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Mode of Action of the Competence-Inducing Factor of *Bacillus stearothermophilus*[1]

ULDIS N. STREIPS[2] AND FRANK E. YOUNG[2]

Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, California 92037

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Development of competence in the noncompetent mutant of *Bacillus stearothermophilus* 4S requires a soluble competence-inducing factor (CF). This study describes several observations that define the mechanism of action of this factor. (i) CF interacts with cells, protoplasts, or cytoplasmic membranes in a temperature-dependent reaction; incubation of CF with cells alters the sedimentation properties of the cells. (ii) Association between CF and the cytoplasmic membrane appears to be the first and rate-limiting step in the process of transfection with deoxyribonucleic acid (DNA) from bacteriophage TP-1C. (iii) The membrane-CF complex is capable of removing infectious DNA from the supernatant fraction in vitro. (iv) Twenty phage-resistant mutants are unable to undergo the above reactions. A model is developed in which CF serves as a dynamic mediator of the reaction between the transfecting DNA and the cell surface.

Although genetic transformation was discovered in 1928 by Griffith (8), competence, the process by which cells develop the capacity to transport deoxyribonucleic acid (DNA), remains obscure. The identification of competence-provoking factor(s) in *Streptococcus* (13) and activator(s) in *Pneumococcus* (19) clearly demonstrated that extracellular substances were involved in eliciting competence in these cocci. More recently, competence-inducing factors have been reported in bacilli as well (2, 6). Studies with various strains of *Streptococci* and mutants of *Pneumococcus* (18) have demonstrated that the noncompetent strains can be classified according to the ability to produce competence-inducing factor (CF) and to interact with CF (CF site mutants). The CF in most of the mesophilic transformation systems varies from 5,000 to 10,000 in molecular weight and is labile to proteolytic enzymes (12, 20). The instability and the physical properties of CF have complicated purification. Despite studies which suggest that CF functions as a nuclease (10) or an autolysin (1), there is still no well-defined characterization of the mode of action of these factors.

Because of the basic similarities and difficulties encountered in the mesophilic systems, we turned our attention to the transfection system of *Bacillus stearothermophilus* (17). This thermophilic organism produces a stable CF and possesses a relatively well-defined cytoplasmic membrane. The data presented demonstrate that CF interacts with the cell in a temperature-dependent reaction, altering the sedimentation properties of the cell. This interaction appears to occur primarily on the cytoplasmic membrane.

MATERIALS AND METHODS

Bacteria and bacteriophage. *B. stearothermophilus* 4S and two spontaneous mutants isolated by Streips and Welker (*manuscript in preparation*), 4S Ton-r and 4S Com$^-$, were used throughout this study. 4S Com$^-$ was isolated as a spontaneous mutant which cannot be transfected with bacteriophage DNA unless CF is added to the culture. This strain does not produce CF even after extended periods of incubation. 4S Ton-r was isolated as a strain resistant to phage TP-1C. This mutant produces CF at levels higher than the wild-type strain but cannot be infected with bacteriophage DNA. Transfecting DNA was obtained from bacteriophage TP-1C (21) by the method described previously (17).

CF was present in the supernatant fluids of stationary-phase cultures of strain 4S Ton-r after removal of the cells (Streips and Welker, *manuscript in preparation*).

Media. The standard TYG medium (21) was used for all liquid cultures. Trypticase agar (TA) was used as the plating medium (21).

Transfection. Procedures for the development of competence and the assay for transfection were as described by Streips (Ph.D. Thesis, Northwestern Univ., 1969). Strain 4S Com$^-$ cells become maximally susceptible to stimulation by exogenous CF after 2 hr of incu-

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[2] Present address: Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. 14620.

bation of 55 C in TYG medium. At this time, CF and phage DNA was added to the cells, the mixture was incubated for 80 min, and the reaction was terminated with deoxyribonuclease (0.05 μg/ml). Infectious centers were assayed on strain 4S Com⁻ cells as described previously (17).

Preparation of protoplasts and protoplast membranes. Cells from a 12- to 15-hr growth on TA plates were suspended in 100 ml of TYG medium in a 500-ml baffled-bottom Erlenmeyer flask. After 2 hr of incubation at 55 C in a rotary incubator shaker, the cells were harvested by centrifugation; the pellets were suspended in 10 ml of 0.04 M tris(hydroxymethyl)aminomethane buffer (*p*H 7.5) containing 0.01 M $MgCl_2$ (TM buffer) and incubated with 50 μg of lysozyme per ml with occasional agitation at 37 C (4). Samples were examined under a phase contrast microscope to ascertain the extent of conversion of rods to spheres. Complete conversion usually occurred after 30 min of incubation. The protoplasts were washed in TM buffer by successive centrifugations at 5,000 rev/min for 10 min at 25 C until the supernatant fluid was free of lysozyme (as evidenced by failure of the supernatant fraction to lyse a bacterial lawn). The final pellet was suspended in 10 ml of TM buffer.

To obtain membranes, 10 ml of the protoplast sample was centrifuged at 5,000 rev/min for 20 min, suspended in 10 ml of 0.15 M NaCl, centrifuged at 15,000 rev/min for 30 min, and suspended in 10 ml of 0.05 M NaCl. As reported by Bodman and Welker (4), rupture of the spheroplasts occurs after 12 to 16 hr at 5 C. The membranes were washed three times by centrifugation at 15,000 rev/min for 20 min in 10 ml of TM buffer. The final pellet was suspended in 10 ml of TM buffer.

Separation of competent cells in Renografin. Renografin-76 (Squibb Methyl Glucamine Diatrizoate Injection USP, E. R. Squibb and Sons, New York, N.Y.) was diluted in TYG medium at room temperature. The step gradient in a final volume of 5.0 ml was prepared as described previously (9), except that a 40% layer was added to the 50, 30, and 12.5% layers. Thus, the final gradient was composed of 1.0 ml of 50% Renografin, 0.5 ml of 40% Renografin, 2.0 ml of 30% Renografin, and 1.0 ml of 12.5% Renografin. The sample was layered on the gradient in 0.5 ml of 12.5% Renografin. The gradients were formed at room temperature and then centrifuged at 0 C for 30 min at 15,000 rev/min in an SW50L swinging bucket rotor in a Beckman model L preparative ultracentrifuge.

Determination of binding of CF. *B. stearothermophilus* 4S Com⁻ cells were grown to competence and diluted to the final assay mixture in a final volume of 2.4 ml as described by Streips and Welker (*manuscript in preparation*). The tubes were divided into two groups (A and B) which were treated as shown in Fig. 1. Because the concentration of CF is limiting, the number of infectious centers is directly proportional to the amount of CF bound to the cells (Streips and Welker, *manuscript in preparation*). Therefore, the extent of binding was calculated from the following ratio: infectious centers per milliliter (tube A)/infectious centers per milliliter (tube B) × 100.

Determination of recovery of CF. *B. stearothermophilus* 4S Com⁻ cells were grown to competence as

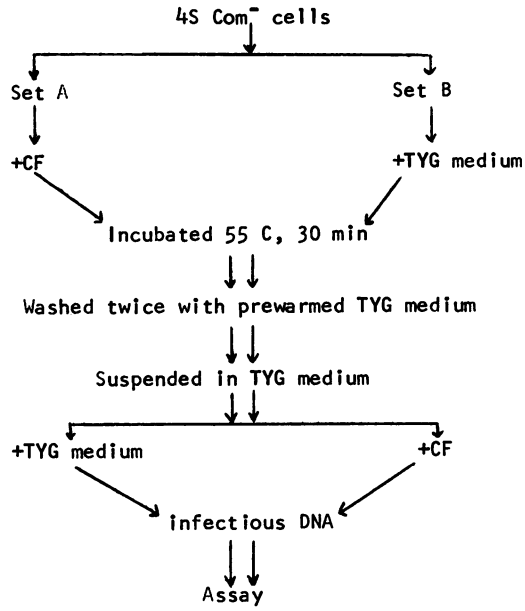

FIG. 1. *Method for the determination of binding of competence-inducing factor to strain 4S Com⁻ cells. Either 0.1 ml of CF or 0.1 ml of TYG medium was added in the experimental procedure to maintain a constant volume of 2.5 ml in all tubes before addition of infectious DNA.*

above. All tubes received 0.1 ml of CF. One group of tubes was incubated at 55 C for 30 min on a rotary incubator shaker; the tubes were centrifuged, and the pellets were washed twice with TYG medium. The other group of tubes was immediately centrifuged, and the pellets were washed twice with TYG medium. The supernatant fluids from the initial inoculum and the two washings (designated as supernatant I, II, and III) were collected and assayed for CF activity. The CF activity was determined by mixing 0.5-ml samples of 4S Com⁻ cells from cultures grown to the mid-exponential phase of growth with 2.0 ml of trypticase-soft agar and 0.5 ml of the solution to be tested for CF activity. To this mixture was added 0.1 ml of bacteriophage TP-1C DNA (final concentration, 0.1 μg/ml), and the contents of the tube were overlaid onto a TA plate and incubated for 12 to 15 hr at 55 C. The control level for CF activity was determined by assaying, as above, 0.5-ml samples of TYG medium containing 0.1 ml of CF. The number of infectious centers per milliliter produced by the presence of the supernatant fluids from each tube was totaled. The percentage of recovery was calculated by the following formula: recovery (%) = (sum of infectious centers per milliliter for all supernatant fluids)/(infectious centers per milliliter of control) × 100. The resulting ratio was designated as per cent recovery.

Amino acid and amino sugar analysis of protoplast membranes. A sample (3.0 ml) of the protoplast preparation was centrifuged in a Sorvall angle centrifuge at 5,000 rev/min for 10 min. The resulting pellet was suspended in 1 ml of 4 N HCl, placed in a Pyrex ampoule,

sealed, and incubated at 105 C for 16 hr. The sample was subsequently dried and rehydrated twice, diluted in a standard *p*H 2.2 buffer, and analyzed on a Beckman Spinco amino acid analyzer (22).

Chemicals. Deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.), 1 mg/ml, was dissolved in 1 M $MgSO_4$ and stored at −20 C. Lysozyme (Worthington Biochemical Corp., Freehold, N.J.) was added as crystals.

RESULTS

Association of CF with cells. The direct association between CF and the cells was investigated as the first step in the elucidation of the role of CF in transfection. *B. stearothermophilus* 4S Com$^-$ cells were grown at 55 C under conditions which induce maximal competence. In these experiments, cells were incubated with CF at various temperatures, centrifuged, washed three times with TYG medium, and assayed as described for transfection. As shown in Table 1, the capacity of cells to bind CF is temperature dependent. To demonstrate that the remainder of the CF was not inactivated during incubation at 55 C, the supernatant fraction from the cells grown and incubated with CF at 55 C (Table 1) was assayed for residual CF activity. As shown in Table 2, 53% of the CF was present in the supernatant fraction after incubation for 30 min at 55 C with 4S Com$^-$. If the 9% of CF activity which cannot be accounted for at zero time in Table 2 was lost due to experimental conditions, the CF activity which is not bound to the cells (Table 3) can be quantitatively recovered in the supernatant fraction (Table 2). When cells were grown at 67 instead of 55 C, the ability to bind CF at 67, 55, or 0 C was lost (Table 4). Cells of the phage-resistant strain (4S Ton-r) did not bind CF at 55 or 67 C. These cells also could not be transfected at either temperature. Therefore, strains Ton-r and 4S Com$^-$ can be designated as CF$^+$ CF site$^-$, and CF$^-$ CF site$^+$, respectively, as suggested by Tomasz for the pneumococcal and streptococcal systems (19).

Previous studies on *B. subtilis* have shown that the competent fraction of the population sediments differently in gradients of sucrose (15) or

TABLE 1. *Kinetics of CF binding to Bacillus stearothermophilus*[a]

Temp (C)	Incubation with CF (min)	Binding (%)
55	0	1
	10	11
	20	19
	30	30
	40	32
37	0	0
	10	0
	20	0
	40	7
0	0	0
	10	1
	30	2
	45	0

[a] The assay for binding was as described in Materials and Methods.

TABLE 2. *Recovery of CF from Bacillus stearothermophilus*[a]

Exposure to CF (min)	Fraction assayed	Infectious centers (per ml)	Recovery[b] (%)
0	Supernatant I	2,300	
	Supernatant II	520	
	Supernatant III	20	
	Total	2,840	91
30	Supernatant I	1,140	
	Supernatant II	300	
	Supernatant III	190	
	Total	1,630	53

[a] The assay for recovery of CF was as described in Materials and Methods.

[b] Recovery calculated on the basis of the CF control without cells which yielded 3,110 infectious centers per ml.

TABLE 3. *Association of CF with Bacillus stearothermophilus at 55 C*[a]

Exposure to CF (min)	Sample	Infectious centers (per ml)	Binding (%)
0	A	2,860	
	B	30	1
30	A	10,280	
	B	3,370	33

[a] The assay for binding was as described in Materials and Methods.

TABLE 4. *Association of CF with Bacillus stearothermophilus at 67 C*[a]

Exposure to CF (min)	Binding (%)	Recovery[b] (%)
0	0	100
30	5	100

[a] The assays for binding and for recovery of CF were as described in Materials and Methods, except that the cells were initially grown at 67 C for 60 min.

[b] Recovery calculated on the basis of the CF control without cells which yielded 2,570 infectious centers per ml.

Renografin (5, 9). The effect of the CF on the population of *B. stearothermophilus* 4S Com⁻ was studied by a modification of the Renografin technique. As shown in Fig. 2, all of the 4S Com⁻ cells sedimented as a single band in the gradient at the top of the 50% Renografin layer, but after incubation with CF for 30 min at 55 C a portion of the population was found at the interface of the 12.5 and 30% Renografin layers, and another portion was found at the interface of the 30 and 40% Renografin layers. Samples of these bands were assayed for transfection. Although transfection was obtained only in the upper bands without the addition of more CF, it was not possible to quantitate the frequency of transfection because growth of cells was inhibited by 50% Renografin. These preliminary experiments suggest that competent cells are confined to the upper bands. This interpretation was strengthened by examining the sedimentation pattern of 4S Com⁻ which was grown at 67 C and incubated with CF at either 67 or 55 C for 30 min. Under these conditions, the cells sedimented as a single band at the top of the 50% layer of Renografin. In *B. subtilis* it was also noted that the competent cells sediment more slowly in Renografin (5, 9).

Association of CF with protoplasts and protoplast membranes. In an attempt to determine the site of binding, we examined binding of CF by protoplasts and protoplast membranes obtained from *B. stearothermophilus* 4S Com⁻ cells. Diaminopimelic acid, glucosamine, and muramic acid were not detected in the hydrolysates of these cytoplasmic membranes. Since these protoplasts could not be transfected, the binding of CF could be quantitated only by measuring the residual CF in solution after incubation with membranes. As shown in Table 5, only 21% of the CF activity remained in the supernatant fraction after incubation for 30 min at 55 C. Because incubation of the protoplasts with CF at 55 C produces some lysis after prolonged exposure, it was important to determine whether the DNA from these protoplasts or from the supernatant fractions of these protoplasts interfered with the assay. Therefore the protoplasts were lysed by the NaCl method of Bodman and Welker (4). Samples of the supernatant fraction were then added to the standard transfection reaction mixture. These samples did not influence the extent of transfection. Thus, the loss of CF appears to be related to binding of the CF to the protoplasts. Furthermore, since results identical to those shown in Table 5 are obtained with membranes isolated from protoplasts, it appears that the reaction occurs between some membrane component and exogenous CF.

Previous studies have demonstrated that CF would not react with cells preincubated at 67 C (Streips and Welker, *manuscript in preparation*). To investigate further the binding between protoplasts or membranes and CF, protoplasts and membranes were incubated for 30 min with CF at 67 C and assayed as described previously. As shown in Table 6, there was no binding of CF to

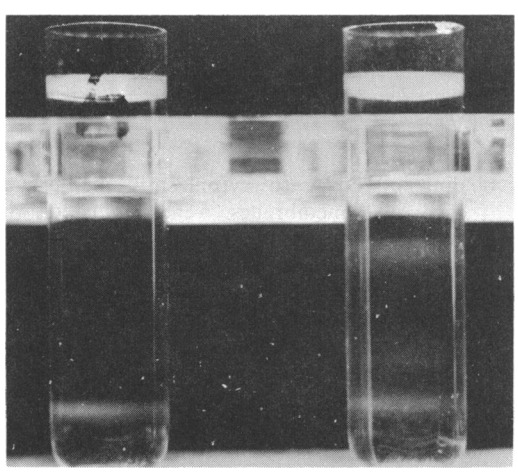

FIG. 2. *Effect of competence-inducing factor on sedimentation of strain 4S Com⁻ cells in Renografin gradients. Left, control; right, experimental.*

TABLE 5. *Recovery of CF from protoplasts at 55 C*[a]

Exposure to CF (min)	Fraction assayed	Infectious centers (per ml)	Recovery[b] (%)
0	Supernatant I	1,990	
	Supernatant II	100	
	Supernatant III	20	
	Total	2,110	82
30	Supernatant I	460	
	Supernatant II	70	
	Supernatant III	10	
	Total	540	21

[a] Samples (0.5 ml) of the protoplast suspension described in Materials and Methods were added to 2.0 ml of TM buffer in capped tubes (18 by 150 mm). Both tubes received 0.1 ml of CF and were kept at 0 C. One tube was washed twice immediately with cold TM buffer in a Sorvall RC-2B refrigerated centrifuge at 0 C for 10 min at 5,000 rev/min. The other tube was incubated for 30 min in a rotary incubator shaker at 55 C and then washed twice with cold TM buffer as above. The supernatant fraction were assayed for CF activity and the recovery was calculated as described in Materials and Methods.

[b] Recovery calculated on the basis of the CF control without cells which yielded 2,580 infectious centers per ml.

TABLE 6. *Recovery of CF from protoplasts at 67 C*[a]

Exposure to CF (min)	Fraction assayed	Infectious centers (per ml)	Recovery[b] (%)
0	Supernatant I	3,200	
	Supernatant II	160	
	Supernatant III	40	
	Total	3,400	100
30	Supernatant I	3,210	
	Supernatant II	110	
	Supernatant III	10	
	Total	3,330	100

[a] The protoplasts were treated as described in Table 5, except that exposure to CF was at 67 C. The assay for CF activity and calculation of recovery was as described in Materials and Methods.

[b] Recovery calculated on basis of the CF control without cells which yielded 3,310 infectious centers per ml.

protoplasts under these conditions. Similar results were obtained with isolated membranes.

Effect of CF on the kinetics of infection. Recent studies with antibodies to single-stranded DNA (7) and with metabolic inhibitors (16) have suggested that the DNA may exist for a finite period of time between the wall and the membrane or bound to the membrane before integration into the genome. In the presence of CF, the *B. stearothermophilus* transfection system becomes insensitive to deoxyribonuclease by 35 min (Streips, Ph.D. Thesis, Northwestern University, 1969). Therefore, the dissociation of DNA from the cell by washing and cold shock at 25 C was studied by incubating the cells for various times at 55 C with DNA. The number of transfectants in samples washed at 25 C were compared with those obtained in samples incubated with deoxyribonuclease. As shown in Fig. 3, similar kinetics were obtained with chilling and deoxyribonuclease.

The time between the addition of CF and DNA to cells and the initial appearance of transfectants at 3 min could be related to the time of transport of DNA, the formation of a complex between DNA and the receptor site, or the formation of a complex between CF and the receptor site. To test these possibilities, transfection was compared in samples of cells of 4S Com$^-$ which had been preincubated with CF or DNA for 30 min with samples in which the DNA and CF were added simultaneously. As shown in Fig. 4, preincubation with CF eliminated this lag. The preincubation with DNA had no effect.

Because the binding of CF to the cell is the rate-limiting step in vivo, it was important to determine whether the CF would promote

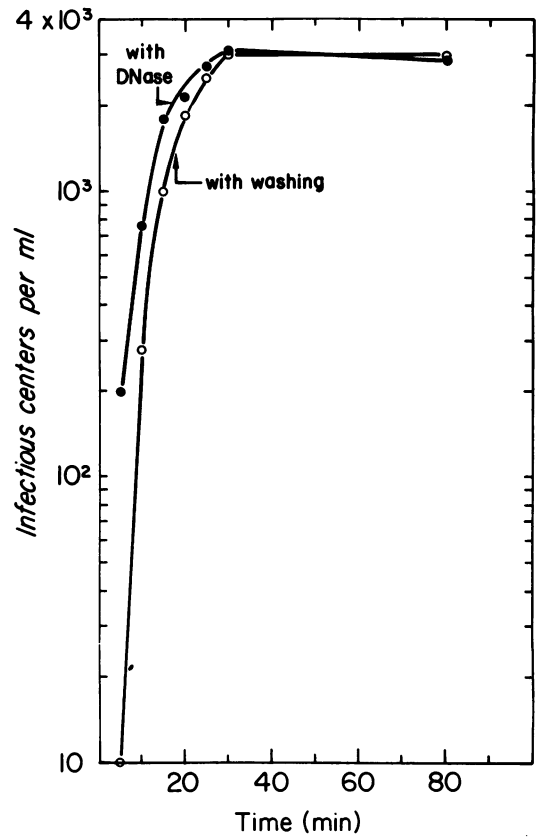

FIG. 3. *Effect of washing and deoxyribonuclease on transfection. Cells were grown to competence. All tubes received 0.1 ml of TP-1C DNA (final concentration, 0.8 μg/ml). CF (0.1 ml) was added to each tube, and the tubes were shaken on a rotary incubator shaker at 55 C. At the indicated times, either 0.2 ml of deoxyribonuclease (0.1 μg/ml) was added to the tube or the cells were washed twice at 25 C for 10 min with TYG (2.8 ml volumes). The cells were suspended in 2.6 ml of TYG, and all tubes were incubated at 55 C with shaking for 80 min. At this time, samples were assayed for infectious centers, in duplicate, on B. stearothermophilus 4S Com$^-$ cells.*

binding of DNA to cytoplasmic membranes. Accordingly, membranes were incubated with DNA in the absence of CF to determine the control level of binding and with CF to ascertain specific binding of DNA. Additional controls included incubation of CF and DNA at various temperatures. As shown in Table 7, approximately 80% of the transfecting activity of the DNA is recovered when the complete mixture is incubated for 30 min. When CF is added and incubated for only 10 min, only 16% of the biologic activity is recovered. In contrast, 10 min of incubation of CF with DNA does not result in inactivation, whereas significant inactivation is

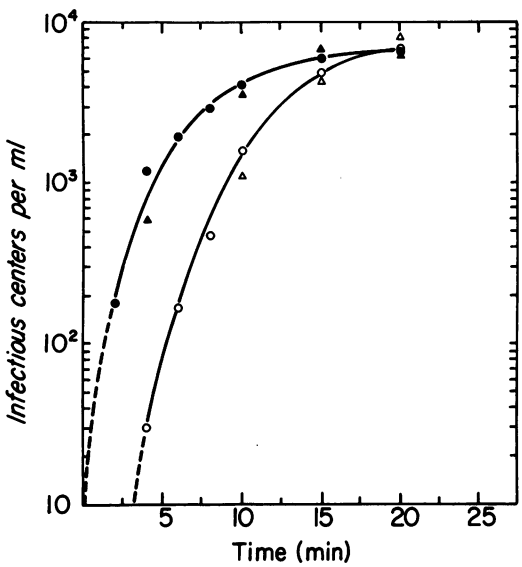

FIG. 4. *Effect of preincubation of 4S Com⁻ cells with CF on the development of resistance of infectious centers to deoxyribonuclease. Cells were grown to competence. CF (0.1 ml) was added to only one group of tubes. Both groups of tubes were shaken on a rotary incubator shaker at 55 C for 30 min. At this time, 0.1 ml of CF was added to the group which had not previously received CF, and 0.1 ml of TP-1C DNA (final concentration 0.8 μg/ml) was added to all tubes. At the indicated times, 0.2 ml of deoxyribonuclease (0.1 μg/ml) was added to tubes from both groups. All tubes were incubated at 55 C for 80 min before assaying for infectious centers on B. stearothermophilus 4S Com⁻ cells.*

observed after 30 min of incubation at 55 C. At 37 C this inactivation is not observed.

DISCUSSION

The early studies of Pakula and Walczak (13) and Tomasz and Hotchkiss (19) clearly demonstrated that soluble competence-inducing factors exist in *Streptococcus and Pneumococcus*. More recently it has been possible to distinguish noncompetent mutants on the basis of either loss of production of CF (CF^-) or of capacity to react with CF ($CF\ site^-$) (18). Although such soluble competence-inducing factors were reported in *B. subtilis* (2), the phenomenon was not reproduced in many laboratories. At present it is not possible to ascertain whether this is due to the instability of the CF or related to tight binding of the CF with the cells. Furthermore, a lack of CF^- $CF\ site^+$ mutants has severely inhibited further work in this system. In addition to these observations, numerous studies have implicated the integrity of the cell wall (14) or autolysis of the cell wall (1, 23) in the complex process of induction of competence in bacteria. At present, the interaction between CF and CF binding site during the period of competence cannot be described in molecular terms.

Because of these difficulties with the *B. subtilis* transformation system, we have chosen the *B. stearothermophilus* transfection system to explore the relationship between CF, CF site, and DNA. The present study adds several critical observations that should aid in unraveling the events resulting in competence.

(i) There is a physical association between CF and cells which results in the alteration of the sedimentation properties of these cells. (ii) The site for CF association appears to be on the cytoplasmic membrane. (iii) The association between CF and cytoplasmic membrane can be modified by incubation at elevated temperatures (67 C). (iv) The association between CF and the cytoplasmic membrane appears to be the first and rate-limiting step in the development of resistance of transfectants to extracellular deoxyribonuclease and washing (irreversible binding). (v) Addition of CF to membranes of CF^- $CF\ site^+$

TABLE 7. *Effect of B. stearothermophilus membranes and CF on removal of biologically active DNA from the supernatant fraction*

Sample	Time of incubation[a] (min)	Incubation temp (C)	Residual transfecting activity[b] (%)
Complete[c]	0	55	100
Complete	30	55	80
Complete-membranes	30	55	100
DNA preincubated with CF[d]	10	55	100
DNA preincubated with CF[d]	20	55	79
DNA preincubated with CF[d]	30	55	22
DNA preincubated with CF[d]	30	37	100
Complete + CF[e]	0	55	99
Complete + CF[e]	0	37	99
Complete + CF[e]	10	55	16
Complete + CF[e]	20	55	7
Complete + CF[e]	30	55	4
Complete + CF[e]	30	37	32

[a] Time of incubation with various constituents before assay of residual DNA activity by transfection.

[b] Residual biological activity as measured by transfection; 100% = 1.8×10^3 infectious centers/ml.

[c] The complete incubation mixture consisted of 0.1 ml of DNA (2.5 μg/ml), 0.5 ml of protoplast membranes (from 1.5×10^9 viable cells/ml), and 2.0 ml of TM buffer.

[d] Samples in which DNA was preincubated with CF for the indicated periods of time and then assayed for residual DNA activity by transfection.

[e] Competence-inducing factor (0.1 ml) was added to the membranes in TM buffer for 30 min before addition of DNA for the indicated times of incubation.

cells results in the removal of infectious DNA from the supernatant fraction in vitro. (vi) There is interaction between DNA and CF in vitro which is slower than the reaction between the CF and the membranes. (vii) Twenty mutants resistant to bacteriophage TP-1C produced CF but could not be transfected. None of these mutants bound CF at 37, 55, or 67 C. It is possible that phage resistance results in the alteration of the cellular membranes which abolishes interaction with exogenous CF.

The following model would encompass these observations with those noted previously (Streips and Young, 10th Int. Congr. Microbiol., p. 202).

(a) CF + site $\rightleftarrows$ CF-site
(b) CF-site + DNA $\rightleftarrows$ DNA-CF-site
(c) DNA-CF-site $\rightleftarrows$ DNA-site + CF
(d) DNA-site $\rightleftarrows$ infectious centers + site

At present it is not possible to determine whether active CF is released from the site in step (c). Similarly, in step (d) it is not known whether the site is regenerated or if it can adsorb more CF. The binding of the CF and subsequently of DNA to the cytoplasmic membrane does not appear to require the synthesis of an additional protein in vivo or in vitro (U. N. Streips and F. E. Young, *unpublished data*). However, the uptake of DNA before replication (step d) may depend on such a synthesis.

The interaction between DNA and CF appears to be complex. From the data shown (Table 7), it is not possible to distinguish between the following three possibilities. (i) The DNA is inactivated by the CF; (ii) the DNA is bound to the CF, making the complex too bulky for access to the membrane; or (iii) The DNA changes the ability of the CF to function normally. Experiments are in progress to determine whether one or more of these possibilities is operative. It is clear that, once the CF is adsorbed to the membrane, the reaction with DNA is extremely rapid and temperature independent, thus differing markedly from the reaction between DNA and CF.

There are at least two hypotheses which can explain the effects of CF in light of the model presented above. First, the CF might be one of the class of DNA binding proteins described recently by Alberts and Frey (3). Such a protein released at a specific time in the growth cycle could associate with DNA and a site on the cell membrane surface. The multiple bands observed in the Renografin gradient might be related to the number of molecules of CF bound to the cell. Alternatively, the CF could inhibit macromolecular metabolism in a fashion similar to the colicins (11). This inhibition could give the newly acquired molecule of DNA a selective advantage for intracellular survival and replication. Therefore, studies on the influence of CF on macromolecular metabolism should aid in establishing the precise relationship between the binding of CF and the alteration of sedimentation properties noted in *B. subtilis* and *B. stearothermophilus*. The elucidation of these hypotheses should provide guidance for future studies on the action of the CF in the *B. stearothermophilus* transfection system.

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ADDENDUM IN PROOF

Since the presentation of a part of these data at the 10th International Congress for Microbiology, Mexico City, 1970, and the submission of this manuscript for publication, Ziegler and Tomasz (Biochem. Biophys. Res. Commun. **41**:1342-1349) have demonstrated that the CF in the *Pneumococcus* transformation system also has a membrane receptor site.

LITERATURE CITED

1. Akrigg, A., and S. R. Ayad. 1970. Studies on the competence-inducing factor of *Bacillus subtilis*. Biochem. J. **117**:397-403.
2. Akrigg, A., S. R. Ayad, and B. R. Barker. 1967. The nature of a competence-inducing factor in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **28**:1062-1067.
3. Alberts, B. M., and L. Frey. 1970. T_4 Bacteriophage 32: a structural protein in the replication and recombination of DNA. Nature (London) **227**:1313-1318.
4. Bodman, H. A., and N. E. Welker. 1969. Isolation of spheroplast membranes and stability of spheroplasts of *Bacillus stearothermophilus*. J. Bacteriol. **97**:924-935.
5. Cahn, F. H., and M. S. Fox. 1968. Fractionation of transformable bacteria from competent cultures of *Bacillus subtilis* on Renografin gradients. J. Bacteriol. **95**:867-875.
6. Charpak, M., and R. Dedonder. 1965. Production d'un' facteur de competence' soluble par *Bacillus subtilis* Marburg ind 168. C. R. H. Acad. Sci. **260**:5638-5640.
7. Erickson, R. J., F. E. Young, and W. Braun. 1969. Binding of rabbit gamma globulin by competent *Bacillus subtilis* cultures. J. Bacteriol. **99**:125-131.
8. Griffith, F. 1928. Significance of pneumococcal types. J. Hyg. **27**:113-159.
9. Hadden, C., and E. W. Nester. 1968. Purification of competent cells in the *Bacillus subtilis* transformation system. J. Bacteriol. **95**:876-885.
10. Kohoutová, M., H. Braná, and I. Holubová. 1968. Isolation and purification of a substance inducing competence and inactivating transforming DNA in pneumococcus. Biochem. Biophys. Res. Commun. **30**:124-129.
11. Nomura, M. 1967. Collicins and related bacteriocins. Annu. Rev. Microbiol. **21**:257-284.
12. Osowiecki, H., J. Nalecz, and W. T. Dobrzanski. 1969. The mechanism of competence in the transformation of

streptococci of serological group H. Purification and some properties of the competence factor. Mol. Gen. Genet. **105**:16-20.
13. Pakula, R., and W. Walczak. 1963. On the nature of competence of transformable streptococci. J. Gen. Microbiol. **31**:125-133.
14. Prozorov, A. A. 1965. The effect of egg-white lysozyme on the permeability of cells of *B. subtilis* to transforming DNA. Dokl. Akad. Nauk SSSR **160**:472-474.
15. Singh, R. N., and M. P. Pitale. 1967. Enrichment of *Bacillus subtilis* transformants by zonal centrifugation. Nature (London) **213**:1262-1263.
16. Strauss, N. 1970. Early energy-dependent step in the entry of transforming deoxyribonucleic acid. J. Bacteriol. **101**: 35-37.
17. Streips, U. N., and N. E. Welker. 1969. Infection of *Bacillus stearothermophilus* with bacteriophage deoxyribonucleic acid. J. Bacteriol. **99**:344-346.
18. Tomasz, A. 1969. Some aspects of the competent state in genetic transformation. Annu. Rev. Genet. **3**:217-232.
19. Tomasz, A., and R. D. Hotchkiss. 1964. Regulation of the transformability of pneumococcal cultures by macromolecular cell products. Proc. Nat. Acad. Sci. U.S.A. **51**: 480-487.
20. Tomasz, A., and J. L. Mosser. 1966. On the nature of the pneumococcal activator substance. Proc. Nat. Acad. Sci. U.S.A. **55**:58-66.
21. Welker, N. E., and L. L. Campbell. 1965. Induction and properties of a temperature bacteriophage from *Bacillus stearothermophilus*. J. Bacteriol. **89**:175-184.
22. Young, F. E., J. Spizizen, and I. P. Crawford. 1963. Biochemical aspects of competence in the *Bacillus subtilis* transformation system. I. Chemical composition of cell walls. J. Biol. Chem. **238**:3119-3125.
23. Young, F. E., and J. Spizizen. 1963. Biochemical aspects of competence in the *Bacillus subtilis* transformation system. II. Autolytic enzyme activity of cell walls. J. Biol. Chem. **238**:3126-3130.