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Genetic Transformation of *Neisseria gonorrhoeae* to Streptomycin Resistance

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ABSTRACT

SPARLING, PHILIP F. (Communicable Disease Center, Atlanta, Ga.). Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J. Bacteriol. 92:1364–1371. 1966.—Eight strains of *Neisseria gonorrhoeae* were transformed to streptomycin resistance by deoxyribonucleic acid (DNA) extracted from a streptomycin-resistant strain of *N. gonorrhoeae*. In all strains, competence was greatest in the naturally occurring, virulent clonal types 1 and 2, which gave transformation frequencies up to 1%. Clonal types 3 and 4, which arise on laboratory transfer and are avirulent, gave maximal transformation frequencies of 0.00005%. Competence was maximal in lag and early log phases of growth, but was maintained throughout the growth cycle. A complex broth was required for the physiological expression of competence. The kinetics of DNA uptake, dose-response curve of DNA versus transformants, time required for phenotypic expression, and other features were similar to those in other bacterial transformation systems.

In bacterial transformation, competence refers to the ability of the bacterium to take up exogenous deoxyribonucleic acid (DNA) and to be genetically transformed by it. The nature of competence is poorly understood (8). It may be affected by physiological and environmental factors such as the growth phase of the recipient cells and the medium in which they are exposed to DNA, and also by genetic determinants in the recipient cells (11).

The *Neisseria* are among the many bacteria competent to be transformed by DNA from the same or related organisms. Traits transformed in the *Neisseria* include synthesis of specific capsular polysaccharide (1), antibiotic resistance (2), and nutritional requirements (10).

Virtually the only species of *Neisseria* which has not been investigated for possible transformability is *N. gonorrhoeae* (3). This, plus renewed interest in the physiology of the gonococcus after the demonstration by Kellogg et al. (13) of hereditable clonal variants associated with virulence, has prompted this investigation.

This paper describes the results of transformation to streptomycin resistance in eight strains of *N. gonorrhoeae*, with reference to the relationship of clonal types and other factors to competence to be transformed.

MATERIALS AND METHODS

Strains. The one donor and eight recipient strains of *N. gonorrhoeae* (Table 1) were all from this laboratory's collection and were obtained from male and female patients with gonorrhea in Atlanta, Ga. All were gram-negative diplococci of typical colonial morphology, oxidase-positive, able to ferment glucose but not maltose, and were positive by the fluorescent-antibody test for *N. gonorrhoeae* (4). Clonal types were determined by the criteria and method of Kellogg et al. (13).

Media. Primary isolations were made on GC Medium Base (Difco) with 1% agar (GCBA), plus hemoglobin and 1% Difco Supplement B (Chocolate Agar). Clonal type determinations and subsequent laboratory passages were made on GCBA plus 1% of each of two defined supplements (GCBA-DS). Supplement 1 was composed of (per 100 ml of distilled water): glucose, 40 g; glutamine, 1 g; and cocarboxylase, 2 mg (C. E. Lankford, Bacteriol. Proc., p. 40, 1950). Supplement 2 was composed of (per 100 ml of distilled water): $Fe(NO_3)_3 \cdot 9H_2O$, 50 mg (13). The defined supplements were filter-sterilized and added to the autoclaved (15 min, 121 C) GCBA medium after it cooled to 48 C. The final *p*H was 7.2. GCBA-DS plates were poured 1 to 7 days before use.

Carbohydrate fermentations were performed on medium composed of (per 100 ml of distilled water): GCBA, 3.6 g; 0.04% aqueous solution of phenol red, 4.25 ml; 20% carbohydrate solution, 5 ml. All incubations were at 36 C in candle jars in a water-jacketed incubator unless otherwise specified.

TABLE 1. *Strains of Neisseria gonorrhoeae used in transformation study*

Strain	Date isolated	Passages[b]	Frozen[c]	Clonal types	Streptomycin MIC[d]
					μg/ml
St28[a]	December, 1963	—	—	4	>5,000
F62	February, 1962	>840	300 days[e]	1, 2, 3, 4	12.5
Pr6	December, 1962	10	3 years	1, 2, 3, 4	12.5
Pr7	December, 1962	30	3 years	1, 2, 3, 4	12.5
CFM87u	March, 1964	15–18	1 year	1, 2, 3, 4	25.0
CF299c	March, 1965	5–8	0–1 month	1, 2, 4	<6.25
CF393c	May, 1965	3–6	0–6 months	2, 3	12.5
CF397c	May, 1965	3–15	0–6 months	1, 2, 3, 4	12.5
CF398c	May, 1965	3–6	0–6 months	1, 2, 3, 4	<6.25

[a] Donor of DNA.
[b] Number of selective passages of clonal types on GCBA-DS before used in transformation test.
[c] Duration at −70 C in glycerine-broth before use in transformation test.
[d] Minimal inhibitory concentration at which no visible colonies appeared.
[e] 150 weekend freezings.

Strains were stored at −70 C in a 20% (v/v) glycerine-broth for up to 3 years with satisfactory maintenance of viability, and for over 1 year with stability of specific clonal types. The broth (GCBB), which contained the ingredients in GCBA, minus agar, was composed of (per liter of distilled water): Proteose Peptone No. 3 (Difco), 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 0.5g; cornstarch, 1.0 g (*p*H 7.2).

None of the strains used grew in GCBB medium, whether supplemented with defined supplements 1 and 2 or with Supplement B, but full viability was maintained for about 3 hr. Brain Heart Infusion broth (Difco) and Trypticase Soy Broth (BBL), with or without addition of supplements or CO_2, also failed to support growth. Slow, nondisperse growth was obtained in 0.1% agar-broth tubes. Addition of serum to broth medium gave inconstant growth.

Turbid growth of all strains was obtained in 24 hr or less with initial inocula of 10^5 to 10^6 colony-forming units (CFU) per milliliter in a diphasic medium similar to that of Gerhardt and Heden (6). A 30-ml amount of GCBA-DS was poured into a 250-ml Erlenmeyer flask, and, just before use, 70 ml of GCBB with 1% of each of defined supplements 1 and 2 was poured over the solid agar bottom layer. The cotton-stoppered flask was incubated without added CO_2 at 35 to 36 C in a walk-in incubator. Growth was equal whether or not starch was in the broth; starch was usually omitted to facilitate observation of growth in a clear broth. Generation times were shortest (about 45 min) when the flask was rotated slowly on an inclined multiple dialyzer apparatus.

DNA preparations. The partially purified DNA used for most of the studies was prepared by a modification of the method of Catlin (2) from the streptomycin-resistant strain ST-28, type 4. The 24-hr growth obtained on 40 GCBA-DS plates was washed off with standard buffer (0.14 M NaCl, 0.015 M sodium citrate). Most of the cells were lysed by addition of powdered sodium dodecylsulfate to a final concentration of 5%, with stirring at room temperature for 3 hr. Addition of two volumes of 95% ethyl alcohol resulted in a fibrous precipitate, which was collected on glass rods and dissolved in 1 M NaCl. This solution was centrifuged at 3 C for 60 min at 34,800 × *g*, with a resultant supernatant fluid and residue. The fibers precipitated from the supernatant fluid with two volumes of 95% ethyl alcohol were washed in 75% ethyl alcohol and dissolved in standard buffer. The residue was redissolved in 1 M NaCl, and the above procedure was repeated twice. The collected fibers in standard buffer were deproteinized twice by adding 0.41% sodium dodecylsulfate, with stirring at room temperature for 3 hr. After each deproteinization, the concentration of NaCl was raised to 1 M, and the solution was centrifuged at 3 C for 60 min at 34,800 × *g*. The DNA fibers were then precipitated from the supernatant fluid with 95% ethyl alcohol, washed in 75% ethyl alcohol, and dissolved in standard buffer. The final DNA solution was quantitated by the diphenylamine reaction (5). It was stored at 4 C and retained its full activity for over 1 year. Treatment at 37 C for 30 min with 50 μg/ml of ribonuclease (Worthington Biochemical Corp., Freehold, N.J.), preheated to 80 C for 10 min to destroy any contaminating deoxyribonuclease, did not diminish the activity of the transforming DNA.

Transforming DNA from strain ST-28, prepared according to the general method of Marmur (15) after cell lysis with 5% dodecylsulfate, was of equal activity.

DNA from the streptomycin-sensitive strain of CF299c was prepared by the modified method of Catlin (2) for use as a control.

Deoxyribonuclease assay. Strains were grown on GCBA-DS plates containing 0.2% sperm DNA (Nutritional Biochemicals Corp., Cleveland, Ohio). The presence of deoxyribonuclease was tested by the addition of a solution made by dissolving 15 g of $HgCl_2$ in 120 ml of 1 N HCl (12). Pancreatic deoxy-

ribonuclease (Worthington Biochemical Corp.) was added as a control.

Standard transformation procedure. The clonal types of the recipient strains were grown for 18 hr on GCBA-DS slants and were collected into warm GCBB, with resultant turbid suspensions. Purity of clonal types was checked on a GCBA-DS plate inoculated with the same loop with which the slants were inoculated. Experiments with clonal types 3 and 4 were not conducted when the plate showed any clones of type 1 or type 2. Clonal type 2 and some type 3 gave particulate suspensions. All suspensions were vigorously agitated on a Vortex test tube mixer, and were centrifuged briefly. The homogenous supernatant fluid was diluted 1:10 to 1:15 into warm GCBB to give slight visible turbidity, containing about 2×10^7 CFU/ml. An 0.85-ml amount of this suspension was added to screw-cap tubes (100 by 13 mm) containing 0.1 ml of streptomycin-resistant DNA and 0.05 ml of $CaCl_2$ at final concentrations of 10 μg/ml and 0.002 M, respectively. These reaction mixture tubes were incubated without shaking at 36 C for 30 min, after which 0.05 ml (50 μg) of pancreatic deoxyribonuclease (Worthington Biochemical Corp.) with 0.002 M magnesium was added to destroy unbound DNA (2). Serial 10-fold dilutions were made 5 min later in GCBB.

The number of colony-forming units (E) exposed to DNA was determined by adding a sample of 0.3 to 1.5 ml of the appropriate dilution to 30 ml of soft GCBA-DS, kept liquid in a 48 C water bath after autoclaving; from this, 4-ml samples were pipetted onto the surface of each of five plates containing a previously poured hard base layer of approximately 20 ml of GCBA-DS. The plates were rotated gently to disperse the soft agar overlay uniformly. After the overlay hardened, the plates were incubated at 36 C in candle jars for at least 42 hr before counts were made.

Determination of the number of transformants (T) was done in similar fashion. The plates were incubated for 7 hr before each was overlaid with 4 ml of soft GCBA-DS containing enough streptomycin sulfate (Nutritional Biochemicals Corp.) to give 300 μg/ml after diffusion throughout the plate. Counts were made after 66 hr or more of incubation because of the small size of the subsurface colonies.

Any deviations from this standard protocol are indicated in the Results section. Soft agar overlays with less than 1% agar gave excessive plate moisture and increased contamination. Counts on five identical plates usually varied less than 20% when more than 100 colonies per plate were present. Transformation frequencies were expressed both as the percentage of exposed colony-forming units transformed, and by the ratio $T/E \times 10^5$ (2).

Controls consisted of streptomycin-resistant DNA treated with 50 μg/ml of deoxyribonuclease for at least 5 min at 36 C before the cells were added, and use of streptomycin-sensitive DNA rather than streptomycin-resistant DNA in otherwise identical reaction mixture tubes. Mutations to streptomycin resistance were occasionally observed. When numbers of transformants were low, final values were determined by subtracting the background number of mutants.

Estimates of the extent of cellular aggregation in clumps were made from Gram stains of the reaction mixture tube suspensions and from phase microscopy of wet mounts.

Testing for streptomycin sensitivities. Selected transformants were subcultured on streptomycin-free GCBA-DS, and were tested by means of the overlay technique described above for the levels of streptomycin resistance attained. Determinations of the sensitivities of the recipient and donor strains were made by the same method.

RESULTS

Clonal types related to competence. All eight of the streptomycin-sensitive strains in Table 1 were transformed to streptomycin resistance. All transformants were resistant to $>5{,}000$ μg/ml of streptomycin, which level of resistance was maintained for at least 15 passages on streptomycin-free media.

Higher frequencies of transformation were obtained with clonal types 1 and 2 than with types 3 and 4 (Table 2). The figures given in Table 2 are the means of two or more experiments, in most instances. The determinations of transformation frequencies for types 3 and 4 were based on counts of zero to a few colonies per plate and should be accepted only as estimates.

The greatest amount of cell clumping was observed in clonal types 1, 2, and 3. This resulted in some artifactual increase in the calculated transformation frequencies for these types. The low number of transformants obtained from clonal type 3 cells, despite their high degree of cell clumping, indicated that the difference in competence of types 1 and 2 as compared with 3 and 4 was not due to cell aggregation. There was no significant difference between types 1 and 2, or between types 3 and 4.

Most of the strains had typically mucoid, viscid type 3 clones; all type 4 clones were mucoid and viscid, and all types 1 and 2 clones were morphologically smooth. Strain CF393c was unusual in that its type 3 clones were quite friable.

Different strains gave results of varying reproducibility, when results of experiments from separate days were compared. The relative values for the clonal types did not change, however. Strain CF397c, type 2, gave results which usually varied less than twofold; the mean transformation frequency of 12 separate experiments was 3.6×10^{-3}, with a standard error of the mean of 1.1×10^{-3}.

Some strains used in this study were recently isolated, and others were over 3 years old. Number of serial transfers and duration in the frozen state did not appear to influence competence. No

TABLE 2. *Competence of strains of Neisseria gonorrhoeae related to clonal types*

Strain	Clonal type			
	1	2	3	4
F62	0.09[a] (20)[b]	0.20 (20)	0.00005 (30)	0.000006 (5)
Pr6	0.06 (10)	0.10 (20)	0.00003 (20)	0.000006 (5)
Pr7	0.06 (10)	0.03 (10)	0.00002 (10)	<0.00001 (5)
CFM87u	0.10 (20)	0.08 (30)	0.000009 (40)	0.00002 (45)
CF299c	0.05 (20)	0.04 (30)	None[c]	0.00002 (5)
CF393c	None	0.58 (10)	<0.00005 (20)	None
CF397c	0.15 (10)	0.36 (10)	<0.00005 (35)	0.00005 (15)
CF398c	0.04 (5)	0.03 (15)	<0.00001 (20)	<0.00001 (10)

[a] Percentage of exposed colony-forming units transformed.

[b] Figures in parentheses indicate approximate percentage of presumptive colony-forming units with ≥4 cells each.

[c] "None" indicates clonal type not observed.

loss of competence in a given strain after repeated laboratory transfer was observed.

The plate test for deoxyribonuclease production was negative for the four clonal types of strains Pr6, F62, and CF397c, grown for 1 to 7 days. This is in contrast to the findings of Kellogg et al. (13), who reported all four clonal types positive for deoxyribonuclease production. The reason for the discrepancy is unknown, but may be due to the different media employed.

Expression of competence. Transformation frequencies were the same when the recipient strains were grown on GCBA-DS medium, Chocolate Agar, or Brain Heart Infusion agar before they were suspended in broth and exposed to DNA. Likewise, transformation frequencies were the same when the suspension was made in GCBB, Brain Heart Infusion broth, or Trypticase Soy Broth, with or without 10% heat-inactivated (56 C, 30 min) human serum or 1% of defined supplements 1 and 2.

Transformation frequencies were reduced, however, unless the cells were suspended in a complex liquid medium during exposure to DNA (Table 3). Strain CF397c type 2 was suspended in GCBB and divided into two portions. The first was transformed in the usual manner. The second was washed three times in 0.15 M NaCl buffered with 0.01 M K_2HPO_4-KH_2PO_4 (PBS), *p*H 7.0, either by centrifugation or by collection on a membrane filter (HA; Millipore Filter Corp., Bedford, Mass.). The washed cells were suspended in PBS, and transformation was attempted by 30-min exposure to 10 μg/ml of streptomycin-resistant DNA. Few transformants were obtained, even when the elapsed time for cell washing was as little as 2 min. However, when samples of the same PBS suspension were supplemented with 5% (v/v) GCBB and calcium, or 5% (v/v) heat-inactivated (56 C, 30 min) human serum and calcium before exposure to DNA, normal numbers of transformants were obtained. Albumin (0.5%, v/v, Bovine Fraction V, Armour Pharmaceutical Co., Kankakee, Ill.) and calcium had less effect. Dialysis of serum against PBS for 48 hr reduced its activity. Differences in cell viability in the various suspensions as cause of the different transformation frequencies was excluded by determinations of viable counts from the suspensions after addition of deoxyribonuclease. The nature of the factor(s) in serum and broth necessary for the physiological expression of competence was not defined.

Many strains gave equal numbers of transformants when a concentration ranging from 0.00001

TABLE 3. *Influence of suspending medium on transformation of strain CF397c type 2*[a]

Medium	Transformants (T/E × 10^8)
GCBB + 0.002 M Ca^{+2}	230
PBS[b]	0.01
PBS + 0.002 M Ca^{+2}	0.01
PBS + 0.002 M Ca^{+2} + 0.5% albumin	3
PBS + 5% serum	75
PBS + 5% serum + 0.002 M Ca^{+2}	500
PBS + 5% dialyzed serum	28
PBS + 5% dialyzed serum + 0.002 M Ca^{+2}	73
PBS + 5% GCBB	110
PBS + 5% GCBB + 0.002 M Ca^{+2}	340

[a] Cells in suspension exposed to 10 μg/ml of streptomycin-resistant DNA for 30 min at 36 C.

[b] A portion of initial suspension in GCBB was washed and suspended in phosphate-buffered saline and divided into samples, which received the indicated supplements before being exposed to DNA (see text for details).

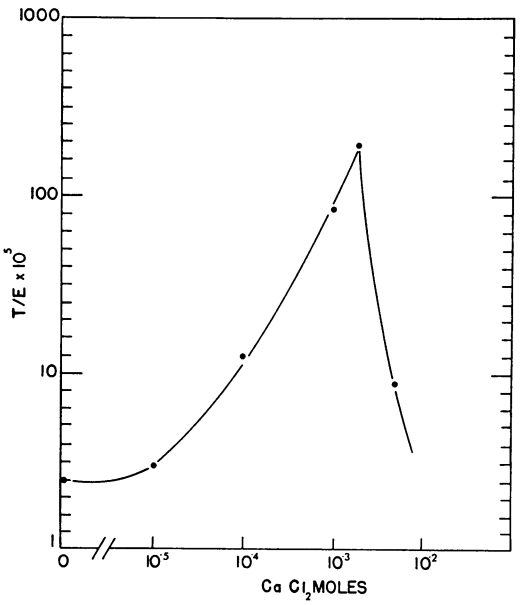

FIG. 1. *Effect of molar concentration of $CaCl_2$ on transformation of strain CF397c type 2.*

to 0.001 M $CaCl_2$ was added to the reaction mixture tubes containing 10 μg/ml of streptomycin-resistant DNA and organisms suspended in GCBB. Strain CF397c was unusual in that increased numbers of transformants were obtained with increasing concentration of $CaCl_2$, up to a peak at 0.002 M $CaCl_2$ (Fig. 1). The decline in transformation frequency above 0.002 M $CaCl_2$ was probably due to precipitation of calcium phosphates from the broth in which the cells were suspended, with coincident decline in available Ca^{+2}.

The temperature at which the cells were incubated with DNA influenced the number of transformants obtained. The optimal temperature was 36 to 37 C (Fig. 2).

The time required for full uptake of 10 μg/ml of streptomycin-resistant DNA by competent cells was investigated by incubating a series of identical reaction mixture tubes for various times before rapidly adding 100 μg/ml of deoxyribonuclease to destroy unbound DNA (Fig. 3). Rapid uptake took place in the first few minutes, with continued uptake until 30 to 45 min. The effect of more than 30-min contact with DNA was variable, and, therefore, 30 min was the time adopted for the standard procedure. The curve was not changed by use of greater concentrations of enzyme.

The dose-response curve of transformation frequency versus concentration of DNA was determined, with the results of a typical experiment plotted in Fig. 4. The saturating level of DNA was 5 to 10 μg/ml when less than 10^8 cells were used.

Although uptake of DNA was nearly complete in 30 min, it took considerably longer for the newly introduced streptomycin-resistant DNA to be integrated, recombined, and expressed as streptomycin resistance (phenotypic expression). The time of incubation of identical sets of five

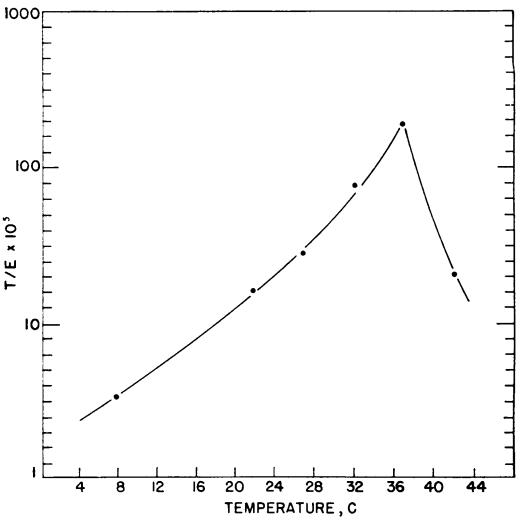

FIG. 2. *Effect of temperature on transformation of strain CF397c type 2.*

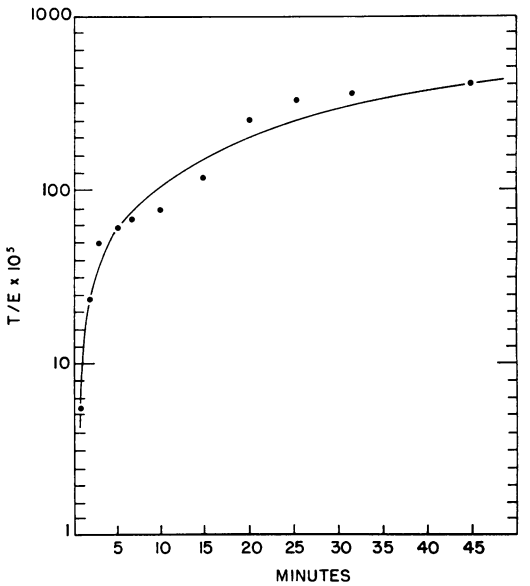

FIG. 3. *Kinetics of uptake of 10 μg/ml of DNA by strain CF397c type 2.*

plates from the same reaction mixture tube was varied before the streptomycin overlay was applied (Fig. 5). Full phenotypic expression required 6 to 7 hr.

An important problem is the relationship of the growth cycle to competence. Initial estimates were obtained by growing the recipient for variable times on solid GCBA-DS slants. Competence was maximal on slants incubated for 12 to 18 hr, with gradual decline to 10 to 20% of younger cultures at 24 to 28 hr.

This problem was investigated more thoroughly when it became possible to obtain rapid growth in the broth of diphasic agar-broth flasks (*see* Materials and Methods). Normally competent CF397c type 2 and Pr6 type 1 were incompetent (T/E = 10^{-5} to 10^{-7}) after being grown to turbidity in this medium and diluted into fresh GCBB before exposure to DNA. This loss of competence was associated with change of the original clonal type 1 or 2 of these strains to type 4, which was complete in 18 to 24 hr. It was not clear whether this was due to phenotypic reversion of clonal types 1 and 2 to type 4, or to selective growth advantage of small numbers of type 4 cells originally present in the inoculum.

The clonal type 1 of strain F62 was stable in this diphasic growth medium, and was therefore used to study the relationship of growth to competence (Fig. 6). Maximal competence was in the lag and early log phases, with apparent decline thereafter when samples of the growing culture were inoculated directly into the reaction mixture tubes containing DNA and calcium. However, if the stationary-phase culture was rapidly diluted 10^{-3} into fresh GCBB and immediately exposed to DNA, transformation frequencies equal to early log cultures were obtained. This effect of dilution was not due to decreased numbers of cells, because exposing variable numbers of washed cells to 10 μg/ml of DNA gave constant transformation frequencies until almost 10^9 cells

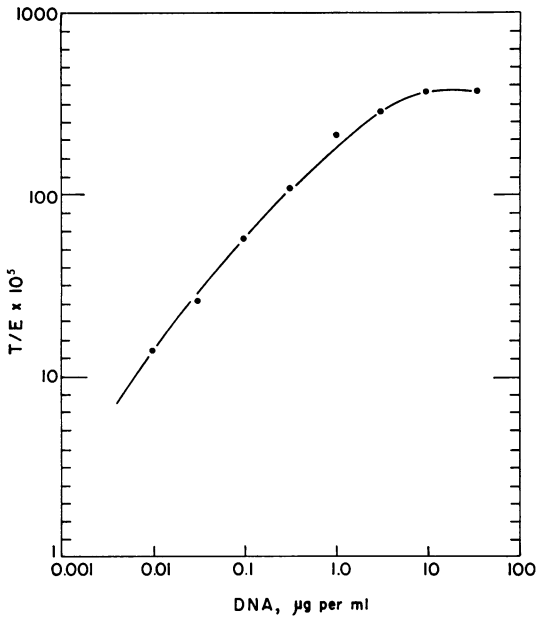

FIG. 4. *Effect of concentration of DNA on transformation of strain CF397c type 2.*

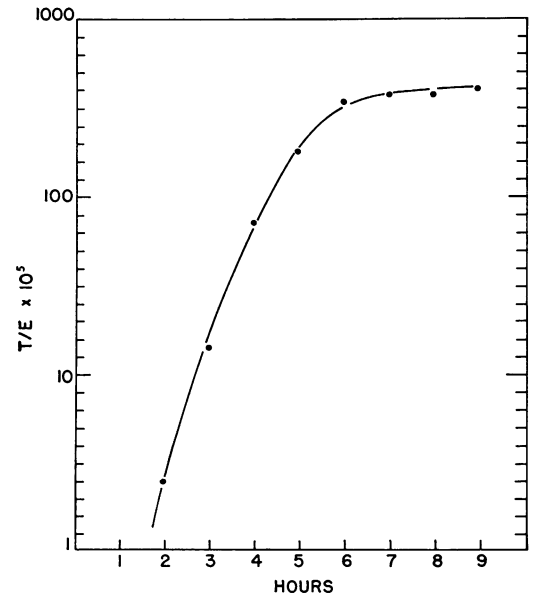

FIG. 5. *Time required for phenotypic expression of streptomycin resistance, strain CF397c type 2.*

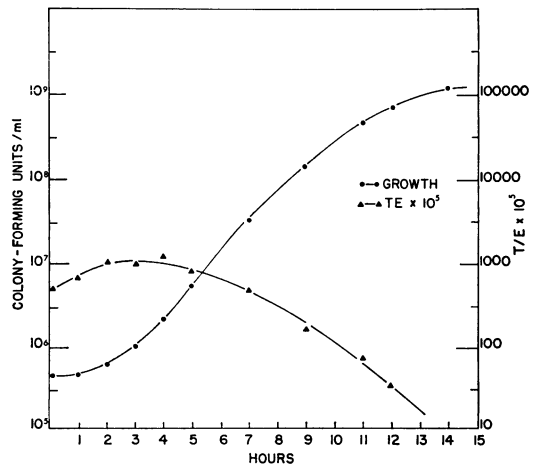

FIG. 6. *Transformation of strain F62 type 1 during growth in broth of diphasic medium.*

were used. It thus appeared that strain F62 type 1 was competent throughout its growth curve, and that the broth of concentrated cultures did not support transformation of this strain. It will be noted that growth in the diphasic medium to early log phase increased transformation frequencies for strain F62 type 1 to about 1%, as opposed to 0.1% when grown for 18 hr on GCBA-DS slants.

The degree of clumping in diphasic cultures was similar to that of suspensions made from slants in GCBB.

DISCUSSION

This study demonstrates that *N. gonorrhoeae* can be genetically transformed to high levels of streptomycin resistance by DNA extracted from a naturally occurring streptomycin-resistant strain of *N. gonorrhoeae*. Competence in all strains is greatest in the morphologically distinct clonal types 1 and 2, which are the only types isolated directly from humans and the only types which are virulent on reinoculation into male volunteers (13; Kellogg, *unpublished data*). Clonal types 3 and 4, which arise spontaneously on laboratory transfer and which predominate in old laboratory cultures in which unselective transfers are made, are relatively incompetent.

A relationship between colonial morphology and competence has been demonstrated in other bacteria. Competence in the pneumococcus was shown by Ravin (17) to be inversely related to the quantity of polysaccharide capsule produced, which suggests that the mucoid capsule may act as a physical barrier to the entry of DNA. A similar phenomenon may occur in *N. meningitidis*. The serogroup C strains studied by Jyssum and Lie (11) were all less competent than the serogroup B strains, which correlates with the presence of capsules in their group C strains, but not in group B strains (K. Jyssum, *personal communication*). *N. gonorrhoeae* cells do not possess a true capsule, and no antigenic differences between the clonal types have been found (Kellogg, *unpublished data*). Clonal types 3 and 4 are more mucoid, however, on plate culture than types 1 and 2, and produce more slime in agar-broth cultures. Since no demonstrable differences in extracellular deoxyribonuclease production were found in this study, or by Kellogg et al. (13), it is possible that the lack of competence of types 3 and 4 is due to their greater extracellular slime layer. There may, of course, be other undetected biochemical or genetic differences among the clonal types which explain both virulence and competence. The only biochemical differences noted to date pertain to the selective growth advantage of types 3 and 4 in vitro (13).

The quantitative results in this study of transformation of *N. gonorrhoeae* are not directly comparable with those published for other *Neisseria* species, in part due to uncertainties regarding the extent of the artifactual increase in estimated transformation frequencies caused by cell clumping. It seems clear, though, that the findings pertaining to kinetics of DNA uptake, the time for phenotypic expression, quantities of DNA required, and competence as a function of the growth cycle are similar in *N. gonorrhoeae* and the more extensively studied *N. meningitidis* (2, 14). Both *N. meningitidis* and *N. gonorrhoeae* are unusual in maintaining competence throughout the growth cycle. Most other transformable bacteria are competent only during certain phases of growth (9, 16, 19).

There do appear to be certain differences between *N. meningitidis* and *N. gonorrhoeae*. The meningococcus requires only calcium or albumin for competence to be expressed in saline (14), whereas the gonococcus requires more complex factors present in serum and broth. The requirement for a complex transforming broth during exposure to DNA, and the inhibition of transformation by temperature shifts, probably indicate that metabolic activity of the recipient cells is prerequisite to successful uptake of DNA. Growth is not required, for the broth media used in this study did not support growth. Similar conclusions in other bacteria were reviewed by Ravin (18).

It cannot be stated whether maximal competence was achieved in these experiments, for this requires study of the frequency of single and double transformations by two unlinked markers (7). The increased competence exhibited by strain F62 type 1 when grown to early log phase in diphasic agar-broth medium suggests that the standard procedure of growing the recipient on agar slants before suspending them in broth did not bring them to full competence.

It is hoped that questions relating to optimal conditions for development of competence, and the difference in competence exhibited by the clonal types, will be elucidated by further study.

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