

Reevaluation of bacteriocinogeny in *Neisseria gonorrhoeae*¹

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SYNOPSIS Bacteriocin typing has been described previously and proposed for typing gonococci. A survey has been made of 150 strains of *N. gonorrhoeae* from various places to determine the feasibility of a gonocin typing system. All strains were found to produce an inhibitory substance which inhibited all strains of gonococci tested, one strain of *Neisseria flavescens*, two strains of *Neisseria meningitidis*, as well as the producing strain itself. The inhibitory activity was enhanced by supplementary glucose, reduced by supplementary serum, and unaffected by the addition of HEPES buffer, by the temperature of incubation, or by the exposure of potential producer strains to sublethal concentrations of mitomycin C. This nonspecific inhibitory activity differed from that of a putative bacteriocin produced by a strain of *N. meningitidis*, in that the latter inhibited most other meningococci but not the producer strain itself. Bacteriocinogeny has not yet been convincingly demonstrated in *N. gonorrhoeae*, and gonocin typing has not yet been shown to be feasible. Production of the nonspecific inhibitor may have obscured past attempts to demonstrate type-specific gonococcal bacteriocin.

Bacteriocins are a unique class of antibiotics which differ from other antibiotics in that bacteriocins elaborated by one species are usually active only against a proportion of strains of the same or closely related species (Hamon and Peron, 1960; Reeves, 1965). This limited spectrum is particularly characteristic of bacteriocins of Gram-negative bacteria. With few possible exceptions (Goodwin, Levin, and Doggett, 1972), bacteriocins are not active against the bacterial strains which elaborate them.

Bacteriocin typing systems have been used widely in epidemiological studies of many bacterial species. Bacteriocinogeny was first described in *N. meningitidis* in 1966 (Kingsbury, 1966), and variation in susceptibility to meningococcal bacteriocins has been used as a tool to identify virulent strains of *N. meningitidis* (Counts, Seeley, and Beaty, 1971). Flynn and McEntegart (1971) detected bacteriocin-like activity in 75% of gonococci examined and found 13

patterns of inhibition of six indicator strains. They suggested that these patterns were sufficiently distinctive to permit a typing system, based upon bacteriocin production, to be applied in epidemiological studies of gonococcal infections. This report describes a search for gonocin production, and the detection of a nonspecific inhibitor produced by *N. gonorrhoeae*.

Materials and Methods

SELECTION OF ISOLATES TESTED

Strains of *N. gonorrhoeae* tested for bacteriocin production included 88 fresh isolates recovered from patients in Seattle (two had gonococcal arthritis and one had salpingitis), four isolates from the Philippines, 17 provided by Dr James Plorde from Ethiopia, 19 provided by Dr James Gale from Taiwan, 21 provided by Dr Alice Reyn from Denmark, and one previously reported to produce bacteriocin (gonocin) and provided by Drs W. D. Lawton and H. A. Gaafar (Lawton and Gaafar, 1973). These isolates were tested in groups of 19 together with a strain of *N. meningitidis* serogroup B, strain 51, which produces a highly active inhibitor of meningococci and gonococci, and was provided by Dr Harry Beaty for use as a control in each experiment. Each set of strains was tested for production of bacterio-

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cin-like activity against every other strain in that set, as well as against a group of five indicator strains which had previously been found by Flynn and McEnteggart to be susceptible to gonocin and were kindly provided by Dr Flynn. Other *Neisseria* species tested as indicators for susceptibility to gonocin were provided by Dr B Wesley Catlin.

METHOD OF TESTING FOR BACTERIOCIN PRODUCTION

Each isolate was grown on chocolate blood agar or on Difco GC base medium containing 1% defined supplement (GCBDS; White and Kellogg, 1965) at 35°C in air plus 5% CO₂. All strains were maintained at -70°C in a 50:50 solution of gamma globulin-free horse serum (BBL) and tryptic soy broth (Difco).

Preliminary tests for gonocin were made on GCBDS medium and on chocolate blood agar, using the method of Flynn and McEnteggart, (1971) and employing the five indicator strains provided by these authors. As described by Flynn and McEnteggart, inhibitory activity on chocolate blood agar was weaker and less consistent than on the GCBDS medium, and the use of chocolate blood agar was discontinued. Inhibitory activity on GCBDS medium was rarely observed after 24 hours' incubation, was only sometimes observed after 48 hours' incubation, and the results for the same strains tested under the same conditions were not always reproducible. On occasion, however, complete removal of the growth from the agar surface after 48 hours was difficult, and it was discovered that all indicator strains were consistently inhibited around any remnants of growth, with an inhibition zone of 1 to 3 mm width. Remnants of growth of any given strain also gave a similar zone of inhibition even when that same strain was employed as an indicator. Subsequently, therefore, tests for inhibitory activity of *N. gonorrhoeae* strains were made after 24-hr and 48-hr incubation periods, both when growth was and was not removed before inoculation with the indicator strain.

All tests were made on GCBDS medium dispensed in 50-ml volumes in 150-mm petri plates. Inocula were prepared from overnight growth on solid medium, which was suspended in Difco nutrient broth to an optical density of 0.2 at 540 nm in a Bausch and Lomb spectronic 20 colorimeter. A Steers replicator was employed to inoculate 0.0025 ml of each culture suspension containing approximately 1×10^8 colony-forming units per ml onto four sets of identical plates which were incubated for 24 or 48 hours at 35°C in air plus 5% CO₂.

All growth was removed from the agar surface with a glass slide, followed by a cotton swab from

two sets of plates at 24 and 48 hours, respectively. The plates were then exposed to chloroform vapour for 15 min, followed by exposure to air for 30 minutes. The remaining two sets of plates were similarly exposed to chloroform at 24 and 48 hours without removal of the growth.

Indicator strains were prepared in the same manner as the initial inocula, but were adjusted to an optical density of 0.1 at 540 nm. After exposure to chloroform and air the plates were flooded with 5.0 ml of the indicator suspension, and the excess was decanted before incubation at 35°C in air plus 5% CO₂. Plates were examined at 24 hours for inhibition of the indicator strains.

EFFECT OF GLUCOSE, SERUM, HEPES BUFFER, AND TEMPERATURE OF INCUBATION ON INHIBITORY ACTIVITY

In separate experiments, the effect of glucose, serum, and HEPES buffer on inhibitory activity, observed on GCBDS medium, was evaluated by the omission of supplementary glucose from the GCBDS medium; by the addition of 10% horse serum (BBL) heated at 56°C for 30 min; and by the addition to GCBDS medium of HEPES buffer to a final concentration of 0.03 M, followed by adjustment of the pH to 7.4. Observations were made after 24 and 48 hours without removal of the basal growth, as described above. The effect of temperature of incubation was also determined by comparing the level of activity obtained incubating both the inhibitory and indicator strains on GCBDS medium at 28°C with that obtained by incubating at 35°C.

DIFFUSIBILITY OF INHIBITOR

Because preliminary experiments showed that inhibitory activity was variably removed with removal of surface growth of the producing strain of *N. gonorrhoeae* the above method was modified by an additional step to determine whether the inhibitory activity was cell-associated or diffusible. Following exposure to chloroform of strains being tested for bacteriocinogeny, a 50% dilution of GCBDS medium was cooled to 50°C, then overlaid to a thickness of approximately 1 mm over the producer colonies. When the overlay had hardened, the plates were then flooded with 5.0 ml of the indicator strain suspension, as described above.

TREATMENT WITH MITOMYCIN C

The minimal inhibitory concentrations (MICs) of mitomycin C were determined for eight strains of *N. gonorrhoeae*. Colonies of *N. gonorrhoeae* scraped from the surface of GCBDS medium after 24 hours' incubation were suspended in nutrient broth to an optical density of 0.15 at 540 nm, and then applied

with a Steers replicator to GCBDS medium containing serial dilutions of mitomycin C. The MIC was defined as the concentration which completely inhibited growth after incubation for 24 hours at 35°C in air plus CO₂, and ranged from 0.04 to 0.1 µg per ml for the strains examined. Colonies which grew on 0.02 µg mitomycin C per ml were tested for bacteriocin production, as described above. In addition, these eight strains were tested for bacteriocin production directly on GCBDS medium containing 0.02 µg mitomycin C per ml.

ULTRAVIOLET IRRADIATION

Strains of *Neisseria gonorrhoeae* were suspended in buffered salt solution (Clowes and Hayes, 1968) to an optical density of 0.15 at 540 nm. Seven millilitre aliquots of each strain were placed in a 90 mm diameter petri plate and were exposed to uv irradiation at 1200 ergs per cm² per second. A survival curve was constructed for each of the strains and the doses required to give a 10% survival rate were calculated for each strain. Seven millilitre aliquots of each strain were then exposed to these latter doses and then promptly used to inoculate GCBDS medium and tested for bacteriocin production, as described above.

Results

One hundred and fifty strains of *N. gonorrhoeae* were tested for their ability to produce a bacteriocin. The results are summarized in table I. When growth was removed from the agar surface, antigonococcal activity was shown by six (4.0%) of 150 strains after 24 hours, and by 33 (22.0%) of 150 strains after 48 hours' incubation. Inhibition zones were small, being limited to the area immediately above where the basal strain had grown and no strain produced inhibition of all indicator strains. By contrast, when the growth was not removed from the agar, antigonococcal activity against all indicators was shown by 147/150 strains after 24 hours and by 150/150 strains after 48 hours. When growth was not removed at 48 hours, every strain produced an inhibitor active against itself. The inhibition zones

ranged from 1 to 3 mm, and were often irregular in outline. The size of the inhibitory zones was observed to be correlated with the amount of growth. No colonies resistant to the inhibitory substance were ever observed within the zones of growth inhibition.

The effect of the deletion of glucose, the addition of horse serum, or the addition of Hepes buffer was evaluated for 10 producer strains tested against nine indicator strains. All strains tested inhibited every indicator strain after 24 hours' and 48 hours' incubation on complete GCBDS medium. Growth of *N. gonorrhoeae* on GCBDS medium was moderately reduced when glucose was omitted and was slightly enhanced by the addition of horse serum. The inhibitory activity of the 10 producer strains against all nine indicator strains was markedly decreased on GCBDS medium without added glucose, and on GCBDS medium plus 10% inactivated horse serum. The addition of Hepes buffer to the GCBDS medium had no effect on the inhibitory activity observed in this experiment.

In contrast, as shown in the figure, *N. meningitidis* strain 51 produced a wide symmetrical zone of inhibition against all strains of gonococci employed as indicators. The inhibitory zone was always present after 24 hours' incubation and was not affected by the removal of the surface growth before inoculation with the indicator strains. While *N. meningitidis* strain 51 was itself resistant to this

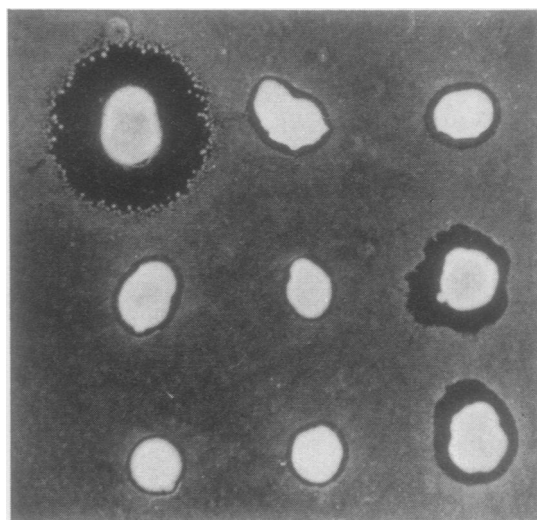


Fig Inhibition of a lawn of a gonococcal indicator strain produced by *N. meningitidis* strain 51 (upper left corner) and by eight strains of *N. gonorrhoeae*. The indicator strain employed in the lawn is also included as one of the eight producer strains (lower right corner).

	Growth Removed		Growth Not Removed	
	24 Hr	48 Hr	24 Hr	48 Hr
Inhibition of any indicator	6	33	148	150
Inhibition of all indicators	0	0	147	150

Table I Inhibition of indicators by 150 strains of *N. gonorrhoeae* in relation to duration of incubation and removal of growth of the inhibiting strains

widely diffusible inhibitor, it did exhibit a small irregular zone of inhibition when employed as an indicator inoculated on 48 colonies of the homologous strain 51. This self-inhibition resembled the nonspecific zones of inhibition produced by all gonococci against all gonococci.

Ten strains of gonococci were tested for inhibition of 10 indicator strains inoculated on the surface of a 1 mm thick layer of GCBDS agar covering 48-hour-old producer colonies. All 10 strains produced an even circular zone of complete inhibition of all indicators in the area immediately above each producer colony, indicating diffusibility of the inhibitor. All 10 strains exhibited self-inhibition by this method.

Tests for bacteriocin production were made using the same methods but incubating the plates at 28°C during growth of both the producer and indicator strains. For those strains which grew at this temperature (nine of 10 tested), the inhibitory effect was the same as that observed at 35°C.

The activity of 10 strains of *N. gonorrhoeae* was tested against two strains of *N. meningitidis* and six nonpathogenic species of *Neisseria*. As shown in table II, both strains of *N. meningitidis* were inhibited by all 10 gonococci, and *N. flavescens* ATCC 13120 was inhibited by nine of 10 strains, when the growth of gonococci was not removed from the agar surface. All zones of inhibition were small and irregular. None of the remaining nonpathogenic strains were inhibited by *N. gonorrhoeae*.

	Growth Removed		Growth Not Removed	
	24 Hr	48 Hr	24 Hr	48 Hr
<i>N. meningitidis</i> 51 (group B)	0/10	0/10	10/10	10/10
<i>N. meningitidis</i> HCS4 (group B)	0/10	0/10	10/10	10/10
<i>N. catarrhalis</i> Ne 11	0/10	0/10	0/10	0/10
<i>N. flava</i> WRI 2105	0/10	0/10	0/10	0/10
<i>N. flavescens</i> ATCC 13120	0/10	0/10	0/10	9/10
<i>N. perflava</i> Ne 16	0/10	0/10	0/10	0/10
<i>N. mucosa</i> CIP 5951	0/10	0/10	0/10	0/10
<i>N. sicca</i> Ne 12	0/10	0/10	0/10	0/10

Table II Inhibition of two strains of *N. meningitidis* and six non-pathogenic *Neisseria* species by 10 strains of *N. gonorrhoeae*

EFFECT OF MITOMYCIN C AND ULTRAVIOLET IRRADIATION ON PRODUCTION OF INHIBITION
Exposure of eight strains of *N. gonorrhoeae* to

mitomycin C and uv irradiation did not increase the size of the zones of nonspecific inhibition produced by these strains against all other gonococci tested as indicators, or affect production of a qualitatively different inhibitor resembling the putative bacteriocin of *N. meningitidis*.

Discussion

The inhibitory substance produced by all strains of *N. gonorrhoeae* tested in this study differed from most previously described bacteriocins, and from the bacteriocin-like inhibitor produced by *N. meningitidis* strain 51, in that the former was active against all other strains of gonococci tested and inhibited the strains which produced it. Treatment with mitomycin C and uv irradiation under conditions employed in this study failed to induce diffusible bacteriocins with type specificity. Although shown to be diffusible, the inhibitor produced by *N. gonorrhoeae* was largely restricted to the surface of the GCBDS medium. The production or expression of this inhibitory substance was independent of incubation temperature, was reduced by the omission of supplementary glucose from the growth medium, and was inhibited by serum. The nature of the inhibiting substance has not been defined. It seems likely that the variable removal of this inhibitor during removal of surface growth previously hindered the detection of production of the inhibitor by all gonococci (Flynn and McEnteggart, 1971).

There may be quantitative differences in production of this nonspecific inhibitor among various strains of *N. gonorrhoeae*. However, under the conditions employed in this study, it is unlikely that such differences will provide the basis for a useful means of differentiating strains of gonococci, since no qualitative difference in production of, or susceptibility to, the inhibitor has been observed among gonococci. It seems likely that this inhibitory substance is not a bacteriocin, but that it is a metabolite produced by all gonococci on GCBDS medium.

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Addendum

Walstad, Reitz, and Sparling (*Infect Immun*, **10**, 481-488, 1974) have also recently observed growth inhibition among strains of *N. gonorrhoeae* due to longchain free fatty acids and monoacylphosphatidylethanolamine, and have been unable to detect bacteriocin production by gonococci.