In Vitro Inhibition of *Neisseria gonorrhoeae* Growth by Strict Anaerobes

A. MORIN,* S. A. SAHEB, J.-G. BISAILLON, R. BEAUDET, AND M. SYLVESTRE

Centre de Recherche en Bactériologie, Institut Armand-Frappier, Université du Québec, Laval-des-Rapides, Ville de Laval, Québec, Canada H7N 4Z3

Thirty two anaerobic bacterial strains were screened for their in vitro inhibitory activity toward 21 *Neisseria gonorrhoeae* strains, including two penicillin-resistant strains. From this screening, six anaerobic bacteria belonging to the genera *Bacteroides*, *Peptostreptococcus*, and *Eubacterium* were selected for their large antagonococcal spectrum of activity. The inhibitory activity of these six strains appears to be specific to the gonococcus as a variety of anaerobes and aerobes were not generally inhibited by these strains. The antagonococcal effect was not due to pH change or nutrient depletion of the media.

Numerous microorganisms have been shown to interfere with the replication of bacteria. This interference is one of the suggested mechanisms by which resistance to gonorrhea is likely to occur (9). Most of the studies dealing with the growth inhibition of *Neisseria gonorrhoeae* by other bacteria have been carried out in vitro and have dealt mainly with the antagonococcal activity of aerobic bacteria (7, 11). However, it is well known that anaerobic bacteria constitute a major proportion of the normal microflora of the human urogenital tract (1). This knowledge led Kaye and Levison (8) to suggest that, in bacterial interference, indigenous microorganisms present in large numbers are more likely to be important than microorganisms present in small numbers. To our knowledge, interference of the in vitro growth of *N. gonorrhoeae* by strict anaerobes is not well documented (7, 8). It has been shown that a few species of anaerobic bacteria from the vaginal flora, including *Gaffkya anaerobia*, *Fusobacterium varium*, *Clostridium tertium*, and *Bifidobacterium* sp., are inhibitory to in vitro-grown *N. gonorrhoeae* (8). However, these anaerobic bacteria are not frequently isolated from the vaginal flora of children and adult women (1, 4). The anaerobic bacteria isolated from the male anterior urethra did not exhibit antagonococcal activity (7). The present study was therefore undertaken to further evaluate the antagonococcal activity of 32 in vitro-grown strict anaerobes which represent the most frequently encountered bacterial species in the human urogenital tract.

MATERIALS AND METHODS

Bacterial strains. The bacteria tested for their antagonococcal activity were *Propionibacterium jensenii* (Pr0), *Propionibacterium acnes* (Pr20, Pr26), *Bacteroides fragilis* (B1A, B15), *Bacteroides disiens*

(B2), *Bacteroides vulgatus* (B4), *Bacteroides asaccharolyticus* (B5), *Bacteroides ovatus* (B24), *Bacteroides melaninogenicus* subsp. *melaninogenicus* (B27), *Bacteroides corrodens* (B30), *Peptococcus asaccharolyticus* (B1, Pc6, Pc23, Pc25), *Peptococcus magnus* (Pc8, Pc10, Pc10A, V12, V12A, Pc19), *Peptococcus prevotii* (Pc8A), *Peptostreptococcus anaerobius* (Pc9, Ps11B, Ps11C), *Streptococcus morbillorum* (Sc13, Sc13A), *Streptococcus intermedius* (B14), *Eubacterium limosum* (Pc9A, Ps11A), *Eubacterium lentum* (E28), and *Eubacterium aerofaciens* (E29). For demonstrating the antagonococcal activity, as well as its specificity, the following different target strains were used: (i) 21 *N. gonorrhoeae* strains, including those numbered G1 to G19 and 2 penicillin-resistant strains (PR 77112, PR 77117); (ii) 11 aerobic and facultatively anaerobic bacterial strains, including *Corynebacterium* sp. (A-9, A-10), *Diplococcus pneumoniae* (A-4), *Escherichia coli* (A-11), *Staphylococcus aureus* (A-1), *Staphylococcus epidermidis* (A-2, A-3), *Streptococcus mitis* (A-5), *Streptococcus agalactiae* (A-6), and *Streptococcus faecalis* (A-7, A-8); and (iii) 9 strict anaerobic bacterial strains, including *P. asaccharolyticus* (B1), *P. prevotii* (Pc8A), *P. magnus* (V12A), *P. anaerobius* (Ps11B), *S. morbillorum* (Sc13A), *P. acnes* (Pr26), *E. lentum* (E28), *B. fragilis* (B15), and *Lactobacillus acidophilus* (L3).

Culture media. The cultivation of the anaerobic bacterial strains was carried out by the methods described in the Anaerobe Laboratory Manual of the Virginia Polytechnic Institute (6). Cultures were carried out on prereduced brain heart infusion agar plates (Difco Laboratories, Detroit, Mich.). Prereduced chopped meat media (Carr Scarborough Inc., Ga.) were used to maintain the strains. The gonococcal strains were cultivated and maintained on GC plates (Difco) supplemented with 1.5% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) and hemolyzed horse blood. *L. acidophilus* was grown on MRS plates (Difco) and stored at 4°C on MRS agar slopes. Aerobic bacterial strains were grown on brain heart infusion agar plates, and their suspensions in brain heart infusion broth containing 10 to 15% glycerol were stored at -80°C.

Demonstration of the antagonococcal activity

and its specificity. Anaerobes were spot inoculated on prereduced brain heart infusion agar plates. Inoculation and incubation were carried out under a gas mixture of 10% CO₂-10% H₂-80% N₂ (Liquid Carbonic Corp., Canada) with the bacteriological glove box procedure (3). After an incubation period of 48 h at 37°C, the bacterial growth was scraped off with a microscope slide, and the plates were exposed to chloroform vapors for 2 h to kill residual cells. The plates were then left in the air for the same period of time and were inoculated, either aerobically or anaerobically, with 0.1 ml of standardized suspensions of the target cells. After 24 h of incubation at 37°C, aerobically with or without 5% CO₂ and 70% humidity or anaerobically under 80% N₂-10% CO₂-10% H₂, inhibitory activity was reported positive when there was no growth of the target strains on and around the spot of growth (Fig. 1).

Determination of the pH effect on *N. gonorrhoeae* growth. The determination of the pH effect on *N. gonorrhoeae* growth was carried out by counting the number of viable cells in standardized suspensions of the 21 gonococcal strains (see below) with chocolate GC plates with pH values ranging from 5.3 to 8.1.

Standardization of inocula. Cells from cultures of *N. gonorrhoeae* target strains grown overnight on GC plates supplemented with 1.5% IsoVitaleX and hemolyzed horse blood (37°C; 5% CO₂-70% H₂O) were collected by flooding the media with 4.5 ml of GC broth. These suspensions were standardized to an optical density of 0.15 to 540 nm (approximately 10⁸ colony-forming units per ml with a Bausch & Lomb Spectrophotometer, Spectronic 21). The aerobic target strains were grown overnight at 37°C on brain heart infusion agar plates and then harvested, and the cell suspensions were standardized as for *N. gonorrhoeae*. In the case of the anaerobic target strains, the cultures were grown overnight at 37°C in prereduced brain heart infusion broth (Carr Scarborough), and the bacterial suspensions were standardized as described above.

pH measurements. The pH values on the surfaces of solid media were measured with a Corning electrode (no. 476216) paired to a pH meter (Corning 125).

RESULTS

The inhibitory activity of the 32 anaerobes tested against 21 strains of *N. gonorrhoeae* is shown in Table 1. It appears that a large variation in the inhibitory activity existed among strains of the same species (e.g., *B. fragilis* [B1A, B15]), as well as among the species of the same genus (*Bacteroides* sp., *Streptococcus* sp., *Eu-bacterium* sp.). The growth of more than 75% of the 21 *N. gonorrhoeae* strains tested, including 2 penicillin-resistant strains, was inhibited by the following six strains: *B. fragilis* (B1A), *B. ovatus* (B24), *P. anaerobius* (Ps11B, Ps11C, Pc9) (see Fig. 1), and *E. limosum* (Ps11A). These six strains were further tested for the specificity of their antagonistic activity with anaerobic and aerobic strains commonly found in human urogenital flora. It can be seen from Table 2 that

TABLE 1. Inhibition of *N. gonorrhoeae* growth by strict anaerobes

Strain designation	Strain tested	No. of strains of <i>N. gonorrhoeae</i> inhibited out of a total of 21 (percentage of total strains inhibited)
Pr0	<i>P. jensenii</i>	7 (33)
Pr20	<i>P. acnes</i>	8 (38)
Pr26	<i>P. acnes</i>	6 (29)
B1A	<i>B. fragilis</i>	18 (86)
B15	<i>B. fragilis</i>	2 (9)
B2	<i>B. disiens</i>	4 (19)
B4	<i>B. vulgatus</i>	1 (5)
B5	<i>B. asaccharolyticus</i>	10 (42)
B24	<i>B. ovatus</i>	16 (76)
B27	<i>B. melaninogenicus</i> subsp. <i>melaninogenicus</i>	9 (43)
B30	<i>B. corrodens</i>	1 (5)
B1	<i>P. asaccharolyticus</i>	0 (0)
Pc6	<i>P. asaccharolyticus</i>	1 (5)
Pc23	<i>P. asaccharolyticus</i>	1 (5)
Pc25	<i>P. asaccharolyticus</i>	0 (0)
Pc8	<i>P. magnus</i>	1 (5)
Pc10	<i>P. magnus</i>	1 (5)
Pc10A	<i>P. magnus</i>	0 (0)
V12	<i>P. magnus</i>	1 (5)
V12A	<i>P. magnus</i>	0 (0)
Pc19	<i>P. magnus</i>	0 (0)
Pc8A	<i>P. prevotii</i>	0 (0)
Pc9	<i>P. anaerobius</i>	20 (95)
Ps11B	<i>P. anaerobius</i>	17 (81)
Ps11C	<i>P. anaerobius</i>	20 (95)
Sc13	<i>S. morbillorum</i>	11 (52)
Sc13A	<i>S. morbillorum</i>	1 (5)
B14	<i>S. intermedium</i>	1 (5)
Pc9	<i>E. limosum</i>	1 (5)
Ps11A	<i>E. limosum</i>	20 (95)
E28	<i>E. lentum</i>	0 (0)
E29	<i>E. aerofaciens</i>	0 (0)

the growth of only a few strains was inhibited by these six anaerobes having large antagonistic spectrum of activity. Anaerobic and aerobic strains *S. mitis* (A-5), *Corynebacterium* sp. (A-10), *E. coli* (A-11), *D. pneumoniae* (A-4), *P. prevotii* (Pc8A), and *P. anaerobius* (Ps11B) were subject to the antagonistic activity of *B. ovatus* (B24) or *B. fragilis* (B1A) or both. *L. acidophilus* (L3) was inhibited by *B. ovatus* (B24) and *P. anaerobius* (Ps11B).

As chloroform-treated media which supported the growth of six inhibitory strains showed the same pattern of inhibition whether or not they were overlaid with GC medium (Table 3), the

FIG. 1. Illustration of the antigenococcal activity detected by the method described in the text. In this case, *P. anaerobius* (Pc9) inhibited the growth of *N. gonorrhoeae* G1.

results strongly suggest that the gonococcal growth inhibition is not due to a mere nutrient depletion. The data in Table 3 show that the inhibition of *N. gonorrhoeae* growth by strict anaerobes is not due to a pH shift as surface pH values range from 6.4 to 7.3. According to previous studies (2, 5), *N. gonorrhoeae* grows well in this pH range. This was further confirmed by the fact that under the growth conditions used in this study (Table 4), all of the 21 gonococcal strains tested (except G6) were able to grow within a pH range of 6.3 to 8.1. Furthermore, a few strains of *N. gonorrhoeae* (G1, G5, G9, G15, G16, G19, and PR 77112) were able to grow at pH 5.8, whereas no growth was detected at pH 5.3.

DISCUSSION

Studies based on the inhibition of in vitro-grown *N. gonorrhoeae* by aerobic and facultatively anaerobic bacteria led to the conclusion that these microorganisms play a role in the resistance to gonorrhea (11). Based on the assay described in this paper, our results clearly show that some strains of anaerobes also inhibit in vitro growth of *N. gonorrhoeae*. This inhibition of the gonococcal growth by anaerobes was not an all-or-none phenomenon as there was variation within genera and species regarding inhibitory activity (Table 1). This observation is in good agreement with those reported for aerobes (10). The inhibitory activity of the six strains selected for their large antigenococcal spectrum of activity, *B. fragilis* (B1A), *B. ovatus* (B24), *P. anaerobius* (Pc9, Ps11B, Ps11C), and *E. li-*

TABLE 2. Specificity of the antigenococcal activity of strict anaerobes^a

Strain designation	Target strain	Inhibition by:
Aerobes		
A-1	<i>S. aureus</i>	None
A-2	<i>S. epidermidis</i>	None
A-3	<i>S. epidermidis</i>	None
A-4	<i>D. pneumoniae</i>	<i>B. ovatus</i> (B24), <i>B. fragilis</i> (B1A)
A-5	<i>S. mitis</i>	<i>B. ovatus</i> (B24)
A-6	<i>S. agalactiae</i>	None
A-7	<i>S. faecalis</i>	None
A-8	<i>S. faecalis</i>	None
A-9	<i>Corynebacterium</i> sp.	None
A-10	<i>Corynebacterium</i> sp.	<i>B. ovatus</i> (B24), <i>B. fragilis</i> (B1A)
A-11	<i>E. coli</i>	<i>B. ovatus</i> (B24)
Anaerobes		
B1	<i>P. asaccharolyticus</i>	None
Pc8A	<i>P. prevotii</i>	<i>B. ovatus</i> (B24)
V12A	<i>P. magnus</i>	None
Ps11B	<i>P. anaerobius</i>	<i>B. ovatus</i> (B24), <i>B. fragilis</i> (B1A)
Sc13A	<i>S. morbillorum</i>	None
Pr26	<i>P. acnes</i>	None
E28	<i>E. lentum</i>	None
B15	<i>B. fragilis</i>	None
L3	<i>L. acidophilus</i>	<i>B. ovatus</i> (B24), <i>P. anaerobius</i> (Ps11B)

^a The following six strict anaerobic bacteria were tested: *B. fragilis* (B1A), *B. ovatus* (B24), *E. limosum* (Ps11A), and *P. anaerobius* (Pc9, Ps11B, Ps11C).

TABLE 3. Effect of pH and nutrient depletion on the antigenococcal activity

Strain tested	Nonoverlaid brain heart infusion agar		Overlaid brain heart infusion agar ^a	
	pH	Inhibitions ^b	pH	Inhibitions ^b
Uninoculated medium	7.2	—	7.3	—
<i>B. fragilis</i> (B1A)	6.6	+	6.6	+
<i>B. ovatus</i> (B24)	6.4	+	6.4	+
<i>P. anaerobius</i> (Pc9)	6.8	+	6.9	+
<i>P. anaerobius</i> (Ps11B)	6.8	+	6.9	+
<i>P. anaerobius</i> (Ps11C)	6.8	+	6.9	+
<i>E. limosum</i> (Ps11A)	6.8	+	6.8	+

^a With 6.5 ml of chocolate GC agar.

^b Tested with *N. gonorrhoeae* strain G1. Symbols: +, Inhibition of growth; —, growth.

mosum (Ps11A), was also specific for *N. gonorrhoeae*. This is clearly shown by the fact that only *B. fragilis* and *B. ovatus* inhibited the growth of a limited number of bacteria other than *N. gonorrhoeae*, whereas for the four remaining strains, the inhibitory activity was

TABLE 4. Effect of pH on *N. gonorrhoeae* growth

<i>N. gonorrhoeae</i> strain tested	Colony-forming units $\times 10^{17}$ ^a at the following pH					
	5.3	5.8	6.3	7.0	7.5	8.1
G1	NG ^b	0.9 $\pm$ 0.1	4.5 $\pm$ 0.4	3.4 $\pm$ 0.4	2.2 $\pm$ 0.5	3.0 $\pm$ 0.3
G2	NG	NG	1.9 $\pm$ 0.3	2.5 $\pm$ 0	2.3 $\pm$ 0	2.6 $\pm$ 0.5
G3	NG	NG	1.7 $\pm$ 0.8	2.2 $\pm$ 0.2	1.9 $\pm$ 0.3	1.9 $\pm$ 0.5
G4	NG	NG	6.0 $\pm$ 0.4	7.0 $\pm$ 0.6	6.5 $\pm$ 1.3	6.0 $\pm$ 0.4
G5	NG	2.0 $\pm$ 0.3	4.6 $\pm$ 0.3	4.8 $\pm$ 0.1	5.1 $\pm$ 0.2	3.8 $\pm$ 0.6
G6	NG	NG	NG	1.0 $\pm$ 0.2	1.5 $\pm$ 0.2	1.3 $\pm$ 0.2
G7	NG	NG	5.7 $\pm$ 0.2	8.8 $\pm$ 0.8	8.6 $\pm$ 1.8	8.3 $\pm$ 0.5
G8	NG	NG	7.9 $\pm$ 0.5	8.2 $\pm$ 0.5	9.8 $\pm$ 0.2	9.2 $\pm$ 0.4
G9	NG	1.1 $\pm$ 1.0	1.2 $\pm$ 0.1	1.4 $\pm$ 0	1.0 $\pm$ 0.1	1.1 $\pm$ 0.2
G10	NG	NG	14.2 $\pm$ 0.2	12.3 $\pm$ 0.2	14.9 $\pm$ 0.4	11.9 $\pm$ 1.2
G11	NG	NG	5.3 $\pm$ 0.1	5.3 $\pm$ 0.4	6.0 $\pm$ 0.7	5.7 $\pm$ 0.2
G12	NG	NG	6.4 $\pm$ 0.5	7.2 $\pm$ 0.4	6.3 $\pm$ 0.2	5.7 $\pm$ 0.7
G13	NG	NG	7.6 $\pm$ 0.7	8.0 $\pm$ 0.1	7.5 $\pm$ 0.6	5.9 $\pm$ 0.6
G14	NG	NG	2.4 $\pm$ 0.5	3.7 $\pm$ 0.4	3.9 $\pm$ 0.2	4.1 $\pm$ 0.2
G15	NG	6.2 $\pm$ 0.9	4.4 $\pm$ 0.3	4.9 $\pm$ 0.2	4.3 $\pm$ 0.6	3.9 $\pm$ 0.4
G16	NG	5.5 $\pm$ 1.0	4.9 $\pm$ 0.2	4.7 $\pm$ 0.7	5.7 $\pm$ 0.1	4.2 $\pm$ 0.4
G17	NG	NG	5.6 $\pm$ 0.4	7.1 $\pm$ 0.7	6.5 $\pm$ 1.1	6.0 $\pm$ 0.6
G18	NG	NG	0.2 $\pm$ 0.3	0.1 $\pm$ 0.1	0.2 $\pm$ 0	0.2 $\pm$ 0.5
G19	NG	4.9 $\pm$ 0.3	6.2 $\pm$ 0.4	7.0 $\pm$ 2.0	8.6 $\pm$ 1.6	4.6 $\pm$ 0.9
PR77112	NG	4.8 $\pm$ 0.3	5.0 $\pm$ 0.1	6.1 $\pm$ 0.6	6.9 $\pm$ 1.0	6.7 $\pm$ 1.3
PR77117	NG	NG	4.1 $\pm$ 0	5.8 $\pm$ 0.7	5.4 $\pm$ 0.4	6.9 $\pm$ 0.8

^a Mean of three values and standard deviation.^b NG, No growth.

mainly restricted to the gonococcus. The fact that members of the genera *Bacteroides*, *Eubacterium*, and *Peptostreptococcus* markedly inhibited the growth of *N. gonorrhoeae* agrees well with the suggestion that predominant members of the vaginal flora could be the organisms most susceptible to showing interfering activity (8). It should, however, be pointed out that this was not true for members of the genus *Peptococcus* as the strains tested have shown no inhibition or very little inhibition of the in vitro-grown gonococcus. Nutrient depletion as a possible mechanism of the observed in vitro inhibitions could be ruled out as, on one hand, the growth inhibition patterns did not differ whether or not the test media were overlaid with fresh GC medium and, on the other hand, the strains belonging to the same species were unequally effective on *N. gonorrhoeae*. In addition, as all the tested gonococcal strains were shown to grow well at pH values around 6.3, the changes in pH could not be the cause of the observed inhibitions. However, it is possible that pH values lower than neutrality could affect the degree of the observed inhibitions.

It must be clear that the observed sensitivity in the artificial milieu of culture media is not necessarily representative of the complex series of interactions that occur in the urogenital tract. In fact, in vivo gonococcal inhibition deals with a quantitative phenomenon related to multiple variables which are not reproducible in vitro.

These variables include the inhibitory capacity of the interfering bacteria, the number of inhibitory bacteria, the number of gonococci, the duration of incubation, and the physiological conditions of the host, such as immunity and the menstrual cycle (11). Unless an animal model could be found in which all of these variables could be reproduced, the in vitro assay, such as that described in this report, will remain the method of choice for the evaluation of the anti-gonococcal activity of viable anaerobes found in the genitourinary tract.

Work is presently being carried out to determine the mechanism by which anaerobes inhibit in vitro growth of *N. gonorrhoeae*, as well as the nature of the inhibitory substances involved. Preliminary results (A. Morin et al., Annu. Meet. Can. Public Health Assoc. Montreal, Canada, abstr. no. VBC-7, 1979) indicate that gonococcal growth inhibition by the above six selected strains of anaerobes is not due to the presence of volatile and nonvolatile fatty acids but to low-molecular-weight inhibitors which can be produced in liquid medium.

ACKNOWLEDGMENTS

We thank L. Wilson for technical assistance.

These studies were made possible by financial support from Institut Armand-Frappier.

LITERATURE CITED

1. Bartlett, J. G., A. B. Onderdonk, E. Drude, C. Goldstein, M. Anderka, S. Alpert, and W. M. Mc-

- Cormack. 1977. Quantitative bacteriology of the vaginal flora. *J. Infect. Dis.* **136**:271-277.
2. Brookes, R., and B. Sikyta. 1967. Influence of pH on the growth characteristics of *Neisseria gonorrhoeae* in continuous culture. *Appl. Microbiol.* **15**:224-227.
3. Dowell, V. R., and T. M. Hawkins. 1974. Laboratory methods in anaerobic bacteriology. Center for Disease Control Laboratory Manual publication no. (CDC) 74-8272. Center for Disease Control, Atlanta, Ga.
4. Hammerschlag, M. R., S. Alpert, A. B. Onderdonk, P. Thurston, E. Drude, W. M. McCormack, and J. G. Bartlett. 1978. Anaerobic microflora of the vagina in children. *Am. J. Obstet. Gynecol.* **131**:853-856.
5. Hebeler, B. H., S. A. Morse, W. Wong, and F. E. Young. 1978. Effect of pH on the chemical and biological properties of the cell envelope of *Neisseria gonorrhoeae*: isolation of a protein-peptidoglycan complex, p. 18-25. In G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), *Immunobiology of Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.
6. Holdeman, L. V., E. P. Cato, and W. E. C. Moore. 1977. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg.
7. Ingraham, L. S., S. J. Kraus, and G. F. Brooks. 1978. Microbial inhibition of *Neisseria gonorrhoeae*, p. 350-355. In G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), *Immunobiology of Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.
8. Kaye, D., and M. E. Levison. 1977. In vitro inhibition of growth of *Neisseria gonorrhoeae* by genital microorganisms. *Sex. Transm. Dis.* **4**:1-3.
9. Kraus, S. J. 1975. Gonococcal immunity mediated by bacterial interference, p. 89-91. In *International symposium on gonorrhea*. Information Canada, Ottawa, Canada.
10. Kraus, S. J., R. C. Geller, G. H. Perkins, and D. L. Rhoden. 1976. Interference of *Neisseria gonorrhoeae* growth by other bacterial species. *J. Clin. Microbiol.* **4**: 288-295.
11. Saigh, J. H., C. C. Sanders, and W. E. Sanders, Jr. 1978. Inhibition of *Neisseria gonorrhoeae* by aerobic and facultatively anaerobic components of the endocervical flora: evidence for a protective effect against infection. *Infect. Immun.* **19**:704-710.