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# Conditions Required for the Attainment of Colony-Type Stability of *Neisseria gonorrhoeae* in Liquid Culture

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Colony-type morphology in *Neisseria gonorrhoeae* is associated with virulence, transformability, and the presence or absence of pili. A reliable method for achieving large populations of cells that are relatively stable with respect to colony type would be valuable, for example, in studies of virulence or for the isolation of pilus-specific phages. Previously described methods designed to achieve type stability in liquid culture were inadequate for a variety of reasons, including their low final cell yields and/or their requirements for prolonged incubation. The success of the procedure described in this communication depends upon the use of an overnight plate harvest to insure a relatively large and stable inoculum for the liquid medium. Yields of as high as $10^{10}$ colony-forming units/ml are routinely obtained after 4 to 5 h of incubation. Such cultures exhibit a colonial-type stability of 85 to 95% with respect to the original colonial type used for inoculation of the start plate.

In 1963, Kellogg et al. (4) reported the isolation of four colonial types of *Neisseria gonorrhoeae* and designated them as T1, T2, T3, and T4. Types T1 and T2 differ from T3 and T4 in several important respects. The former are virulent (3, 4), can be made competent for genetic transformation (6) and produce pili (2, 7); whereas the latter are avirulent, are nontransformable, and do not possess pili. The major difficulty in studying these colonial types results from their inherent instability particularly in broth cultures. A reliable method for achieving large populations of cells that are relatively stable with respect to colony type would be valuable, for example, in studies of virulence or for the isolation of bacteriophages. Such cultures could be used to prepare lawns of indicator cells of a known colonial type against which samples could be screened for phage activity. Lawns that are homogeneous with respect to colonial type offer two major advantages over heterogeneous lawns: (i) a means of screening for phages specific for the pili contained on virulent T1 and T2 but absent from avirulent types T3 and T4, and (ii) a means for obtaining lawns that are uniform and even enough in consistency to reveal the presence of minute and/or turbid plaques. In this communication we describe a simple, highly reproducible procedure which allows one to obtain good growth of broth cultures of *N. gonorrhoeae* while maintaining a high degree of colonial-type stability.

(A preliminary report of this work was presented at the 75th Annual Meeting of the American Society for Microbiology, 27 April to 2 May 1975, New York, N.Y.)

## MATERIALS AND METHODS

**Organisms.** Most of our studies were done with strain F62 types T1, T3, and T4. The colonial types were isolated by us by repeated selective transfer of single colonies (4) from parent strain F62 kindly provided by D. S. Kellogg, Jr. (Center for Disease Control, Atlanta, Ga.). Clinical specimens (urethral exudates and cervical samples) were obtained from the Kansas City, Mo., Health Department, Venereal Disease Center. Clinical isolates were identified as *N. gonorrhoeae* on the basis of the Gram stain, the oxidase reaction, and sugar fermentations (glucose +, lactose −, maltose −, fructose −, sucrose −).

**Media and cultural conditions.** GC medium base (Difco) plus defined supplements (GCBA-DS) (4) was used for the selective transfer of colony types, growth of the inocula, and viable counts. Plates were incubated at 35 C in a Napco $CO_2$ incubator (model 3321). The atmosphere contained 10% $CO_2$ and humidity was maintained by the addition of water to the bottom of the incubator chamber. Broth cultures were grown in the biphasic medium described by Sparling (6), which consisted of a 30-ml GCBA-DS agar base layer and 70 ml of GC broth plus defined supplements (GCBB-DS) as the overlay. The 250-ml Erlenmeyer flasks containing the biphasic medium were inoculated and incubated at 36 C in air in a New Brunswick incubator-shaker model R25 (reciprocating drive) at 90 rpm (50 to 70 back and forth strokes per min).

Stability in biphasic medium was achieved by the following protocol. (i) A frozen stock of cells that had been stabilized with respect to colony type by successive selective transfer (see below) was thawed at room temperature, and 0.1 ml of the suspension ($10^6$ to $10^8$ colony-forming units per ml) was spread on a freshly poured (1 to 3 h old) GCBA-DS plate. The use of a freshly poured plate is critical for the success of the procedure. (ii) After 17 h of incubation (10% $CO_2$, humidity), the start plate was harvested in 5 ml of GCBB-DS, and 0.6 ml was used to inoculate a flask containing biphasic medium.

**Determination of colony type.** Colony types were distinguished by the procedures described by Kellogg et al. (4) utilizing an AO Cycloptic dissecting microscope equipped with a mirror that is matte opal on one side and mirror glass on the other (American Optical Corp.). We depended almost exclusively on the matte opal side of the mirror. However, initially, we had some difficulty in distinguishing between T3 and T4. We alleviated this difficulty by the use of the standard mirror. With this side of the mirror, as opposed to the matte opal side, the granulation and yellow-gold color associated with typical T3 were discernible.

**Preparation and storage of single colony-type stocks.** Strain F62 and clinical strains were first streaked onto plates containing GCBA-DS to reveal colonial morphologies. Under our standard conditions of incubation (see above) differences in colonial morphology are most pronounced after 20 to 22 h of incubation. Colonies that exhibited morphologies typical of each of the types T1 through T4 were then stabilized by successive selective single-colony transfers. In general, 10 to 12 selective transfers were sufficient to obtain 90 to 95% stability with respect to type. After plate stability had been achieved, a colony of the type to be propagated was streaked onto a plate of GCBA-DS. After 20 to 22 h of incubation, the plate was examined for colonial-type stability. Plates exhibiting 90 to 99% stability were harvested in 3 ml of broth containing 1.5% proteose peptone no. 3 (Difco) plus 20% glycerol (vol/vol). The suspension was stored at −76 C until needed as a source of inoculum.

## RESULTS AND DISCUSSION

**Stabilization by means of single colony selection.** Our results using selective transfer of single colonies on plates were essentially the same as described by Kellogg et al. (4). Freshly isolated strains (most of which were T1 and T2 upon isolation) became T3 and T4 (predominantly T4) after as few as five nonselective transfers. T1 and T2 could be maintained as reasonably stable types only by means of selective transfer. On the other hand, we rarely, if ever, observed T1 or T2 in T4 stocks that had undergone 10 selective transfers. Similarly, T3 that was transferred for 2 weeks gave rise to an occasional T4 but rarely to a T1 or T2 colony. It must be emphasized that such observations do not necessarily mean that stabilized T4 and T3 lost the potential for conversion to T1 and T2. In the absence of a selective procedure (e.g., type-specific phages), we relied on observation alone with its low inherent sensitivity to evaluate the potential for type stability. Although reasonably we would expect to observe T4 --> T1 dissociation that occurs at a frequency of $10^{-3}$, we could easily miss the phenomenon were it to take place at a frequency of $10^{-4}$.

**Type stability in biphasic medium.** Once we reproduced the colonial-type stability on agar as reported by Kellogg et al. (3, 4), we began to investigate the conditions required for maintenance of type stability during mass growth in broth. Attempts to initiate growth utilizing suspensions made from a single stabilized colony or from many single colonies resulted in cultures that exhibited extended lag phases prior to the onset of growth. When we plated samples taken from such cultures at various times during growth, we found a high degree of colonial-type heterogeneity. A variety of procedures was tested in which we varied such parameters as the nature, age, and size of inoculum, media components, $CO_2$ concentration, incubation temperature, degree of agitation, etc. The only procedure that gave consistently good results with respect to a high degree of colonial-type stability along with high viable count is described above. The results obtained when this procedure was used to grow strain F62 types T1, T3, and T4 are shown in Fig. 1 through Fig. 3.

An examination of the growth curves shown in Fig. 1 reveals that stabilized types T1, T3, and T4 all exhibited similar patterns of growth

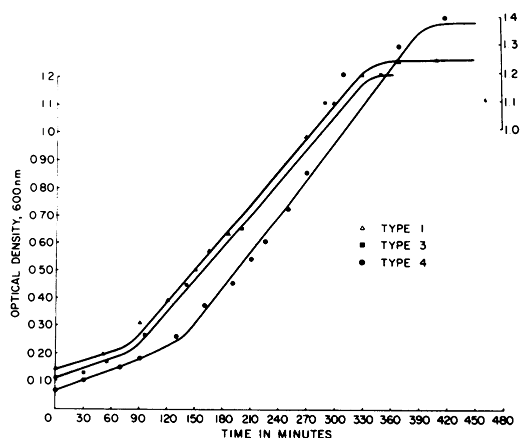


FIG. 1. *Growth of N. gonorrhoeae strain F62 types T1, T3, and T4 in biphasic medium. See text for growth conditions.*

under our conditions in biphasic medium. Growth curves of type T2 are difficult to reproduce because of the extensive micro- and macroscopic clumping exhibited by that dissociation variant. In Fig. 2 we plotted a growth curve using viable counts obtained in the same experiment as was shown in Fig. 1. Although only the numbers obtained for T4 are shown, the data for T1 and T3 fit the same curve. It should be noted that a viable count of greater than $10^{10}$ colony-forming units per ml was obtained under these conditions. Calculation of the generation time from the data plotted in this figure provides a value of approximately 40 min. In Fig. 3, we plotted the percentage of the population that retains the T-type of the inoculum against time. The numbers in parentheses indicate the viable count at the times shown and are presented merely for reference. Samples were plated on GCBA-DS medium and the colonies were typed after the plates had been incubated 20 to 22 h at 35 C with 10% $CO_2$ and humidity. Even with type T1, the least stable type shown, better than 88% stability was retained for a minimum of 4.5 h. The T1 curve, indicated by a solid line, is not one of our best. We used it to be consistent because these data were obtained from the same culture shown in the previous figures. However, the data indicated by a dotted line represents a more typical T1 stability curve. After 6 h of growth in liquid, type T1 exhibited better than 90% type stability.

The success of our protocol depended on the use of a relatively large inoculum that was homogeneous with respect to type. Such an inoculum can be easily prepared from overnight growth on a plate that was freshly poured prior to inoculation with a suspension of stabilized cells. To our knowledge, this procedure represents the best yet devised for the maintenance

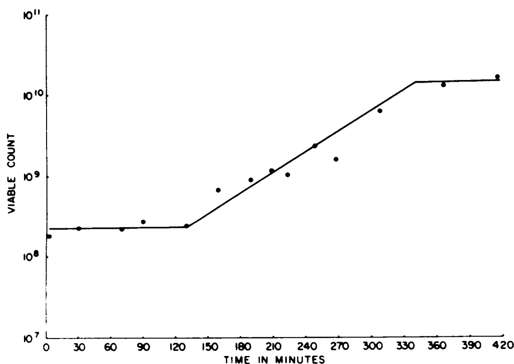


FIG. 2. *Growth of N. gonorrhoeae strain F62 type T1 in biphasic medium.*

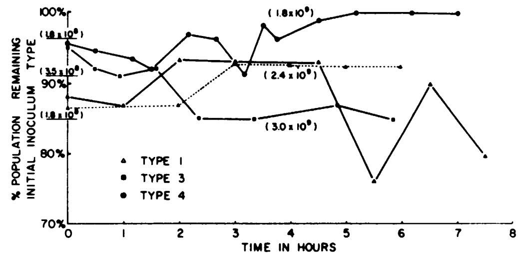


FIG. 3. *Stability of N. gonorrhoeae strain F62 types T1, T3, and T4 in biphasic medium. The solid and dotted lines shown for type T1 represent two separate experiments. Each point represents 300 to 500 colonies examined under the dissecting microscope to determine percentages of colony types.*

of colonial-type stability under conditions that allow growth to very high cell yields. Jephcott (1) was able to maintain 75% stability for type T1 in a biphasic medium consisting of a dextrose-starch agar base and a dextrose-starch-peptone broth. However, a 10% $CO_2$ atmosphere was required; generation times were unusually long and the maximum viable counts achieved were low (approximately $10^8$ colony-forming units per ml). Recently we became aware of a parallel study by LaScolea et al. (5) in which stability of strain F62 type T1 was achieved in a liquid minimal medium. Although the maximum viable counts obtained were an order of magnitude lower than those of our system, approximately 90% stability of T1 was maintained.

Our method is a simple, highly reproducible procedure which allows one to obtain good growth of broth cultures of *N. gonorrhoeae* while maintaining a high degree of colonial-type stability. We successfully applied this procedure to a large number of clinically isolated strains of *N. gonorrhoeae*.

## ACKNOWLEDGMENTS

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