Effect of Filtrates from Transformable and Nontransformable Streptococci on the Transformation of Streptococci

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ABSTRACT

PERRY, DENNIS (Northwestern University Medical School, Chicago, Ill.), AND HUTTON D. SLADE. Effects of filtrates from transformable and nontransformable streptococci on the transformation of streptococci. *J. Bacteriol.* 91:2216-2222. 1966.—The nature of the transformation competence factor from a group H streptococcus was investigated. The activity of competence factor reached a maximum at the time that optimal competence was attained, the maxima of both occurring in the early log phase of growth. The decrease in competence factor was much more gradual than the decrease in number of competent cells. No inhibitor, however, was detected as being responsible for the decrease in either competent cells or competence factor activity. Efforts to induce transformation in other serological groups of streptococci with the use of group H competence factor were unsuccessful. The development of competence in group H when grown in the presence of nontransformable group A strains resulted in a significant increase in the number of transformants. Culture filtrates from early log phase group A cells also caused an increase in the number of transformants from the group H strain. The addition of 10^{-4} M ethylenediaminetetraacetic acid to group A (or group H) culture filtrates caused significant increases in the number of transformants. These results thus indicate that group A streptococci, although nontransformable, produce low levels of "competence factor." Late culture filtrates from the group H streptococcus and several strains of group A streptococci possessed deoxyribonuclease-like activity which inhibited the transformation of the group H strain. This activity in the A filtrates, however, was not prevented by group A anti-deoxyribonuclease sera. Instead, these sera also inhibited transformation. Evidence indicates that the lack of transformation of group A streptococci is due to factors other than the production of deoxyribonuclease.

The acquisition of transforming ability (competence) by several species of bacteria is accompanied by the production of a substance which is capable of inducing competence in incompetent bacterial cells of the same strain (2, 8, 12). This substance, termed "competence factor" (2), has not been found to be produced by transformable cells in which competence was not allowed to develop or by nontransformable cells. The discovery that competence factor from a transformable group H streptococcus could markedly increase the rate of transformation in

another group H strain (8) led to speculations that perhaps nontransformable streptococci could be rendered transformable. Thus, an investigation into the properties of competence factor and its effect on nontransformable streptococci (9) was performed. Studies of the effect on transformation of culture filtrates from nontransformable streptococci also were undertaken to elucidate the reason for lack of transformation in some strains.

MATERIALS AND METHODS

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Bacterial strains. Group H streptococcus strain Challis, was used as donor and recipient in all transformation experiments. Group A strains T14m and

T1g from our laboratory collection were also employed. A Challis strain, resistant to over 1.0 mg of streptomycin per ml, was isolated according to procedures previously described (9).

Preparation of deoxyribonucleic acid (DNA). DNA was isolated by rupturing the cells with a Sagers press (11) as described previously (10), and was purified by the method of Young and Spizizen (14).

Media. The transforming medium (PYG), modified from that of Pakula and Walczak (8), contained 1% Proteose Peptone (Difco), 0.2% yeast extract (Difco), and 0.2% glucose. The pH of the medium was adjusted to 7.8 with 1 N NaOH.

Transforming procedure. A 0.1-ml quantity of a $1:10$ dilution (5×10^7 to 9×10^7 colony-forming centers per milliliter) of an 18-hr culture [grown in Todd Hewitt Broth (Difco) plus 4% sheep blood] was inoculated into 1.8 ml of PYG plus 10% human serum. The human serum had been heated at 60 C for 30 min to destroy any deoxyribonuclease activity. The cells were incubated at 37 C for 2 hr to reach the stage of optimal competence. DNA was then added in a volume of 0.1 ml to a concentration of 5 μ g/ml. The remainder of the procedure was as described previously (9). The yield of transformants was 1 to 5%.

Culture filtrates. Competence factor was obtained from strain Challis by growing the cells to competence (2 hr) and filtering the culture on ultrafine-porosity sintered-glass filters (Aloe Scientific). Culture filtrates from group A strains were collected at different times by the same procedure. All filtrates were used immediately or stored at -20 C until tested.

Determination of the effect of culture filtrates on transformation. Strain Challis was inoculated for transformation as described, except for the omission of human serum. No transformation occurred when serum was omitted. After 1.5 hr of incubation at 37 C, the filtrate to be tested was added (0.2 ml) to give a final concentration of 10%, and incubation was continued for another 30 min. The remainder of the procedure was as described above. Filtrates suspected of possessing inhibitory activity were added simultaneously with competence factor or immediately before the addition of DNA. As controls, culture filtrates were replaced with 0.2 ml of PYG containing 10% human serum.

RESULTS

Characterization of competence factor from strain Challis. As stated previously, no transformation occurred in PYG medium when human serum was omitted. Since the addition of competence factor to cells involved adding 10% (v/v) of the normal amount of serum, controls were devised in which culture filtrates were replaced with the same amount of PYG containing 10% serum. Only a negligible amount of transformation occurred in the controls (0.001% or less as compared to 0.02 to 0.05% when competence factor was added). When the normal concentration of serum (10%) is added to Challis

cells which have been grown for 1.5 hr in the absence of serum, 0.5 to 1% transformants occurred as compared to 1 to 5% when serum was added at the beginning of the 2-hr incubation period. The transformation of strain Challis was characterized by the sudden onset of competence, with a maximum at 2 hr of incubation (Fig. 1). A decrease in the level of competence occurred after 2 hr, although somewhat more gradually than the rate of increase. Filtrates from strain Challis were collected at different times and tested for their ability to induce competence in the absence of serum. The increase in competence factor followed the rise in the number of competent cells (Fig. 1). Maximal factor activity also occurred at 2 hr. The rate of decrease in competence factor activity, however, was considerably lower than the rate of decrease in number of competent cells. The relation of time of appearance of competent cells and competence factor to the growth of the culture can also be seen in Fig. 1. The maximum of each occurred in the early log phase.

The induction of competence in Challis by the addition of culture filtrates generally yielded 0.02 to 0.05% transformants, as compared to a yield of 1 to 5% when competence was allowed to develop in the presence of serum. The competence factor activity of several different batches of culture filtrate varied as much as 10-fold,

FIG. 1. Development of competence and production of competence factor in relation to growth (optical density) of group H strain Challis. Symbols: ○, development of competence by cells grown in PYG plus 10% human serum; ●, induction of competence by the addition of strain Challis culture filtrates to cells grown in PYG in the absence of human serum; ▲, growth (optical density).

while the percentage of transformants of the cells from which the filtrates were taken remained fairly constant. On occasions, competence factor was obtained which produced 0.5% transformants. Attempts to purify competence factor by isolation from culture filtrates showed that maximal activity was precipitated when $(\text{NH}_4)_2\text{SO}_4$ was added to 80 to 90% saturation. The activity of these preparations, however, was decreased about 10-fold in the process.

The effect of competence factor on strain Challis cells in which competence was allowed to develop normally was also investigated. A 10 to 30% increase in the number of transformants was observed. In three such experiments, an average of 2.1% transformants was obtained when competence was developed in PYG plus 10% human serum. However, an average of 3.0% transformants was obtained when competence factor was added.

The time at which strain Challis reaches optimal competence can be controlled by varying the number of cells. With an inoculum of 5×10^7 to 9×10^7 colony-forming centers per milliliter, optimal competence occurred at 2 hr, yielding 1 to 5% transformants. When the inoculum was decreased approximately 10-fold, however, optimal competence did not occur until after 3 hr, with a yield of 0.5 to 1.0% transformants. The number of transformants with the same inoculum was only 0.04% after 2 hr of incubation. Exposure of the culture to competence factor for 30 min, however, resulted in an increase in the number of transformants to 0.2% after 2 hr.

It was also of interest to determine whether group H competence factor could be employed to induce transformation of other serological groups of streptococci. Ten different strains of group A streptococci were exposed to 10 to 100% concentrations of competence factor (culture filtrates) from strain Challis. No transformation resulted in these experiments. Efforts were made to determine whether competence factor from one transformable streptococcal strain could be used to induce competence in another transformable strain of a different serological group. When two transformable group F strains of streptococci (10), grown in the presence or absence of serum, were exposed to competence factor from group H, strain Challis, no increase in the rate of transformation occurred.

Since a decrease in activity of competence factor occurred after the optimum had been reached (Fig. 1), filtrates from strain Challis which had been growing for 5 hr were tested for their ability to inhibit transformation. No inhibition occurred under these conditions. Furthermore, when 4- and 5-hr culture filtrates

of strain Challis were incubated at 37 C for 30 min with transforming DNA, no decrease in transformation occurred. Late culture filtrates (18 hr) of strain Challis, however, caused over 90% loss in transformation.

Although competence factor appeared rather stable, some loss in activity was noted after storage for several days at -20 C. Thus, various agents were employed in efforts to stabilize the activity of the factor. Initially, experiments were performed to test the effect of ethylenediaminetetraacetic acid (EDTA) on the growth and transformation of strain Challis. A concentration of 5×10^{-4} M EDTA or greater inhibited growth and transformation (Table 1). In contrast, the activity of competence factor was increased approximately eightfold upon addition of 10^{-4} M EDTA (Table 2). This concentration of EDTA had no effect on the number of transformants obtained from cells in which competence was allowed to develop normally. When 10^{-4} M EDTA was added to cells at different times before maximal competence occurred, it had no effect on the development of competence (Table 3). Similar results were obtained with cysteine. Thioglycolate, on the other hand, had no effect on competence factor at concentra-

TABLE 1. *Effect of EDTA on growth and transformation of strain Challis**

EDTA concn	OD (550 m μ)	Transformants
M		%
0	0.445	2.7
0.00005	0.450	2.3
0.0001	0.455	2.1
0.0005	0.420	0.8
0.001	0.015	0

* Various concentrations of EDTA were added at the time of DNA addition. Serum was incorporated in the PYG medium. Optical density (OD) readings were taken after 5 hr of incubation. The initial OD was 0.01.

TABLE 2. *Effect of EDTA on the activity of strain Challis competence factor**

Addition	No. of transformants/ 10^6 cells
Competence factor.....	330
Competence factor + EDTA (10^{-4} M) ..	2,700

* Strain Challis was grown for 1.5 hr in PYG (serum omitted) before competence factor was added to a final concentration of 10%. Results are averages of four experiments.

TABLE 3. *Effect of time of addition of EDTA on the transformation of strain Challis**

Time	Transformants
<i>min</i>	<i>%</i>
—†	1.3
120	1.5
90	1.3
60	1.4
30	1.3
0	1.1

* EDTA was added to 10^{-4} M at the times indicated before optimal competence occurred. Serum was incorporated in the PYG medium.

† No EDTA.

tions up to 10^{-2} M, whereas higher concentrations were inhibitory.

Effect of culture filtrates from group A streptococci on transformation of strain Challis. Initially, efforts to obtain transformation in group A streptococci involved developing competence of strain Challis in the presence of several group A strains. Strain Challis was inoculated for transformation as usual into PYG plus 10% human serum. In addition, approximately 10^7 cells (18 hr) of either strain T14m or T1g were added at the same time. The remainder of the procedure was as described for streptomycin-resistant Challis DNA. Again, no transformation of the group A strains occurred (Table 4). The development of competence in strain Challis resulted in a higher number of transformants when the strain was grown in the presence of either of the group A strains. The development of competence of strain Challis in the presence of group A strains T14m and T1g resulted in an approximate 1.3-fold and 4.5-fold increase, respectively, in the number of transformants.

These observations led to subsequent investigations concerning the effect of group A culture filtrates on transformation of strain Challis. Approximately 10^7 cells per milliliter of group A strain T14m or T1g were inoculated for transformation, and samples of the filtrates were collected hourly for 5 hr. These filtrates were tested at 10% concentrations on Challis cells in which competence was induced with Challis competence factor. The group A culture filtrates were added simultaneously with competence factor. Experiments also were performed in which the group A culture filtrates were used in the absence of Challis competence factor. In Fig. 2, lines A and B represent the number of transformants obtained in PYG medium. In A, 0.2 ml of competence factor was included; in B, 0.2 ml of PYG containing 10% human serum

TABLE 4. *Development of competence in strain Challis in the presence of group A streptococci**

Strains	No. of transformants/ 10^6 cells	
	Expt 1	Expt 2
Challis.....	160	90
Challis + T14m.....	220	110
Challis + T1g.....	620	470

* Challis DNA (streptomycin-resistant) was employed at a concentration of $0.1 \mu\text{g/ml}$. Challis: 8×10^7 cells per milliliter. T14m and T1g: 0.5 to 2.0×10^7 cells per milliliter. Serum was incorporated in the PYG medium.

FIG. 2. *Effect of culture filtrates from group A streptococci on the development of competence in group H strain Challis.* Cells were grown in PYG in the absence of human serum. After 1.5 hr of incubation, the following were added: ●, 0.2 ml of T14m culture filtrate; ○, 0.2 ml of T1g culture filtrate. In addition, for curves 1 and 2, 0.2 ml of strain Challis competence factor also was included; for curves 3 and 4, 0.2 ml of PYG plus 10% human serum. Line A represents the average number of transformants obtained with Challis competence factor (2-hr filtrate); line B, the average number of transformants obtained with PYG plus 10% human serum (control).

(control). A definite increase in the number of transformants occurred when culture filtrates from 2-hr and 3-hr cells of T14m and T1g, respectively, were employed. The T14m culture filtrate caused an approximate threefold and twofold increase in the number of transformants over that obtained in the presence of competence factor (line A) and in the control (line B), respectively. The amount of increase with T1g

culture filtrate was approximately 3.5-fold and 2.5-fold. In contrast, culture filtrates from 4- and 5-hr cells of strains T14m and T1g inhibited transformation. A 5-hr filtrate of strain T14m caused complete inhibition, whereas a 5-hr filtrate of strain T1g caused only 32% inhibition. Incubation of transforming DNA with these filtrates at 37 C for 30 min also resulted in a loss of transformation. Heating at 65 C for 30 min destroyed this inhibitory activity. Culture filtrates from 5-hr cells of five other strains of group A streptococci inhibited transformation from 25 to 60%.

The observation that EDTA increased the activity of competence factor prompted a study of the effect of EDTA on culture filtrates from strain T14m. The addition of 10^{-4} M EDTA to filtrates from T14m cells, incubated for various times, caused a significant increase in the "competence factor" activity of the 1-, 2-, and 3-hr culture filtrates (Fig. 3). In addition, the inhibitory activity of the 4- and 5-hr culture filtrates was decreased.

The effect of group A streptococcal anti-deoxyribonuclease sera also was tested in further efforts to show that the inhibitory activity of 5-hr group A filtrates was due to deoxyribonuclease. Group A streptococcal antisera, prepared from rabbits, contained a mixture of anti-deoxy-

ribonucleases A, B, C, and D in initial titers (13) of 1:20, 1:80, 1:160, and 1:160, respectively. Normal rabbit serum and anti-deoxyribonuclease sera were heated at 60 C for 30 min before use at a concentration of 1:1 (v/v). The final concentration of all components (sera, filtrates) in the reaction tubes was 5% (v/v). As shown in Table 5, T14m filtrate reduced the rate of transformation 13-fold. However, no decrease in the inhibitory activity of the filtrate occurred when it was preincubated with anti-deoxyribonuclease or normal rabbit sera. Unexpectedly, it was found that the anti-deoxyribonuclease sera possessed inhibitory activity of the same degree as the T14m filtrate. Normal rabbit serum increased the number of transformants approximately fivefold. The addition of DNA, pretreated with anti-deoxyribonuclease sera, to competent cells also resulted in a decrease in the number of transformants. Pretreatment of DNA with normal rabbit serum had no significant effect on transformation.

TABLE 5. Effect of streptococcal anti-deoxyribonuclease on the transformation of strain *Challis**

Components added in addition to strain <i>Challis</i> competence factor	No. of transformants/ 10^6 cells
None.....	550
T14m filtrate (5 hr).....	43
T14m filtrate (5 hr) + antideoxyribonuclease sera†.....	12
T14m filtrate (5 hr) + normal rabbit serum†.....	48
Anti-deoxyribonuclease sera.....	48
Normal rabbit serum.....	2610
Anti-deoxyribonuclease-treated DNA††.....	48
Normal rabbit serum-treated DNA†.....	820

* All components were added simultaneously with competence factor, except the anti-deoxyribonuclease and normal rabbit serum-treated DNA which were added after the cells had incubated for 2 hr (time at which DNA was usually added). The group A streptococcal antisera contained a mixture of anti-deoxyribonucleases A, B, C, and D in initial titers of 1:20, 1:80, 1:160, and 1:160, respectively. Normal rabbit and anti-deoxyribonuclease sera were heated at 60 C for 30 min before use at a concentration of 1:1 (v/v). All components were employed so that the final concentrations in the reaction tubes were 5% (v/v).
† Pre-incubated at 37 C for 30 min before addition to cells.
†† Anti-deoxyribonuclease A was not included.

FIG. 3. Effect of EDTA (10^{-4} M) on culture filtrates from a group A streptococcus (strain T14m). Solid lines represent T14m filtrates (0.2 ml) containing 10^{-4} M EDTA; broken lines, T14m filtrates (0.2 ml) without EDTA. Symbols: ●, 0.2 ml of *Challis* competence factor was included; ○, 0.2 ml of PYG plus 10% human serum. Lines A and B are the same as those described in Fig. 2. Filtrates were tested on strain *Challis*.

DISCUSSION

Knowledge of the mechanism by which transformable strains of microorganisms attain competence is limited. Some insight into this problem was gained, however, upon the discovery of a substance (competence factor) which appears to control this physiological state of the cell. It is interesting that the release of competence factor by cells, at least in group H streptococci, is almost as sudden as the onset of competence. Our results are similar to those of Pakula and Walczak (8), who also observed a sudden increase in competence factor. These investigators, however, observed that optimal competence persisted for several hours, after which a sharp decrease occurred. Culture filtrates from cells incubated for 17 hr were found to retain maximal competence factor activity. In contrast, our results showed an almost immediate decrease in both competence and competence factor after the optima had been attained, the decrease being considerably more gradual for competence factor. Even so, competence factor had lost about 45% of its original activity 1 hr after the optimum had been reached, and about 80% of its activity 3 hr after reaching the optimum (Fig. 1). The differences between our results and those of Pakula and Walczak (8) are not readily explained, although one possibility could be the use of different transforming media. For instance, it was shown that competence remained at a maximum for a much longer period when beef-heart extracts were added to the transforming medium (6).

Tomasz and Hotchkiss (12) noted that the loss in competence by a pneumococcal culture was initiated by the production of an inhibitor in the culture filtrate. They ruled out deoxyribonuclease as the inhibitor, in that fixation of DNA by cells which had already developed competence was not inhibited. It has not been possible to detect an inhibitor in the culture filtrates of strain Challis in the first 5 hr of incubation. Even so, inhibitor-like substances are suspected. This is likely in that a rather rapid decrease in the level of competence occurred, even though much of the activity of competence factor still remained (Fig. 1). It is possible that the level of inhibitor was too low to detect, or that it is more closely associated with the cell. On the other hand, the decrease in activity of competence factor could mean that the continued incubation of highly competent cells results in more of the factor being "fixed" by the cells. Thus, culture filtrates taken later would possess lower activity.

The observation that group H competence factor failed to bring about the transformation of group A streptococci, or to induce competence in transformable group F strains, may be indicative of the specificity of the factor. Pakula and Walczak (8) showed that competence factor was more active on the streptococcal strain from which it was derived.

The increased activity of competence factor upon addition of EDTA or cysteine is probably due to the chelating properties of these agents, with certain inhibitory divalent cations being removed. Anagnostopoulos and Spizizen (1) found that EDTA and other metal-chelating compounds enhanced competence in *Bacillus subtilis* by removing inhibitory cupric ions. It was surprising, however, that the normal development of competence in strain Challis (growing in PYG plus serum) was not affected by the same concentration of EDTA (Table 3). That EDTA caused a decrease in the activity of 4- and 5-hr culture filtrates of the group A strains was not surprising, since it is well known that this agent is an inhibitor of deoxyribonuclease.

Investigations by several workers have implied that competence factor is necessary for uptake of transforming DNA. Osowiecki and Lancow (4) and Pakula and Hauschild (7) showed that the exposure of a weakly transformable group H streptococcus to competence factor (strain Challis) results in an increase in the uptake of isotopically labeled DNA and in the number of transformants. The latter investigators also noted that significant uptake of the label occurred in the absence of added competence factor. Since no transformation occurred, this uptake was said to be due to labeled "impurities." It is probable, however, that some competence factor was produced, because this strain is weakly transformable. Furthermore, Perry and Slade (9) demonstrated the uptake of P^{32} -labeled DNA by a nontransformable group A streptococcus. The observation that culture filtrates (early log phase) from two group A streptococcal strains caused an increase in transformation also indicates that some group A strains, although nontransformable, can produce low levels of non-specific "competence factor." Thus, failure to produce competence factor (or failure to take up DNA) does not seem to be a primary reason for the lack of transformation in these strains. More likely, the absence of transformation in the group A strain is due to an inability to integrate DNA once it is taken up. The nature of DNA after uptake by a group A streptococcus is now under study.

The inhibitory activity of 4- and 5-hr culture

filtrates from group A streptococci may be due, to some extent, to deoxyribonuclease. Group A streptococci are known to produce deoxyribonuclease (3, 13), and it has been suspected, although not demonstrated, that deoxyribonuclease is one of the major reasons for their lack of transformation. McCarty (3), employing viscosimetric methods, noted that deoxyribonuclease is released into the medium by group A streptococci as early as the beginning of the log phase of growth. Ottolenghi and Hotchkiss (5) also observed that small amounts of deoxyribonuclease appear in filtrates of young transformable pneumococcal cultures, whereas larger inhibitory amounts occurred in late culture filtrates. Similarly, our results indicate that late culture filtrates of strain Challis possess deoxyribonuclease-like activity, since these filtrates prevented transformation. Culture filtrates from group A streptococcal cells in the early log phase (1 to 3 hr), however, did not inhibit transformation. On the contrary, transformation was stimulated. In addition, it was shown that deoxyribonuclease-treated P^{32} -labeled DNA was not taken up (9). In view of these observations, it thus appears that extracellular deoxyribonuclease may not be a major factor in the lack of transformation in some strains of streptococci.

It is quite apparent that further studies on the inhibitory activity of the anti-deoxyribonuclease sera are necessary before any definite conclusions can be drawn. Since a decrease in the number of transformants occurred upon preincubation of DNA with anti-deoxyribonuclease sera, it seems that the inhibiting substance affects transformation either by degrading DNA or by preventing its interaction with competent cells. The presence of an "anti-competence factor" in the antisera, however, has not been ruled out. In addition, deoxyribonuclease has not been completely excluded, even though the antisera were heated at 60 C for 30 min. The presence of serum may have afforded some protection for the deoxyribonuclease.

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