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Siderophore Production by Pathogenic *Neisseria* spp.

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Previous studies have established the importance of iron acquisition to the growth and virulence of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Although preliminary evidence that the *Neisseria* spp. produce siderophores has been presented, the exact mechanism of iron acquisition has remained obscure. Siderophore production by *N. gonorrhoeae* and *N. meningitidis* was induced in two different low-iron media. The iron-reactive siderophores, "gonobactin" and "meningobactin," were partially purified by ion exchange chromatography followed by extraction with phenol-chloroform-ether or by gel filtration. The compounds were of low molecular weight, their synthesis was repressed by iron in the medium, and they appeared to be hydroxamic acids since they were stimulatory for *Arthrobacter flavescens* JG-9 (a hydroxamate auxotroph) and gave a positive Csáky reaction for bound hydroxylamine. In the iron form, the compounds had an absorption maximum of approximately 420 nm. Although meningobactin stimulated growth of the gonococcus in low-iron media and vice versa, the homologous activity was more marked, indicating that the compounds, though similar, were probably not identical. As determined by *A. flavescens* assay the meningococcus produced three to five times more siderophore than did the gonococcus; however, the amount of siderophore present in the culture fluids of even the meningococcus was 100- to 1,000-fold lower than the concentration of hydroxamate siderophores reported to be produced by *Bacillus megaterium* or *Aerobacter aerogenes*. Virulent, colony type 1 *N. gonorrhoeae* produced significantly more gonobactin than did the avirulent colony type 3 gonococci.

Iron is required for the growth of nearly all microbes (12, 15); *Neisseria gonorrhoeae* and *Neisseria meningitidis* are not excluded. However, to acquire their essential iron the pathogenic *Neisseria* spp. must compete, as do other pathogens, with the host's iron-binding and storage proteins such as transferrin in serum, lactoferrin in secretions, and ferritin in tissues. Certain microorganisms have been shown to obtain iron by production of powerful, low-molecular-weight, iron-binding compounds, siderophores (12), which enable the parasite to compete successfully with the host's iron-sequestering mechanisms (27).

The siderophores which have been characterized chemically are of two classes; hydroxamate and phenolate (15). The fungi, actinomycetes, and several bacteria produce siderophores of the hydroxamate class, whereas only the true bacteria, such as *Escherichia coli* (17), *Salmonella typhimurium* (24), and *Vibrio cholerae* (21), produce phenolate-type siderophores.

Although it has been demonstrated previously that the ability of gonococci and meningococci to acquire iron is important both in vitro (3, 16, 20, 28) and in vivo (19, 22, 23), no siderophore has been clearly demonstrated for either organism. Payne and Finkelstein (22), however, presented preliminary evidence for siderophore production by the two organisms. In contrast to their observations, others (3, 16) have been unable to detect siderophore production by either of the pathogenic *Neisseria* spp.

The present paper describes the partial purification and some of the properties of the siderophores produced by the gonococcus and the meningococcus. The compounds appear to be hydroxamic acid in nature, and although they are similar to each other, they are not identical. For the sake of brevity, the siderophore(s) of the meningococcus and the gonococcus are referred to as "meningobactin" and "gonobactin," respectively. This does not imply, however, that these compounds are completely novel—although this may be the case—nor does it imply anything about the structure of these compounds.

MATERIALS AND METHODS

Bacterial strains. Neisseria strains were as previously described (22, 28). *Bacillus megaterium* ATCC 19213 and *Arthrobacter flavescens* JG-9 were obtained from C. E. Lankford, University of Texas, Aus-

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tin (6). *E. coli* 711 (F1LT), a transformed K-12 derivative bearing an LT gene(s) of the *Ent* plasmid from porcine strain P307, was provided by S. Falkow (7). *V. cholerae* 569B, a hypertoxinogenic strain from our culture collection, produces a phenolate siderophore (21). *N. gonorrhoeae* strains 6555 and 2686 and *N. meningitidis* serogroup strains A-17 and B-11 were as previously described (22, 28). Strains were maintained by lyophilization and by storage at −70°C in buffered peptone-saline broth (GC medium base formulation as per Difco Laboratories, without agar and starch) containing 20% glycerol. All *Neisseria* spp. cultures were incubated at 36°C with 5% CO_2. *A. flavescens* JG-9 was incubated at 30°C, and all other cultures were incubated at 37°C.

Media and bioassays. Imferon agar (20) was used routinely for the growth of all pathogenic *Neisseria* spp. *A. flavescens* JG-9 was cultured on hydroxamate assay medium (described below) supplemented with 5 μg of Desferal per ml of medium. All other strains were cultured on meat extract agar (18).

Conalbumin agar (CA) medium was as described previously (28). Briefly, this was composed of GC medium base with 2% defined supplement (as described by C. E. Lankford, Bacteriol. Proc., p. 40, 1950; omitting the ferric nitrate), and 500 μg of "iron-free" conalbumin (type 1; Sigma Chemical Co., St. Louis, Mo.) per ml of medium. When this medium was used for siderophore production by the pathogenic *Neisseria* spp., the CA medium was overlaid with broth equal to two times the volume of the agar medium. The broth was the same formulation as the CA agar medium, except that the agar and starch were omitted, and it contained 500 μg of iron-free conalbumin per ml to effect iron limitation.

In some cases for isolation of siderophore(s) produced by the meningococcus, the *Neisseria* spp. defined medium of Archibald and DeVoe (1) was used. As determined by the ferrozine method of Stookey (25), the medium contained less than 0.18 μM residual iron, and *N. meningitidis* would respond in a dose-related fashion to iron addition. For maximum siderophore production the medium was supplemented with 0.54 μM iron as $FeCl_3$.

CA medium also was used to assay siderophores (28). The molten medium was seeded with assay organisms (*N. gonorrhoeae* 6555 or *N. meningitidis* B-11) at 5×10^4 to 10×10^4 colony-forming units per ml. After the seeded CA plates had solidified, 4.5-mm wells were cut in the plates, and 50 μl of test solution, diluted in buffered peptone-saline broth where required, was added to the wells. The zones of stimulation or inhibition were measured at 20 and 48 h of incubation (28). For purposes of quantitation 1 U of activity in the CA bioassay was arbitrarily defined as the reciprocal of the dilution required to produce a 10-mm zone of stimulation for the homologous species of assay organism; 1 U of activity also was equivalent to approximately 1.2 and 0.9 μg of schizokinen (SK) for the gonococcus and meningococcus, respectively. As shown previously, SK was one of the few exogenous siderophores that could stimulate growth of the pathogenic *Neisseria* spp. in the presence of conalbumin (28).

For bioassay of hydroxamic acid siderophores, the basal medium, (hydroxamate assay medium, Table 1) was seeded with 10^6 to 10^7 colony-forming units of *A. flavescens* JG-9 per ml of medium, and the mixture was poured in 25-ml samples into 100- by 15-mm plates. The inoculum for this was a 72-h culture grown on hydroxamate assay medium supplemented with 5 μg of Desferal per ml of medium. After the plates were allowed to solidify, 4.5-mm wells were cut in the agar, and the compounds to be tested were added in 50-μl samples to the wells. The assay plates were then incubated at 30°C for 96 h, at which time the zones of stimulation were measured. Additionally, strain JG-9 was seeded into molten CA medium at this inoculum level; after the plates solidified, suspected hydroxamate-producing test strains were plated onto the surface of the agar. A halo of yellow *A. flavescens* colonies surrounding the colonies of the test strains was considered to be a positive response.

Chemical assays. Bound hydroxylamine was determined by the method of Csáky (8) with unhydrolyzed and acid-hydrolyzed samples. Hydroxylamine hydrochloride (Sigma) was used as a standard, and the absorbance was measured at 526 nm.

Siderophores. Desferal, the methane sulfonate of iron-free ferrioxamine B, was purchased from Ciba Pharmaceutical Co., Summit, N.J. Schizokinen and arthrobactin were a gift from C. E. Lankford, University of Texas, Austin (6).

RESULTS

Growth stimulation of *A. flavescens* JG-9. *A. flavescens* JG-9 has an absolute requirement for hydroxamic acids or hemin and has been used for a number of years to assay micro-

TABLE 1. *Hydroxamate assay medium*[a]

Solution[b]	Compound	Amount (g)	Other
A	Sucrose	10.0	100 ml of deionized water
B	K_2HPO_4	2.0	900 ml of deionized water
	KH_2PO_4	2.0	
	Yeast extract	1.0	
	Casamino Acids	1.0	
	$MgSO_4 \cdot 7H_2O$	0.1	
	Noble agar	10.0	
C	$FeSO_4 \cdot 7H_2O$	0.10	100 ml of 0.001 N HCl
	$MnCl_4 \cdot 4H_2O$	0.07	
	$ZnSO_4 \cdot 7H_2O$	0.02	
	$CuSO_4 \cdot 7H_2O$	0.01	

[a] Modified from Burnham and Neilands (5) and Byers et al. (6).

[b] Solutions A and B were sterilized by autoclaving. Solution C was filter sterilized. After cooling components A and B to 45°C, 10 ml of solution C was added. For bioassay the medium was seeded with 0.1 ml of a solution containing approximately 10^9 colony-forming units of *A. flavescens* JG-9 per ml and poured into 100- by 15-mm plates.

organisms for the presence of hydroxamate-type siderophores (5, 6). CA medium was seeded with strain JG-9, and then test organisms were diluted and plated for discrete colony formation on this iron-deficient medium. Halos of *A. flavescens* formed around the colonies of *N. gonorrhoeae*, *N. meningitidis*, and *B. megaterium* 19213, whereas none formed around *E. coli* 711 (F1LT) or *V. cholerae* 569B (Table 2). *E. coli* (17) and *V. cholerae* (21) have been shown to produce phenolate-type siderophores which were not stimulatory for JG-9. *B. megaterium* 19213 produced the dihydroxamate SK (6). Halo formation around the colonies of *N. gonorrhoeae* and *N. meningitidis* was a good indication that these organisms also produced a hydroxamate-class siderophore.

Further evidence of hydroxamate production was obtained with *A. flavescens* JG-9 growing on hydroxamate assay medium plates. As seen in Fig. 1 the assay is quite sensitive with an endpoint of stimulation by Desferal at approximately 500 pg/well (0.8 pmol/well). However, the dihydroxamate siderophores, SK and arthrobactin, were approximately 100-fold less effective than Desferal, with endpoints of activity at 50 ng/well (100 pmol/well). Additionally, the halo pattern was less dense for SK and arthrobactin. Byers et al. (6) also found that SK was less active than Desferal for *A. flavescens* JG-9. Hemin was much less stimulatory than the siderophores in this assay: more than 1 μg of hemin per well was required. By this assay it was again determined that both *N. meningitidis* and *N. gonorrhoeae* produce JG-9-stimulatory material, presumably a hydroxamic acid siderophore.

Cultures of *N. meningitidis* B-11 and *N. gonorrhoeae* 6555 on CA medium overlaid with broth containing conalbumin were harvested at 36 h. After the cells were removed by centrifugation and filtration through 0.45-μm pore size membrane filters (Millipore Corp., Bedford, Mass.), the conalbumin was removed by ultrafiltration over a PM-30 membrane, and the dialysates were concentrated 30-fold by freeze-drying and suspending the residue in the appropriate amount of deionized water. In three separate experiments, *N. meningitidis* B-11 and *N. gonorrhoeae* 6555 produced an average of 4.6 nM and 1.7 nM Desferal equivalents, respectively. Since the pattern of stimulation by these culture supernatant fluids was more similar to that of SK than that of Desferal (i.e., the halo was less dense than with Desferal) and since of the numerous microbial siderophores tested only dihydroxamic acid siderophores (such as SK) were stimulatory in the CA assay for the pathogenic *Neisseriae* spp. (9), it was deemed most appropriate to express the supernatant stimulatory activity in terms of SK equivalents. By this criterion, the meningococcus and gonococcus produced approximately 0.58 μM and 0.21 μM SK equivalents, respectively, a quantity of SK 100- to 1,000-fold lower than the SK concentrations produced in late-log cultures of *B. megaterium* (6). Also, it should be noted that in all cases and with all strains of *N. meningitidis* and *N. gonorrhoeae* tested, meningococci produced three to five times more JG-9-stimulatory material than did gonococcus strains.

TABLE 2. *Survey of various bacteria for siderophore production: growth stimulation of A. flavescens JG-9*

Organism	Stimulation (halo formation) at 96 h[a]
N. gonorrhoeae 6555	++
N. gonorrhoeae 2686	+
N. meningitidis B-11	++
N. meningitidis A-17	+++
B. megaterium 19213	+++
E. coli 711 (F1LT)	−
V. cholerae 569B	−

[a] +++, ++, +, and − indicate strong, medium, weak, and zero response, respectively.

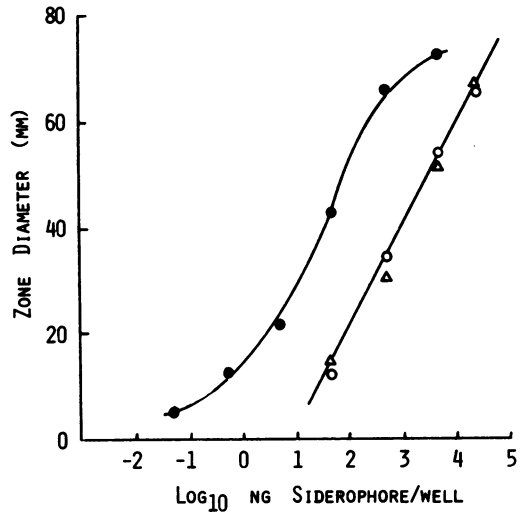

FIG. 1. *Response of A. flavescens JG-9 to various concentrations of Desferal (●), SK (○), and arthrobactin (△).*

Hydroxylamine assay of culture supernatant fluids. With hydroxylamine hydrochloride as a standard, the Csáky assay (8) was used to determine the quality of hydroxamate in the above-mentioned culture supernatant fluids of *N. gonorrhoeae* and *N. meningitidis*. In addition, a 50-fold concentrate of a 48-h culture filtrate of *N. meningitidis* B-11 from *Neisseria* spp. defined medium was assayed (Table 3). While the assay has been reported as not strictly

TABLE 3. *Assay for bound hydroxylamine in the supernatant fluids of Neisseria spp.*

Organism	Culture condition	Concn of hydroxylamine nitrogen[a] (μM)	
		Concentrate	Original (calculated)
N. gonorrhoeae 6555	Biphasic culture, 36 h, 20-fold concn	21.5	1.1
N. meningiditis B-11	Biphasic culture, 36 h, 20-fold concn	30.0	1.5
N. meningiditis B-11	*Neisseria* spp. defined medium culture, 48 h, 50-fold concn	68.0	1.4

[a] Determined by the method of Csaky (8) as modified by Gibson and Magrath (10). Average determination on two different culture supernatant fluids.

quantitative (6, 10), relative quantities can be compared. Both species of *Neisseria* gave a positive reaction for bound hydroxylamine, indicative of production of hydroxamate siderophores by these microorganisms. Again, the quantity of material produced by the meningococcus was greater than that produced by the gonococcus, although the amount produced by both organisms was considerably lower than that produced by other microorganisms (6, 10, 26), and it could not be detected in unconcentrated culture supernatant fluids.

Siderophore production by the pathogenic *Neisseria* spp. As described previously, the effectiveness of various iron compounds and siderophores for the *Neisseria* spp. could be assayed with the CA bioassay (28). In this assay, conalbumin was incorporated into the medium at a concentration adequate to chelate all of the iron present, providing a theoretical iron saturation of the conalbumin of 96.5%. This saturation level of conalbumin caused growth inhibition of 20 h which was overcome by the addition of various iron compounds or siderophores. The sizes of the zones were proportional to the amount of iron added to the assay wells. The assay was also used to determine conditions for optimum production of siderophore by the meningococcus and the gonococcus.

As is typical of siderophore production by other bacteria (12, 15), production of the siderophore(s) of the *Neisseria* spp. was influenced by the iron content of the medium (Fig. 2). Synthesis of gonobactin by a variety of strains of *N. gonorrhoeae* (and of meningobactin by the meningococcus, not shown) was enhanced when the content of available iron in the medium was decreased by adding the iron-binding protein conalbumin to the supernatant fluids. In this experiment CA medium was overlaid with broth with or without 500 μg of iron-free conalbumin per ml of medium. After 48 h of incubation, the supernatant fluids were harvested, the conalbumin was removed by ultrafiltration, and the supernatants were concentrated by lyophilization. The rehydrated concentrates were filter sterilized and assayed by the CA bioassay. With all of the strains the presence of conalbumin in the medium (i.e., when the iron content of the medium was reduced) enhanced gonobactin production, often more than twofold.

Additionally, virulent Kellogg et al. (11) colony type 1 (T1) *N. gonorrhoeae* colonies produced significantly more gonobactin than did their isogenic, avirulent T3 counterparts (Fig. 3). Further, the two strains isolated from disseminated gonococcal infections, strains 1975 and 6555, produced slightly more siderophore than did the urogenital isolates.

Since little or no meningobactin or gonobactin could be detected before the cultures were 36 h old, a 36- to 48-h harvest time was established.

Isolation of gonobactin and meningobactin. The CA bioassay also provided the basis for the siderophore purification sequence, although the *A. flavescens* and Csáky assays were also used. For isolation of meningobactin from *N.*

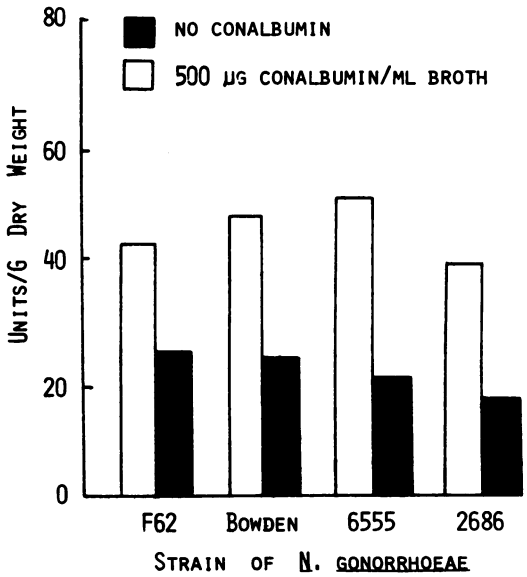

FIG. 2. *Influence of medium iron concentration on siderophore production by N. gonorrhoeae. CA medium was overlaid with broth with or without 500 μg of iron-free conalbumin per ml of medium, and the 48-h culture filtrates were analyzed as described in the text. One unit was defined as the reciprocal of the dilution required to produce a 10-mm zone of stimulation for the homologous species in the CA bioassay.*

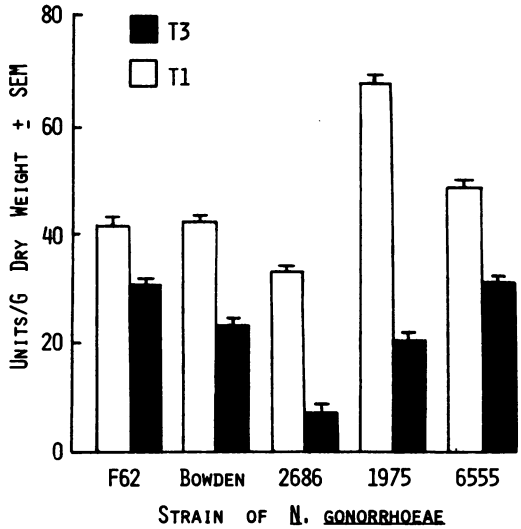

FIG. 3. *Effect of colony type on gonobactin production. T1 or T3 colony types (11) of each strain were inoculated into biphasic culture medium (with conalbumin in both the broth and solid phase) and harvested by centrifugation after 48 h of incubation. The conalbumin was removed by ultrafiltration, the culture supernatants were concentrated by freeze-drying, and the rehydrated supernatants were analyzed in the CA bioassay. Each bar represents the mean of the determination for four cultures.*

meningitidis strains B-11 and A-17, in some cases the low-iron *Neisseria* spp. defined medium was used. Four liters of the medium (1 liter per 2,800-ml Fernbach flask) were inoculated with approximately 10^7 colony-forming units per ml. The cultures in *Neisseria* spp. defined medium were incubated on a gyratory shaker (200 rpm) for 48 h, at which time the cells were removed by centrifugation followed by filtration. The culture supernatant fluids were then passed over a Bio-Rad AG2-X10 (chloride form) anion exchange column (2.5 by 40 cm).

For *N. gonorrhoeae* strains 6555 and 2686 and most often for *N. meningitidis* strains, supernatants of iron-limited (conalbumin-containing) biphasic cultures were used. Three to four liters of conalbumin-supplemented broth were inoculated with 10^7 colony-forming units of bacteria per ml, and the broth was distributed onto 1-liter tissue culture flasks into which CA medium had been previously poured and allowed to solidify on the culture side of the flask. After 48 h of incubation, the cultures were harvested, the cells were removed, and the conalbumin was removed by ultrafiltration. The ultrafiltrates were then passed over a Bio-Rad AG2-X10 column. The content of siderophore in the biphasic filtrates before and after passage through the column was monitored by CA bioassay, and essentially all of the stimulatory material was removed by the column passage. Although *Neisseria* spp. defined medium could be used for siderophore production by *N. meningitidis*, it should be noted that the uninoculated medium itself was strongly inhibitory for the *Neisseria* spp. in the CA bioassay and for *A. flavescens* JG-9 in the hydroxamate medium assay. The nature of the inhibitory factor was not determined, but siderophore could only be detected after concentration on and elution from the anion exchange resin. This inhibitory factor could be responsible for the inability of previous workers (3) to detect siderophore activity.

Siderophore was eluted from the columns with a gradient of 0 to 1 M NH_4Cl (Fig. 4), and the eluate was assayed. Both meningobactin and gonobactin (not shown) eluted from the column between 0.15 and 0.50 M NH_4Cl. Material stimulatory for *A. flavescens* JG-9 also eluted in this position on the NH_4Cl gradient. Another region of *A. flavescens*-stimulatory material eluted at a higher molarity of NH_4Cl, but since this material was not active for the *Neisseria* spp. in the CA bioassay, no further characterization was attempted. The CA assay active fractions were pooled and concentrated 10-fold by flash evaporation at 60°C.

The concentrates were then either desalted by Bio-Gel P-2 chromatography or extracted by phenol-chloroform followed by ether-water. In either case the desalted material was concentrated to approximately 5 ml and tested for CA bioassay activity, *A. flavescens* activity, iron reactivity, and bound hydroxylamine. In all cases the material was positive.

Upon gel filtration on Bio-Gel P-2 the active material was found to elute after the exclusion volume, but before the salt fraction (Fig. 5). It should also be noted, as has been reported previously (22), that an inhibitory factor which comigrates with the siderophore peak was present in these fractions. Both the gonococcus and the meningococcus produced the factor(s); however, it was more inhibitory for the gonococcus than for the meningococcus. The nature of that compound was not further invested.

Meningobactin and gonobactin were found to be poorly extractable in ethyl acetate and chloroform. However, a procedure similar to that of Mullis et al. (14) as derived from Byers et al. (6) was successful in extracting the compounds. The pH of the concentrated (10×) eluate fractions containing gonobactin or meningobactin was adjusted to 7.0 with 6 N KOH. In a typical experiment, 20 ml of the concentrate was extracted three times for 5 min each with a 50-ml volume of 85% chloroform-phenol (1:1, vol/vol). To the combined chloroform-phenol extracts was added

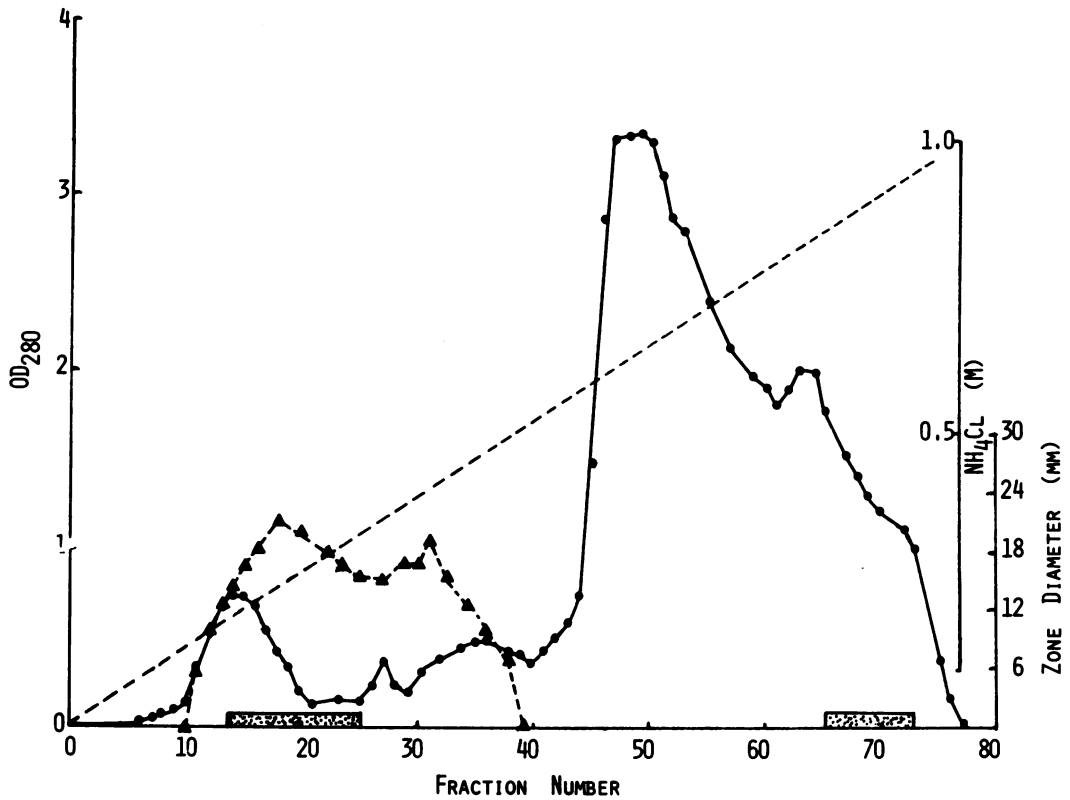

FIG. 4. *Elution profile of meningobactin from a Bio-Rad AG2-X10 (chloride form) anion exchange column (2.5 by 40 cm). Iron-limited biphasic cultures of N. meningitidis B-11 were harvested at 48 h, the conalbumin was removed, and the culture supernatants were passed through the column. After rinsing of the column with 3 void volumes of deionized water, the adherent material was eluted with a 0 to 1.0 M NH_4Cl gradient at a rate of 18 ml/h. The fractions were collected in 7-ml volumes, and the optical density at 280 nm (●) was recorded. The fractions were sterilized by autoclaving and then assayed in the CA bioassay with N. meningitidis B-11 (▲). The stippled bars represent the fractions stimulatory for A. flavescens JG-9.*

a 200-ml volume of ethyl ether, and the mixture was extracted three times with 50-ml volumes of deionized water. At this point, the siderophore was again the aqueous phase. This was washed with 3 equal volumes of ether and was concentrated by flash evaporation to approximately 5 ml, titrated to pH 7.0, and filter sterilized.

Properties of meningobactin and gonobactin. The phenol-chloroform-ether extract was stimulatory in both the CA and *A. flavescens* bioassays. The siderophores were probably hydroxamic acid derivatives since they were positive for bound hydroxylamine. Like other siderophores, the compounds were of small molecular weight since they were easily dialyzable; within a few hours meningobactin from a phenol-chloroform-ether extract dialyzed quantitatively through dialysis tubing with a molecular weight cutoff of 6,000 to 8,000. The same was true of gonobactin from a P-2 desalted fraction.

Both meningobactin and gonobactin in their partially purified states were iron reactive (Fig. 6). To 20 U of phenol-chloroform-ether-extracted meningobactin (pH 5.0) was added 500 μM iron as $FeCl_3$. There was an immediate reaction with the complex exhibiting an absorption maximum at approximately 420 nm.

Although the compounds produced by the pathogenic *Neisseria* spp. were very similar in their properties, they are probably not identical since each was most stimulatory for the homologous species (Table 4). The phenol-chloroform-ether extract of *N. meningitidis* B-11 was more stimulatory for itself than for *N. gonorrhoeae* 6555, and in the reciprocal experiment the differences were even more pronounced.

DISCUSSION

To acquire the necessary iron from their environment, many microorganisms produce

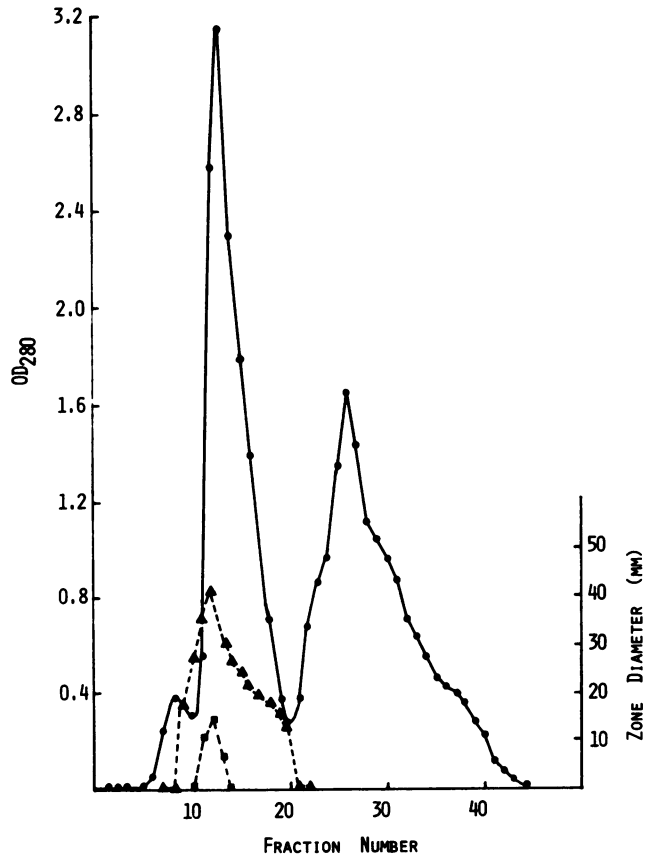

FIG. 5. *Gel filtration profile of meningobactin on a Bio-Gel P-2 column (1.0 by 25 cm) equilibrated with water. The pooled and 10-fold-concentrated, bioassay-active fractions from the AG2-X10 column were chromatographed at a rate of 5 ml/h, the optical density at 280 nm (●) was recorded, and the fractions were collected in 1.5-ml samples. These factions were assayed in the CA bioassay for stimulatory (▲) and inhibitory (■) activity. Inhibition was seen as a zone of inner clearing surrounded by a larger zone of stimulation.*

small-molecular-weight iron-chelating and -transporting agents which have been termed siderophores. However, a few naturally occurring soil microorganisms, such as *A. flavescens* JG-9, are unable to produce siderophores, but depend upon compounds produced by other microbes to acquire their iron. This dependence on hydroxamates or hemin has been exploited for a number of years and was used in this study to assay microbes for production of hydroxamate-type siderophores.

Burnham and Neilands (5) analyzed a number of bacteria and fungi for their ability to stimulate this bacterium and found a 100% correlation between stimulation of strain JG-9 and production of bound hydroxylamine, a feature indicative of hydroxamate production. Both the meningococcus and the gonococcus produced compounds, designated meningobactin and gonobactin, respectively, which were stimulatory for *A. flavescens* JG-9 and gave a positive test for bound hydroxylamine.

The amount of siderophore produced by the pathogens under the best conditions was determined by two different assays to be 100- to 1,000-fold lower than the amount of hydroxamate produced by *B. megaterium* (6) or *Aerobacter aerogenes* (10). This low level of production was no doubt responsible, in part, for the inability of other investigators to detect siderophore in the culture fluids of either *N. gonorrhoeae* (16) or *N. meningitidis* (3). Indeed, the greater portion of the siderophore produced by these pathogens may remain cell associated since it was found that simply washing the cells with 0.05 M NaCl or NH_4Cl released a very high titer of CA and *A. flavescens* active material (Yancey and Finkelstein, unpublished data). It is possible that the hydroxamate siderophore found in the supernatant fluids of the late cultures was released

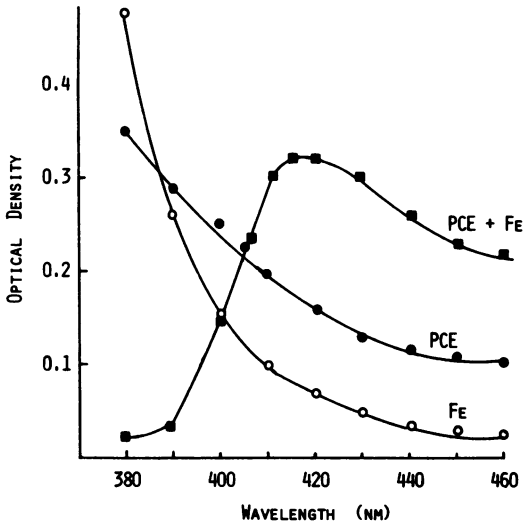

FIG. 6. *Visible spectra of an aqueous phenol-chloroform-ether extract of meningobactin (20 U) at pH 5.0 in the absence (●) or presence (■) of 500 μM iron; control, 500 μM iron only (○).*

TABLE 4. *Specificity of gonobactin and meningobactin*

Material tested	U of activity[a] for strain:	
	B-11	6555
PCE[b] extract of *N. meningiditis* B-11 (meningobactin)	20	10
PCE extract of *N. gonorrhoeae* 6555 (gonobactin)	3	25

[a] One unit of activity was defined as the reciprocal of the dilution required to produce a 10-mm zone of stimulation in the CA bioassay.

[b] PCE, Phenol-chloroform-ether.

upon lysis of the cells. Other microbes have been reported to contain high levels of cell-associated chelating agent. Sonic lysates of *B. megaterium* grown in low-iron medium contained significant amounts of SK (6, 13). Synthesis of an unknown hydroxamate by *E. coli* carrying the Col V virulence plasmid was recently analyzed, and those investigators found that a majority of hydroxamate in their preparation was cell associated (26). The presence of siderophore at the cell surface would seem a much more conservative and efficient mechanism of iron acquisition than would active secretion of large quantities of siderophore that would diffuse rapidly away from the cells. Archibald and DeVoe (2) found that under their experimental conditions, iron bound specifically to transferrin was available only through direct contact with the cells. Siderophore present at the surface of the cells, bound to a specific transport receptor, might serve as the vehicle of removal of iron from iron-transferrin complexes.

We found previously that the only types of exogenous siderophores effective in relieving conalbumin-mediated bacteriostasis of *N. gonorrhoeae* and *N. meningitidis* were the dihydroxamates, SK, aerobactin, and arthrobactin (9). The similarities in the properties of these structurally related compounds and meningobactin and gonobactin should be recognized. Both neisserial siderophores: (i) were extracted with phenol-chloroform in a manner similar to SK (14); (ii) gave patterns of *A. flavescens* JG-9 stimulation similar to those of SK and arthrobactin (6); (iii) adhered readily to anion exchange resins used for SK and aerobactin isolation (10, 14); and (iv) gave visible spectra absorption maxima at pH 5.0, in the presence of iron, at approximately 420 nm, similar to those of SK (6). The differences in specificity of meningobactin and gonobactin for the homologous species suggested, however, that even if the compounds were similar to the dihydroxamate compounds, they are not identical; no such specificity for these dihydroxamates has been found. SK, aerobactin, and arthrobactin were all slightly more stimulatory for the meningococcus than for the gonococcus (9). This difference in specificity also was consistent with the observations of Payne and Finkelstein (22) that whereas culture filtrates of *N. meningitidis* were stimulatory for *N. gonorrhoeae*, the converse was not true. In the experiments reported here (Table 4) the same quantity of gonobactin produced zones of stimulation 10 times smaller for the meningococcus than for the gonococcus. However, meningobactin produced zones only twofold smaller for *N. gonorrhoeae* than for *N. meningitidis*.

The finding that piliated, virulent T1 gonococci produced more gonobactin than did the nonpiliated T3 strains was interesting and consistent with our observations that piliated gonococci were more virulent and better able to acquire iron in the chicken embryo model (19).

Payne et al. (23) reported that strains of *N. gonorrhoeae* isolated from disseminated gonococcal infection were better able to acquire iron in vivo than were urogenital isolates from patients with uncomplicated infections and also observed (22) that strain 6555 from a disseminated gonococcal infection produced more stimulatory activity in iron-limited culture fluids than did a urogenital isolate. In the experiments reported here (Fig. 2 and 3, Table 2) the disseminated gonococcal infection strains tested, 1975 and 6555, did produce slightly more stimulatory

material than did the urogenital isolates F62, Bowden, and 2686, although those differences in some cases were small.

This is the first demonstration that siderophores produced by the pathogenic *Neisseria* spp., probably hydroxamates, permit the growth of *N. meningitidis* and *N. gonorrhoeae* in iron-restricted environments. Meningobactin and gonobactin have also been found to enhance virulence of the gonococcus in chicken embryos (9). By implication, gonobactin and meningobactin might also be important to the growth of these organisms on mucosal surfaces, in body fluids, or within phagocytes, environments which are iron limited because of the host's iron-binding proteins, lactoferrin and transferrin.

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