REQUIREMENTS FOR TRANSFORMATION IN *BACILLUS SUBTILIS*[1]

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The finding (Spizizen, 1958, 1959) that a number of auxotrophic mutants of *Bacillus subtilis* could be transformed to prototrophy has provided a simple system for the study of transformation of biochemical characters. This is made possible by the fact that *B. subtilis* can be grown in simple chemically defined media. Nutritional and other biochemical markers can thus be readily employed. In addition, it is possible to define the nutritional conditions required in the transformation process itself.

It is the purpose of the present investigation to present data on the requirements for transformation in *B. subtilis* strain 168, indole requiring, which is the most highly transformable strain so far examined. These studies were made with cells in the vegetative stages of growth rather than with germinating spores as previously employed (Spizizen, 1958) since the latter require complex factors. Vegetative cells were found to become optimally competent for transformation when grown in simple media and at a particular stage of the growth cycle. In addition to glucose and an ammonium salt as carbon and nitrogen sources, the requirements for competency in strain 168 include indole or L-tryptophan and a chelator for cupric ions.

MATERIALS AND METHODS

Isolation of deoxyribonucleic acid (DNA). DNA was isolated from wild-type organisms, such as *B. subtilis* strain 23 or from prototrophic transformants. Organisms were grown for 12 to 16 hr at 37 C in flasks on a rotary shaker in a medium containing 1% lactate, 0.25% yeast extract (Difco), and minimal components.[4] The cells harvested by centrifugation at 4 C were resuspended at $\frac{1}{10}$ to $\frac{1}{20}$ volume in a 20% sucrose, 0.1 M sodium chloride, and 0.05 M sodium citrate solution. The suspension was warmed to 37 C and lysozyme (crystalline, egg-white, Armour) added to give a concentration of 50 μg per ml. Incubation at 37 C with gentle agitation for 30 min was followed by a second addition of 50 μg per ml lysozyme. Complete conversion of cells to protoplasts was usually obtained after 20 min at 37 C as observed by phase-contrast microscopy. Sometimes when the density of cells was low, rapid protoplast formation could be obtained with only one addition of lysozyme. A reduction in flow birefringence and an increase in viscosity of the suspension accompanies the formation of protoplasts.

The protoplasts were centrifuged at 18,000 $\times$ *g* for 30 min at 0 to 4 C. The supernatant liquid was decanted and the sediment suspended to the original volume in cold 0.1 M sodium chloride solution containing 0.05 M sodium citrate. This treatment disrupted the protoplasts without extracting DNA. The suspension was again centrifuged at 18,000 $\times$ *g* for 30 min, and the supernatant fluid discarded. The sediment was then suspended to the original volume in cold 2 M sodium chloride solution by means of a loose fitting Ten Broeck homogenizer. The viscous suspension was gently agitated by means of a magnetic stirrer at 4 C for 1 to 2 hr. It was then centrifuged at 15,000 $\times$ *g* at 0 to 4 C for 20 min. The supernatant fluid was saved for DNA precipitation. The sediment was again suspended in 2 M sodium chloride solution and

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[4] The composition of the minimal medium employed in our studies is: ammonium sulfate, 0.2%; dipotassium phosphate, 1.4%; monopotassium phosphate, 0.6%; sodium citrate·$2H_2O$, 0.1%; magnesium sulfate·$7H_2O$, 0.02%. When glucose was employed as a carbon source, it was added aseptically to a final concentration of 0.5%.

held at 4 C overnight. It was again centrifuged at 15,000 × *g* and the supernatant saved for DNA precipitation. The sediment may be extracted 3 to 4 times.

The supernatant fluids from the 2 M sodium chloride extracts (one volume) were slowly added to cold 95% ethanol (at least two volumes) and the fibers of DNA-protein removed with hooked glass rods. The fibers were dried on filter paper and suspended in small volumes of 2 M sodium chloride with the aid of a Ten Broeck homogenizer. After 24 hr at 4 C, the insoluble protein was removed by centrifugation, the supernatant liquid was warmed to 37 C and $\frac{1}{20}$ volume of 2% sodium deoxycholate solution added to remove protein. After 1 hr at 37 C the suspension was placed at 4 C overnight, centrifuged to remove insoluble protein and some deoxycholate and the DNA precipitated with two volumes of cold 95% ethanol. The DNA fibers were suspended in 2 M NaCl and the sodium deoxycholate treatment repeated. After alcohol precipitation and resuspension in 2 M NaCl, little or no protein could be detected by the Lowry method (Lowry et al., 1951). Ribonucleic acid (RNA) was removed by treatment for 2 hr at 37 C with 50 μg per ml of ribonuclease followed by alcohol precipitation.

DNA was determined by the colorimetric diphenylamine method of Burton (1956) using a thymus DNA preparation as standard, and RNA by the orcinol method (Ashwell, 1957) using D-ribose as standard.

Cell growth for transformation. Unless otherwise specified, the following conditions were employed in the tests:

B. subtilis strain 168 (Spizizen, 1958) was grown on tryptose blood agar base (Difco) for about 16 hr at 37 C and transferred to liquid minimal medium containing 0.5% glucose, 50 μg per ml L-tryptophan, and 0.02% casein hydrolyzate acid. The inoculum was at least 1 × 10^8 cells per ml. The cell suspensions were placed in tubes with 17 mm inner diameter, the total volume of culture per tube being not more than 2.5 ml. The tubes were slanted on a rack and shaken on a reciprocal shaker for 4 hr at 37 C. The cells were next centrifuged at 3,500 rev/min in a Servall type SP angle centrifuge for 5 min and diluted 10-fold in fresh minimal medium containing 0.5% glucose, 5 μg per ml L-tryptophan, 0.01% casein hydrolyzate, and an additional amount of $MgSO_4$ (5 μmoles per ml). The total volume of culture per tube during this period was 1.0 ml. Tubes of the same size as above and the same shaking device were used. DNA from wild-type strains was usually added at the beginning of this period and incubation at 37 C (with shaking) continued for 90 min.

Assay for transformants. The transformation mixture was diluted in minimal medium and plated on minimal glucose agar plates. After 24 hr incubation at 37 C the number of colonies were counted. The total number of viable cells was determined by plating with added L-tryptophan (0.05 ml of 2 mg per ml L-tryptophan per plate). When small numbers of transformants were formed the transformation mixture was first centrifuged to remove the small amount of L-tryptophan. Casein hydrolyzate was sometimes added to the minimal plates to speed the growth of the colonies.

RESULTS

Requirement for L-tryptophan (or indole) and chelator. Our initial studies (Spizizen, 1958) had indicated that conditions for optimal transformation of *B. subtilis* strain 168 to prototrophy could be achieved by using recipient cells grown in a minimal medium containing yeast extract. Two periods of growth were employed under aerobic conditions at 37 C. The first period was for 4 hr in the absence of transforming DNA. The cells were then centrifuged and resuspended in 10 times the volume of fresh medium with DNA added for a 90-min period of growth at 37 C.

It was found that the yeast extract could be replaced by indole or L-tryptophan and acid casein hydrolyzate. The requirement for L-tryptophan during the 4-hr growth period is shown in Table 1. It is seen that at least 5 μg L-tryptophan are required for optimal transformation to occur.

Casein hydrolyzate seemed to be a specific re-

TABLE 1

L-Tryptophan requirement for competence

L-Tryptophan*	% Transformation to Prototrophy
μg/ml	*0.8 μg/ml DNA*
0	0
2	1.4
5	2.6
20	2.2

* Addition to minimal glucose medium containing 0.02% acid casein hydrolyzate during the 4-hr growth period (see Materials and Methods).

TABLE 2

Compounds active in competence

Addition to Minimal Medium + 5 μg L-Tryptophan per Ml*	% Transformation to Prototrophy
20 μg/ml	*8 μg/ml DNA*
None	0.01
L-Histidine	0.55
D-Histidine	0.57
Histamine	0.64
EDTA,† Na	0.32
Histidyl-histidine	0.82
Carnosine	0.32
Histidine methyl ester	0.39
2,2′-Bipyridyl	1.66
β-Mercaptoethanol	0.72

* These compounds replace the casein hydrolyzate during the 90-min period. For other details, see Materials and Methods.

† EDTA = ethylenediaminetetraacetic acid.

quirement for the last stages of competence (during the 90-min period). (The term "competence" is used to denote susceptibility of the population of cells to transformation.) It was found that this effect was due to the histidine content of the mixture. Several histidine derivatives were tested and those possessing free amino groups were able to replace histidine (histidine-methylester, histidyl-histidine, carnosine, histamine). Moreover, D-histidine exhibited the same activity. It then became apparent that the histidine effect was related to the chelating power of the compound. All chelators that form complexes of high stability with heavy metals were found subsequently to be active in promoting competence: 2,2′-bipyridyl, β-mercaptoethanol, ethylenediamine, ethylenediaminetetraacetate (EDTA) (Table 2). All the metal chelates of EDTA, the stability constants of which are lower than that of EDTA-Cu^{++}, were also active (chelates of Mg^{++}, Ca^{++}, Mn^{++}, Fe^{++}, Co^{++}, Cd^{++}, Zn^{++}). (The EDTA-chelates used were obtained from Geigy Industrial Chemicals, Ardsley, New York.) EDTA-Cu^{++} and EDTA-Ni^{++} (with a stability constant higher than EDTA-Cu^{++}) were inactive. EDTA-Na-Fe^{+++} was active although its stability constant is higher than that of the Cu^{++} chelate but it is possible that the state of the iron may have been modified under the conditions of our experiments. The results suggest that the action of these compounds on competence could be explained as a complexing effect on cupric ions and it was shown that indeed cupric compounds inhibited the development of competence and that this effect was counteracted by the chelators (Table 3).

The time of the addition of the chelator to the medium appears to be rather critical. It was found that optimal competence occurred when the compound was added during the first half of the 90-min period and its presence maintained for about 45 min.

During the first stages of competence (4-hr period) the effect of amino acids appears to be mainly a stimulating one on the growth of the

TABLE 3

Cupric ion inhibition of competence and chelator enhancement

Addition to Minimal Medium + 5 μg L-Tryptophan per Ml*	% Transformation to Prototrophy
	8 μg/ml DNA
None	0.05
Casein hydrolyzate, 0.01%	0.24
L-Histidine, 50 μg/ml	0.24
$CuSO_4$, 10^{-5} M	0.005
L-Histidine, 50 μg/ml + $CuSO_4$, 10^{-5} M	0.23
Fe^{+++}Na EDTA,† 10^{-4} M + $CuSO_4$, 10^{-5} M	0.20

* During the 90-min period. Other details as described in Materials and Methods.

† EDTA = ethylenediaminetetraacetic acid.

TABLE 4

Transformation of Bacillus subtilis strain 168 at different times following 4-hr growth in minimal transformation medium

DNA Addition* (Min after 4-Hr Growth Period)	% Transformation to Prototrophy
0	0.11
15	0.15
30	0.36
45	1.64
60	1.77
75	2.5
90	2.0
105	1.8
120	1.1

* DNA (8 μg/ml) added for 15 min during the second growth phase (see Materials and Methods). DNA uptake terminated by deoxyribonuclease (see text).

organism. Approximately 0.02% solution of casein hydrolyzate was found to be optimal for the rapid appearance of competence. Higher concentrations were found to be inhibitory (probably due to the synthesis of excess cell-wall components). The absence of amino acids during the 4-hr period results in delay of the appearance of competence.

Growth studies on the appearance of competence. Growth of *B. subtilis* strain 168 in glucose minimal medium containing 5 to 50 μg per ml of L-tryptophan and 0.02% acid hydrolyzed casein provides optimal conditions for the development of competence. It was found that the cells became highly transformable when wild-type DNA was added after 5 hr or longer under these growth conditions.

The cells were grown as outlined in Materials and Methods. When DNA from a wild-type donor strain was added to cells after the initial 4-hr growth period for 15-min intervals, the results of Table 4 were obtained. The DNA action was terminated by the addition of 50 μg of crystalline deoxyribonuclease (DNAase, 1 × crystallized, Worthington) and 5 μmoles per ml magnesium sulfate. The number of transformants was determined by the colony count on glucose minimal agar after 24 hr of incubation following plating. The transformation was relatively low during the first 45 min of the second growth period, but became optimal after 60 min. The cells retained optimal sensitivity to transformation for an additional 60 min in this experiment. Usually competence was maintained for 3 to 4 hr under these conditions of growth.

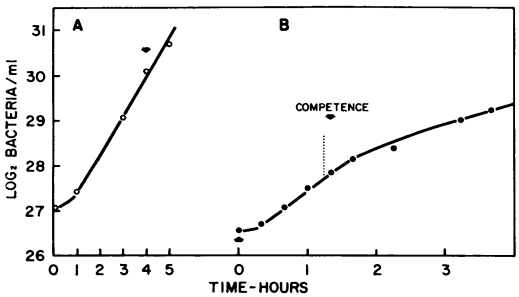

Fig. 1. Growth of *Bacillus subtilis* strain 168 under the conditions employed for the development of competence. Growth as determined by viable counts. Conditions as described in Materials and Methods. *A*, Growth during the first stage (4 hr). The *arrow* indicates when the cells were centrifuged. *B*, Growth during the second stage (90 min). The *arrow* indicates the time when maximal competence was reached.

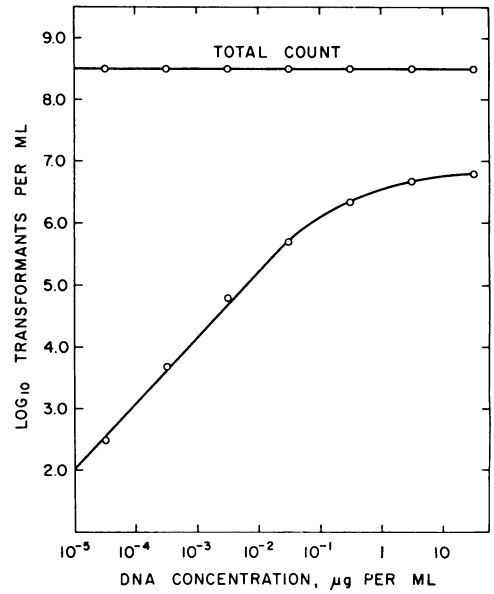

Fig. 2. DNA concentration and transformation of *Bacillus subtilis* strain 168 to prototrophy. DNA uptake terminated by deoxyribonuclease (see text). All other details as described in Materials and Methods. Total count determined by colony count on minimal medium to which L-tryptophan was added to give a final concentration of 4 μg/ml.

Fig. 1 represents the growth of the cells of *B. subtilis* strain 168 as determined by viable counts in the glucose-minimal medium containing L-tryptophan and casein hydrolyzate. The curves depict growth during the 4-hr period followed by centrifugation and dilution in fresh medium prior to the second growth period. The appearance of competence occurred toward the end of the logarithmic growth period and was retained for a further 3 to 4 hr when growth of the culture had ceased.

Although competence was reached at the end of the logarithmic growth period under these conditions, it was found that it was relatively much lower in the same period of growth in medium containing higher concentrations of amino acids. It would appear that limited amino acid content of the medium is a prerequisite for transformability in this strain.

Transformation for other markers in mutants of strain 168 (Spizizen, 1959) is similarly dependent on these specific growth conditions.

Relation of DNA concentration to transformation. Fig. 2 represents data from an experiment in which different concentrations of DNA isolated from a wild-type donor were incubated with the cells during the 90-min period (see Materials and Methods) followed by the addition of 50 μg deoxyribonuclease and 5 μmoles per ml magnesium sulfate. As little as 10^{-5} μg per ml of DNA produced transformation and the response up to 10^{-1} μg of DNA was linear. This is a similar response curve to that obtained with pneumococcal transformation (Hotchkiss, 1957).

Although the response curve was similar in shape for most preparations of DNA employed, the level of the response with high concentrations varied with the quality of the DNA preparation. With some preparations and with highly competent cells, the percentage of transformation was as high as 3.5, but more frequently it was found to be 1 to 2%.

DISCUSSION

The development of sensitivity to transformation in *B. subtilis* strain 168 occurs toward the end of the logarithmic phase. In addition to the growth factor, L-tryptophan or indole, a metal-ion chelating compound such as L-histidine is required. The presence of relatively large amounts of amino acids, such as are present in acid hydrolyzed casein, reduces the sensitivity to transformation, presumably by permitting the synthesis of wall components which may prevent DNA uptake. These facts would suggest that some kind of unbalanced growth is achieved under the special growth conditions required for optimal transformation.

The role of the chelating compound in the formation of competent cells has not been clarified by the present experiments. It may be involved in the synthesis of cellular components inhibited by traces of cupric ions, as suggested by the requirement for a contact interval with the growing cells of at least 60 min. The requirement for serum albumin (Fox and Hotchkiss, 1957) in the development of competence in pneumococcal transformations may be analogous.

Although the nutritional conditions have thus been defined for competence the nature of the physiological state underlying this condition has not been revealed. It has been shown in this laboratory (Young and Spizizen, 1961) and by others (Lerman and Tolmach, 1957) that when cells become sensitive to transformation they are capable of taking up irreversibly highly polymerized DNA. The ability to take up DNA may involve some structural changes in the cell wall which permit the penetration of the macromolecules. In addition, some enzymatic mechanism for the active uptake of DNA may be developed, as suggested by recent experiments (Anagnostopoulos, *unpublished data*) which show that certain metabolic poisons inhibit uptake.

Finally, some genetic control of the attainment of the competent state has been suggested by recent studies (Young and Spizizen, 1961) which indicate that sensitivity to transformation occurs at a stage of growth in which presporulating events occur. Strains of *B. subtilis* unable to undergo sporulation (and presumably also presporulation) do not become competent. It is suggested that some alterations in the structure of the cell wall associated with presporulation physiology are the basis for competency.

SUMMARY

The conditions required to develop optimal sensitivity of *Bacillus subtilis* strain 168 (indole dependent) to transformation by deoxyribonucleic acid from wild-type strains have been studied. Optimal sensitivity to transformation to prototrophy was achieved by growth in a glucose minimal medium containing L-tryptophan and acid-hydrolyzed casein. Two growth periods were employed. In the first 4-hr period, the casein hydrolyzate provided growth stimulation and high concentrations inhibited the formation of competent cells. During the second period (of 90 min), the casein hydrolyzate could be replaced by L-histidine or any of a number of metal chelating compounds.

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