TRANSFORMATION OF *BACILLUS SUBTILIS* USING HYBRID DNA MOLECULES CONSTRUCTED BY ANNEALING RESOLVED COMPLEMENTARY STRANDS

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THE question of whether one or both strands of the transforming deoxyribonucleic acid (DNA) molecule is inserted into the host chromosome of *Bacillus subtilis* has been the subject of several recent reports. BODMER and GANESAN (1964) presented physical evidence for the incorporation of one strand. AYAD and BARKER (1969) in contrast, presented physical evidence suggesting that both strands of the duplex are incorporated. Evidence bearing on this question using *D. pneumoniae* (FOX and ALLEN 1964) and *H. influenzae* (NOTANI and GOODGAL 1966) favored the incorporation of only one strand of the donor duplex. BRESLER *et al.* (1964) and VESTRI, FELICETTI and LOSTIA (1966) have reported genetic evidence in *B. subtilis* transformation for the use of both strands with low frequency.

With the advent of methods for separating the complementary strands of *B. subtilis* DNA (ROGER, BECKMANN and HOTCHKISS 1966; RUDNER, KARKAS and CHARGAFF 1968), it became possible to prepare pure hybrid duplexes each strand of which contained a unique genetic marker. The use of such preparations permits a more critical evaluation of the role of the duplex in transformation. The transformants which arise from the use of these duplexes are easily analyzed and permit one to examine the behavior of hybrid duplex free of its mirror image. (GABOR and HOTCHKISS 1966, 1969; PETERSON and GUILD 1968).

METHODS

Organisms: The following mutant derivatives of *Bacillus subtilis* strain 168 (try_2^-) were used for these experiments: strain 301 ($try_2^-his_{12}^-tyr_1^-$) (STRAUSS 1966), strain 302 ($try_2^-his_{12}^-$), strain 303 ($his_{12}^-tyr_1^-$), strain SB 100 ($try_2^-his_2^-tyr_1^-$) (kindly supplied by E. NESTER). The his_{12} locus is unlinked to the other markers and is not relevant to the present study. The try_2, his_2, and tyr_1 loci are linked to each other in the listed order. Strains 302 and 303 were prepared by transformation of strain 301 with wild-type DNA. In addition, a *pur⁻leu⁻met⁻* strain (kindly supplied by N. SEOUKA) was used as a recipient for determining the location of transforming activity of 301 DNA strands obtained from hydroxyapatite columns.

Transformation: Competent cells were prepared by the method of ANAGNOSTOPOULOS and SPIZIZEN (1961) as modified by CAHN and FOX (1968). Strain SB 100 cells were grown for 4.5 to 5 hr in Medium I, concentrated 10-fold in Medium II containing 10% glycerol, and frozen in liquid nitrogen. For use as recipients, they were thawed, diluted 1/10 in Medium II and grown at 30°C for 2.5 hr. $MgCl_2$ was added to a final concentration of 0.02M. The cells were then added to the transforming DNA.

Preparation of transforming DNA: The method of MARMUR (1961) was used with the exception that the isopropanol step was omitted. The last step in the purification consisted of a 10 min exposure to phenol followed by alcohol precipitation and extensive dialysis against 0.15M NaCl – 0.05M Na citrate. Preparations were stored at 4°C over chloroform at concentrations of 0.2 to 0.4 mg/ml of DNA.

Purification of complementary strands

Melting the DNA: Transforming DNA was diluted 1/10 with double-distilled water to a concentration of 20 to 40 μg/ml. The solution was chilled in an ice bath, and 1/10 volume of cold 1N NaOH was added dropwise with gentle swirling. The solution was warmed to room temperature in a 40°C water bath, allowed to stand for 10 min, chilled, and neutralized carefully to pH 7.3 with 1N HCl. The solution was immediately diluted with 0.8 volumes of 1.2M buffered saline (0.05M Na phosphate buffer, pH 6.7, containing 1.2M NaCl.) (MANDEL and HERSHEY 1960). The final pH of this DNA solution was 6.3. This solution was applied to the methylated albumen–Kieselguhr (MAK) column.

Separation of the strands on the MAK column: The MAK bed was prepared according to the method of MANDEL and HERSHEY (1960). The water-jacketed column (3.1 cm diameter) was cooled to 8°C. Elution was carried out by the method of ROGER, BECKMANN and HOTCHKISS (1966) as modified by RUDNER, KARKAS and CHARGAFF (1968), with the exception that the elution of the second, heavy (H) peak was carried out gradually by re-levelling the salt-gradient reservoirs after elution of the first light (L) peak. The elution was accomplished with 400 ml of a 0.60M to 1.2M buffered saline gradient. Elution was scanned at a wavelength of 254 mμ using an ISCO scanner. Tests of the resulting fractions for transforming capacity before and after self-annealing revealed, as RUDNER, KARKAS and CHARGAFF (1968) had found, that the light (L) fractions showed little or no increase in residual transforming activity after self-annealing, while the heavy (H) fractions showed a 5–10 fold increase in transforming activity. To test the possibility that the low transformation capacity of each self-annealed single strand was a result of contamination by the complementary strand, the *self-annealed* strands were dialyzed against 0.05M phosphate buffer, pH 6.7, and passed over hydroxyapatite (HA) to separate out any double-stranded material.

Purification of the partially resolved strands on HA: HA was prepared according to the method of TISELIUS, LEVIN and HJERTEN (1956). Columns (2.5 cm diameter) were prepared at room temperature by packing 25 ml of HA under 1.10 atm (16.2 psi). Each strand was adsorbed on the HA, and eluted with 300 ml of a 0.05M to 0.40M Na phosphate buffer gradient, pH 6.7 (BERNARDI 1965). 6 ml fractions were collected and stored in the cold over chloroform until they could be tested for transforming activity. A typical elution pattern is shown in Figure 1. In the case of both the L and H strand, the transforming activity was confined exclusively to the double-stranded region of the elution pattern. The single-stranded region showed a transforming activity of less than 1 in 10^7 recipient cells. Note in particular that residual transforming activity of the L strand appeared in the double-stranded regions.

Preparation of hybrid duplexes: Purified strands were mixed, and 1/10 volume of 2.9M NaCl–0.15M Na citrate solution was added to each mixture before annealing. Annealing was carried out by heating at 70°C for one hour, and then cooling slowly overnight. Table 1 contains a list of all the duplexes prepared. The data of Table 2 indicate the change in linkage characteristics of three traits. Note that resolution of the strands caused a 14% decrease in the *tyr–his* liaison and a 57% decrease in the *try–his* liaison. Nevertheless, observed frequency of double transformants conforms to that calculated from the product of the frequencies of each single trait. This finding suggests that renatured and resolved-renatured material are subject to the same mechanism of recombination as is native DNA.

RESULTS

Transformation by hybrid duplexes: The transformation of a cell to histidine independence can result from the insertion of one or both strands of the donor DNA into the recipient chromosome. The number of histidine-positive strands

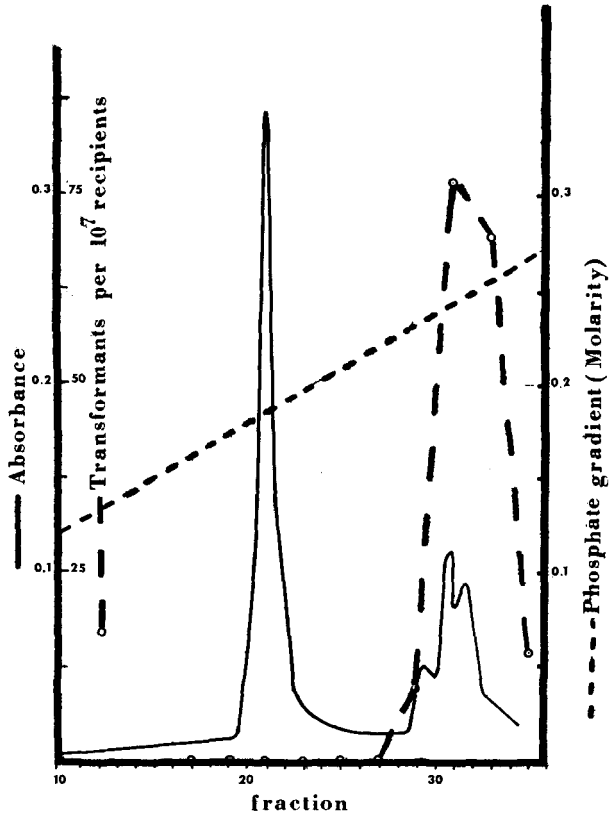
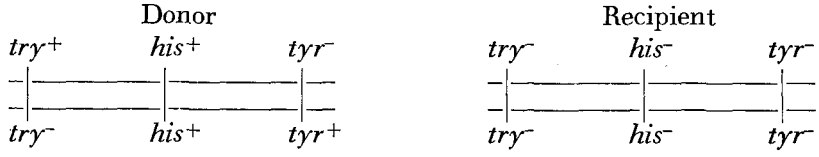


FIGURE 1.—Elution pattern of a MAK fraction from HA. The L or H fraction obtained from a MAK column was self-annealed and applied to the HA column as described in the text. Double-stranded material is known to elute at approximately 0.23M phosphate and single-stranded material at 0.18M phosphate. Elution was carried out with a 0.05M to 0.40M phosphate buffer (pH 6.7) gradient. Recovery was uniformly 100%.

inserted can be determined from the pattern of linked marker cotransfer using a hybrid DNA and a recipient of the types:



One can select for the histidine-positive trait and subsequently determine which of the histidine-independent colonies have received the tyrosine trait, which have received the tryptophan trait, and which have received both the tyrosine and tryptophan trait simultaneously. If only one histidine-containing strand is used, the frequency of tyrosine cotransfer will be a measure of the linkage of tyrosine to histidine in that strand. Similarly, the frequency of cotransfer of the tryptophan trait will be a measure of the linkage of tryptophan to histidine

TABLE 1

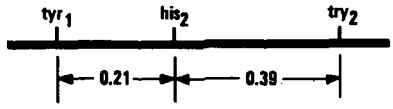
List of hybrid duplexes prepared

Genotype	Strand designation	μg mixed	Total DNA/ml (μg/ml)	Maximum total duplex* (μg/ml)
tyr_1+ his_2+ try_2-	302L	5.300		
			4.9	0.078
tyr_1- his_2+ try_2+	303H	0.043		
tyr_1+ his_2+ try_2-	302H	9.000		
			15.2	14.000
tyr_1- his_2+ try_2+	303L	7.700		
tyr_1+ his_2+ try_2-	302L	6.400		
			12.4	11.600
tyr_1+ his_2+ try_2-	302H	7.200		
tyr_1- his_2+ try_2+	303L	7.700		
			7.0	.095
tyr_1- his_2+ try_2+	303H	.052		
tyr_1+ his_2+ try_2+	wt L	0.160		
			4.3	0.440
tyr_1- his_2+ try_2-	301H	2.900		
tyr_1+ his_2+ try_2+	wt H	3.500		
			3.9	0.190
tyr_1- his_2+ try_2-	301L	.110		
tyr_1+ his_2+ try_2+	wt L	0.160		
			2.5	0.360
tyr_1+ his_2+ try_2+	wt H	2.100		
tyr_1- his_2+ try_2-	301L	.160		
			6.7	0.290
tyr_1- his_2+ try_2-	301H	7.230		
tyr_1+ his_2+ try_2-	302L	6.400		
			16.0	12.800
tyr_1- his_2+ try_2-	301H	9.600		
tyr_1+ his_2+ try_2-	302L	6.200		
			19.8	15.500
tyr_1+ his_2+ try_2+	wt H	9.600		
tyr_1+ his_2+ try_2+	wt L	.160		
			0.20	0.080
tyr_1- his_2+ try_2+	303H	.040		
tyr_1+ his_2+ try_2+	wt L	0.190		
			6.7	0.420
tyr_1+ his_2+ try_2-	302H	5.800		

* Based on stoichiometry only. This figure indicates only the maximum amount of duplex which is possible, and not that which is actually present.

TABLE 2

Comparison of linkage characteristics of native, renatured, and resolved-renatured DNA



DNA	% Cotransfer			
	$\frac{tyr^+}{his^+}$	$\frac{try^+}{his^+}$	$\frac{try^+tyr^+}{his^+}$ (Observed doubles)	$\frac{tyr^+}{his^+} \times \frac{try^+}{his^+}$ (Expected doubles)
Native	0.88	0.76	0.68	0.67
Renatured	0.86	0.59	0.52	0.51
Resolved-Renatured	0.76	0.33	0.25	0.25

Competent cells were exposed to the various DNA samples for 30 min. Deoxyribonuclease was added and the cells plated on minimal medium containing tyrosine and tryptophan. The resulting histidine-positive colonies were toothpicked to minimal medium containing only tyrosine or tryptophan. At least 200 histidine-positive colonies were analyzed.

in the other strand. Finally, the method of clonal analysis demands that if both the tryptophan and tyrosine traits are found in the same histidine-positive clone, then the histidine regions in both strands must have been incorporated by the recipient (see below). Such clones should, in fact, be mixed clones of genotypes *his*$^+$*tyr*$^+$ and *his*$^+$*try*$^+$. At first glance one would not expect to find the genotype *his*$^+$*tyr*$^+$*try*$^+$ because of early segregation. This aspect will be discussed in a later section.

The experimental procedure involved exposure of a histidine-tyrosine-tryptophan-requiring recipient (SB 100) population to the various hybrid duplexes for 30 minutes. The cells were then diluted and plated on a supplemented minimal medium containing tyrosine and tryptophan. Histidine-independent colonies were toothpicked to tyrosine-supplemented minimal medium to test for tryptophan independence, to tryptophan-supplemented minimal medium to test for tyrosine independence, and to minimal medium to test for either double transformants (growth), or mixed clones (no growth). The possible growth responses of the *his*$^+$ colonies are shown below:

	Plating medium		
	Minimal +tyrosine	Minimal +tryptophan	Minimal
Double transformants	+	+	+
Mixed clones	+	+	−

The results of Table 3, columns 1 and 2, clearly support the contention that the predominant mechanism of transformation involves only one strand of an entering duplex. It is evident that markers which are *trans* to one another in a heteroduplex appear simultaneously in a *his*$^+$ colony with a frequency less than 0.7% (columns 1 and 2). For the case of 618 histidine-positive colonies tested, for example, (column 1, experiment 1), none was a double transformant and only four

TABLE 3

Transformation by hybrid duplexes

Column		1	2	3	4	5	6	7
DNA type		302L: 303H	302H: 303L	302L: 302H	303L: 303H	wt L: 301H	wt H: 301L	wt H: wt L
DNA configuration*		ty+ L H tr+	tr+ L H ty+	ty+ L H ty+	tr+ L H tr+	ty+ L H	L H ty+ tr+	ty+ L H tr+
his+ colonies tested	expt 1	618	645	118	118	304	396	72
	expt 2	240	—	232	—	239	241	239
Tyr+	expt 1	0.37	0.41	0.74	<0.0085	0.35	0.18	0.76
	expt 2	0.37	—	0.78	—	0.33	0.17	0.79
Try+	expt 1	0.20	0.15	<0.0085	0.41	0.12	0.061	0.33
	expt 2	0.21	—	<0.0043	—	0.14	0.083	0.31
Try+Tyr+	expt 1	<0.0016** (0.074)	0.0016(0.062)	<0.0085	<0.0085	0.076*** (0.042)	0.045(0.011)	0.25(0.25)
	expt 2	<0.0042 (0.078)	—	<0.0043	—	0.092(0.046)	0.058(0.014)	0.21(0.24)
Mixed clones	expt 1	0.0065(0.074)	0.0032(0.062)	<0.0085	<0.0085	<0.0033	<0.0025	0.014
	expt 2	<0.0042 (0.078)	—	<0.0043	—	<0.0042	<0.0041	<0.0042

Column		8	9	10	11	12	13
DNA type		301L: 301H	Native	302L: 301H	302L: wt H	wt L 303H	wt L: 302H
DNA configuration*		L H	ty+ L H ty+ tr+ tr+	ty+ L H	ty+ L H ty+ tr+	ty+ ty+ L H tr+ tr+	ty+ L H ty+ tr+
his+ colonies tested	expt 1	.83	238	240	241	180	343
	expt 2	—	219	—	—	—	—
Tyr+	expt 1	<0.012	0.87	0.32	0.73	0.32	0.65
	expt 2	—	0.88	—	—	—	—
Try+	expt 1	<0.012	0.86	<0.0042	0.17	0.27	0.17
	expt 2	—	0.76	—	—	—	—
Try+Tyr+	expt 1	<0.012	0.74(0.75)	<0.0042	0.13(0.12)	0.10(0.086)	0.11(0.11)
	expt 2	—	0.68(0.67)	—	—	—	—
Mixed clones	expt 1	<0.012	0.0084	<0.0042	<0.0042	<0.0056	<0.0029
	expt 2	—	—	—	—	—	—

Competent cells were exposed to the various DNA samples for 30 min. Deoxyribonuclease was added and the cells plated on minimal medium containing tyrosine and tryptophan. The resulting histidine-positive colonies were toothpicked to minimal medium, minimal medium containing tyrosine, or to minimal medium containing tryptophan.

* Corresponding negative traits not shown; his_2+ marker not shown.

** Indicates no colonies of this type found of 618 *his*+ colonies tested.

*** Figures in parentheses are expected values obtained by multiplying the frequencies of occurrence of the *try*+ and *tyr*+ traits. For example, in column 5, (0.042) is the product of the frequency of the *tyr*+ trait (0.35) and the frequency of the *try*+ trait (0.12).

were mixed clones. The possibility that these four mixed clones arose from a congressive event, i.e., from a simultaneous penetration of two separate DNA fragments into the same cell, is diminished by the finding that although the amount of DNA in duplex form which was used for the crosses of columns 1 and 2 (Table

3) differed by a factor of 180, the number of mixed clones obtained was similar. Moreover, the concentration of DNA used for the cross of column 2 was in the saturating range of the transformant-concentration response. Assuming then, that the mixed clones arose from a single DNA fragment, one can calculate the frequency with which *his*$^+$ colonies resulted from the insertion of both histidine-containing strands.

An examination of the control crosses (columns 3–13) sufficed to establish that in general, the product *tyr*$^+$/*his*$^+$ × *try*$^+$/*his*$^+$ was identical to the observed number of double transformants. The use of hybrids in which the tryptophan and tyrosine traits were on the same strand resulted in a significant number of *try*$^+$ *tyr*$^+$ double transformants. In this case, however, the product *tyr*$^+$/*his*$^+$ × *try*$^+$/*his*$^+$ was higher than that observed (columns 5 and 6, Table 3). The explanation for this finding probably stems from the presence among the *his*$^+$ colonies of some clones which arose from insertion of that strand which did not contain the *try* and *tyr* markers. The observed frequency of each marker would then be an underestimate, the extent of which would be a function of the relative frequency with which each of the *his*$^+$-containing strands was used.

It is apparent that in most cases (except the crosses of columns 5 and 6, Table 3), the sum of the frequencies for a single trait approximates the frequency of that trait in a homoduplex. This finding indicates that there is no gross effect of the negative and wild-type alleles on each other.

DISCUSSION

The results reported in this communication are in basic agreement with those of BODMER and GANESAN (1964), BRESLER *et al.* (1964), and VESTRI, FELICETTI and LOSTIA (1966) concerning the number of strands used for transformation. The approach described in this report is similar to that of BRESLER *et al.* (1964). In this study however, we have utilized hybrid DNA prepared from the purified, resolved, complementary strands. The data demonstrate that the use of both strands is a rare event. The frequency of this event was obtained from the following consideration:

$$B = \frac{r}{f_{ty_L} \times f_{try_H}} = \frac{(ty^+try^+)/his_t^+}{ty_L^+/his_L^+ \times try_H^+/his_H^+}$$

where B is the frequency of *his*$^+$ colonies which have incorporated both *his*$^+$ containing strands.

r is the *frequency* of mixed colonies, i.e., colonies containing both *his*$^+$*tyr*$^+$*try*$^-$ and *his*$^+$*tyr*$^-$*try*$^+$ cells.

(ty^+try^+) is the *number* of mixed clones.

f_{ty_L} is the frequency with which the tyrosine locus of the L strand is cotransferred with the adjacent his_L locus of the light (L) strand.

f_{try_H} is the frequency with which the tryptophan locus of the heavy (H) strand is cotransferred with the adjacent his_H locus.

his_t^+ is the total number of histidine-independent colonies tested.

his_L^+ is the number of histidine-independent colonies arising from the L strand.

his_H^+ is the number of histidine-independent colonies arising from the H strand.

ty_L^+ is the number of tyrosine-independent colonies arising from the L strand.

try_H^+ is the number of tryptophan-independent colonies arising from the H strand.

This expression is valid only for the duplexes shown in columns 1 and 2, Table 3.

It is evident from this equation that the values of f_{ty} and f_{try}, or their product, are necessary in order to determine B, the frequency of utilization of both strands. Although the precise values for f cannot be determined within the framework of this approach, the observed values for ty_L^+/his_t^+ and try_H^+/his_t^+ (column 1, Table 3) permit one to assign surprisingly narrow limits to the product $f_{ty_L} \times f_{try_H}$. For example, the observation that 37 out of every 100 histidine-positive colonies tested is tyr^+ indicated that a *minimum* of 37 of these his^+ colonies *must* have arisen from the L strand. Similarly, the finding that 20 out of every 100 his^+ colonies tested were try^+ indicated that a *minimum* of 20 of these his^+ colonies *must* have been derived from the H strand. These two experimental observations are sufficient to define the limits of the ratio his_L/his_H. This ratio must vary between 0.37/0.63 and 0.80/0.20 for the case of column 1, Table 3. These limits, in turn, determine the limits of cotransfer, f. If one assumes that 37% of the his^+ colonies were derived from the L strand, then f_{ty_L} must have been 1.0 and f_{try_H} must have been $0.20/0.63 = 0.32$. Similarly, if one supposes that 20% of the his^+ colonies were derived from the H strand, then f_{try_H} must have been 1.0 and f_{ty_L} must have been $0.37/0.80 = 0.46$. The product $f_{ty_L} \times f_{try_H}$ varies, therefore, between 0.32 and 0.46. It can be calculated, however, that this variation is not strictly linear but reaches a minimum of 0.30 when the strands are used with equal frequency, i.e., $his_L^+/his_t^+ = 0.5$. The product actually varies, therefore, between 0.30 and 0.46.

Substituting these values into the equation, one obtains for the case of column 1, Table 3:

$$B = \frac{0.0065}{f_{ty_L} \times f_{try_H}} .$$

For $f_{ty_L} \times f_{try_H} = 0.30$, B = 1.4%; and for $f_{ty_L} \times f_{try_H} = 0.46$, B = 2.2%.

Similar considerations for the case of the mirror-image duplex of column 2, Table 3 yield

$$B = \frac{0.0032}{f_{ty_L} \times f_{try_H}} = 0.66\% \text{ to } 1.39\%$$

when the product $f_{ty_L} \times f_{try_H}$ varies from 0.23 to 0.48.

These values of B mean that of the his^+ colonies resulting from the use of the hybrid duplexes of columns 1 and 2, approximately 2 and 1%, respectively, had received both his^+-containing strands of the donor duplex.

These values of B contraindicate the possibility that the frequency with which both strands of a duplex are used is the product of the frequencies with which each strand is used separately. The frequency of use of the L strand (i.e., his_L^+/his_t^+) (column 1, Table 3) varies between 0.37 and 0.80, and of the H strand correspond-

ingly from 0.63 to 0.20. The products of these frequencies vary therefore from a minimum of 0.16 to a maximum of 0.25, values differing by an order of magnitude from those calculated on the basis of the number of mixed clones. The use of a given strand, therefore, virtually precludes the use of the other strand.

The validity of the foregoing approach depends on a demonstration that the cotransfer of either the *try* or *tyr* traits with the *his* marker is unaffected by the presence of a different allele or by the presence of other genes. The absence of any effect of other genes can be seen from Table 3 in which, for example, a tyr^+ trait in a hybrid duplex appears with about one-half the frequency of tyr^+ in a homoduplex. In columns 1 and 2 the sum of the tyr^+/his^+ frequencies for the mirror-image duplexes is $0.37 + 0.41 = 0.78$, which is in good agreement with a tyr^+/his^+ ratio of 0.74 in column 3. One also may conclude from the data that the *try* trait and the *tyr* trait do not interfere with each other in a transformation event. This is evident from the observation that in general, the product $tyr^+/his^+ \times try^+/his^+$ agrees well with the observed number of double transformants. The evidence suggests therefore that the association of either the *try* trait or the *tyr* trait, respectively, with the *his* trait on a given strand is constant and dependent solely on the probability of a break occurring in the strand during the preparation of the DNA or during the recombination event.

Although one would not expect to find double transformants in the case of the crosses of columns 1 and 2, Table 3, one was found. VESTRI and his coworkers (1966) postulated that this resulted from the duplication of the inserted region followed by a crossover event. This model is not particularly appealing because of the likelihood that the chromosome of *B. subtilis* is circular. Another circumstance which could give rise to a try^+tyr^+ double transformant is that in which one trait is inserted immediately behind a replicating fork and the other traits immediately in front of the fork. Subsequent replication of the fork region would give rise to a homoduplex.

The reason for the low values of cotransfer of the *try* and *tyr* traits when both are on the same strand of a heteroduplex (columns 5 and 6, Table 3) is not clear. The possibility that the aberrant behavior of the wild-type H : 301L and wild-type L : 301H heteroduplexes was a result of extensive degradation of the wild-type DNA was lessened by the finding that the wild-type L : wild-type H duplex (column 7, Table 3), prepared from the same wild-type strands used for the experiments of columns 5 and 6, behaved according to expectation. The possibility remains that the 301 DNA preparation was extensively degraded. This problem is under study.

The presence of cross-linked duplexes in the single-stranded preparations obtained from MAK columns (ROGER, BECKMANN and HOTCHKISS 1966; RUDNER, KARKAS and CHARGAFF 1968) is strongly implied by the finding that the transforming activity of the light strand which is obtained from the MAK column is not increased by self-annealing, and by the observation that this transforming activity elutes from HA in the double-stranded region. The conclusion that the strands are resolved is based on the absence of transforming activity of the self-annealed strands, and on the restoration of transforming activity when comple-

mentary fractions are annealed. The HA-purified single strands have no detectable transforming activity (less than 10^{-5}% of the recipient population under the conditions used in this laboratory). It is recognized by the author that although this observation is compelling, it does not constitute rigorous evidence for a separation of the strands.

The findings reported here indicate that in *B. subtilis* the use of both strands of a donor DNA duplex is a rare event, and one which occurs with a ten-fold lower probability than one might expect from the frequency with which each of the strands is used separately. This finding implies that the use of one strand of a duplex virtually excludes the use of its complement. Recent experiments have indicated that the recombination event for the case of pneumococcus is preceded by conversion of the donor DNA to the single-stranded state (LACKS 1962). This conversion has been demonstrated to result from degradation of one of the strands. A process of this type, if entirely effective, would preclude the occurrence of mixed clones arising from the crosses depicted in columns 1 and 2, Table 3. The observation that mixed clones do result, signifies either that the degradative process leading to the single-stranded state is not entirely effective, and that double-stranded insertion occurs with low frequency, or that the single-stranded condition of the donor DNA is achieved by simple denaturation and that the insertion process itself precludes the use of both strands, with rare exception, in the same region. The reports of DUBNAU (personal communication) stress the conversion of donor DNA to the single-stranded state immediately prior to its insertion into the *B. subtilis* chromosome. The question of how this single-stranded material arises, whether by simple denaturation, or by destruction of one of the strands of each duplex, has still to be resolved for the case of *B. subtilis*.

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SUMMARY

Hybrid duplexes have been prepared from the purified, resolved, complementary DNA strands of *Bacillus subtilis* mutants. An examination has been made of the frequency with which both strands of a hybrid duplex are used in a transformation event by scoring the frequency with which two unselected traits linked in the *trans* position are cotransferred with a third interjected selected trait. It was found that the frequency with which both strands were used in a single event was approximately 1% for the case of one heteroduplex and 2% for the mirror-image heteroduplex. This frequency is well below that expected from the product of the frequencies with which each strand is used separately. The implication of these findings with regard to the process of recombination is discussed.

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