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Effects of Gaseous CO_2 and Bicarbonate on the Growth of *Neisseria gonorrhoeae*

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The quantitative and qualitative growth response of *Neisseria gonorrhoeae* strains was assessed under the following conditions: incubation in a candle jar (~2.5% ambient CO_2) on medium without bicarbonate, incubation in air on medium without bicarbonate, incubation in air on medium with bicarbonate, and incubation in air in a sealed jar on medium with bicarbonate. Incubation in the presence of ambient CO_2 (candle jar) resulted in the highest plating efficiencies for the three laboratory strains 9, 62, and 2686. The addition of $NaHCO_3$ to the medium enhanced the growth response in air of all three strains, particularly if incubation was carried out in a closed environment (sealed jar). The qualitative growth response of 34 clinical isolates and laboratory strains was assessed under the same conditions of incubation after the plating of an inoculum containing approximately 2×10^6 bacteria. The strains were divided into different classes based on their growth responses. About 40% of the strains grew as well on bicarbonate-containing medium incubated in air in sealed jars as they did on medium without bicarbonate incubated in a candle jar. Ten percent of the strains showed only slight growth on bicarbonate-containing medium incubated in sealed jars and appeared to have an almost obligate requirement for ambient CO_2. Twenty percent of the strains apparently had partially lost their requirement for gaseous CO_2 and showed slight growth in air on medium without bicarbonate and slight to moderate growth in air on medium containing $NaHCO_3$. The remaining 30% seemed to have completely lost their requirement for gaseous CO_2 and/or the bicarbonate anion and grew almost as well in air on medium without bicarbonate as they did in either ambient CO_2 (candle jar) or on medium containing bicarbonate incubated in a sealed jar. These results suggest that *N. gonorrhoeae* strains may vary widely in their requirements for CO_2 and/or the HCO_3^- anion. Incubation in the presence of ambient CO_2 tends to maximize the growth response on solid medium of those strains, which require it for growth. The presence of ambient CO_2 is particularly important if growth is to be obtained after the plating of small inocula. Medium containing 0.1% $NaHCO_3$, if incubated in a closed environment (sealed jar), appears to be equivalent to medium without bicarbonate incubated in ambient CO_2 in supporting the growth of some but not all strains of *N. gonorrhoeae*.

A requirement for small amounts of CO_2 by heterotrophic bacteria for the initiation of growth is well established (1, 8, 15, 16). The pathways and enzymes used in the fixation of CO_2 and/or the HCO_3^- anion have been identified for some species (5, 11, 17, 18, 21, 22).

Neisseria gonorrhoeae and other pathogens require an increased CO_2 concentration for the initiation of growth. The reason for this increased requirement for CO_2 is not known, and the pathways by which pathogens such as *N. gonorrhoeae* fix CO_2 and/or the HCO_3^- anion have not been studied.

The fact that pathogenic *Neisseria* require an increased CO_2 tension for primary isolation is well documented (4, 7, 9, 12, 23). It has been suggested that this CO_2 requirement can be replaced by incorporating certain purines, pyrimidines, metabolic intermediates, or certain types of yeast extract into the culture medium (6, 9, 10). Also, it has been suggested that the addition of bicarbonate to culture medium can replace the CO_2 requirement of *N. gonorrhoeae* (20). Most of these studies are not comparable because investigators used different strains, which we now know may vary widely in their nutritional requirements, sensitivity to agar toxicity, and requirement for CO_2 (2, 3, 12–14). Also media of different compositions were used, which may have added additional variables.

Much of the earlier growth response data and also a large portion of the more recent data are based on qualitative measurements of growth which are unable to distinguish small differences in growth response. We have previously shown that quantitative data on the growth response of *N. gonorrhoeae* can be obtained if nutritional and toxic variables are carefully controlled (13).

In the present study we attempt to quantitatively and qualitatively assess the roles gaseous CO_2 and the HCO_3^- anion play in supporting the growth of both well-characterized laboratory strains and recent clinical isolates of *N. gonorrhoeae*. The medium used for these experiments was essentially nontoxic and satisfied the nutritional requirements of most *N. gonorrhoeae* strains tested (13).

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. Strains 1, 2, and 3 are laboratory strains obtained from D. S. Kellogg, Jr. (Center for Disease Control, Atlanta, Ga.). Strains 4 through 31 are clinical isolates from different geographical locations in the United States. These were also obtained from D. S. Kellogg, Jr. Strains 32 through 34 are auxotypes of *N. gonorrhoeae* obtained from the American Type Culture Collection, Rockville, Md. All strains were stored at −70°C in buffered proteose peptone no. 3 (Difco) broth containing 20% (vol/vol) glycerol.

TABLE 1. *N. gonorrhoeae strains used*

Stock no.	Strain	Type and source[a]
1	9	Laboratory strain, CDC
2	62	Laboratory strain, CDC
3	2686	Laboratory strain, CDC
4	A-1	Clinical isolate, CDC
5	A-2	Clinical isolate, CDC
6	A-3	Clinical isolate, CDC
7	A-4	Clinical isolate, CDC
8	A-5	Clinical isolate, CDC
9	A-6	Clinical isolate, CDC
10	B-1	Clinical isolate, CDC
11	CD-1	Clinical isolate, CDC
12	E-1	Clinical isolate, CDC
13	H-2	Clinical isolate, CDC
14	H-3	Clinical isolate, CDC
15	K-1	Clinical isolate, CDC
16	K-4	Clinical isolate, CDC
17	K-5	Clinical isolate, CDC
18	K-6	Clinical isolate, CDC
19	J-1	Clinical isolate, CDC
20	J-2	Clinical isolate, CDC
21	J-3	Clinical isolate, CDC
22	J-4	Clinical isolate, CDC
23	J-5	Clinical isolate, CDC
24	J-6	Clinical isolate, CDC
25	O-2	Clinical isolate, CDC
26	O-3	Clinical isolate, CDC
27	Va-1	Clinical isolate, CDC
28	Va-2	Clinical isolate, CDC
29	Va-3	Clinical isolate, CDC
30	Va-4	Clinical isolate, CDC
31	Va-5	Clinical isolate, CDC
32	27628	Catlin auxotype, ATCC
33	27629	Catlin auxotype, ATCC
34	27630	Catlin auxotype, ATCC

[a] CDC, Center for Disease Control; ATCC, American Type Culture Collection.

Media. TTU complete medium contained the following, in grams per liter: polypeptone peptone (BBL), 15; K_2HPO_4, 4; KH_2PO_4, 1; NaCl, 5; soluble starch (Difco), 1; and purified agar (Difco), 10. The following supplements (micrograms per milliliter) were added: glucose, 5,000; thiamine (B_1), 2; ferric nitrate, 5; cysteine, 55; hypoxanthine, 5; uracil, 10. The diluent used in all experiments was buffered saline gelatin (13). In the experiments to be described, 0.1% $NaHCO_3$ was added to some sets of TTU complete agar medium.

Quantitative plating experiments. For each experiment, strains were thawed and streaked to TTU complete medium and incubated in a candle jar (~2.5% ambient CO_2) at 35°C. After incubation for 24 h, suspensions were made in warm buffered saline gelatin, vortexed for 15 to 20 s to break up clumps, and adjusted to an absorbance reading of 1 on a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 550 nm. The suspension was then diluted in warm buffered saline gelatin, and 0.05-ml portions of each dilution were plated in duplicate. The inoculum was spread to dryness with a glass spreader. Plates were incubated in a candle jar (~2.5% ambient CO_2), in a mechanical convection incubator (ambient air), or in a sealed jar (ambient air). The incubation temperature in all cases was 35°C.

Qualitative plating experiments using the streak plate method. The qualitative growth response was determined as follows: suspensions of each strain were prepared as described for quantitative platings. Plates were divided into four sectors, and each strain was streaked within a sector using a calibrated loop (0.001 ml) so as to obtain isolated colonies in the more dilute areas. The total number of viable bacteria delivered by the calibrated loops was approximately 2×10^6. Plates were incubated under the same atmospheric conditions as described above for quantitative plating experiments. The growth response was qualitatively ranked as heavy, moderate, light, or no growth. Coded plates were scored by three different individuals in order to make the results as objective as possible. Agreement among individuals was good.

RESULTS

Plating efficiency of *N. gonorrhoeae* strains on TTU complete medium with and without 0.1% $NaHCO_3$, incubated under different atmospheric conditions. The results of this experiment are shown in Table 2. The efficiency of plating (EOP) of the three laboratory strains 9, 62, and 2686 on the TTU complete medium

without $NaHCO_3$ and incubated in candle jar (~2.5% CO_2) was set at 1. When an identical set of plates was incubated in air in a mechanical convection incubator, the EOP for strains 9 and 62 was reduced to almost undetectable levels. Strain 2686 was less sensitive to incubation in air, but its EOP was still reduced almost 100,000-fold below that of control plates incubated in the presence of ambient CO_2. If 0.1% $NaHCO_3$ was incorporated into the medium and the plates were incubated as above in a mechanical convection incubator in air, the EOP for strains 9 and 62 increased about 1,000-fold. Strain 2686 showed no increase in EOP. If medium containing 0.1% $NaHCO_3$ was incubated in a sealed jar, the EOP for strains 9 and 62 were further increased about 1,000-fold. After incubation in a sealed jar, the EOP for strain 2686 was almost equal to that of controls.

TABLE 2. *EOP of three Neisseria gonorrhoeae strains on TTU complete medium with and without 0.1% $NaHCO_3$, incubated under different atmospheric conditions*[a]

Medium	EOP of strain:		
	9	62	2686
TTU complete without $NaHCO_3$[b]	1	1	1
TTU complete without $NaHCO_3$[c]	9.5×10^{-9}	7.6×10^{-9}	3.7×10^{-5}
TTU complete with 0.1% $NaHCO_3$[d]	4.8×10^{-5}	3.8×10^{-5}	3.7×10^{-5}
TTU complete with 0.1% $NaHCO_3$[e]	2.9×10^{-2}	5.4×10^{-2}	0.41

[a] EOP was determined by dividing the number of colony-forming units (CFU) observed on experimental plates (represented by *c*, *d*, and *e* above) by the number of CFU observed on control plates (represented by *b* above). Each value represents the mean of three plating experiments. The mean colony counts on control plates (*b* above) used to compute EOP were as follows: 9, 2.1×10^9; 62, 2.6×10^9; 2686, 2.7×10^9.

[b] Plates incubated in a candle jar (~2.5% ambient CO_2).

[c] Plates incubated in air in a mechanical convection incubator.

[d] Same as *c*.

[e] Plates incubated in air in a sealed jar.

The growth response of all three strains on TTU complete medium without bicarbonate after incubation in air in sealed jars was only slightly (~10-fold) better than that observed after incubation in air in a mechanical convection incubator (data not shown in Table 2).

Qualitative growth response of *N. gonorrhoeae* strains on TTU complete medium with and without 0.1% $NaHCO_3$, incubated under different atmospheric conditions. Most of the *N. gonorrhoeae* strains initially tested grew well on TTU complete medium after incubation in a candle jar (~2.5% ambient CO_2). A few strains grew very slowly under these conditions, and it is thought that they represent strains with unusual growth requirements not fully satisfied by TTU complete medium. Their exact nutritional requirements are presently being determined. These strains were not included in this study, because maximal or near maximal growth in the presence of ambient CO_2 was desirable in comparing growth responses under different incubation conditions (see Table 3).

Four plates of TTU complete medium, two with and two without 0.1% $NaHCO_3$, were divided into four sectors, and each sector was

TABLE 3. *Qualitative growth response of Neisseria gonorrhoeae strains on TTU complete medium with and without 0.1% $NaHCO_3$, incubated under different atmospheric conditions*

Classes based on growth response	Growth response[a] on:				Strains belonging to different growth classes
	TTU complete without $NaHCO_3$		TTU complete with 0.1% $NaHCO_3$		
	CO_2[b]	Air[c]	Air[d]	Air in Jar[e]	
1	3+	0	0	2+ or 3+	4, 8, 9, 11, 12, 13, 20, 21[f]
2	3+	0	1+	2+ or 3+	16, 18, 19, 27, 30, 31
3	2+ or 3+	0	0 or 1+	1+	5, 6, 17, 25
4	3+	1+	1+ or 2+	3+	1, 2, 3, 7, 14, 23
5	3+	2+ or 3+	2+ or 3+	3+	10, 15, 22, 24, 26, 28, 29, 32, 33, 34

[a] The growth response of the strains under different incubation conditions was as follows: 3+, heavy confluent growth in area of heaviest inoculum, isolated colonies in more dilute areas; 2+, moderate confluent growth in area of heaviest inoculum, isolated colonies in more dilute areas; 1+, light confluent growth in area of heaviest inoculum, few or no colonies in more dilute areas; 0, no growth or light haze or <10 small colonies in area of heaviest inoculum.

[b] Plates incubated in a candle jar (~2.5% ambient CO_2).

[c] Plates incubated in air in a mechanical convection incubator.

[d] Same as *c*.

[e] Plates incubated in air in a sealed jar.

[f] Stock numbers of strains used. See Table 1.

streaked with a standard inoculum of each strain (~2×10^6 bacteria) using a calibrated loop (see Materials and Methods). Plates without $NaHCO_3$ were incubated either in a candle jar (~2.5% ambient CO_2) or in air in a mechanical convection incubator. Plates containing $NaHCO_3$ were incubated either in air in a mechanical convection incubator or in air in a sealed jar. Based on growth response (see footnote *a*, Table 3) under these four conditions of incubation, the 34 strains could be divided into five classes (see Table 3). Class 1 strains showed no growth in air on medium with or without $NaHCO_3$. These strains grew almost as well on medium with $NaHCO_3$ after incubation in air in a sealed jar as they did on medium without $NaHCO_3$ incubated in a candle jar. Class 2 strains were similar to class 1, except they showed slight growth on $NaHCO_3$-containing medium incubated in air in a mechanical convection incubator. Class 3 strains seem to have an almost absolute requirement for gaseous CO_2 and showed no or only slight growth under other conditions of incubation. Class 4 was similar to class 2, except these strains showed slight growth on medium without $NaHCO_3$ when incubated in air and somewhat heavier growth on $NaHCO_3$-containing medium. Class 5 strains grew almost as well in air on medium with or without $NaHCO_3$ as they did either in the presence of gaseous CO_2 or on $NaHCO_3$-containing medium incubated in a sealed jar. This class apparently has partially or completely lost its requirement for gaseous CO_2 and/or the HCO_3^- anion.

DISCUSSION

The data in Table 2 suggest that the HCO_3^- anion can almost completely replace the CO_2 requirement of strain 2686 and partially replaces the CO_2 requirement of strains 9 and 62 if incubation is carried out in a sealed jar. Since incubation of bicarbonate-containing medium in air in a mechanical convection incubator resulted in EOPs intermediate between those observed on medium without bicarbonate incubated in ambient CO_2 and those on bicarbonate-containing medium incubated in sealed jars (see Table 2), it seems that the presence of the HCO_3^- anion plus a closed environment is additive with respect to enhancement of growth. Possibly small amounts of CO_2 are produced endogenously by the inoculated bacteria before cell division begins, and in a closed environment (sealed jar) this CO_2 is utilized along with the HCO_3^- anion to stimulate growth. Such endogenously produced CO_2 would probably be quickly lost due to air flow when plates are incubated in a mechanical convection incubator, thus leaving only the HCO_3^- anion present in the medium to serve as partial substitute for gaseous CO_2. This is only speculation at this time because the pathway(s) *N. gonorrhoeae* uses in the fixation of gaseous CO_2 and/or the HCO_3^- anion and their metabolic function(s) have not been studied.

To obtain high plating efficiencies, conditions (presence of essential nutrients, optimal pH, temperature, and CO_2 concentration) must be such that growth is obtained when small inocula are plated. If such optimal conditions are not met, it is reflected quantitatively in a lowering of the EOP. By comparing differences in EOP, one can assess quantitatively the growth response resulting from both a large inoculum, i.e., platings of lower dilutions, and a small inoculum, i.e., platings of high dilutions. It is obvious that a gaseous CO_2 atmosphere was superior to $NaHCO_3$-containing medium incubated in sealed jars in supporting the growth of strains 9 and 62 when small inocula were plated (reflected by ~100-fold decrease in EOP; see Table 2). Bicarbonate-containing medium incubated in a sealed jar was almost equivalent to medium without bicarbonate incubated in ambient CO_2 in supporting the growth of strain 2686 (EOP almost equal to 1; see Table 2).

When the growth response of laboratory strains and clinical isolates was assessed qualitatively after the plating of a rather large inoculum (~2×10^6 bacteria) and incubated as described above, it was possible to divide the strains into 5 growth classes (see Table 3). Classes 1, 3, and 5 were the most different. Class 2 resembled class 1 and class 4 was similar to class 5. Most class 1 strains grew as well on bicarbonate-containing medium incubated in air in sealed jars as they did in a candle jar (~2.5% ambient CO_2) on medium without bicarbonate. Essentially no growth was noted on medium with or without $NaHCO_3$ when plates were incubated in air in a mechanical convection incubator. For this class of strains apparently the combination of the HCO_3^- anion and a closed environment (sealed jar) was adequate to support growth from this size inoculum, i.e., ~2×10^6 bacteria. Class 3 strains showed only slight growth on bicarbonate-containing medium incubated in sealed jars. For some reason this class seems to have an almost obligate requirement for gaseous CO_2. Class 5 strains appear to have either partially or completely lost their requirement for gaseous CO_2 and/or the HCO_3^- anion and grew almost as well on medium without bicarbonate in air as they did in either ambient CO_2 (candle jar) or on bicar-

bonate-containing medium incubated in a sealed jar. These results suggest that *N. gonorrhoeae* strains may vary widely in their requirement for CO_2 and/or the HCO_3^- anion.

The presence of ambient CO_2 appears to maximize the growth response of those strains that require it for growth, particularly if the number of bacteria plated is small (see Table 2). Medium containing 0.1% $NaHCO_3$ when incubated in a sealed jar was equivalent to medium without bicarbonate incubated in ambient CO_2 in supporting the growth of most *N. gonorrhoeae* strains tested after the plating of rather large numbers of cells, i.e., 10^6 or greater (see Table 3). Since clinical specimens may contain either small or large numbers of viable *N. gonorrhoeae* and, further, since strains seem to vary in their ability to utilize the HCO_3^- anion as a partial or complete substitute of CO_2, we conclude that incubation in the presence of ambient CO_2 (in either a candle jar or CO_2 incubator) remains the best method for primary isolation.

Although it has been suggested that *N. gonorrhoeae* quickly loses its CO_2 requirement after passage in vitro (19), our data indicate that this is not true for the laboratory strains 9, 62, and 2686. These strains have been transferred repeatedly in vitro but still have a rather stringent requirement for ambient CO_2 when their growth response is determined quantitatively (see Table 2). Also, the clinical isolates used in our qualitative growth response study (Table 3) have all been transferred several times in vitro and still some apparently have a stringent CO_2 requirement, i.e., those strains falling into classes 1 and 2. These results suggest that there may be basic genetic and/or biochemical differences in the way various *N. gonorrhoeae* strains use gaseous CO_2 and/or the HCO_3^- anion.

To our knowledge, the superiority of ambient CO_2 in enhancing the growth response of *N. gonorrhoeae* has never been demonstrated by quantitative plating experiments. It is our contention that a quantitative assessment of the growth response of *N. gonorrhoeae* must be developed before one can design meaningful experiments to study the roles CO_2 and the HCO_3^- anion play in the basic physiology and metabolism of this organism. This study shows that the effects of different atmospheric conditions on the growth response of *N. gonorrhoeae* can be distinguished both qualitatively and quantitatively. The specific roles of gaseous CO_2 and the HCO_3^- anion in the metabolism and growth of *N. gonorrhoeae* are presently under investigation.

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