orthodox linkage and, therefore, should be more sensitive to deoxyribonuclease degradation. The survival of *ind tyr*, *met ileu*, and *ind met* linkage as a function of deoxyribonuclease concentration was, therefore, determined. The data (Fig. 5) indicate that sensitivity to deoxyribonuclease and cotransfer frequency are related in the order expected. Taking the deoxyribonuclease concentration which halves the frequency of cotransfer as an approximate measure of the relative molecular distance between two sites, the data would indicate that this distance is about 10 times greater between *ind* and *met* than between *ileu* and *met*, and about 100 times greater than between *ind* and *tyr*.

Similar relative values for the molecular distance between these pairs of markers were obtained when their sensitivity to hydrodynamic shear was compared (Table 3).

Activity of other enzymes on ind met linkage. Although linkage between *ind* and *met* is most simply interpreted on the assumption that the two loci are located on a single DNA molecule, other modes of association are not ruled out. The two sites might be located on different molecules attached end to end, or through cross-links with material other than DNA. In an attempt to distinguish between these possibilities, prototrophic DNA extracted with benzoate and phenol was treated with a number of enzymes (Table 4), and the effect of such treatment on the frequency of cotransfer of ind^+ and met^+ was determined. No evidence for selective destruction of linkage by any of these enzymes was found.

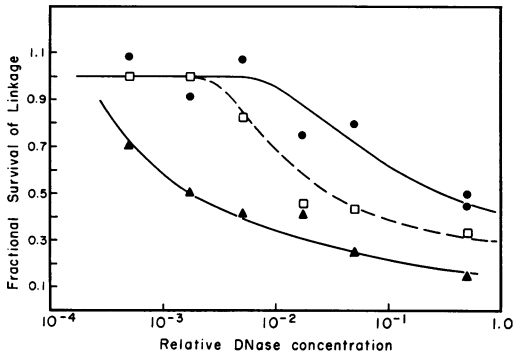

FIG. 5. *Relative sensitivity of ind met, met ileu, and ind tyr linkage to deoxyribonuclease. DNA [0.1 μg/ml in phosphate buffer (pH 7.0) with 0.15 M NaCl and 0.01 M $MgSO_4$] extracted from a prototroph with benzoate and phenol was mixed with an equal volume of a solution of deoxyribonuclease at the indicated concentration. The mixture was incubated for 15 min at 37 C, and reaction was terminated by 20-fold dilution into a competent culture of strain I. Double transformants (expressed as percentage of ind^+ transformants) are plotted as a fraction of the percentage in a control culture (no deoxyribonuclease). Symbols: ●, ind^+ tyr^+; □, met^+ $ileu^+$; ▲, ind^+ met^+.*

TABLE 3. *Effect of hydrodynamic shear on ind^+ tyr^+, met^+ $ileu^+$, and ind^+ met^+ cotransfer frequency**

Marker	Flow rate (ml/min)				
	0	0.25	1	2	4
ind^+ tyr^+.....	1.00†	1.06	0.90	0.85	0.80
met^+ $ileu^+$.....	1.00	0.90	0.90	0.66	0.66
ind^+ met^+.....	1.00	1.00	0.70	0.27	0.20

* DNA extracted with benzoate and phenol from a prototroph was diluted to 0.1 μg/ml in saline-citrate and expelled from a syringe through a 27-gauge(0.4 mm) needle at various flow rates. Competent cells of strain I were added, and transformants were assayed on appropriate media.

† Cotransfer frequency at indicated flow rate. Doubles were calculated as percentage of ind^+ transformants and expressed as a fraction of the corresponding frequency in the unsheared control.

Mapping of markers showing unstable linkage. A new mutant requiring riboflavine for growth (rib^-) was found to show linkage with ind^- of a type similar to that between ind^- and met^-. The rib^- marker was therefore introduced into strain I to make strain II, to examine the behavior of all three markers in a single experiment. This new strain was transformed with DNA extracted by benzoate and phenol from a prototroph in the presence and absence of carrier DNA (Table 5). The frequency of ind^+ rib^+ transformants was greater than that of ind^+ met^+ transformants, both in the presence and absence of carrier DNA, and, in addition, the ind^+ met^+ transformants appeared to be somewhat less sensitive to dilution in the absence of carrier DNA. Ind^- and rib^- are apparently more closely linked than ind^- and met^-.

The frequency of cotransfer of met^- and rib^- was then determined to see whether the data were compatible with a linear arrangement of the three loci (Table 6). With donor DNA extracted by benzoate and phenol in the absence of carrier DNA, the number of met^+ rib^+ transformants is, in most cases, less than the corresponding number for the unlinked pairs ind^+ arg^+, met^+ arg^+, and rib^+ arg^+. In the presence of carrier DNA,

TABLE 4. *Effect of treatment with different enzymes on ind+ met+ and met+ ileu+ cotransfer frequency*

Treatment*	Mean number of transformants (per ml)				*met+ ileu+* (per cent *ind+*)	*ind+ met+* (per cent *ind+*)
	ind+	*met+ ileu+*	*ind+ met+*	*ind+ arg+*		
Control	1,230	490	130	4	40	10.6
Chymotrypsin	1,790	790	119	2	44	6.6
Trypsin	1,450	590	113	0	40.7	7.8
Ribonuclease	2,120	900	152	2	41	7.2
Lipase	1,570	620	96	3	39.5	6.1
Phospholipase	1,360	630	97	2	46	7.1

* Benzoate-phenol DNA from a prototroph was diluted to 0.2 μg/ml in *Escherichia coli* DNA (100 μg/ml in saline-citrate) and mixed with an equal volume of enzyme solution (enzymes were dissolved in phosphate buffer at *p*H 7.0 containing 0.2 M NaCl). Trypsin, lipase, and phospholipase solution contained 10^{-3} M $CaCl_2$. Ribonuclease was preheated (80 C at *p*H 5.0) to inactivate any contaminating deoxyribonuclease. Exposure to enzyme was for 15 min at 37 C, and reaction was terminated by 20-fold dilution into competent culture of strain I.

TABLE 5. *Comparative cotransfer frequencies for ind+ met+ and ind+ rib+**

DNA	Carrier DNA	*ind+ met+*	*ind+ rib+*	*ind+ arg+*
μg/ml				
0.1	+	13.7	20.0	1.2
0.1	−	1.3	2.4	0.6
0.01	−	1.1	3.3	0.16

* DNA (benzoate-phenol extraction) was diluted in *Escherichia coli* carrier DNA (100 μg/ml). This was then diluted 20-fold into a culture of strain II. The transformants were selected on appropriate media. Frequencies of double transformants are given as per cent *ind+*. (N.B., *met+* is integrated with an efficiency of 1.6 relative to *rib+*).

met+ rib+ and *ileu+ rib+* transformants are, in all cases, greater in frequency than double transformants for the unlinked pairs, the frequency of *met+ rib+* doubles being about 10% that of *ind+ met+* doubles.

These results are clearly consistent with linkage of all three markers in a linear sequence with the order *met-ind-rib*.

The linkage relationships in this complex of markers are summarized in Fig. 6. *Ind* and *tyr* are only 2 of the 13 genes identified in this region of the genome by Nester et al. (1963). The order of markers within one group showing stable linkage relative to those in neighboring groups could not be established, since the frequencies of cotransfer between the different members of one group and any marker in a second group were not significantly different. Linkage of *ileu*, *ilva*, and *met* has also been reported by Anagnostopoulos, Barat, and Schneider (1963). Anagnostopoulos and Barat (*personal communication*) have also found that this group is linked to ind_{-168} by transduction, the suggested order of markers being *ileu-ilva-met-ind*.

DISCUSSION

The behavior of the three mutants *met−*, *ind−*, and *rib−* is clearly consistent with a linear association on a single DNA molecule, the instability being accounted for by the fact that the molecular distance between them is sufficiently great for linkage to be preserved only by mild DNA extraction procedures and with self-protecting concentrations of DNA. If this interpretation is valid, then the DNA isolation procedures used here represent a useful extension to current methods of mapping in transformation. Other possibilities are not ruled out, however, particularly since all DNA preparations in which linkage was demonstrable contained 10 to 20% protein, as well as some ribonucleic acid (RNA). The markers might be association (i) on different DNA molecules, attached end to end by other material or (ii) on different molecules cross-linked side by side by other material. The evidence against (ii) is provided by the genetic data presented here, which suggest a linear relationship of the three sites, and data of Anagnostopoulos and Barat (*personal communication*) which indicate a linear order for *ileu*, *met*, and *ind*. The absence of sensitivity to various proteolytic enzymes, lipase, phospholipase, and ribonuclease do not support (i), although this alternative cannot be ruled out on the basis of evidence available at present.

Using an elegant and novel mapping technique, Yoshikawa and Sueoka (1963*a*, *b*) have suggested that *B. subtilis* has a single chromosome which replicates sequentially as a unit. They have mapped *ileu* and *met* close to the terminal end of

TABLE 6. *Cotransfer frequencies for met, ileu, and rib**

Carrier DNA	Expt no.	Mean no. of transformants (per 0.1 ml)					
		met$^+$ *rib*$^+$	*ileu*$^+$ *rib*$^+$	*met*$^+$ *arg*$^+$	*arg*$^+$ *rib*$^+$	*ind*$^+$ *arg*$^+$	*ind*$^+$ *met*$^+$
Present	1	41.5 (2)	—	—	97 (1)	118 (2)	251 (1)
	2	134 (4)	66.5 (4)	210	91.3 (4)	180 (3)	—
Absent	1	82.6 (5)	—	50 (2)	45 (2)	45 (2)	412 (1)
	2	68.2 (5)	48.0 (5)	31.6 (5)	—	—	—

* DNA extracted by use of the benzoate-phenol method was used at a concentration of 0.1 μg/ml with or without *Escherichia coli* DNA (100 μg/ml). The recipient was strain II. Numbers in parentheses are the number of plates counted.

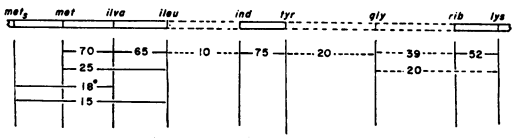

FIG. 6. *Map based on stable and unstable linkage relationships. Numbers are frequencies of cotransfer of markers expressed as percentages. Solid lines indicate stable linkage. Broken lines indicate unstable linkage, the value given being that obtained with DNA prepared by the benzoate-phenol method protected by dilution in carrier DNA. The relative order of markers in different groups connected by unstable linkage has not been unequivocably established. Symbols: gly, requirement for glycine; lys, requirement for lysine; met$_s$, methionineless mutant obtained from N. Sueoka; 18°, value obtained with PAS DNA diluted without carrier.*

this chromosome, and *ind*$_{-168}$ about 30% (Oishi, Yoshikawa, and Sueoka, 1964) of the chromosome length from the terminus. It was therefore surprising that a phenotypically similar linked pair should prove to be cotransformable with *ind*$_{-168}$. Through the kindness of N. Sueoka, we were able to determine whether the *met*$^-$ mutant used by Yoshikawa and Sueoka (1963*a*) was linked to the *ilva* mutant used in the present paper. The data in Fig. 6 show that they are, indeed, linked, giving 18% cotransfer compared with 65% between *ilva* and our *met*$^-$ mutant.

If Yoshikawa and Sueoka have interpreted their data correctly, then it would follow that extraction procedures used in the present paper must yield DNA with a molecular weight equivalent to about one-third of the total genetic length. A preliminary estimate of the amount of DNA per genome in *B. subtilis* (Kelly, 1964) gives a value of approximately 10^{10} Daltons. Hence, our preparation would necessarily contain molecules with a molecular weight of 3×10^9. Attempts to obtain an estimate of the molecular weight of molecules carrying both *ind*$^+$ and *met*$^+$ from the sedimentation velocity of *ind*$^+$ *met*$^+$ transforming activity in a sucrose gradient were not successful. The data suggest that if a higher than average molecular weight component exists in these preparations, it is degraded when it separates from the bulk of the DNA during sedimentation, presumably because the concentration falls below self-protecting levels. It seems unlikely, however, that the molecular distance between *met* and *rib* represents one-third of the genome, since, in preliminary tests with 15 new mutants phenotypically different from those described in this paper, none of the mutants showed linkage with *met* and *rib*. Moreover, it seems unlikely that DNA fragments with a molecular weight in excess of 10^9 could be incorporated into transducing phage particles as Anagnostopoulos and Barat have found. Therefore, it seems necessary to consider other possible interpretations of the data of Yoshikawa and Sueoka. One which has been considered by them (Sueoka and Yoshikawa, 1963) and which must clearly be entertained as a possible alternative is that *B. subtilis* has more than one chromosome, or a single chromosome consisting of several independently replicating subunits. The molecular distance it would be necessary to postulate between unstably linked sites would depend on what assumptions were made concerning the structure, relative rates of replication, and relative times of replication of each subunit. None of this information is at present available.

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