Cultivation of *Neisseria gonorrhoeae* Under Low-Oxygen Conditions

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Sixteen *Neisseria gonorrhoeae* strains were examined for their abilities to grow under reduced oxygen tension. Low oxygen tensions were developed by evacuation-replacement procedures in which anaerobes and oxidation-reduction indicators were used as controls. All strains survived 96 h in a medium reduced to below −125 mV without visible growth. Detectable growth occurred at 0.05% oxygen, and 33% of normal colony size under air (21% oxygen) was obtained at 0.15% oxygen. Population selection did not determine survival and growth, but carbon dioxide tension was required. Characteristic colony morphologies were not evident at the lower oxygen concentrations. Colonial variation was not influenced during survival under anaerobic conditions or growth under low oxygen levels (0.15%). Medium differences were not significant affectors. We concluded that *N. gonorrhoeae* will grow under tensions suitable for anaerobes, and will demonstrate certain modifications of behavior under these conditions.

The genus *Neisseria* is described as having aerobic to facultative anaerobic oxygen relationships in *Bergey’s Manual of Determinative Bacteriology, 8th ed.* (1). No identification of species oxygen relationships is given. *Neisseria* spp. that Prevot and Fredette considered anaerobes (*N. reinitmis, N. orbiculata, N. vulvovaginitis*, and *N. dicoioes*) are now listed under the genus *Veillonella* in *Bergey’s* (11). *Neisseria gonorrhoeae* has been considered an aerobic requiring carbon dioxide for primary isolation. Growth of one of its colony types (T2) under oxygen tensions below 17+% (candle jar) was strongly influenced by the degree of carbon dioxide tension (7). James-Holmquest et al. did not obtain anaerobic growth of gonococci (6), and according to Morse et al. (9), gonococci do not have the appropriate metabolism for anaerobic growth. However, gonococci have been isolated from body sites (such as joint fluids) which have a low-free-oxygen character, and there are no data to suggest that gonococci can obtain oxygen from the various oxygen transport compounds in human fluids. This would indicate potential growth capability under oxygen levels much lower than that currently used in in vitro work. Over the last several years, we have observed gonococcal survival for various periods under anaerobic conditions. The presence of pinpoint colonies after 48 h of incubation indicated that some growth had occurred. An extension of this work has been previously reported (12; H. B. Short, V. L. Clark, D. S. Kellogg, and F. E. Young, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D39, p. 44). Here we report the growth of *N. gonorrhoeae* under low oxygen tensions without the use of oxidation-reduction poising agents.

(This study has been presented previously in part [D. S. Kellogg, Abstr. Annu. Meet. Soc. Microbiol. 1981, D57, p. 55].)

MATERIALS AND METHODS

**Media.** *N. gonorrhoeae* strains were maintained and studied on either GC agar base (GBCA) (Difco Laboratories, Detroit, Mich.) or Catlin’s *Neisseria* defined medium (2) (GIBCO Diagnostics, Madison, Wis.) with agar to which a defined growth supplement, stabilized defined supplement (3), was added. Early work also used GCB-stabilized defined supplement supplemented with 10% sheep erythrocyte lysate. The anaerobic organisms were maintained and studied on brain heart infusion agar plus 10% sheep blood.

**Bacteria.** Sixteen *N. gonorrhoeae* strains were used: eight laboratory strains (190, 167, F62, 9, LB999, 005, L6, and 461) and eight recent isolates (Sioux, 060991), FC1, FC2, FC4, FC5, FC6, and FC11). All strains were maintained as T1 or T2 colonies during the studies. Several of these strains have special characteristics, such as requirements for proline (190, LB999) and arginine (461, 005) or the production of β-lactamase (L6). Other *Neisseria* spp. examined were *N. mucosa* B135, *N. flavescens* N-155, and two *N. meningitidis* strains, 1854 (serogroup A) and KC795 (nongroupable). The four anaerobes used to monitor oxygen tensions were *Clostridium tetani* 14339, *Clostridium hemolyticum* A-740, *Veillonella parvula* 18085, and *Bacteroides fragilis* subsp. *vulgatus* 14464. The
latter were kindly provided by George Lombard of the Anaerobic Infections Laboratory, Hospital Infections Program, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga.

Gas phase containers. Routine cultivations at standard gas conditions were developed in candle jars (3+% carbon dioxide plus 17+% oxygen). Anaerobic conditions were developed in Brewer Anaerobic Jars (Becton, Dickinson & Co., Rutherford, N.J.) with a GasPack system (BBL Microbiology Systems, Cockeysville, Md.). Low-oxygen conditions (<17% oxygen) were generated in a vacuum chamber (medical prototype, design EL1018A for flexible anaerobic glove box; Coy Laboratories, Ann Arbor, Mich.). The chamber was evacuated with a Duo-Seal vacuum pump (model 1397) to 30 inches (76.2 cm) of mercury vacuum. The chamber was refilled with a gas mixture consisting of 4.0% carbon dioxide and 96% nitrogen. Four such evacuations and refills left a calculated 0.06% oxygen in the chamber. Measured volumes of air were put into the chamber during the fourth refill to develop the desired oxygen levels. On the fourth refill, excess CO₂:N₂ gas mixture was put into the chamber to produce 1 inch (2.54 cm) of gas pressure, which served as an indicator of tank integrity. Carbon dioxide was present for all incubations except where its omission is specifically indicated. Survival or growth (or both) without exogenous carbon dioxide was accomplished by incorporating beakers of 1 N sodium hydroxide with filter paper wicks in the growth containers.

Indicators. Oxidation-reduction levels were checked with chemical indicators such as methylene blue (+11 mV), resazurin (∼42 mV), indigo-carmine (∼125 mV), and phenosafranine (∼252 mV). The millivoltage level given for each dye is that at which the indicator is 50% reduced. Each dye was dissolved at levels of 1 mg/ml and added to a NaHCO₃-glucose solution (6 and 4 mg/ml) in minimal amounts to produce a visible color against a white background.

Incubations. All incubations were performed at 36°C, except when specific temperature tests were performed (25 and 30°C). Brewer Anaerobic Jars lacking the catalyst granules were used in tests carried out at 25 and 30°C. These jars and the inoculated plates were placed in an anaerobic glove box; the plates were opened, closed, and placed in the jars; and the jars were closed securely. After the jars were removed from the anaerobic glove box, measured volumes of air were introduced by syringe into each jar, which were then placed at the appropriate temperature for incubation. Incubation times were 20 h, except where otherwise specified.

Tests. Catalase and oxidase tests were performed by procedures described in the Manual of Clinical Microbiology, 2nd ed. (10).

The identities of the organisms growing under low-oxygen conditions were confirmed by (i) transfer to fresh medium and incubation under 17+% oxygen—3+% carbon dioxide, (ii) examination by Gram stain, (iii) rapid carbohydrate degradation (10), and (iv) fluorescent-antibody staining, where appropriate. The fluorescent-antibody reagent had been developed with N. gonorrhoeae 9 grown under 17+% oxygen.

Electron microscopy. Cultures were fixed and stained with ruthenium red by the procedure of Springer and Roth (14). The bacteria were dehydrated in successive 10-min steps of graded ethanol and embedded in maraglas (15). Thin sections were cut on a Reichert OM2 ultra microtome fitted with a diamond knife, mounted on bare copper grids, and examined in a model 200 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.).

RESULTS

The oxidation-reduction indicators were used to determine relative oxygen levels during the early part of this study. The methylene blue indicator changed from blue to bluish-green specks on a white background at a calculated 2
to 3% oxygen level. At a calculated oxygen level of ≤1%, methylene blue was reduced (white) and indigo-carmine was reduced from blue to dark green. At 0.15% oxygen, indigo-carmine became a light green to yellow-green, or slightly more than 50% reduced (−125 mV). Some variability with indicator response at low levels of calculated oxygen necessitated changing from chemical to microbiological indicator systems. The anaerobes used in these experiments responded to the calculated oxygen levels in a manner similar to that reported in the literature (Fig. 1) (8, 13). C. tetani and C. hemolyticum grew well enough at 0.15% oxygen to detect colonies without the aid of a microscope. As expected, B. fragilis and V. parvula demonstrated a lower sensitivity to oxygen.

The relationship of gonococcal colony diameter to oxygen level is shown in Fig. 2. Colony size decreased with declining oxygen to the 5% level. Further reductions of oxygen to a level of 0.15% did not decrease colony sizes; however, below an oxygen level of 0.15%, colony sizes were variable but unrelated to the colony type of inoculum and averaged less than 0.05 mm in diameter. Colonies incubated for longer periods showed a twofold increase in average size after 40 h but no further increase with another 24 h (for a total of 64 h). All strains survived 96 h at 36°C under anaerobic conditions (Brewer Anaerobic Jar) with the production of pinpoint colonies (less than 0.05 mm diameter maximum). Removal of plates from either anaerobic or low-oxygen (0.15%) gas phases into candle jars (17+% oxygen–3+% carbon dioxide) resulted in growth to normally sized (0.45 mm), morphologically typical colonies in 20 h. Periodic examina-

tion of these plates after removal to an atmosphere containing 17+% oxygen determined that, after 2 h, colony type morphology was beginning to be observable. After 5 h under 17+% oxygen, colony morphology was characteristic for each colony type. None of the strains used survived incubation for 4 to 8 h in 17+%
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Oxygen followed by 20 h at 0.15% oxygen and then another 20 h under 17+% oxygen. The usual colony size differential between growth on GCBA and Neisseria defined medium was also obtained under low-oxygen conditions (Fig. 2). Growth on GCBA plus 10% sheep erythrocyte lysate gave slightly larger colonies than GCBA alone but otherwise followed the same colony diameter-oxygen percentage curve. Storage and inoculation of media in an anaerobic glove box did not affect the results of subsequent incubation under 0.15% oxygen.

Morphological distinction between the four colonial types of N. gonorrhoeae were retained at oxygen levels down through 1 to 3%, as shown by T1 morphology (Fig. 3). Morphological distinctions disappeared below this level, and a distinct morphology unlike any of the usual morphologies appeared (Fig. 4). Transfer of these plates to candle jars for 20 h resulted in growth to a normal size and the development of a morphology similar to that obtained from the same inoculum grown in candle jars. Growth occurring under 17+ and 0.15% oxygen, followed by 17+% oxygen, was nearly identical in the number of colonies and the percentages of colony types. Survival under 0.15% oxygen tension at 25 and 30°C was the same as that obtained under anaerobic oxygen tensions, nearly 100% of the inoculum of each strain over a 48-h period. Increased oxygen (26%) did not influence growth or colony morphology.

Gonococcal colonies grown under 0.15% oxygen were much more viscous than those grown under 17+% oxygen. Gram stains of ethanol-acetone (50:50)-fixed cells from each growth condition showed the presence of much more intercellular material between 0.15% oxygen-grown cells than between 17+% oxygen-grown cells. After transfer of 0.15% oxygen-grown cells to 17+% oxygen, this intercellular material declined in amounts parallel to the development of typical colonial morphology. Examining individual gonococcal cells by fixed-phase optics (×600) revealed that the cells were less refractive and had indistinct outlines, in contrast to cells grown in candle jars. Electron microscopical examination of cells grown under 0.15 and 17+% oxygen did not reveal any differences in intercellular materials. The random distribution of ruthenium red particles indicated that neither type of cells had polysaccharide capsule. There were no differences in the cell walls and membranes of the two types of cells. However, the cytoplasmic material of cells grown under 0.15% oxygen had a uniform density (Fig. 5), in contrast to cells grown under 17+% oxygen, in which there were substantial areas of very low density (Fig. 6).

N. gonorrhoeae colonies grown under 0.15% oxygen gave oxidase reactions equivalent to those grown under 17+% oxygen. The oxidase reactions produced by the two N. meningitidis strains and N. mucosa and N. flavescens under 0.15% oxygen were much weaker than those produced under 17+% oxygen. Catalase activity with N. gonorrhoeae strains was equally strong under either oxygen level. N. meningitidis 1854 (A) was catalase positive under both 0.15 and 17+% oxygen; however, KC795 (NT) was catalase positive only under 17+% oxygen. N. flavescens and N. mucosa were catalase negative under both oxygen conditions. Carbohydrate degradation patterns were the same for all strains except N. flavescens, regardless of whether they were grown under 0.15 or 17+% oxygen. N. flavescens cells grown under 0.15% oxygen produced acid reactions with glucose, maltose, and fructose, in contrast to the lack of activity of the cells of this strain grown under 17+% oxygen.

Fluorescent-antibody reactivity was increased qualitatively by growth under 0.15% oxygen. Removal of plates from a 0.15% oxygen atmo-
sphere and incubation of the plates in a 17+\% oxygen atmosphere resulted in a decrease in the fluorescent staining of the gonococcal cells over time which paralleled the reduction in intercellular material and development of typical colonial morphology. *N. meningitidis* cells reacting with anti-gonococcal fluorescent-antibody reagent increased by sevenfold when grown under 0.15\% oxygen levels as compared with those grown under 17+\% oxygen.

**DISCUSSION**

The altered colony morphologies and reduced colony diameters occurring as a result of cultivation under 0.15\% oxygen are phenotypic in character, as shown by the resumption of their basic characteristics upon return to 17+\% oxygen. There was no difference in these responses between virulent and avirulent cell types. Since the unbound oxygen supply in body fluids is low and variable between tissues and avirulent cells do not survive in the body, the virulence characteristic is not related to oxygen level effects per se.

Although the only morphological difference between 17+ and 0.15\% oxygen-grown cells is in cytoplasmic density, there are differences in the intercellular material, both quantitatively and qualitatively. This intercellular material is antigenic, as shown by the enhanced fluorescent-antibody staining of low-oxygen-grown cells over 17+\% oxygen-grown cells. This material is a direct result of growth under low-oxygen conditions, as shown by its decline quantitatively when 0.15\% oxygen-grown cells are transferred to 17+\% oxygen. Taking into consideration the tremendous differences in the metabolic climate between in vivo and in vitro conditions, we concluded that this reduction in intercellular material when 0.15\% oxygen-grown cells are transferred to 17+\% oxygen parallels the decline in fluorescence between in vitro transfers from primary isolates seen by Deacon et al. (4). In the host, this material may serve a function related to low-free-oxygen tension and secondarily in-
terfere with the defensive mechanisms of the host.

Survival under anaerobic conditions and growth under low-oxygen conditions should not be surprising when the isolation of gonococci from tissues such as the cervix, rectum, and joint fluids, where free oxygen should be even lower than the 0.3 to 0.4% oxygen of the human buccal folds (5), is considered. The optimum oxygen levels for most bacteria are those which allow growth either most rapidly or in the largest numbers or with a character that is useful for identification. In terms of bacterial relationships to hosts, this artificially high oxygen may influence adversely those very factors we are most interested in examining. An examination of the effects of low-oxygen cultivation on characteristics involved in the interaction of gonococci and host mechanisms is under way at present.

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LITERATURE CITED