DEMONSTRATION OF A COMPETENCE-ENHANCING FACTOR IN SUPERNATANTS OF NEISSERIA GONORRHOEAE F62 TYPE T1.

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SUMMARY

Evidence is presented for the existance of a competence-enhancing factor (CEF) in the supernatants obtained from competent N. gonorrhoeae F62, type T1 cell populations. The factor restores competence for transformation to T1 cells that have undergone a one thousand-fold decrease in transformability as a result of being washed. Fresh broth partially restores competence to washed cells. CEF is destroyed upon exposure to trypsin, heat or membrane filtration. The ability of supernatant to enhance competence is inhibited in the presence of chloramphenicol.

INTRODUCTION

Competence in bacterial transformation refers to the ability of cells to take up and incorporate exogenous DNA, which is subsequently integrated into the resident genome. The development of competence is often proceeded by the production of an extracellular competence factor (CF). Investigations into the biochemical nature of this important physiological state have led to the isolation of CF in several species of bacteria (7, 10). The first report of genetic transformation in Neisseria gonorrhoeae (8), revealed that among the four clonal (colonial-morphology) types designated as T1, T2, T3, and T4, (5) only T1 and T2 are competent. As a result of renewed interest in the concept that virulence and competence in N. gonorrhoeae have a common basis (2), a number of laboratories have attempted the isolation of a gonococcal competence factor. Conceivably such a factor might render normally avirulent and noncompetent type T4 cells both virulent and competent. The data presented in this communication suggest the release of a competence-enhancing factor (CEF) into the medium by competent cultures of T1. T1 gonococcal cells that lose competence after having been washed with saline buffered with KPO_4 (PBS, pH 7.0), can be made transformable upon resuspension in supernatants derived from competent cultures that contain CEF. CEF shares a number of the prop-

erties common to the competence factors of other strains: sensitivity to proteolytic enzymes and heat, inactivation by membrane filtration, and a requirement for protein synthesis for expression of its activity. A component of fresh broth, although able to restore partial competence of PBS-washed cells, differs from CEF in that the broth activity is heat stable and insensitive to trypsin.

MATERIALS AND METHODS

Strains and Growth Conditions.

N. gonorrhoeae F62 clonal types (T1, T2, T3, and T4) were obtained from D.S. Kellogg (Communicable Disease Center, Atlanta, Georgia). The solid medium used to obtain colonies of N. gonorrhoeae was GC Medium Base (Difco) containing 1% agar and enriched with defined supplements 1 and 2 (GCBA-DS). The composition of the defined supplements has been described by Sparling (8). Liquid cultures were grown in 250-ml flasks in diphasic medium (8), which consisted of 30 ml of GCBA-DS (solid layer) and 70 ml of GCBB-DS (liquid broth). Plates containing GCBA-DS agar were incubated at 36C in a CO_2 (10%) atmosphere under conditions of controlled humidity (Napco Incubator Model 3321). Liquid cultures were incubated with slow shaking at 36C in the absence of CO_2 in an incubator-shaker (New Brunswick Inc., Model R25).

Clonal-Type Stability.

The method used to stabilize the clonal types of N. gonorrhoeae was by selective transfer on solid (GCBA-DS) medium (5). T1 could be maintained as a reasonably stable type after 10-15 such transfers that resulted in a culture exhibiting 96-98% stability. For use in transformations a GCBA-DS plate containing selectively transferred T1 cells was harvested and the harvest was used to inoculate the GCBB-DS (liquid) medium. Incubation at 36C for 3-4 hr resulted in a maximally competent culture.

DNA.

Transforming DNA was prepared by a modification of Marmur's method (11). The DNA concentration was determined by the diphenylamine method of Burton (3).

Transformation.

Cells to be used as recipients for transformation were grown in GCBB-DS medium at 36C. An aliquot (0.9 ml) of broth culture was mixed with donor DNA at a final concentration of 5 or 10 μg/ml and 0.05 ml of $CaCl_2$ (0.002 M final concentration) in a test tube. In most cases the donor DNA carried the streptomycin-resistance marker. The transformation tubes were incubated at 36C, without shaking in a 10% CO_2 atmosphere for 30 min, after which 0.05 ml (50 μg) of pancreatic (Worthington Biochemicals Corp.) deoxyribonuclease with 0.002 M $MgSO_4$ was added to destroy unbound DNA, and the tubes were incubated for an additional 10 min. The number of colony-forming units exposed to donor DNA was determined by plating appropriate serial dilutions on GCBA-DS medium. The plates on which transformants were to be scored were incubated at 36C for 5-6 hr (for phenotypic expression), and then were overlayed with 2.5 ml of soft agar (0.5% agar) containing enough streptomycin so that the final concentration was 500 μg/ml plate medium.

All transformation experiments included two controls: (1) recipient cells without DNA, (2) recipient cells plus DNA that had been pretreated with DNase (50 μg/ml) for 15 min. Controls were uniformly negative.

CEF Assay.

A 25 ml culture of T1 (early logarithmic-phase cells at a concentration of 2-4 X 10^7 cells/ml) were centrifuged at 27,000 X g for 10 min in the cold and the supernatant carefully decanted into a sterile flask and maintained at 4C. The pelleted cells were washed by centrifugation three times in 0.15 M NaCl buffered with 0.01 M KPO_4 (PBS) pH 7.0. The cells were then resuspended to give the required cell density of 2-4 X 10^7 cells/ml. The resuspended cells were transformed as described above using donor DNA to assay for competence. A 0.9 ml sample of the growing culture was always used to test the level of competence before the CEF assay regimen was undertaken. When washed cells resuspended in PBS were transformed, they exhibited an extremely low transformability, whereas cells resuspended in their original supernatant showed an immediate recovery of competence.

RESULTS

Our results for the kinetics of competence development in gonococcal cultures grown in GCBB-DS (broth) medium were essentially the same as those reported by Sparling (8). The initial observation that competent gonococcal cells exhibited a marked reduction in percentage transformation after they were washed in PBS was made by Sparling (8). The data in Table 1 confirm that finding and further in-

Table 1

Effect of Washing Competent Cells of N. gonorrhoeae F62-T1-str^S on Subsequent Transformation to Streptomycin Resistance.

Source of Recipient Cells	Recipient cells/ml	Transformants/ml	%T[d]
T1 supernatant[a].	3.0×10^4	3.1×10^2	1.0
T1 cells washed in PBS and resuspended in PBS[b].	3.0×10^7	1.2×10^3	0.004
T1 cells washed with PBS and resuspended in filtered T1 supernatant[c].	4.0×10^7	1.7×10^5	0.4
T1 cells washed with PBS and resuspended in the T1 supernatant.	3.0×10^7	3.3×10^5	1.1

a. T1 supernatant was obtained by centrifuging a culture that exhibits maximum competence, at 27,000 x g for 20 min.
b. The T1 pellet obtained in 'a' was washed three times with PBS and finally the cells were resuspended in PBS and saturating levels of DNA were immediately added to the suspension.
c. Filtration was done through a Millipore membrane (0.45μm).
d. Percentage transformation = $\frac{\text{number of transformants per ml}}{\text{number of recipient cells per ml}} \times 100$.

spection reveals that upon filtration through a Millipore filter (0.45μm), there is partial inactivation of the competence-enhancing property of the supernatant. It is also evident from Table 1 that CEF is released into the medium by competent cells. Thus, upon resuspension in the supernatant derived from a competent culture, PBS-washed cells regain maximum competence (1% transformation). It should be noted,

however, that the supernatant obtained by centrifugation of a T1 competent culture retained a few (3×10^2 cells/ml) competent cells.

An examination of the results shown in Table 2 reveals that the competence-enhancing activity contained in the supernatant is sensitive to both trypsin and heat.

Table 2

Sensitivity of Competence-Enhancing Factor to Trypsin and Heat

PBS-washed cells resuspended in:	Recipients/ml[a]	Transformants/ml	%T[c]
T1 supernatant	2.9×10^7	2.5×10^5	0.90
T1 supernatant + trypsin[b]	3.2×10^7	8.9×10^4	0.27
T1 supernatant preheated[d]	7.0×10^7	2.0×10^5	0.28*
PBS	2.4×10^7	1.0×10^3	0.004

a. Recipient cells of N. gonorrhoeae F62-T1-str^S were grown using the standard conditions described under Materials and Methods.
b. T1 supernatant was treated with freshly prepared solution of trypsin (10µg/ml) for 30 min before donor DNA was added.
c. Percentage transformation.
d. T1 supernatant was heated at 95 C for 20 min before donor DNA was added.
* Corrected value obtained from a parallel experiment.

It was demonstrated by Sparling (8) that when PBS-washed cells were resuspended in fresh broth (5% GCBB-DS), an increase in the percentage transformation was obtained. We attempted to determine the nature of the component of broth responsible for restoring partial competence to washed cells. The results of this experiment are shown in Table 3. Proteose peptone-3 (Difco) is apparently the component of fresh GCBB-DS broth.

In our experience, the stimulation of competence in washed cells obtained with fresh broth never exceeded more than one third to one fourth the effect obtained with supernatant derived from a competent culture. A similar effect was also ob-

served upon resuspension of washed cells in yeast extract; a casamino acids preparation was less effective (Table 3). The results shown in Table 4, demonstrate that the ability of fresh broth to partially reverse the loss of competence in washed cells is unaffecteded by treatment with trypsin or heat. Results obtained by Tomasz (9) and Lacks and Greenberg (6) suggest that protein synthesis, in addition to being required for the synthesis of competence factor itself, also is essential for anothe

Table 3

Effects of the Components of GCBB-DS[a] and Other Supplements on Competence for Transformation

PBS-washed cells were resuspended in:	Recipients/ml*	Transformants/ml	%T[b]
A:			
T1 supernatant	2.6×10^7	2.58×10^5	1.00
Complete broth	6.0×10^7	1.60×10^5	0.27
Broth (-supplement #1)	6.0×10^7	1.60×10^5	0.27
Broth (-supplement #2)	6.0×10^7	1.70×10^5	0.28
Broth (both supplements)	6.0×10^7	1.65×10^5	0.27
Broth (- supplements 1 and 2 and NaCl)	6.0×10^7	1.68×10^7	0.27
Supplement #1 only	4.0×10^7	2.00×10^2	0.0005
Supplement #2 only	4.0×10^7	1.00×10^2	0.00025
NaCl (0.5 g/l) only	4.0×10^7	1.30×10^3	0.003
Saline buffered with phosphate (PBS)	2.0×10^7	2.50×10^3	0.012
B:			
Yeast Extract (0.3%)	7.0×10^7	1.80×10^5	0.25[c]
Casamino Acids (0.5%) (vitamin free)	6.0×10^7	6.00×10^5	0.10[c]

a. GCBB-DS (broth) consists of GC Medium Base (Proteose-peptone; NaCl buffered with phosphate) plus defined supplements 1 and 2.
b. Percentage Transformation.
c. Corrected value from another experiment.
* See the legend of Table 2.

step involved in the induction of competence. Similarly, we have found that restoration of competence to PBS-washed cells of N. gonorrhoeae that have been resuspended in supernatant containing CEF appears to be dependent upon further protein synthesis. In the presence of chloramphenicol (5µg/ml) washed cells suspended in

CEF exhibited a significant reduction in competence (Table 4). As expected, chloramphenicol also reduced the competence of washed cells that had been resuspended in fresh broth. Variability in cell viability in the suspensions as a cause of the different transformation frequencies obtained in supernatant and broth was ruled out by determination of viable counts before and after the addition of DNA.

Table 4

Effect of Various Treatments on the Ability of Competence-Enhancing Factor and Broth to Restore Competence to Washed Cells

PBS-washed cells resuspended in:	Recipients/ml*	Transformants/ml	%T[d]
T1 supernatant	3.0×10^7	2.8×10^5	0.93
T1 supernatant + CAM[a]	3.0×10^7	4.0×10^4	0.13
Broth	6.5×10^7	2.0×10^5	0.3
Broth + CAM[a]	6.5×10^7	4.0×10^4	0.06
Preheated Broth[b]	2.6×10^7	1.0×10^5	0.3
Broth + Trypsin[c]	7.0×10^7	2.0×10^5	0.28
PBS	3.0×10^7	2.0×10^3	0.007

a. Chloramphenicol (CAM) at a final concentration of 5 µg per ml was added to cells at the same time as donor DNA.
b. Broth was heated at 95°C for 20 min before donor DNA was added.
c. Broth was treated with trypsin (10µg/ml) for 30 min before donor DNA was added.
d. Percentage Transformation.
* See Table 2.

DISCUSSION

Previous studies concerned with the isolation of soluble factors responsible for eliciting the competent state in other bacterial genera have been reviewed by Tomasz (9). We initiated the present investigation in order to ascertain whether or not such factors are involved in the achievement of competence in N. gonorrhoeae. Our studies indicate that competent T1 cultures release CEF into the medium during its growth. Washing of competent gonococcal cells causes an immediate loss of competence; however, upon resuspension of such washed cells in the original supernatant, the loss is reversed. Such an effect of washing has been reported for H. influenzae

(4) and B. subtilis (1). The gonococcal CEF is heat labile, sensitive to proteolytic enzyme treatment and is inactivated by filtration through membrane filters.

The partial increase in competence conferred to washed cells by resuspension in fresh broth (GCBB-DS) is extremely reproducible with respect to the degree of restoration. The value (0.3 - 0.4% transformation) was always at least 3-fold below the full recovery of competence brought about by the active component of T1 supernatant which we refer to as CEF.

A careful examination of the results shown in Tables 3 and 4 reveal one of the difficulties that we have encountered in interpreting our results. In most experiments a lower viable count is obtained after cells are resuspended in supernatant than when they are resuspended in fresh broth. We have compared the effect of fresh broth and supernatant on growth and viability during the time that elapses between resuspension of the cells and the plating of the samples and we have observed no significant differences. With extraordinary care one can insure equal numbers of recipient cells in both broth and supernatant. Under these circumstances (data not shown) supernatant (containing CEF) stimulates competence to a value of 3 to 4-fold higher than that obtained with fresh broth. We have concluded on the basis of these and other experiments that the differences in viability that we have observed are probably the result of more pronounced clumping in supernatant compared to fresh broth. Such clumping apparently affects the total population and the transformable portion of it to the same extent. Furthermore, the active component of supernatant is quite different from the material contained in fresh broth. The latter activity is not destroyed by trypsin or heat; the former is sensitive to either treatment.

Induction of competence in gonococcal cells is inhibited by chloramphenicol. We interpret this observation to imply that competence in N. gonorrhoeae, as has been shown in other systems (9), requires protein synthesis for its expression.

We have thus far been unable to significantly purify CEF by such procedures as $(NH_4)_2SO_4$ precipitation and/or gel filtration; the activity seems relatively unstable. The crude material can be diluted 10-fold with full retention of activity. Attempts to induce competence in noncompetent, avirulent type T1

cells (8) by resuspension of such cells in T1 supernatant and by growing differentially genetically marked T1 and T4 cells in mixed cultures have thus far failed.

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