JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1975, p. 165-170
Vol. 1, No. 2

Printed in U.S.A.

Stability of Pathogenic Colony Types of *Neisseria gonorrhoeae* in Liquid Culture by Using the Parameters of Colonial Morphology and Deoxyribonucleic Acid Transformation

LEONARD J. LA SCOLEA, JR.,* MICHAEL J. DUL,[1] AND FRANK E. YOUNG

Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received for publication 8 November 1974

This investigation describes the surveillance of the colonial stability of the pathogenic type 1 from the gonococcal strain F_{62} to the nonvirulent types 3 and 4 in different liquid media. The maintenance of the colony types was monitored by the parameters of colonial morphology and deoxyribonucleic acid-mediated transformation. During growth in a complex medium, Mueller-Hinton broth, only 46.7% of the gonococcal population remained as type 1 after 12 h. The greatest change in the type 1 colony-forming units correlated with the decline in viable count. The conversion process could not be prevented by the continual maintenance of the gonococcus in logarithmic growth. The frequency of transformation from PRO^- (proline) to PRO^+ was proportional to this decrease in type 1 colony-forming units. In contrast to Mueller-Hinton medium, the chemically defined minimal medium Gonococcal Genetic Medium (GGM) was capable of maintaining approximately 90% of the gonococcal population in the type 1 colonial form after 16 h of growth, despite a decrease in the viable count. Although the percentage of type 1 appeared to remain constant in GGM, the apparent transformation frequency increased approximately 24-fold from 0 to 12 h of growth. GGM appears to stimulate or maintain competence, as evidenced by an eightfold increase in transformation when cells are exposed to deoxyribonucleic acid in GGM as compared to Mueller-Hinton.

In 1963 Kellogg and co-workers (14) classified the gonococcus into four major types (T_1, T_2, T_3, and T_4) based on colonial morphology characteristics. It was observed that types 1 and 2 produced disease in human volunteers when inoculated intra-urethrally, whereas this did not occur with types 3 and 4 (13, 14). Furthermore, gonococci freshly isolated from clinical material most often have type 1 or 2 colonial morphology. However, upon repeated, nonselective transfers these gonococci will give rise to a population of predominantly colonial types 3 and 4. Further studies established that types 1 and 2 and the laboratory-adapted colony types differ not only in colonial morphology and pathogenicity but also in autoagglutinability (13, 14), ability to irreversibly bind naked deoxyribonucleic acid (DNA) by genetic transformation (18), possession of pili (11, 20), resistance to phagocytosis (21), sensitivity to the bactericidal action of antiserum plus complement (22), infectivity for the chicken embryo (3), and attachment to human sperm (9). A major hindrance in studying the pathobiology of the gonococcus is the rapid conversion of the virulent forms (types 1 and 2) to the nonvirulent laboratory-adapted types 3 and 4. Stabilization of types 1 and 2 would greatly aid studies on the mechanism of pathogenesis of the gonococcus. In this study we investigated the stability of colony type 1 of strain F_{62} in a defined liquid and biphasic minimal medium, termed Gonococcal Genetic Medium (GGM), that has been previously described by La Scolea and Young (15) and a complex liquid growth medium, Mueller-Hinton (MH), with specific supplements (16). The maintenance of the colony types was monitored by two parameters: (i) colony morphology and (ii) DNA-mediated transformation. It was noted that in the defined liquid minimal medium (GGM) type 1 colonies were highly stable through a complete growth cycle, whereas this was not the case in the complex medium, MH. Furthermore, GGM appears to be a highly satisfactory medium for DNA-mediated transformation. This communication will also present some additional observations on the stability of gonococcal colonial

[1] Present Address: University of Massachusetts Medical Center, 55 Lake Ave. North, Worcester, Mass. 01605.

types in liquid culture and DNA transformation with *Neisseria gonorrhoeae*.

MATERIALS AND METHODS

Bacterial strains. In this investigation two gonococcal laboratory strains were used, RD5 and F_{62} type 1, which were kindly provided by D. S. Kellogg (Center for Disease Control, Atlanta, Ga.) and F. J. Tyeryar (Naval Medical Research Institute, Bethesda, Md.). The isolation of these strains was accomplished by three successive single-colony selections. The gonococcal colony types were determined by the procedure outlined by Kellogg et al. (13, 14). All the strains were confirmed as *N. gonorrhoeae* by colonial morphology (13, 14), Gram stain, oxidase reaction, and fermentation of glucose but not maltose, lactose, or sucrose. The genotype designations, phenotypes, and origin of these two strains has been previously described (15).

Maintenance of bacterial strains. Strains were stored at −76 C in Trypticase soy broth containing 15% (vol/vol) glycerol, with satisfactory maintenance of viability and stability of specific colonial types. For use, the cultures were thawed at room temperature and inoculated onto the appropriate media.

Media. The defined liquid and solid minimal medium routinely used in this investigation was complete GGM (15). The biphasic medium contained liquid and solid GGM (15). The complex liquid growth medium was MH broth (BBL; 16) supplemented with 0.5% (vol/vol) glucose, 0.2% (vol/vol) yeast extract, 1.0% (vol/vol) isovitalex, and 5 μg of $CaCl_2$ per ml. All dilutions were made in a minimal salts solution which was composed of two solutions each at a final concentration of 7.5% (vol/vol) (15). Minimal salts I contained (in grams per liter of distilled, demineralized water): KCl, 4; NaCl, 70; $Na_3C_6H_5O_7 \cdot 2H_2O$, 15; $MgCl_2 \cdot 6H_2O$, 6; NH_4Cl, 4; Na_2SO_3, 10; K_2SO_4, 12; and $NaC_2H_3O_2 \cdot 3H_2O$, 20. The second minimal salts solution contained (in grams per liter of distilled, demineralized water): K_2HPO_4, 140; and KH_2PO_4, 60. Saline was not utilized for dilutions in this investigation because of its detrimental affect on the gonococci, as noted by Hunter and McVeigh (8). The solid medium routinely used for all the colony morphology determinations, as described by Kellogg et al. (13, 14), was GC medium base (Difco) supplemented with isovitalex to a final concentration of 1% (vol/vol).

Growth experiments in liquid media. Gonococcal colonial types were inoculated from frozen stock onto GC base supplemented with 1% (vol/vol) isovitalex (GCI). After 20 h at 37 C in 8 to 10% CO_2, a single colony of the desired colonial type was selected and inoculated onto GCI plates. After 20 h at 37 C a heavy suspension of the cells grown on GCI (approximately 10^8 cells/ml) was prepared in either liquid GGM or MH medium that was preincubated overnight at 37 C in an atmosphere of 8 to 10% CO_2. A sample of this inoculum was transferred to an acid-washed nephelometer flask (250 ml) containing 34 ml of complete GGM or MH medium that had also been preincubated overnight at 37 C in 8 to 10% CO_2. The nephelometer flask used for the GGM biphasic medium contained 34 ml of complete GGM and a 30-ml layer of the solid phase of this same medium. Usually the inoculum was diluted 1:35 to obtain an initial turbidity of 6 to 9 Klett units (Klett Summerson spectrophotometer, filter no. 54). Samples were removed at various time intervals for the determination of colony types, DNA-mediated transformation, and colony-forming units (CFU) per milliliter. Cultures were incubated at 37 C in a New Brunswick G76 waterbath at 120 rpm in air. With the preincubation of the media in a CO_2 atmosphere, no further CO_2 was required for growth. These growth experiments were repeated a minimum of two times, with the monitoring of cell morphology by phase microscopy. It is important to note that there is a tendence of type 1 gonococci to autoagglutinate or clump. Therefore, the quantitation of colony morphology and frequency of transformation are subject to this artifact.

Phenotypic determinations. The phenotypes of the gonococcal strains used in this investigation were determined by streaking on agar composed of the complete GGM minus the individual amino acids (15).

Colony morphology determinations. At specific time intervals, samples were removed and diluted in a minimal salts solution (see above) that had been prewarmed to 37 C in 8 to 10% CO_2. The dilute suspensions were vortexed for 15 s to reduce clumps. The dilutions were plated on GC medium base plates supplemented with 1% isovitalex. After 20 to 24 h at 37 C in 8 to 10% CO_2, the colonial types were determined by the procedure of Kellogg et al. (13, 14). The percent of each colonial type was calculated by counting the number of a specific colony type or plate divided by the total gonococcal colonies per plate (× 100). The total colonies per plate were also used for the viable count (CFU per milliliter).

DNA-mediated transformation. DNA was prepared from gonococcal strain RD5 using the procedure of Yasbin and Young (23). A sample of cells from each of the growth media was removed at various times during growth and assayed for DNA transformation by a minor modification of the method developed by Sparling (18). Essentially, each sample was diluted 1:10 to 1:15 in the homologous medium that had been prewarmed to 37 C in 8 to 10% CO_2 (approximately 10^7 CFU/ml) and vortexed for 30 s to reduce clumps; a 0.85-ml sample was removed and added to a screw-cap tube containing $CaCl_2$ (final concentration, 2 mM), and 0.1 ml of DNA (final concentration 5.6 μg/ml) isolated from strain RD5. After incubation at 37 C for 30 min in 8 to 10% CO_2 without shaking, deoxyribonuclease (50 μg/ml) containing $MgCl_2 \cdot 6H_2O$ (2 mM) was added, and incubation was continued for an additional 15 min. The cells were subsequently washed by centrifugation for 10 min at 10,000 × *g* in a Sorvall RC2B centrifuge in minimal salts solution to remove the excess nutrients, and the transformants were determined by spreading samples on GGM minus proline (15). The viable count was determined on GCI plates.

RESULTS

Stability of T_1. A major hindrance in studying several avenues of experimentation with *N. gonorrhoeae* is the rapid conversion of the

pathogenic colonial types 1 and 2 to the nonvirulent, laboratory-adapted types 3 and 4 (13, 14). Therefore, the first objective of this investigation was to monitor the colonial stability of T_1 from the gonococcal strain F_{62} in three different types of media: liquid GGM, biphasic GGM, and MH medium. Two parameters were utilized in monitoring the stability of T_1 in the strain F_{62}. The first parameter was colonial morphology. As a result of the characteristic autoagglutinability of type 1, this is not a strictly quantitative technique (13, 14). To give greater significance to our calculations substantially high numbers of colonies were screened (Tables 1-5). Since gonococcal colony types 1 and 2 have the ability to undergo DNA-mediated transformation in contrast to types 3 and 4 (18), the second parameter employed to monitor T_1 stability was the ability of the cells to be transformed.

As indicated by the increase in optical density and viable count (Fig. 1A and B; Tables 1-3), the media employed in this study were able to support growth of gonococcal strain F_{62}, type 1. As expected, the more rapid growth rate was obtained in the complex medium, MH, than in the defined minimal medium (GGM; Fig. 1A and B; Tables 1-3). After 12 h of growth in GGM, 88.3% of the gonococcal population was still T_1 colonial morphology (Table 1). Thus, there was only a 7.7% decrease from the initial T_1 inoculum. The T_1 stability in liquid GGM was also reflected in the frequency of DNA-mediated transformation (Table 1). Although the percentage of type 1 appeared to remain constant, the frequency of transformation increased approximately 24-fold from 0 to 12 h (Table 1).

It was observed that the stability of T_1 from gonococcal strain F_{62} appeared to be lower in biphasic GGM (Table 2). After 12 h of incubation in the biphasic medium, the culture contained 78.4% T_1, representing a 17.6% decrease from the initial inoculum (Table 2). The transformation frequency in GGM biphasic medium increased from 0 to 12 h of growth; however, the increment was only threefold as compared to the dramatic increase of 24-fold in GGM liquid (Tables 1 and 2). This difference in the increment of the transformation frequency in GGM liquid and biphasic may be influenced by the relative autoagglutinability of the T_1 gonococcus in both media.

Unlike complete GGM, the stability of T_1 varied markedly in MH medium (Table 3). According to the parameter of colonial morphology, the frequency of T_1 colonies declined 14.5% in the first 8 h of growth (Table 3). However, at the specific time in which there was a decline in

TABLE 1. *Colony morphology and DNA-mediated transformation frequencies of N. gonorrhoeae strain F_{62} (T_1) in liquid GGM*[a]

Time of incubation (h)	CFU/ml ($\times 10^7$)	Total colonies examined for % colony morphology	T_1 (%)	DNA transformation (%)
0	3.0	3,183	96	0.16
4	4.06	4,451	91.8	0.14
8	10.1	4,091	91.6	1.2
12	60.0	1,863	88.3	3.8

[a] The medium used in these experiments was complete liquid GGM (15). The procedure for the inoculation and growth of F_{62} (T_1) in GGM is given in Materials and Methods. Determinations of viable count, percent colonial morphology, and DNA-mediated transformation were as described in Materials and Methods. These experiments were repeated a minimum of two times.

TABLE 2. *Colony morphology determinations and DNA-mediated transformation frequencies of N. gonorrhoeae strain F_{62} (T_1) in GGM biphasic medium*[a]

Time of incubation (h)	CFU/ml ($\times 10^7$)	Total colonies examined for % colony morphology	T_1 (%)	DNA transformation (%)
0	3.0	3,183	96	0.13
4	6.4	6,159	92.9	0.15
8	39.0	3,617	79.6	0.35
12	100.0	1,381	78.4	0.37

[a] In these experiments GGM biphasic medium (15) was utilized for the preparation, inoculation, and growth of the gonococcus. The CFU, colonial morphology, and DNA transformation determinations were performed as described in Materials and Methods. Experiments were repeated a minimum of two times.

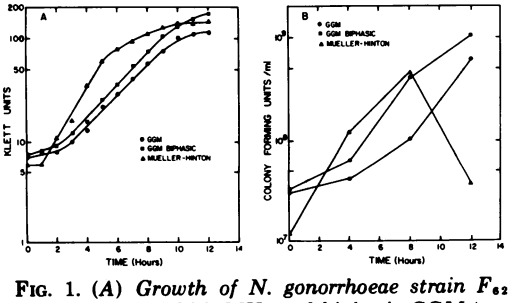

FIG. 1. *(A) Growth of N. gonorrhoeae strain F_{62} type 1 in liquid GGM, MH, and biphasic GGM (see Materials and Methods). (B) CFU of gonococcal strain F_{62} type 1 per ml in liquid GGM, MH, and biphasic GGM.*

TABLE 3. *Colonial morphology and DNA-mediated transformation of gonococcal strain F_{62} (T_1) in MH liquid medium*[a]

Time of incubation (h)	CFU/ml ($\times 10^7$)	Total colonies examined for % colony morphology	T_1 (%)	DNA transformation (%)
0	1.2	3,271	95	0.02
4	12.0	5,170	80.6	0.24
8	44.0	3,106	80.5	0.29
12	3.7	1,770	46.7	0.17

[a] The preparation, inoculation, and growth of the gonococcal strain F_{62} (T_1) in this medium were performed as described in Materials and Methods. Determinations of viable count, percent colonial morphology, and DNA transformation are also described in the test. These experiments were repeated a minimum of two times.

the viable count the incidence of T_1 decreased dramatically from 80.5 to 46.7% (Table 3). The frequency of DNA transformation correlated with this decrease in gonococcal T_1 colony morphology (Table 3). Furthermore, there was an eightfold decrease in the initial frequency of transformation when cells from a standard inoculum were exposed to DNA in MH as compared to GGM (Tables 1 and 3). It was also noted that in MH there was a 15-fold increase in the frequency of DNA-mediated transformation from the initial inoculum to 8 h of growth (Table 3). However, the increase in the frequency of transformation occurred earlier in MH than in GGM liquid or biphasic media (Tables 1-3). Therefore, after 12 h of growth in MH medium, 46.7% of the gonococcal population consisted of T_1 in contrast to 88.3 and 78.4% in liquid GGM and biphasic media, respectively.

Relationship of autolysis to change in colony morphology. To determine whether the rapid decrease in the percent of T_1 CFU in MH medium was related to autolysis in the stationary phase of growth, the culture was maintained in the logarithmic phase by transferring a standard inoculum when the culture reached 45 to 60 Klett units (Fig. 2). The gonococcal population was maintained for 18 h in exponential growth (Table 4; Fig. 2). The results in Table 4 indicate that the conversion process from T_1 to T_3 and T_4 is not prevented by the continual maintenance of the gonococcus in logarithmic growth. Thus, after three successive subcultures in MH medium, the percentage of T_1 in the gonococcal population decreased from 91.6 to 33.1%.

Because it was possible that autolysis or cell death could also occur in liquid GGM and produce a reduction of T_1 CFU, F_{62} T_1 were grown for 16 h in GGM (Fig. 3; Table 5). In contrast to MH medium, the percentage of T_1 remained constant despite the decline in viable count in GGM (Tables 3 and 5). Consequently, after 16 h of growth in GGM, 92.5% of the gonococcal population are still T_1, even though there was a marked decrease in CFU. These results suggest that GGM may provide an environment that maintains or promotes the stability of strain F_{62} T_1 as opposed to MH medium.

DISCUSSION

The results described in this investigation demonstrate that strain F_{62}, composed predominantly of colonial type 1, behaves differently in

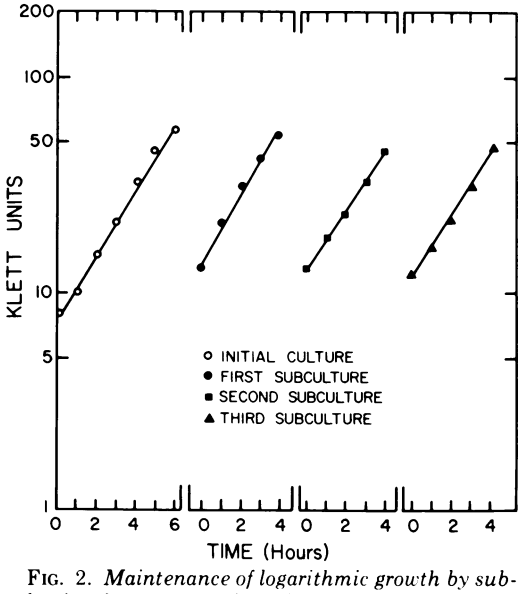

FIG. 2. *Maintenance of logarithmic growth by subculturing for gonococcal strain F_{62}, type 1, in liquid MH medium.*

TABLE 4. *Colonial morphology of N. gonorrhoeae strain F_{62} (T_1) during repeated subculture in MH medium*[a]

Subculture	Time of incubation (h)	CFU/ml ($\times 10^7$)	Total colonies examined for % colony morphology	T_1 (%)
1	0	1.4	2,749	91.6
	4	4.3	1,299	85.4
2	0	0.43	1,299	85.4
	4	6.1	1,823	67.0
3	0	0.61	1,823	67.0
	4	7.9	2,379	33.1

[a] These experiments were repeated a minimum of two times.

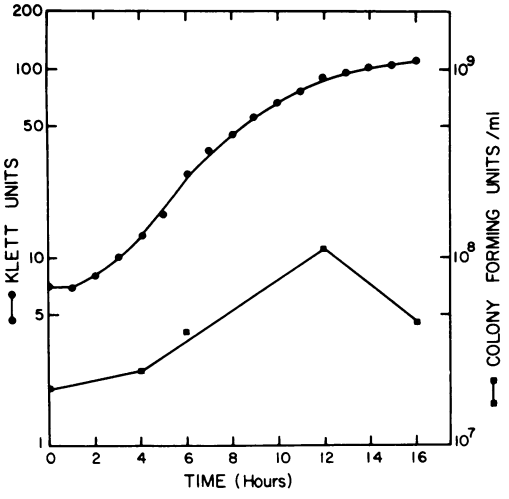

FIG. 3. *Growth of N. gonorrhoeae strain F_{62} type 1 in liquid GGM.*

TABLE 5. *Colonial morphology of N. gonorrhoeae strain F_{62} (T_1) grown for 16 h in liquid GGM medium*[a]

Time of incubation (h)	CFU/ml ($\times 10^7$)	Total colonies examined for % colony morphology	T_1 (%)
0	2.0	3,611	98.6
4	2.5	404	95.5
6	4.0	725	95.5
12	11.0	2,291	91.9
16	4.5	2,244	92.5

[a] These experiments were repeated a minimum of two times.

various media. According to the parameters of colonial morphology and DNA-mediated transformation, it was noted that 88.3% of the gonococcal population gave rise to T_1 CFU after 12 h of growth in liquid GGM. Even when autolysis occurred after 16 h of growth, the culture remained predominantly T_1 (92.5%). However, only 46.7% of the gonococcal population remained as T_1 after growth for a comparable time in MH medium. The greatest change in the T_1 CFU correlated with the decline in viable count. The frequency of DNA-mediated transformation agreed with this dramatic decrease of T_1 CFU. Analysis of the data obtained by Jephcott (10) revealed a similar correlation in the decrease in T_1 with viable count. It was further observed that the conversion process from T_1 to T_3 and T_4 was not prevented by the continual maintenance of the gonococcus in logarithmic growth by subculturing in MH medium. Therefore, the type 1 colonial morphology of strain F_{62} was highly stable in liquid GGM, whereas this was not the case in MH broth. Experiments are in progress to determine whether the stability of T_1 in liquid GGM is strain specific or a common property of gonococcal strains. A medium such as GGM, which has the ability to maintain approximately 90% of the gonococcal population of F_{62} in the T_1 colonial form after 16 h of growth, presents a variety of advantages in studying the pathobiology of the gonococcus. Furthermore, as a result of the maintenance of very high percentages of T_1 CFU for F_{62}, GGM may have the potential advantage of increasing the yields of gonococcal pili.

Traits transformed by DNA in the *Neisseria* genospecies include synthesis of specific capsular polysaccharide (1), antibiotic resistance (4, 5, 18), and nutritional requirements (6, 12). However, until recently only transformation of antibiotic resistance has been demonstrated for the gonococcus (18). With the development of a defined, minimal media, it has been possible to transform a nutritional requirement for *N. gonorrhoeae*, as recently noted by Catlin, using a complete chemically defined medium for auxotyping (6). Furthermore, based on the results obtained in this study GGM appears to stimulate or maintain competence. For instance, there was an eightfold decrease in transformation when cells were exposed to DNA in MH as compared to GGM. This observation can be explained by either or both of two possibilities. (i) GGM offers *N. gonorrhoeae* a favorable environment for DNA-mediated transformation, or (ii) there is an ingredient in MH which acts in an inhibitory fashion to transformation. Experiments are in progress to distinguish between these possibilities.

Although the percent of F_{62} T_1 in liquid GGM appeared to remain constant, the transformation frequency increased approximately 24-fold from 0 to 12 h of growth. This occurred to a lesser extent in GGM biphasic and MH media. In MH medium the increase in DNA-mediated transformation appeared earlier than in the other media. At present, it is difficult to determine whether there is a marked increase in the real frequency of transformation at a unique phase of the growth cycle as in other transformable bacteria (2, 7, 17, 19, 24). Cell clumping could cause a decrease in the apparent CFU, resulting in an apparent increase in the percent of transformants.

ACKNOWLEDGMENTS

We would like to thank Vicki Ploscowe for excellent technical assistance.

This study was supported by Public Health Service grant 1 ROI AI-11709 from the National Institute of Allergy and Infectious Diseases. L.J. La S. and M.J.D. are postdoctoral

fellows supported by Public Health Service grant 5T01 AI-00427 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Alexander, H. E., and W. Redman. 1953. Transformation of type specificity of meningococci. J. Exp. Med. **97**:797-806.
2. Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741-746.
3. Buchanan, T. M., and E. C. Gotschlich. 1973. Studies on gonococcus infection. III. Correlation of gonococcal colony morphology with infectivity for the chick embryo. J. Exp. Med. **137**:196-200.
4. Catlin, B. W., 1960. Transformation of *Neisseria meningitidis* by deoxyribonucleates from cells and from culture slime. J. Bacteriol. **79**:579-590.
5. Catlin, B. W. 1967. Genetic studies of sulfadiazine-resistant and methionine-requiring *Neisseria* isolated from clinical material. J. Bacteriol. **94**:719-733.
6. Catlin, B. W. 1974. Genetic transformation of biosynthetically defective *Neisseria gonorrhoeae* clinical isolates. J. Bacteriol. **120**:203-209.
7. Hotchkiss, R. D. 1954. Cyclical behavior in pneumococcal growth and transformability occasioned by environmental changes. Proc. Nat. Acad. Sci. U.S.A. **40**:49-55.
8. Hunter, K., and I. McVeigh. 1970. Development of a chemically defined medium for growth of *Neisseria gonorrhoeae*. Antonie van Leeuwenhoek J. Microbiol. Serol. **36**:305-316.
9. James-Holmquest, A. N., J. Swanson, T. M. Buchanan, R. D. Wende, and R. P. Williams. 1974. Differential attachment by piliated and nonpiliated *Neisseria gonorrhoeae* to human sperm. Infect. Immun. **9**:897-902.
10. Jephcott, A. E. 1972. Preliminary study of colony type stability of *Neisseria gonorrhoeae* in liquid culture. Br. J. Vener. Dis. **48**:369-375.
11. Jephcott, A. E., A. Reyn, and A. Birch-Andersen. 1971. *Neisseria gonorrhoeae*. III. Demonstration of presumed appendages to cells from different colony types. Acta Pathol. Microbiol. Scand. Sect. B **79**:437-439.
12. Jyssum, K., and S. Lie. 1965. Transformation of auxotrophs of *Neisseria meningitidis*. Acta Pathol. Microbiol. Scand. **63**:445-455.
13. Kellogg, D. S., Jr., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising. 1968. *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. **96**:596-605.
14. Kellogg, D. S., Jr., W. L. Peacock, W. E. Deacon, L. Brown, and C. I. Pirkle. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85**:1274-1279.
15. La Scolea, L. J., Jr., and F. E. Young. 1974. Development of a defined minimal medium for the growth of *Neisseria gonorrhoeae*. Appl. Microbiol. **28**:70-76.
16. Mueller, J. H., and J. Hinton. 1941. A protein-free medium for primary isolation of the gonococcus and meningococcus. Proc. Soc. Exp. Biol. Med. **48**:330-333.
17. Pakula, R. 1965. Factors regulating competence in transformation of streptococci. J. Bacteriol. **90**:1320-1324.
18. Sparling, P. F. 1966. Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J. Bacteriol. **92**:1364-1371.
19. Spencer, H. T., and R. M. Herriott. 1965. Development of competence of *Haemophilus influenzae*. J. Bacteriol. **90**:911-920.
20. Swanson, J., S. Kraus, and E. Gotschlich. 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. J. Exp. Med. **134**:886-906.
21. Thongthai, C., and W. D. Sawyer. 1973. Studies on the virulence of *Neisseria gonorrhoeae*. I. Relation of colonial morphology and resistance to phagocytosis by polymorphonuclear leukocytes. Infect. Immun. **7**:373-379.
22. Watt, P. J., A. A. Glynn, and M. E. Ward. 1972. Maintenance of virulent gonococci in laboratory culture. Nature (London) New Biol. **236**:186-187.
23. Yasbin, R. E., and F. E. Young. 1972. The influence of temperate bacteriophage ϕ105 on transformation and transfection in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **47**:365-371.
24. Young, F. E., and J. Spizizen. 1961. Physiological and genetic factors affecting transformation in *Bacillus subtilis*. J. Bacteriol. **81**:823-829.