Factors Affecting Genetic Transformation of *Neisseria gonorrhoeae*

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Piliated gonococci were competent in genetic transformation in all stages of growth in minimal and enriched media, but nonpiliated cells were almost totally incompetent. Uptake of deoxyribonucleic acid into a deoxyribonuclease-insensitive state was observed only in competent piliated cells. Competence was not affected by washing of competent cells or treatment of competent cells with proteolytic enzymes. Expression of competence required presence of any of several different monovalent or divalent cations, as well as a utilizable source of energy. Efforts to produce genotypically or phenotypically competent derivatives of nonpiliated cells were unsuccessful. These experiments are consistent with the idea that pili may play a role in the irreversible uptake of transforming deoxyribonucleic acid by the gonococcus, but fail to provide evidence for other types of competence factors.

Competence in genetic transformation in several bacterial species occurs only in certain strains, and often only transiently or during conditions suboptimal for normal growth (25). In addition, competence has been shown to be markedly enhanced in many instances by the effects of soluble protein "competence factors" (25). Competence in transformation is thus often an unstable and relatively atypical physiological state.

In contrast, *Neisseria gonorrhoeae* was reported to be transformable throughout growth, without sudden waves of competence (19). Virulent colony types T1 and T2 (11) of all tested strains were transformable at relatively high frequencies (10^-4 to 10^-2) under all tested conditions of growth, but nonvirulent colony types T3 and T4 were transformable only at very low frequencies (transformation frequencies of approximately 10^-7) (19). Subsequently, transformable virulent colony types of the gonococcus have been shown to be piliated, whereas nonpiliated, nonvirulent gonococci lack pili (10). Presence of pili has also been correlated with competence in transformation in *Moraxella* (5) and in *Neisseria meningitidis* (6). Whether pili are necessary for competence in these organisms, and if so, by what mechanism, is unknown.

Recently, it was reported that competence of piliated type 1 gonococci was enhanced significantly by a soluble factor (18). In addition, the necessity of pili for competence in gonococcal transformation has been questioned by reports of high-frequency transformation of nonpiliated colony type 4 strains (E. Baron and A. K. Saz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, H11, p. 98). Because of these observations, and because of the paucity of information about the mechanisms of transformation in this organism, we have undertaken systematic study of factors that affect transformation of the gonococcus.

MATERIALS AND METHODS

Strains. Bacterial strains FA19 and F62 were described previously (16, 19). F62 is a proline auxotroph. FA248 is a double auxotroph, requiring proline and methionine for its growth, which was isolated in this laboratory by mutagenizing F62 with ethyl methane sulfonate (EMS). All strains were stored in Trypticase soy broth (Difco) containing 20% glycerol at -70°C.

Media and growth. Routine cultures were made on Difco GC base agar medium (GCBA-DS) or GC base broth (GCBB-DS) (16). Each received 1% (vol/vol) of Kellogg defined supplement I and 0.1% of supplement II (11) immediately before use. Minimal medium Davis (Difco) or GCBB without supplements was used for serial dilutions. Defined growth media (NEDA or NEDF) were as described by Catlin (4). All other conditions of growth were as described elsewhere (16). Liquid cultures were grown at 37°C in a shaking incubator supplemented with 5% CO₂. Double-distilled deionized water was used to make minimal media and all reagents for studies of ionic requirements for transformation.

Transformation. The routine methods of obtaining competent recipient cells from GCBA-DS plates, incubation with deoxyribonucleic acid (DNA) in
complex broth medium (GCBB-DS) and selection for drug resistance markers, have been described elsewhere (16). For transformation in tri(hydroxy-
methyl)aminomethylene (Tri) or phosphate buffer, cells grown on GCBA-DS or NEDA medium were incubated with the appropriate buffer, washed once or twice by centrifugation, and resuspended in the transformation buffer (10 mM Tris-hydrochloride, pH 7.4, or 20 mM potassium phosphate buffer, pH 7.4, each containing 0.25% glucose). Various salts were added to the buffers as described in the text. Transformation in defined liquid NEDF medium (4) was done with cells passed twice on defined NEDA medium (4). NEDF was modified for this purpose by omission of the amino acid(s) used as donor markers. All transformation reaction mixtures were incubated at 37°C for 20 min before the reaction was terminated by addition of deoxyribonuclease (DNase) (16). Numbers of viable colony-forming units (CFU) exposed to DNA and of transformants were determined by plating appropriate dilutions of a single sample of the DNase-treated reaction mixture. All platings were completed within 90 min (usually less) of onset of incubation of the reaction mixture. Selection for nutritional markers was made by spreading aliquots of the transformation mixture onto NEDA lacking the particular amino acid(s) used as the selective marker, followed by incubation at 37°C for approximately 44 h. Cells in transformation reaction mixtures containing GCBB-DS were washed once by centrifugation and suspended in an equal volume of medium A before selection for nutritional markers.

Transforming DNA was prepared by the method of Marmur (12). The concentration of DNA was estimated by the diphenylamine reaction (3). The concentration of DNA used in the transformation reaction mixture was 1 µg/ml unless stated otherwise. The pH of complete transformation reaction mixtures was determined with a pH meter (Beckman SS-1) after cells and DNA were added.

Mutagenesis. Eighteen-hour-old cells were scraped from a GCBA-DS plate and suspended in Davis minimal medium A (Difco). A suspension of approximately 10^8 CFU/ml was incubated with EMS, 1% (vol/vol, final concentration) at 37°C. Samples were withdrawn at 20, 30, and 40 min, and placed in an ice bath. They were washed three times with medium A by centrifugation at 4°C, and finally resuspended in Trypticase soy broth + 20% glycerol. An aliquot from each sample was plated for viable count, and the rest was stored frozen at −70°C. Samples exhibiting 99.0 to 99.9% killing by EMS were thawed and plated on NEDA medium. After incubation for 2 days at 36°C, colonies were replica-plated onto NEDA and NEDA lacking various amino acids. Colonies failing to grow on the latter were purified to colony type 1, restested for nutritional requirements, and frozen.

Mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NGN) was utilized in attempts to select competent mutants of nonpiliated T4 cells. Approximately 10^6 CFU of T4 cells suspended in GCBB-DS were incubated with 5 µg of NGN per ml at 37°C for 20 min, which resulted in 90 to 99% killing. Mutagenized cells were washed twice by centrifugation, resuspended in their original volume of GCBB-DS, and incubated at 37°C for 8 h. Aliquots were then transformed as usual, and any transformants that appeared were isolated, screened for T4 DNase, and carefully restested for competence in transformation. This procedure increased frequencies of mutation to high-level streptomycin resistance to about 5 × 10^-4, compared with about 10^-6 for control cells.

Electron microscopy of pili. Electron microscopy was done by a modification of Sharp's method (17). Cells grown 18 h at 37°C on GCB agar plates were suspended in water by adding 1.0 ml of distilled water to the agar surface, followed by gentle agitation of the plate. The suspension was poured into a test tube, and after being allowed to settle briefly, a drop was placed on an agar square and dried. The agar square was then stained with 0.75% colloidin and immersed in water. The film of cells that floated to the surface was picked up with a copper grid, dried, shadow-coated with platinum-carbon, and examined under a microscope (AEI EM6B) with a 20-µm objective aperture at 6 kV, at a magnification of 10,000 to 30,000.

Preparation of radioactive DNA. Cells were labeled with [3H]adenine or [14C]adenine as previously described (2). DNA was isolated and purified by Marmur's method (12), and was dialyzed against SSC (0.15 M NaCl plus 0.015 M sodium citrate) at pH 7.0. Specific activities were generally about 10^6 cpm per µg of DNA. Radioactivity of DNA, which was digested with ribonuclease during purification (12), was unchanged by alkaline digestion (2).

DNA uptake. One volume of labeled DNA was added to 9 volumes (0.9 to 4.5 ml) of cells to give a final DNA concentration of 1 µg (10^4 cpm) per ml. Cells were suspended to a density of 5 × 10^10 to 1 × 10^11 CFU per ml in GCBB or, in some experiments, in 20 mM potassium phosphate buffer, pH 7.4, or 10 mM Tris-hydrochloride, pH 7.4, as described in the text. Cells and DNA were incubated 20 min at 37°C, after which samples to be used for analysis of total DNA bound were immediately placed in ice. Samples to be assayed for DNase-resistant (DNase') DNA uptake were incubated for an additional 20 min at 37°C with 50 µg of DNase I per ml, after which they were placed in ice. All subsequent steps were performed at 0 to 4°C unless stated otherwise. Aliquots were taken, as appropriate, for determination of transformants and viable count. The remainder was centrifuged (10 min, 15,000 × g), suspended in 5 ml of transforming medium, or in 5 ml of SSC, washed twice by centrifugation, and suspended in SSC. Resuspension was either vigorous (blending in a Vortex Genie, Scientific Industries, speed setting 5, for 15 s) or mild (gentle tapping of tube at intervals over 10 min). The pellet obtained from the final centrifugation was resuspended in 1 ml of SSC, immediately collected by filtration on a membrane filter (Schleicher and Schuell B6), rinsed with cold SSC, dried, and counted in a toluene-based scintillant. All values were corrected for radioactivity bound under similar conditions, using DNA digested with DNase I before addition to the cells.

Transformation of lysozyme-EDTA-treated cells.
FA19 T4 cells were suspended to a cell density of approximately $2 \times 10^8$ to $4 \times 10^8$ CFU/ml in TYS broth (10 mg of tryptone per ml, 5 mg of yeast extract per ml, and 0.16 M sucrose). Lysozyme was added to a final concentration of 200 $\mu$g/ml, and disodium ethylene-diaminetetraacetic acid (EDTA) was added to a final concentration of 1 mM. After incubation for 20 min at 37°C, CaCl$_2$ was added to 10 mM final concentration, transforming DNA was added, and transformation was carried out as usual.

Transformation of Tris-EDTA-treated cells. FA19 T4 cells were suspended to a density of $2 \times 10^8$ to $4 \times 10^8$ CFU/ml in 10 mM Tris-hydrochloride, pH 7.4, containing 5 mM EDTA, and incubated 20 min at 37°C. To this was added an equal volume of double-strength GCBB and CaCl$_2$ to a final concentration of 10 mM. Transformation was then carried out as usual.

Chemicals. EMS and trypsin inhibitor were from Sigma. NGN was from Aldrich Chemicals. Lysozyme was from Schwarz Bioresearch. Pronase and trypsin were from Calbiochem. DNase I was from Worthington. [$^{14}$C]adenine and [$^{3}$H]adenine were from New England Nuclear Corp. All other chemicals were reagent grade from commercial sources. Antibiotics were from sources described previously (18).

RESULTS

Relationship of growth phase to competence. It was previously reported that gonococci were maximally competent during log and early-logarithmic phases of growth in complex broth, but were transformable throughout all growth phases, as long as they remained in their piliated phase (19). If competence of piliated gonococci depends at least in part upon production of a soluble competence factor, as has been reported (18), one might expect peaks of competence during growth in liquid media.

Accordingly, we reinvestigated competence in liquid medium. Strain FA248, which is a met-1 pro-1 auxotroph, was grown in defined liquid (NEDF) medium and, at hourly intervals, aliquots were removed and assayed for total viable count and numbers of Pro$^+$ transformants (Fig. 1). Results showed no evidence for sudden waves of increased or decreased transformability, suggesting that competence was essentially uniform during all phases of growth.

The levels of competence of growing cells were estimated more precisely by analyzing frequencies of single and double transformation for the unlinked markers met-1 and pro-1, using the Goodgal and Herriott formula (7). (In experiments not shown, it was established by DNA dilution curves that met-1 and pro-1 were not linked.) Cells of FA248 were grown from log to stationary phase in both defined NEDF medium and in peptone broth (GCBB) medium. At hourly intervals, samples were removed and assayed for viable count, and for numbers of single and double transformants. Results showed that cells were somewhat more competent when grown and transformed in GCBB than when grown and transformed in defined NEDF medium, but levels of competence were essentially identical at all stages of growth in either medium (Table 1).

In other experiments, viable counts were compared to direct Petroff chamber counts. Most cells from both NEDF and GCBB medium were found to be in units of two (diplococci); up to 20% of cells were in units of four or more. Thus, estimates of the percentage of cells that were competent may be fallaciously high, by a factor of at least twofold.

Lack of soluble competence factor. The evidence for unchanging competence throughout growth suggests that soluble competence factors similar to those found in the pneumococcus (24) and streptococcus (13) are not present in the gonococcal transformation system. More direct evidence was sought by two means.

In one set of experiments, competent piliated
gonococci were washed by repeated centrifugation and resuspension in either fresh GCBB medium or in 10 mM Tris-hydrochloride, pH 7.4, containing 0.25% glucose and 20 mM K$_2$SO$_4$ (TGK medium). Transformation efficiencies were affected only slightly by repeated washing (Table 2). Resuspension of washed cells in the supernatant from the first centrifugation, or in the supernatant from cultures of T1 cells grown in GCBB, failed to significantly increase transformation efficiencies over that seen when cells were resuspended in fresh medium.

In a second set of experiments, competent piliated cells were incubated in proteolytic enzymes before exposure to transforming DNA. Digestion with 1.0 to 2.5 mg of trypsin per ml for 30 min, before addition of transforming DNA and during exposure to DNA, resulted in an approximate 10-fold decrease in viable count, but transformation efficiencies were unchanged or slightly increased (Table 3). Results were similar in other experiments in which Pronase was used instead of trypsin. Trypsin had no appreciable effect on numbers and morphology of pili under the conditions studied. These experiments, therefore, do not add to understanding of whether pili are involved in the transformation process, but do provide additional evidence against a soluble or surface-bound (nonpilus) protein competence factor.

**Effect of chloramphenicol.** In many transformation systems, inhibition of protein synthesis with chloramphenicol reduces phenotypic expression of competence (25). Simultaneous addition of 10 μg of chloramphenicol per ml and transforming DNA to competent colony type T1 cells of FA19 or F62 in GCBB medium had no effect on transformation frequencies (results not shown). However, addition of 10 μg of chloramphenicol per ml to a culture of the same cells during growth in GCBB, before addition of transforming DNA, did result in an approximate 10-fold reduction in transformation efficiency over a period of 60 min (Fig. 2). When chloramphenicol-treated cells were washed and allowed to resume normal growth, transformation efficiencies gradually increased again, and were essentially normal after 30 min of incubation (Fig. 2). Thus, concentrations of chloramphenicol that had minimal effect on viability had a much greater effect on phenotypic development of competence, presumably due to interruption of synthesis of one or more proteins essential to the transformation process. Examination of chloramphenicol-treated cells by electron microscopy showed normal numbers and appearance of pili.

**Ionic requirements for transformation.** To better understand the transformation process, and in hopes of being able to reduce variables affecting transformation efficiencies, we studied the ionic requirements for transformation.

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**Table 1. Estimation of competence of N. gonorrhoeae strain FA248, colony type T1**

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Growth phase</th>
<th>Total CFU/ml (×10$^9$)</th>
<th>Transformation efficiency$^a$</th>
<th>% Competent$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Met$^+$ Pro$^+$</td>
<td>Met$^+$ Pro$^+$</td>
</tr>
<tr>
<td>NEDF</td>
<td>Inoculation</td>
<td>0.47</td>
<td>2.1 × 10$^{-3}$</td>
<td>2.7 × 10$^{-5}$</td>
</tr>
<tr>
<td></td>
<td>Log</td>
<td>2.4</td>
<td>1.7 × 10$^{-3}$</td>
<td>1.7 × 10$^{-5}$</td>
</tr>
<tr>
<td></td>
<td>Stationary</td>
<td>10.6</td>
<td>2.8 × 10$^{-3}$</td>
<td>2.0 × 10$^{-5}$</td>
</tr>
<tr>
<td>GCBB-DS</td>
<td>Inoculation</td>
<td>0.25</td>
<td>3.2 × 10$^{-4}$</td>
<td>4.2 × 10$^{-7}$</td>
</tr>
<tr>
<td></td>
<td>Log</td>
<td>1.8</td>
<td>6.6 × 10$^{-3}$</td>
<td>1.2 × 10$^{-6}$</td>
</tr>
<tr>
<td></td>
<td>Stationary</td>
<td>13.0</td>
<td>7.7 × 10$^{-4}$</td>
<td>8.4 × 10$^{-7}$</td>
</tr>
</tbody>
</table>

$^a$ Cells were transformed in the same medium in which they were grown.

$^b$ Calculated according to the method of Goodgal and Herriott (7).

**Table 2. Effect of repeated washing on competence of F62 colony type T1**

<table>
<thead>
<tr>
<th>No. of washes</th>
<th>Cells resuspended in:</th>
<th>Exposed CFU/ml</th>
<th>Transformation efficiency (Pro$^+$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Fresh TGK$^*$</td>
<td>5 × 10$^{10}$</td>
<td>2.8 × 10$^{-4}$</td>
</tr>
<tr>
<td>3</td>
<td>Fresh TGK</td>
<td>6 × 10$^{10}$</td>
<td>1.3 × 10$^{-4}$</td>
</tr>
<tr>
<td>3</td>
<td>First supernatant + 20 mM K$_2$SO$_4$</td>
<td>4.5 × 10$^{10}$</td>
<td>1.0 × 10$^{-4}$</td>
</tr>
<tr>
<td>2</td>
<td>Fresh GCBB$^+$ + 20 mM Ca$^{2+}$</td>
<td>2.1 × 10$^{10}$</td>
<td>1.6 × 10$^{-3}$</td>
</tr>
<tr>
<td>2</td>
<td>GCBB + 2 mM Ca$^{2+}$ (mother liquor)</td>
<td>1.7 × 10$^{10}$</td>
<td>2.7 × 10$^{-3}$</td>
</tr>
</tbody>
</table>

$^a$ Competent F62 T1 cells obtained from an NEDA plate were suspended in 10 mM Tris-hydrochloride + 0.25% glucose, and were washed in the same medium by repeated centrifugation at 4°C. Washed cells were re-suspended in either fresh 10 mM Tris-hydrochloride (pH 7.4) containing 0.25% glucose and 20 mM K$_2$SO$_4$ (TGK), or in the first Tris-glucose supernatant supplemented with 20 mM K$_2$SO$_4$, before transformation.

$^b$ Competent F62 T1 cells obtained from a mid-logarithmic-phase culture in GCBB-DS were washed twice and resuspended in either fresh GCBB, or in the supernatant from the original broth culture ("mother liquor").
TABLE 3. Lack of effect of trypsin on transformation of strain FA19 colony type T1

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Treatment</th>
<th>Viable CFU/ml (x10^6)</th>
<th>Str^+ transformants/ml (x10^6)</th>
<th>Transformation efficiency</th>
<th>Pili</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris + glucose + 5 mM Mg^2+</td>
<td>None</td>
<td>3.5</td>
<td>6.8</td>
<td>1.9 x 10^-4</td>
<td>ND^a</td>
</tr>
<tr>
<td></td>
<td>TI + trypsin</td>
<td>7.0</td>
<td>28.0</td>
<td>4 x 10^-4</td>
<td>+c</td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>0.66</td>
<td>2.9</td>
<td>4.3 x 10^-4</td>
<td>+</td>
</tr>
<tr>
<td>GCBB</td>
<td>None</td>
<td>4.1</td>
<td>13.0</td>
<td>3.1 x 10^-4</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>TI + trypsin</td>
<td>4.6</td>
<td>13.0</td>
<td>2.9 x 10^-4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>0.28</td>
<td>5.0</td>
<td>1.8 x 10^-3</td>
<td>+</td>
</tr>
</tbody>
</table>

^a Cells washed once in 10 mM Tris-hydrochloride (pH 7.4) were suspended in the indicated incubation medium, to which was added either no trypsin, 1 mg of trypsin per ml, which had been pretreated with 1.0 mg of trypsin inhibitor (TI) per ml, or 1 mg of trypsin per ml. The mixture was incubated at 37°C for 30 min, after which Str^+ DNA (1 μg/ml) was added. After 15 min at 37°C, 50 μg of DNase per ml was added, and incubation was continued for an additional 5 min at 37°C. A sample was then spread on an agar square, dried, and prepared for electron microscope examination for pili as described in Materials and Methods. Trypsin inhibitor (1.0 mg/ml) was added, and cells were plated for viable count and Str^+ transformants.

^c ND, Not done.

^c +, Pili present by electron microscopy.

Piliated, competent cells grown for 16 to 18 h on enriched GCBA-DS plates, or on NEDA plates, were washed in 10 mM Tris-hydrochloride, pH 7.4, by centrifugation, and resuspended in fresh 10 mM Tris buffer. In the absence of added cations or glucose, maximal efficiencies of transformation to Str^+, Met^+, or Pro^+ were approximately 3 x 10^-7. Addition of 0.02% glucose increased efficiencies of transformation to Str^+ to approximately 10^-5. Thus, transformation was sharply limited by absence of a readily utilizable source of energy.

Addition of any of several divalent cations to transformation reaction mixtures in 10 mM Tris + 0.25% glucose, or 20 mM potassium phosphate containing 0.25% glucose, resulted in concentration-dependent stimulation of transformation. Results of typical experiments are shown in Fig. 3. Maximal transformation frequencies were approximately 5 x 10^-4 at the optimal concentration of each cation. The most effective divalent cation on a molar basis was Mg^2+, which exerted maximal stimulation at concentrations of 2 mM. In contrast, maximal stimulation by Ca^2+ was achieved only at concentrations of 20 mM. Results were identical when sulfate rather than chloride salts were used, and were independent of numbers of cells (10^4 CFU/ml to 10^6 CFU/ml) present in the transformation reaction mixture.

Not all divalent cations stimulated transformation. Addition of MnCl_2 in concentrations from 0.001 to 1.0 mM slightly increased transformation frequencies occasionally, but usually was without effect. Similarly, CuSO_4 or CoCl_2 in concentrations up to 1 mM did not increase transformation efficiency, but did result in ap-
preciable decreases in total viable counts at salt concentrations of >0.01 mM. The stimulatory effect of MgCl₂ on transformation was completely blocked by 0.01 mM CuSO₄, but MnCl₂ and CoCl₂ did not have this effect (results not shown).

Several monovalent cations also resulted in concentration-dependent stimulation of transformation, when cells were suspended in either Tris or phosphate buffer containing 0.25% glucose. Maximum transformation frequencies were generally higher in phosphate than Tris buffer. Results of typical experiments in phosphate buffer are shown in Fig. 4. Transformation frequencies (2 × 10⁻⁴ to 5 × 10⁻⁴) were similar to those obtained with divalent cations, although peak effects required concentrations of 50 to 100 mM of the monoivalent cations. Both chloride and sulfate salts were equally effective.

Combinations of cations frequently resulted in additive stimulation of transformation. For instance, addition of small concentrations of Mg²⁺ to transformation reaction mixtures containing 20 mM K⁺ resulted in greater transformation efficiency than was achievable with either K⁺ or Mg²⁺ alone (results not shown). Combinations of Ca²⁺ and Mg²⁺ exerted similar additive effects. Greater stimulation was not achieved by various combinations of three or more cations, and frequently resulted in decreases in transformation.

We considered the possibility that DNA precipitated in solutions of very low ionic strength, and that the effect of cations on transformation was due to increasing the solubility of DNA. [³H]adenine-labeled DNA (10⁴ cpm/μg) was added in final concentration of 1 μg/ml to 10 mM Tris-hydrochloride, pH 7.4, containing 0.25% glucose, and either no additional cation, MgCl₂ (0.1 and 2.0 mM), or K₂SO₄ (1 and 20 mM). The solutions were then filtered through 0.45-μm membrane filters (Millipore Corp., type HA), and radioactivity was determined in both the filtrate and on the filter. Results showed that approximately 20% of DNA was retained on the filter, regardless of salt concentration in the DNA solution. We concluded that the stimulatory effect of cations on transformation was not due to increasing the solubility of DNA. It should be noted that DNA was dissolved in SSC and, since DNA was always added in 0.1 volume, all transformation reaction mixtures contained 0.015 M NaCl in addition to other stated cations.

Two problems were encountered in these experiments on the effects of cations on transformation: clumping of cells, and accelerated rates of cell death. Both Mg²⁺ and Ca²⁺ resulted in increased clumping of colony type T1 cells suspended in 10 mM Tris-hydrochloride (pH 7.4), or in GCBB, as determined by phase microscopy. The size of the cell clumps was considerably greater in 2 mM CaCl₂ than in 2 mM MgCl₂. In contrast, T1 cells suspended in Tris buffer alone or in Tris buffer containing either 100 mM NaCl or 20 mM KCl were almost entirely composed of either single cells or diplo-
cocc. Thus, quantitative studies were facilitated by use of monovalent, rather than divalent, cations.

On the other hand, rates of cell death were accelerated by the monovalent cations, with over 90% reduction in viable count after 90-min incubation at 37°C in 10 mM Tris + 0.25% glucose + 100 mM NaCl or 20 mM KCl. Cell death was less marked in similar solutions containing 2 to 5 mM MgCl₂. Because of these factors, all platings for determination of viable count were done after incubations were completed, and at the same time as platings for selection of transformants.

Effect of pH. Transformation in Tris-hydrochloride over a pH range from 5.8 to 9.0 revealed a pH optimum of about 7.5, with sharply reduced transformation efficiencies below pH 7.0 and above pH 8.0 (Fig. 5).

Uptake of DNA. Binding and uptake of [³H]DNA or [¹⁴C]DNA were studied in competent piliated colony type T1 and noncompetent nonpiliated colony type T4 cells of FA19 and F62, under various conditions. Results when cells were suspended in GCBB are shown in Fig. 6. Total DNA bound was nearly identical in both T1 and T4 cells, and showed linear increases over a wide range of DNA concentrations. Approximately 50% of added DNA was bound under these conditions. Results were unaffected by temperature (4 to 37°C) or by the degree of agitation of cells during the washing procedures.

In contrast, only about 0.5% of added DNA was taken up into a DNase-resistant state, and this was observed only in competent colony type T1 cells. No DNase-resistant uptake was seen with colony type T4 cells. DNase-resistant uptake and transformation efficiency followed essentially parallel curves, with saturation at about 0.5 μg of DNA per ml.

Results were variable when cells were suspended in either Tris buffer or in phosphate buffer, each containing 0.25% glucose and any of several combinations of cations, or in defined NEDF medium. Most markedly affected was total (DNase-sensitive) binding of DNA, which was only slightly greater under these conditions than DNase-resistant uptake (Table 4). Increasing the ionic strength of the Tris buffer system with added NaCl, up to 200 mM, did not result in increased total DNA binding, even with meticulous attention to the possibility.

![Fig. 5. Effect of pH on transformation of F62 T1 to Str⁺. Cells were transformed in 10 mM Tris-hydrochloride containing 0.25% glucose and 5 mM MgCl₂, adjusted to the indicated pH.](http://jb.asm.org/)

![Fig. 6. Uptake and Str⁺ transformation by [¹⁴C]DNA in T1 and T4 cells of FA19. Cells were transformed in GCBB. Specific activity of added DNA was 10⁴ cpm/μg. Cells were added to initial density of approximately 5 × 10⁸ CFU/ml. Results were normalized to amounts per 10⁴ CFU, for purposes of comparison between T1 and T4 cells. (a) Results with piliated T1 cells. (b) Results with nonpiliated T4 cells. Symbols: (O) Total DNA bound, (Δ) DNase-resistant DNA uptake, (□) Str⁺ transformants.](http://jb.asm.org/)
that DNA might be sheared during washing of cells.

Competence of colony type T4 cells. We attempted to select competent mutants from nonpiliated type 4 cells of FA19 by NGN mutagenesis. NGN-treated cells were exposed to spectinomycin-resistant (Spc) transforming DNA, and all resulting Spc transformations were scored for colony type and for competence in a subsequent transformation to streptomycin resistance. Results of four experiments were entirely negative: control and NGN-treated colony type T4 cells were equally transformable to Spc (transformation frequencies of $6 \times 10^{-8}$), and increased competence was not demonstrated in any of 24 tested Spc transformations isolated from NGN-treated cells. All transformants were of colony type T4 morphology.

Moderate increases in phenotypic competence of T4 cells were achieved, however, by treatment with various regimens that increase cell envelope permeability (Table 5). Both Tris-EDTA and EDTA-lysozyme treatment resulted in significant decreases in viable count, although the apparent decreases in viability with EDTA-lysozyme might have been due in part to increased cell clumping. That these were transformants was confirmed by relatively frequent (28%) cotransformation for the linked marker str-7, when selection was made for spc-3.

Through the generosity of A. K. Saz, we obtained colony type T4 cells of strain EB16577, which was reported to yield transformants to colony type T1 morphology with frequencies of at least 1%, and which therefore appeared to be unusually competent in transformation (E. Baron and A. K. Saz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, H11, p. 98). Stable colony type T4 cells were purified by two subcultures, and were confirmed to be nonpiliated by electron microscopy. Standard transformations to Str were performed, with cells suspended in GCB containing 2 mM CaCl$_2$. Maximum frequency of transformation was $1.6 \times 10^{-6}$ (mean, $8 \times 10^{-7}$, six experiments), which was 10-fold higher than observed with nonpiliated cells of other strains under the same conditions, but only 0.1% of the transformation frequency of piliated T1 cells of strain EB16577.

**DISCUSSION**

These experiments confirmed the ease with which piliated gonococci can be transformed. Naturally occurring gonococci are all transformable at relatively high frequencies, regardless of the medium in which they are grown. Most cells in an enriched broth culture are competent, and competence is maintained throughout all growth phases. There is, thus, little or no evidence for the necessity of special conditions of growth for development of competence in the gonococcus, as is true in the *Haemophilus influenzae* (1) and *Bacillus subtilis* (20) transformation systems.

Our experiments provide little support for the existence of soluble competence factors in the gonococcus. Siddiqui and Goldberg have

### Table 4. Effect of transforming medium on uptake of DNA by colony type T1 or T4 cells of *N. gonorrhoeae* F62

<table>
<thead>
<tr>
<th>Transforming medium</th>
<th>Total DNA uptake (cpm/10$^6$ CFU)$^a$</th>
<th>DNAse-resistant</th>
<th>T1</th>
<th>T4</th>
<th>T1</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris + 20 mM Ca$^{++}$</td>
<td>260</td>
<td>126.5</td>
<td>42.5</td>
<td>0</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>NEDF</td>
<td>232</td>
<td>78</td>
<td>25</td>
<td>4</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>GCBB + 2 mM Ca$^{++}$</td>
<td>5000</td>
<td>7800</td>
<td>75</td>
<td>8</td>
<td>100</td>
<td>75</td>
</tr>
</tbody>
</table>

* $^a$ (H)DNA (10$^6$ cpm/μg) and colony type 1 or 4 cells of strain F62 were added to final concentrations of 1 μg/ml and 10$^6$ CFU/ml, respectively, in 5 ml of the indicated medium. All values are corrected for uptake of DNAse-pretreated DNA.

$^b$ Transformation frequencies (Str or Pro)$^+$ of T1 cells were nearly identical in all transforming media.

$^c$ Ten millimolar Tris-hydrochloride (pH 7.4), containing 0.25% glucose and 20 mM CaCl$_2$.

### Table 5. Effects of EDTA on transformation of nonpiliated colony type T4 cells of FA19

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reduction in viable count after treatment (%)</th>
<th>Extent of cell clumping after treatment</th>
<th>Transformation medium</th>
<th>Transformation efficiency (Spc)$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>GCBB + 2 mM Ca$^{++}$</td>
<td>$6 \times 10^{-8}$</td>
</tr>
<tr>
<td>10 mM Tris-hydrochloride + 5 mM EDTA</td>
<td>90</td>
<td>Minimal</td>
<td>GCBB + 10 mM Ca$^{++}$</td>
<td>$1.9 \times 10^{-8}$</td>
</tr>
<tr>
<td>Lysozyme, 200 μg/ml, + 1 mM EDTA</td>
<td>98</td>
<td>Marked</td>
<td>Trytopane + 10 mM Ca$^{++}$</td>
<td>$1.2 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

* $^a$ Transformation was with 1 μg of DNA per ml from FA257, which is a spc-3 str-7 derivative of FA19. Values are corrected for frequency of spontaneous mutation to Spc in control experiments, utilizing DNAse-treated DNA. Values are means of three to seven experiments each.

* Of 71 spc-3 transformants, 20 also acquired the linked donor str-7 marker.
hypothesized that colony type T1 cells elaborate a "competence enhancing factor," since transformation frequencies of washed T1 cells suspended in supernatants of T1 broth cultures were threefold higher than when the same cells were suspended in fresh broth (18). They noted, however, that clumping was increased when cells were suspended in culture supernatants, and this could easily account for two- or three-fold increases in transformation frequencies. Experiments similar to those of Siddiqui and Goldberg (18), utilizing the same strain (F62), showed no more than twofold variations in transformation frequency resulting from washing of piliated cells (Table 2); these differences are probably too small to warrant serious consideration of specific gonococcal competence factors. Moreover, competence was not reduced by extensive treatment with proteolytic enzymes (Table 3), which makes it unlikely that gonococci produce competence factors of the type described in pneumococcus (24) and streptococcus (13).

In contrast to the relatively uniform competence of piliated gonococci, there was only very low-level competence in nonpiliated cells. Attempts to select stable competent mutants from nonpiliated cells were unsuccessful. It was possible to increase phenotypic competence 10- to 30-fold in nonpiliated cells by use of regimens that disrupt cell envelope integrity (23) (Table 5), but the resultant transformation frequencies ($2 \times 10^{-6}$) were too low to be of much practical use. Availability of genotypically or phenotypically competent but nonpiliated cells would, of course, greatly facilitate genetic studies of the control of pilus formation in the gonococcus. Further refinements of the Tris-EDTA regimen, or use of this method on nonpiliated strains of inherently higher competence than the one used throughout these studies, might result in significantly higher transformation frequencies. Unfortunately, we were not able to confirm reports (E. Baron and A. K. Saz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, H11 p. 98) of highly competent nonpiliated strains.

Only competent (piliated) cells were able to take up radioactive DNA into a DNase-resistant state. There was close correlation between amount of DNA taken up into a DNase-resistant state and transformation efficiency, but little correlation between total (DNase-sensitive) DNA uptake and transformation (Fig. 6). Total DNA uptake continued to increase linearly above the saturation point for transformants on the DNA dose-response curve, and both competent and noncompetent cells showed equal total DNA uptake (Fig. 6). In addition, total DNA uptake by competent cells varied enormously, depending on the medium used for transformation, even though DNase-resistant uptake and transformation frequencies were very similar in all media (Table 4). Thus, although uptake of DNA into a DNase-sensitive state is undoubtedly the requisite first step in the transformation process (25), most DNase-sensitive uptake appears to be quite nonspecific.

Uptake of DNA into a DNase-resistant state in several transformation systems requires presence of a divalent cation such as Ca$^{2+}$ or Mg$^{2+}$ (15, 25, 26). The experiments reported here showed that divalent cations were stimulatory to transformation of the gonococcus, but a variety of monovalent cations were also effective at relatively high concentrations (Fig. 4). Similar results were reported in H. influenzae by Barnhart and Herriott (1) and by Stuy (21). They concluded that the various cations acted to neutralize net negative charge on the cell surface, thereby allowing binding of negatively charged DNA.

The most effective cation on a concentration basis was Mg$^{2+}$, which exerted maximal stimulation at ionic strengths of 0.01. In contrast, maximal effectiveness of other cations was achieved only at ionic strengths of 0.06 to 0.10. These results could be due to specific stimulation by Mg$^{2+}$ of enzymes involved in the transformation process; evidence in B. subtilis has indicated that Mg$^{2+}$ is necessary for uptake of DNA into a DNase-resistant state (26). As yet, however, there has been no study of the fate of DNA after uptake in the gonococcus, and no conclusions can be drawn about the mechanisms for stimulation of transformation by cations.

Two possibilities might be considered as explanations for the widespread distribution of competence among clinical gonococcal isolates, and for the physiological stability of competence. Hebeler and Young have recently shown that gonococci are highly autolytic, with rates of peptidoglycan turnover of up to 50% per generation during exponential growth (9). Competence in many bacterial systems has been related to local breaks in wall integrity and to autolytic enzyme activity (14, 25). Second, all gonococci have numerous pores in their outer membrane (22), and these pores could provide a means for uptake of macromolecules through the outer membrane. Neither of these possibilities would explain why DNase-resistant uptake is restricted to piliated cells, however, since autolytic activity (8) and distribution of pores (22) are similar in piliated and nonpiliated cells.

The relationship between presence of pili and ability to take DNA into the cell is intriguing.
A similar strong correlation between presence of pili and competence for DNase-resistant DNA uptake has been well described in the meningococcus (6) and also in Moraxella (5). It is conceivable that penetration of the outer membrane by pili provides a mechanism for uptake of macromolecular DNA, either along the exterior surface of the pilus, or through the interior of the pilus. The latter seems quite unlikely in view of the narrow diameter of gonococcal pili and absence of a hollow central core (C. Brinton, personal communication). It is also possible, or perhaps even likely, that pili do not play a direct or indirect role in DNA uptake, and that other features of piliated cells are responsible for uptake. Further understanding of the mechanism of DNA uptake may be obtained by study of transformation-defective mutants of piliated gonococci, some of which show markedly reduced DNase-resistant uptake, but essentially normal numbers and morphology of surface pili (G. Biswas and F. F. Sparling, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, H7 p. 97).

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LITERATURE CITED