Configuration of Transforming Deoxyribonucleic Acid During Entry into Bacillus subtilis

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ABSTRACT

STRUSS, NORMAN (Yale University, New Haven, Conn.). Configuration of transforming deoxyribonucleic acid during entry into Bacillus subtilis. J. Bacteriol. 89:288-293. 1965.—A correlation was obtained between map distance and the length of the lag period preceding the appearance of pairs of genetic traits after the addition of deoxyribonucleic acid (DNA) to a competent culture of Bacillus subtilis. The results are taken to indicate that DNA enters competent cells in a lengthwise fashion. The smallest length of transforming DNA which may participate in a recombination event, and the number of nucleotide pairs which enter the cell per unit time, have been estimated. The evidence indicates that only part of the lag period is devoted to the transport of DNA into the cell. The significance of these results with respect to the mechanism of entry of DNA into the cell is discussed.

Levine and Strauss (1965) described and characterized, for the case of Bacillus subtilis, a temperature-sensitive delay in the appearance of transformants as a function of duration of contact of deoxyribonucleic acid (DNA) with a competent culture. This delay was evident only when deoxyribonuclease was used to terminate the transformation. Use of a washing and dilution procedure to terminate the transformation abolished the delay and permitted the appearance of transformants from zero time. These findings strongly indicated that the entry of DNA into the cell is initiated immediately when the cells and DNA are mixed, and that the delay period represents a phase of the entry process during which the genetic information is susceptible to attack by deoxyribonuclease. The delay period might be interpreted in either of two ways: it might represent the time necessary for the cell to coil the DNA in such a manner that it can be taken into the cell en masse, or, in the event the DNA molecules are entering the cell in lengthwise fashion, it might represent the time necessary for a fragment to enter the cell which is of sufficient length to function in a recombination event. A logical consequence of the latter model is that, during the process of penetration into the cell, part of the DNA molecule will be protected from attack by deoxyribonuclease. As will be seen, this circumstance can be detected by an examination of the joint appearance of linked genetic traits as a function of time of exposure to transforming principle.

MATERIALS AND METHODS

Organisms. Table 1 indicates the strains of B. subtilis employed. Genetic markers are indicated as follows: tryptophan, try; histidine, his; tyrosine, tyr; aromatic, aro; methyl tryptophan, MT.

Media. Media for the maintenance of stocks and for the transformation procedure were prepared as previously described (Levine and Strauss, 1965), except that appropriate supplements were added to permit the growth of the strains indicated in Table 1.

Transformation procedure. The transformation procedure was as previously described (Levine and Strauss, 1965), except that, after 100 min in medium S-3 at 37 C, the culture was transferred to a water bath (28 C). After 5 min, DNA was added. Samples were removed at intervals to tubes containing deoxyribonuclease and MgSO4, such that the final concentrations of the enzyme and MgSO4 were 50 µg and 5 µmoles per ml, respectively. The transformation was carried out at a lower temperature to improve the resolution of time of appearance of transformants. All recipient populations were sampled just prior to the addition of DNA, so that corrections could be made for revertants in the population. Appropriate controls were included for detection of contamination.

Transformants were detected by inoculation of appropriate selective media. In general, double transformants were detected by spreading cultures on solid minimal media. Whenever possible, the appearance of double transformants also was determined by assay of selected single transformants.
for the unselected second trait. Slightly higher values were obtained by the first technique, although the kinetics were not affected. Single-marker transfers into double mutants were detected by spreading cultures on solid minimal medium supplemented with the appropriate growth factor.

For the case of SB222 (aro\(_{1}\)trp\(_{1}\)) only the double transformant and the aro\(_{1}\) transformant can be assayed readily. The trp\(_{1}\) trait was disregarded in studies employing this mutant.

Preparation of DNA. DNA was prepared as previously described (Levine and Strauss, 1965).

Chemicals. Deoxyribonuclease I, 1 time recrystallized, was obtained from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

If it is postulated that DNA enters the cell in a linear fashion, then the delay in the appearance of transformants as a function of time may be explained as the time necessary for the minimal effective length of DNA to become deoxyribonuclease-insensitive. (The minimal effective length is defined as the smallest length of transforming DNA which can participate in a recombination event.) Moreover, if one observes the simultaneous entry of linked loci, the delay should be a function of the distance between the loci less the minimal effective length. Stated in another manner, the kinetics of appearance of any two linked traits will be characterized by an increase in the length of the lag period, provided the two loci are farther apart than the minimal effective length of DNA.

If the entry of DNA into the cell does not occur in lengthwise fashion, then the delay in appearance of linked traits should be uninfluenced by the degree of linkage of these traits.

Appearance of single markers. Figure 1 shows the kinetics of appearance of a single characteris-

tic as a function of time. Single markers first appeared at approximately 2.5 min after the addition of DNA. Despite the finding that the appearance of traits as a function of time did not conform to any simple mathematical model, the delay was estimated quite reproducibly by extrapolation of the curves to infinite slope on a semilogarithmic plot (Levine and Strauss, 1965). In all cases, cultures were sampled between zero time and 2.5 min, but no transformants were found. These values could not be indicated on a logarithmic scale; 0 transformants correspond in reality to fewer than 1 in 2 \( \times 10^7 \) to 4 \( \times 10^7 \) cells, the total number plated in all cases.

Appearance of linked traits. Figures 1, 2, 3, and 4 depict the kinetics of appearance of four linked pairs of traits, as well as a single trait of each pair. The kinetics are of the same general form regardless of whether one assay a pair simultaneously or assays only one of the pair. In all four instances, single traits appeared after approximately 2.5 min. The only aspect which varied was the length of the delay prior to the appearance of double transformants.

The relationship of the delay in appearance of double transformants to the degree of linkage became evident when the two parameters were compared (Table 2 and Fig. 5). The lag period varied inversely as the degree of linkage. It was also evident that loci which are close together, e.g., try\(_{2}\)his\(_{4}\), appeared at the same time as did single loci. The interpretation of these results will be discussed below. Figure 5 depicts as a function of map distance the relationship of the difference in lag period of the entry of a single trait and a pair of traits. The relationship appeared to be a linear one and revealed, moreover, that the entire lag period is not devoted to the actual transport of DNA.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin</th>
<th>Growth requirement*</th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td>try(_{2})</td>
<td>Burkholder and Giles</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>SB 25</td>
<td>try(<em>{2})his(</em>{4})</td>
<td>Nester</td>
<td>Tryptophan, histidine</td>
</tr>
<tr>
<td>SB 137</td>
<td>aro(<em>{1})his(</em>{4})</td>
<td>Nester</td>
<td>Tyrosine, phenylalanine, tryptophan, PABA, histidine</td>
</tr>
<tr>
<td>SB 188</td>
<td>aro(<em>{1})MT(</em>{8})</td>
<td>Nester</td>
<td>Tyrosine, phenylalanine tryptophan, PABA</td>
</tr>
<tr>
<td>SB 222</td>
<td>tyr(<em>{1})aro(</em>{1})</td>
<td>Nester</td>
<td>Tryptophan, phenylalanine, tyrosine, PABA</td>
</tr>
<tr>
<td>SB 455†</td>
<td>MT(_{8})</td>
<td>Nester</td>
<td>—</td>
</tr>
</tbody>
</table>

* Taken from Nester et al. (1965). PABA = p-aminobenzoic acid.
† Methyl tryptophan-resistant transformants secrete tryptophan and were assayed by transferring colonies to try\(_{1}\) mutant overlays. MT\(_{8}\) colonies were detected by satellite growth of the overlay. This procedure was necessary because MT\(_{8}\) strains grow in the presence of methyl tryptophan, albeit slowly (Nester, personal communication).
DISCUSSION

The conclusion that transforming principle attains insensitivity to deoxyribonuclease in lengthwise fashion is based on the observation that an increased period of time is necessary for the joint emergence of linked genetic traits as compared to the appearance of single markers. The length of this lag period appears to be a function of the distance between the loci. These results rule out the possibility that the DNA molecule attains insensitivity to deoxyribonuclease as a mass. Moreover, it may be assumed that there is a cell boundary which is impermeable to deoxyribonuclease, and through which DNA passes in lengthwise fashion. The nature of this boundary is still a matter of conjecture at present. The two most obvious possibilities are either the cell wall or the cell membrane. A third possibility is provided by recent investigations on the relationship of the mesosome of *B. subtilis* to transformability (Miller and Landman, 1961; Landman, Ryter, and Knott, 1964). It may well be that DNA enters the mesosome prior to passage across the membrane, and that the penetration of the DNA into the mesosome confers deoxyribonuclease insensitivity.

On the basis of the results described above, it is possible to consider in some detail the nature of the lag period which characterizes the entry...
of single genes. It appears that at least 1.3 min of this lag period is devoted to the passage of DNA into the cell (Fig. 5). Therefore, the activation energy for the lag period reported in the previous paper (Levine and Strauss, 1965) need not necessarily represent the activation energy for the passage of DNA into the deoxyribonuclease-insensitive state, but might be the activation energy for some process represented by the period preceding the start of entry of the DNA. The activation energy for the transport process per se can be obtained by observing the effect of temperature on the difference in the lag for a pair

![Graph](image)

**Fig. 3.** Kinetics of appearance of single and moderately linked traits. Wild-type DNA was used to transform SB822 (tyr<sup>-</sup>aro<sup>-</sup>). Arrows indicate times at which no transformants could be detected. The tyr<sup>-</sup>aro<sup>-</sup> transformants were enumerated on minimal medium. The ara<sup>-</sup> transformants were enumerated on tyrosine-containing minimal medium.

![Graph](image)

**Fig. 4.** Kinetics of appearance of single and loosely linked traits. SB455 (MT<sup>+</sup>) DNA was used to transform SB188 (MT<sup>+</sup> are<sup>-</sup>). Arrows indicate times at which no transformants could be detected. The arrow at 3 min indicates that no double transformants were detectable. The ara<sup>-</sup> transformants were assayed on minimal medium. The ara<sup>-</sup> MT<sup>+</sup> transformants were assayed by transferring colonies from the minimal medium to plates containing a lawn of tryptophan-requiring mutants (see Table 1).

of traits and a single marker of that pair. These experiments are now in process.

The finding that the appearance of tyr<sup>+</sup>his<sup>+</sup> transformants occurs at the same time as his<sup>+</sup> and single markers in general suggests that the minimal effective length of DNA is at least as long as the distance between the outer boundaries of these two loci. Nester, Schaffer, and Lederberg (1963b) and Ephrati-Elizur, Srinivasan, and Zamenhof (1965) suggested that there are at least three other genetic loci (try<sub>1</sub>, his<sub>1</sub>, and try<sub>3</sub>) between the try<sub>2</sub> and his<sub>2</sub> regions. Assuming that the average length of a cistron is 1,000 nucleotide pairs, the minimal effective length would be at least 5,000 nucleotide pairs in length, representing 3 × 10<sup>4</sup> daltons, a figure in good agreement with the results of Litt et al. (1958) and Rosenberg, Sirotnak, and Cavalieri (1959) for the pneumococcal system. These workers determined...
Table 2. Relationship between degree of linkage and the lag period

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Characters selected for</th>
<th>Lag (min)</th>
<th>Difference in lag (double minus single)</th>
<th>Map distance*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Expt 1</td>
<td>Expt 2</td>
<td>Expt 1</td>
</tr>
<tr>
<td>SB 25</td>
<td>his\textsuperscript{−}try\textsuperscript{−}</td>
<td>his\textsuperscript{+}</td>
<td>2.4</td>
<td>2.6</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>try\textsuperscript{−}</td>
<td>2.5</td>
<td>2.6</td>
<td>2.5</td>
</tr>
<tr>
<td>SB 222</td>
<td>his\textsuperscript{−}aro\textsuperscript{−}</td>
<td>his\textsuperscript{+}</td>
<td>3.0</td>
<td>3.1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aro\textsuperscript{+}</td>
<td>2.5</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>SB 188</td>
<td>MT\textsuperscript{−}aro\textsuperscript{−}</td>
<td>aro\textsuperscript{+}</td>
<td>2.4</td>
<td>2.4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MT\textsuperscript{−}aro\textsuperscript{−}</td>
<td>3.8</td>
<td>3.8</td>
<td></td>
</tr>
</tbody>
</table>

* Map distance values taken from Nester et al. (1963b).
† Values in parentheses are taken from confirmatory experiments carried out in this laboratory.

They did establish, however, that these two loci included at least 13 cistrons and were the farthest apart of any linked loci yet observed in Bacillus subtilis.

Fig. 5. The relationship between map distance and time of entry for double markers. Data were taken from Table 2. The ordinate is the difference in time of entry of double markers and a single marker of each pair. DNA was added at -2.5 min. Single markers and the try\textsuperscript{−}his\textsuperscript{−} pair appear at zero time. The dotted line indicates the time at which the DNA commences entry.
This model not only explains the lag period, but also the initial nonlinearity of the kinetics curve that is obtained as a result of the use of deoxyribonuclease to terminate the transformation (Levine and Strauss, 1965). The nonlinear kinetics may be explained as a resultant of three factors: (i) the increasing probability with time that the length of DNA which has entered the cell carries the marker; (ii) the increased efficiency of DNA in the recombinational event as a function of the length of DNA which has entered the cell (Iyer and Ravin, 1962); and (iii) heterogeneity of the cell population in respect to the rapidity with which the process is carried out.

It is to be emphasized that the time of entry studies with pairs of markers are not analogous to time of entry studies involving chromosomal transfer in Escherichia coli. Assuming that DNA fragments exhibit no directional preference in their entry into the cell, one is measuring the time of entry of a genetic region into a cell without regard for the direction in which this entry occurs. If one considers the hypothetical linked markers A and B, the following molecular species containing both markers are possible: (1) A—B—, (2) B—A—, and (3) →B—A→. In any population of cell-DNA complexes, there will be present some cells which take up species 1, species 2, or both species 1 and 2 in the directions indicated. The lag period then represents the time necessary for the entry of both the A and B loci, regardless of the order in which it occurs, and presumably involves only molecular species of type 1 and 2 in which one of the loci is located at the end of the molecule. Again, based on the assumption that one cistron corresponds to 1,000 nucleotide pairs, it can be calculated that the ingress of DNA occurs at a rate of about 55 nucleotide pairs per second, a rate considerably slower by more than an order of magnitude than that for the transfer of nucleotide pairs during conjugation in E. coli.

The contribution of double transformants resulting from the simultaneous encounter of a cell with two molecules of DNA, one of which carries only the B locus, the other only the A locus, can be assumed to be negligible.

The significance of these findings in terms of genetic mapping remains to be assessed. The resolution with respect to time is not yet as exact as one would desire. Attempts are being made to refine the resolution and to extend the mapping studies to other genetic regions.

Acknowledgments

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Literature Cited


