Transforming Ability of Bacterial Deoxyribonucleic Acid in Relation to the Marker Efficiencies in Diplococcus pneumoniae During Thymidine Starvation

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Thymidine starvation induces a decrease in transforming activity of pneumococcus deoxyribonucleic acid. The integration of low- and high-efficiency markers seems to be equally affected.

It has been already shown that thymineless death of thymine-requiring strains of Escherichia coli is the result of two processes: the induction of episomes during starvation and a base-line lethal effect (8, 14). Unfortunately, despite many attempts to establish the precise nature of this last effect, no definite conclusions have been reached. However, certain experiments suggest that it must be due to some changes in the deoxyribonucleic acid (DNA) molecule of starved cells. It has been found that the transforming ability of DNA from starved cells is lower than that of DNA from unstarved cells, as if the integrity of the molecule has been destroyed during starvation (13). Since the uptake of DNA from starved cells is normal (15), the decrease of transforming ability of bacterial DNA after thymine starvation could be related to a failure in the recombination process or single-strand breaks (1) caused by a nuclease induced during starvation. It has also been reported that the ability of the DNA to serve as a template for ribonucleic acid polymerase decreases during starvation (7, 11), but the role of prophage induction by thymine starvation in such strains has not yet been eliminated.

In this report, we describe the selection of a thymidine-requiring transformable strain of Diplococcus pneumoniae, which does not contain any phage particle as observed by electron microscopy (Lapchine, personal communication). In that strain, moreover, two classes of markers are found, which differ with respect to their recombination processes (3, 6). We have therefore studied the transforming activity of DNA derived from starved and unstarved cultures of thymidine-requiring D. pneumoniae by following the behavior of two markers that differ in their efficiencies in transformation.

MATERIALS AND METHODS

Original strains. The wild-type strain Cl3 is a clone of Avery R36A strain of D. pneumoniae. Its physiological properties and biological requirements have been previously described (12).

The thymidine-requiring strain (Td') was generously provided by A. Ravin. It requires 40 μg of the thymidine per ml in complete medium. Thymidine cannot be replaced by thymine. This requirement is due to a lack of thymidylate synthetase activity (L. R. Friedman, Ph.D. Thesis, University of Rochester, 1970). That strain is resistant to 8 × 10^{-8} M aminopterin and carries two mutations: one is a mutation at the amiA locus since the strain is sensitive to an imbalance in the relative concentration of isoleucine, leucine, and valine and is resistant to 2 × 10^{-8} M aminopterin. Such properties are specific to amiA mutants (12). The mutation is very close to the site amiA-r24 of the amiA map (4), and the transforming efficiency of the mutant is low (0.13); we refer to this marker below as amiA-r124. The other mutation is a Td' marker which also confers a resistance to 2 × 10^{-8} M aminopterin since after transformation of the parental thymidine-requiring strain by the wild-type DNA we could select in the synthetic medium supplemented with an excess of isoleucine another strain still requiring 40 μg of thymidine per ml and resistant to 2 × 10^{-8} M aminopterin (unpublished data).

Selection of Cl3 Td' mutant. To deal with isogenic strains, we transferred the Td' marker into our standard wild-type strain Cl3. Competent cells of Cl3 were treated with DNA extracted from the Td' mutant. The bacteria were plated in a nonselective medium for complete phenotypic expression. After 2.5 hr of incubation at 37°C, a second layer of nutrient agar containing 10^{-5} M aminopterin was poured on the surface, and the plates were incubated overnight. We isolated the aminopterin-resistant colonies.
They all require 60 μg of thymidine per ml for optimal growth in complete medium and 30 μg/ml in synthetic medium. No growth is observed in a medium supplemented with as much as 1 mg of thymine per ml; even with addition of all four deoxyribonucleotides together or separately, thymine does not seem to enter the cells since there is no uptake of radioactive material when thymine is labeled (L. R. Friedman, Ph.D. Thesis, University of Rochester, 1970).

One mutant called Cl 3 Td' was used in the experiments presented in that report. As further proof that only the Td' marker was introduced into the parental strain and controls both thymidine requirement and resistance to 10^{-8} M aminopterin, we transformed Cl 3 Td' strain with a wild-type DNA and selected for Td' colonies in complete medium without thymidine. The relative efficiency of such transformation compared to the str-r41 marker (0.12) is indeed identical to the reciprocal transformation of wild-type cells with Cl 3 Td' DNA (0.13).

**Starvation experiments.** Cells of Cl 3 Td' amiA-rl24 str-r41 are grown in a peptone-yeast extract medium (12) supplemented with thymidine (60 μg/ml) at 37°C. The cells are collected during logarithmic phase (2 × 10^9 cells/ml) by centrifugation of the culture. The pellet is washed and resuspended at 37°C in the same medium without thymidine. Viable count is followed during 6 hr by plating every half an hour a dilution of the culture on a blood-agar medium supplemented with thymidine (60 μg/ml; reference 12).

**DNA preparations.** To obtain the DNA from starved and unstarved cells, 200 ml of the culture is taken off every 2 hr and centrifuged. The pellet is resuspended in 1 ml of a 0.15 M NaCl, 0.015 M sodium citrate solution at pH 7 and lysed with 0.1 ml of a 1% sodium deoxycholate solution. Two parts are then made for each lysate. One is dialyzed against a 10 times diluted solution of saline citrate to eliminate the deoxycholate which is an inhibitor of transformation; the other is treated with alcohol to eliminate free deoxynucleotides. The amount of DNA is determined in both parts by the method of Burton. The lysates are kept frozen in a 10% saline citrate solution.

**Transformation experiments.** Techniques of transformation are described elsewhere (12). Competent cells (5 ml) are treated with a solution of transforming DNA (0.1 μg/ml) for 60 min. The bacteria were plated in a nonselective medium for complete phenotypic expression. After 2 hr at 37°C, a second layer of nutrient agar containing streptomycin (200 μg/ml) or aminopterin (final concentration 10^{-4} M) was poured on the surface for selection. The plates were incubated at 37°C overnight. The number of resistant colonies was then counted.

**RESULTS**

The crude and alcohol-treated preparations of DNA from thymidine-starved and -unstarved cells were tested for their transforming ability on the wild-type strain Cl 3 with respect to the markers amiA-rl24 and str-r41.

The effect of thymidine starvation on the viability of the cells is shown in Fig. 1. The cell viability decreases by 90 to 95% after 6 hr of starvation. The rate of the decrease is maximum during the first 3 hr and levels off thereafter, which indicates that no episome is induced. Indeed, when a strain is carrying an inducible episome, the final level of surviving cells is much lower (14). The total amount of DNA per milliliter does not change significantly as starvation progresses. However, after 6 hr of starvation the transforming ability of the DNA from starved cells is reduced to 15% as compared to the transforming ability of the DNA from unstarved cells. The crude lysates and the alcohol treated preparations behave similarly. The decrease observed for the number of transformants for each marker is the same. The relative efficiency for aminopterin and streptomycin markers has been calculated (Table 1). No variations appear in this ratio after 4 or 6 hr of starvation.

**DISCUSSION**

As previously reported in *Bacillus subtilis* (13) and recently confirmed (10), our results show that the biological activity of DNA decreases during starvation in *D. pneumoniae*. Therefore, it is likely that the decrease in biological activity is independent of episome induction although it cannot be excluded that our strain of *D. pneumoniae* contains an episome.
Studying the penetration of thymine-starved *B. subtilis* DNA in recipient cells during transformation, we have already shown that its uptake is the same as for DNA from unstarved cells (15). If we assume that the decrease in the transforming ability of the thymidine-starved pneumococcus DNA is not due to a lack of penetration, we can suppose that it is in fact related to one of the steps involved in the integration process. A genetic study of two linked markers in *B. subtilis* has shown that the transfer frequency of both markers simultaneously is not modified during starvation (13); this suggests that the existence of single-strand breaks caused by a nuclease is not the only support of the decrease in the transforming efficiency. We were able to follow the fate of DNA from starved and unstarved cells in recombination by comparing the integration of two markers of different transformation efficiencies in the wild-type strain. That strain integrates markers in two different ways: the marker is integrated in one strand of the DNA (high efficiency) or the complete integration requires also the excision and repair of the opposite strand (low efficiency). The ratio between the number of transformants for low-efficiency marker to the number of transformants for high-efficiency marker is usually 0.1 (4). Since the relative efficiency between aminopterin (LE) and streptomycin (HE) markers is not modified, the integration of low- and high-efficiency markers seems to be equally affected. It is possible that DNA from starved cells does not take part in the integration process of transformation.

The kinds of changes which occur to DNA in starved cells and prevent it from serving as transforming DNA are not actually known. However, evidence has been given for nonconservative replication of DNA from starved cells once thymidine is added to the medium, as if some unrepaird breakage occurred in the DNA during starvation (9). More recently, single-strand breaks have been effectively found (5). The rate of single-strand breaks observed for an *E. coli* episome is 0.029 per 74 × 10⁶ dalton per min at 37 C. Assuming the rate is the same for pneumococcus chromosome, 1.4 breaks/10⁷ dalton could occur after 6 hr of starvation. As the average length of the integrated DNA fragment in pneumococcus is about 10⁴ (2), such breaks do not seem to account for 85% loss of biological activity. However, in the absence of direct measurement of single-strand molecular weight of DNA from starved cells, no definite conclusion can be drawn.

In summary, we could confirm that in a thymidineless strain apparently free of any detectable episome, starvation induces changes in the properties of its DNA. The corresponding changes in the molecule have not yet been elucidated. They seem to prevent it from undergoing normal integration into the DNA of the recipient cell.

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**LITERATURE CITED**


