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# Response of *Neisseria gonorrhoeae* to Iron Limitation: Alterations in Expression of Membrane Proteins Without Apparent Siderophore Production

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**For acquisition of iron, an essential nutrient, most microorganisms produce siderophores (low-molecular-weight iron-chelating compounds) and membrane proteins to serve as receptors for the iron-siderophore complexes. The gonococcus does not appear to produce a siderophore, since the quantity of siderophore detected by bioassays of culture supernatants from strains F62 and FA19 was never greater than the amount present in the uninoculated medium. Iron limitation of the laboratory strains F62 and FA19 and 12 recent clinical isolates resulted in the expression of several iron-repressible membrane proteins. The expression of proteins in the apparent molecular weight range of 70,000 to 100,000 was strain dependent. All strains expressed 36,000-dalton (36K) and 19.5K proteins. FA19 and F62 were also grown in medium containing iron sources commonly encountered in vivo (i.e., transferrin, lactoferrin, hemoglobin, or hemin). Comparison of growth rates indicates that transferrin and lactoferrin were more readily utilized as iron sources than hemin and hemoglobin were. Expression of the iron-repressible proteins varied depending upon the iron source. Fewer iron-repressible proteins were observed when cells were supplied with transferrin or lactoferrin than when the cultures were grown with either hemin or hemoglobin. The 36K protein was expressed with all four iron sources.**

Pathogenic microorganisms, such as *Neisseria gonorrhoeae*, presumably must be able to utilize the iron sources available in the host organism. An obligate human parasite, the gonococcus usually causes disease localized to the mucosal surfaces of the urogenital tract, pharynx, and rectum. Occasionally, it may invade the bloodstream and synovial membranes of joints. Transferrin is probably the principal iron source in serum, whereas lactoferrin is probably the principal iron source on mucosal surfaces (31). Previous studies showed that all strains of the gonococcus can use transferrin and hemin as iron sources; 50% of tested strains were able to remove iron from lactoferrin, whereas 70% could obtain iron from hemoglobin (18, 19). The mechanism by which the gonococcus removes iron from these compounds is not well understood.

Several high-affinity iron acquisition mechanisms which can remove iron from compounds such as transferrin have been extensively studied in *Escherichia coli* and other bacteria (20–22). These systems generally consist of siderophores, low-molecular-weight iron-chelating compounds, and outer membrane proteins that serve as receptors for the iron-siderophore complexes and aid in their internalization. These iron uptake systems are induced as a result of iron limitation. Yancey and Finkelstein (34) reported that *N. gonorrohoeae* and *N. meningitidis* produce a hydroxamate siderophore at concentrations approximately 100-fold lower than the amounts produced by other hydroxamate-producing organisms under similar conditions. However, other investigators (1, 24) have not found any evidence supporting the existence of either a gonococcal or a meningococcal siderophore. Norqvist et al. (23) observed that several outer membrane proteins (apparent molecular weights of 76,000, 86,000, and 97,000) are synthesized by the gonococcus in response to iron deprivation. The role of these proteins in iron acquisition has not been determined.

We initiated this study to aid in defining the mechanism by which *N. gonorrhoeae* acquires iron. We report here evidence that certain culture media contain small amounts of siderophore activity as a normal component; in cultures of iron-starved gonococci additional siderophore activity could not be detected by the bioassays used. In addition to the iron-repressible proteins described by Norqvist et al. (23), we have observed, in response to iron deprivation, the synthesis of additional membrane proteins. The expression of these iron-repressible proteins was variable between strains and was dependent on the iron source supplied to the gonococcus.

(These results were presented at the 84th Annual Meeting of the American Society for Microbiology, St. Louis, Mo., March 1984 [S. E. H. West, E. Blackman, and P. F. Sparling, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B121, p. 37].)

## MATERIALS AND METHODS

**Bacterial strains.** *N. gonorrhoeae* strains F62 and FA19 have been well characterized in this laboratory and elsewhere. Strain F62 can utilize iron on transferrin but not from lactoferrin; strain FA19 can utilize both transferrin and lactoferrin as iron sources (18, 19). FA6028 is a transformant of F62 that is capable of using lactoferrin-bound iron. The transformation was performed as described by Sparling (28). Donor DNA was from strain FA19; transformants were selected on the deferrated defined medium of Mickelsen and Sparling (19) supplemented with lactoferrin as the sole iron source. Lactoferrin-utilizing transformants were obtained at a frequency of $10^{-3}$. Recent clinical isolates (12 strains) were obtained from the Durham County Health Department, Durham, N.C., or from North Carolina Memorial Hospital,

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Chapel Hill. These strains were auxotyped by using the minimal medium of LaScolea and Young (14). The protein I serotype was determined as described by Tam et al. (30). Monoclonal antibodies were obtained from Genetic Systems, Seattle, Wash.

**Iron-containing compounds.** Apo-transferrin, lactoferrin, conalbumin, hemoglobin, and hemin were obtained from Sigma Chemical Co., St. Louis, Mo. Transferrin, 9 and 25% saturated with iron, was prepared by adding an appropriate amount of 0.1 M sodium citrate–0.1 M sodium bicarbonate–5 mM $FeCl_3$ buffer, pH 8.6 (17), to apo-transferrin resuspended in 40 mM Tris-hydrochloride–0.15 M NaCl–20 mM sodium bicarbonate buffer, pH 7.4, followed by dialysis against the Tris-hydrochloride–bicarbonate buffer. Lactoferrin was first deferrated by the method of Mazurier and Spik (17). A quantity of iron, sufficient to obtain 10% saturation, was added as described for transferrin. The percent saturation of these proteins was confirmed by determining their iron content, using the ferrozine assay of Stookey (29), and protein concentration, using the Coomassie blue method as described by Bio-Rad Laboratories, (Richmond, Calif.). Hemoglobin was dissolved in deionized water followed by overnight dialysis against deionized water. Hemin was first dissolved in a small amount of 1 N NaOH and then diluted with deionized water to a final concentration of 767 μM.

**Media and growth conditions.** Iron-stressed cultures were obtained by diluting log-phase cells grown in GC broth (1.5% Difco Proteose Peptone no. 3, 0.5% NaCl, 0.4% $K_2HPO_4$, 0.1% $KH_2PO_4$, 1% Kellogg supplement I [10], 0.8% sodium bicarbonate) into GC broth containing 50 μM Desferal to chelate any available iron. This provided an iron-limited growth medium, since gonococci are unable to utilize Desferal-bound iron (23). Iron-sufficient control cultures contained 50 μM Desferal and 100 μM $Fe(NO_3)_3$. Cells were aerated by shaking at 200 rpm; incubation was at 37°C. The optical density of the cultures was monitored with a Klett-Summerson colorimeter. Cells were harvested at late log phase or at the times indicated in the text.

To determine the effect of specific iron sources on the protein composition of membranes, cells were grown in GC broth containing 50 μM Desferal and a specific iron-containing compound which had been added immediately before inoculation of the culture. These cultures were grown to late log phase and then used to inoculate another flask of the same medium. The iron sources tested were transferrin, 9% and 25% saturated with iron; lactoferrin, 10% and 30% saturated; hemoglobin; and hemin. With the exception of the Desferal cultures and strain F62 grown with lactoferrin, cells were harvested at late log phase from the second flasks of media for preparation of crude membrane samples. Because of depletion of intracellular iron pools, the Desferal and F62 lactoferrin cultures did not reinitiate growth when diluted into a second flask of medium. For these cultures, cells were harvested at late log phase from the initial flask.

**Siderophore production.** Flasks (250 ml) containing 50 ml of GC broth overlaid onto 50 ml of GC base agar (Difco Laboratories, Detroit, Mich.) were inoculated with *N. gonorrhoeae* strain F62 or FA19. In some flasks, conalbumin (500 μg/ml, type I; Sigma Chemical Co.) was included in both the agar and broth phases to deprive the gonococcus of iron. Cultures were incubated for 48 h in a 37°C shaking incubator. Uninoculated medium controls and a flask of Difco Proteose Peptone no. 3 (15 mg/ml) were also incubated. Concentrated culture supernatants were prepared by centrifugation at 12,000 × *g*, filtration through a 0.45-μM membrane filter, ultrafiltration through a PM-10 filter (Amicon Corp., Lexington, Mass.), and lyophilization as described by Yancey and Finkelstein (34). These concentrates were suspended in deionized water to give a final 30-fold concentration and were assayed for siderophore activity, using the *Salmonella typhimurium* LT-2 *enb-7* and the *Arthrobacter flavescens* JG-9 bioassays (15, 34). *S. typhimurium* LT-2 *enb-7* was obtained from J. B. Neilands, University of California, Berkeley. *A. flavescens* JG-9 was obtained from R. A. Finkelstein, University of Missouri, Columbia. Desferal (deferoxamine mesylate; Ciba Pharmaceutical Co., Summit, N.J.) was used as a reference siderophore, and results were expressed as Desferal equivalents.

**Preparation of crude membranes.** Late-log-phase cells were harvested by centrifugation at 12,000 × *g*. The cell pellet was suspended in 5 to 10 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.2, and stored at −20°C. To prepare crude membranes, the cell suspension was disrupted by passage (once) through a French pressure cell at 14,000 to 16,000 lb/in$^2$. The lysed cells were diluted two- to fourfold in 10 mM HEPES and were centrifuged at 12,000 × *g* for 10 min. The supernatant was then centrifuged at 100,000 × *g* for 1 h to pellet the membrane fraction. The membrane pellets were suspended in 100 to 200 μl of HEPES buffer and centrifuged at 3,000 × *g* for 5 min. The supernatant contained the membrane fraction and was stored at −20°C. The protein content was determined as described by Markwell et al. (16).

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using the discontinuous buffer system of Laemmli (12). Separating gels contained either 10% acrylamide and 0.13% bisacrylamide or 15% acrylamide and 0.087% bisacrylamide. The outer membrane preparations were heated in an equal volume of sample buffer (0.125 M Tris-hydrochloride, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue) at 100°C for 5 min immediately before electrophoresis at a constant current of 25 mA. Gels were stained either by the silver staining procedure of Wray et al. (32) or with Coomassie brilliant blue R-250 as described by Schleif and Wensink (27).

## RESULTS

**Lack of siderophore production.** We tested for the presence of siderophores in culture supernatants from strains F62 and FA19 grown in the biphasic medium described by Yancey and Finkelstein (34), using the *S. typhimurium* LT-2 *enb-7* bioassay (15) (Table 1). *S. typhimurium* LT-2 *enb-7* is a mutant which requires either a hydroxamate or a phenolate siderophore for growth in the presence of citrate. Uninoculated culture medium contained small amounts of siderophore-like activity. The quantity of siderophore detected in the culture supernatants was never greater than the amount detected in uninoculated medium and usually was less. We tested three lots of GC agar and found that the quantity of siderophore-like activity varied between lots. Difco Proteose Peptone no. 3 was the medium component which possessed the majority of siderophore-like activity. A 1.5% solution of one lot of Difco Proteose Peptone no. 3 contained a Desferal equivalent concentration of 166 nM. A 1% solution of Bacto-Agar (Difco) possessed less activity (<8 nM).

With the *A. flavescens* JG-9 bioassay (34), which is specific for hydroxamate siderophores, lower Desferal equivalent concentrations were detected than with the *S. typhimurium* assay. Two lots of uninoculated GC biphasic me-

TABLE 1. Siderophore content of uninoculated medium and culture supernatants from *N. gonorrhoeae* strains F62 and FA19

| Medium or medium component | Strain | Desferal equivalent concn (nM)[a] |
|---|---|---|
| GC biphasic medium | | 33–117 |
| GC biphasic medium plus conalbumin | | 26–117 |
| GC biphasic medium | F62 | 17–83 |
| GC biphasic medium plus conalbumin | F62 | 13–83 |
| GC biphasic medium | FA19 | 10–17 |
| GC biphasic medium plus conalbumin | FA19 | 30–83 |

[a] The Desferal equivalent concentration was obtained by comparing the zones of growth of *S. typhimurium* LT-2 *enb-7* around paper disks or wells containing the test sample with the zones of growth stimulation obtained with known concentrations of Desferal. The values have been corrected to represent the amount of siderophore in unconcentrated medium or culture supernatants and represent the range of values obtained with three separate lots of GC agar.

dium contained 1.0 to 3 nM siderophore activity; one other lot contained <0.5 nM siderophore activity. Culture supernatants from strains F62 and FA19 never contained more siderophore activity than the uninoculated media (data not shown).

**Effect of iron starvation on protein composition of gonococcal membranes.** Strains F62 and FA19 were grown in GC broth containing the iron chelator Desferal (50 μM) to bind all ionic iron in the culture medium (approximately 7 μM as determined with the ferrozine assay [29]). Crude membranes were prepared from the initial log-phase inoculum (grown in GC broth) and from cultures in Desferal-containing medium 1, 2, 3, and 4 h after inoculation. The Desferal-treated, iron-deficient culture of FA19 grew at the same rate for two mass doublings as the iron-sufficient control culture (50-min generation time), but the viable cell count at stationary phase was lower ($6.7 \times 10^8$ versus $1.5 \times 10^9$ CFU/ml). Initial viable cell counts for both cultures were $1.2 \times 10^8$ CFU/ml. Similar results were obtained with F62. Several membrane proteins were observed in increased amounts in response to iron starvation over the 4-h incubation period (Fig. 1). With strain FA19, proteins of apparent molecular weights of 104,000, 88,000, 74,000, 70,000, and 36,000 were either increased in amount or first detected after 2 h of iron deprivation. A 19,500-dalton (19.5K) protein was observed on 15% acrylamide gels when stained with silver (see Fig. 3B, lane b). Small amounts of the 36K protein were detected in iron-sufficient cultures by the silver stain, but not by the less sensitive Coomassie blue stain. Crude membranes from iron-deprived cells of strain F62 contained proteins of 70,000, 41,000, 36,000, and 19,500 apparent molecular weights (Fig. 1B; see Fig. 3B, lane b). In some preparations, a 104K protein was expressed in small amounts in F62 (data not shown). The 70K protein was present in more abundant amounts in F62 than was a 70K protein observed in strain FA19. The appearance of a 70K protein coincided with the disappearance of a 71K protein. The protein I serotype of both strains was not affected by iron starvation.

The effect of Desferal on the protein composition of crude membranes was apparently due to iron deprivation, since cells grown in media containing 50 μM Desferal and 100 μM $Fe(NO_3)_3$ had membrane protein compositions identical to those of iron-sufficient cells grown in the absence of Desferal.

To eliminate the possibility that these proteins were induced as a result of generally poor growth conditions, we stressed strains F62 and FA19 in several ways other than iron starvation. For these experiments the medium contained sufficient iron for growth (approximately 20 μM). With reduced aeration (100 rpm versus 200 to 250 rpm) or lowered pH (6.6 versus 7.2) there was no change in the iron-repressible protein composition of crude membranes prepared from late-log-phase cells. However, the generation times were 90 min (100 rpm aeration) and 70 min (pH 6.6) compared to 50 min for fully aerated cells at pH 7.2. We also grew cells to mid-log phase, at which time aeration was stopped for 2 h before cells were harvested for membrane preparation. Cessation of aeration stopped growth as measured by viable cell counts. Again the iron-repressible protein composition of the crude membrane samples was identical to that of cells grown under iron-sufficient conditions (data not shown).

**Effect of specific iron sources on membrane protein composition.** The previous experiments were designed to determine the response of the gonococcus to an absolute unavailability of exogenous iron. However, in vivo, if a mucosal or systemic pathogen were able to use iron bound to lactoferrin, transferrin, or other soluble iron carriers, it might not be exposed to such severe iron limitation. To determine the response of the gonococcus to these iron sources, cells were grown in GC broth containing 50 μM Desferal and a specific iron-containing compound. The growth rates of strains F62 and FA19 when grown with 10 and 25% saturated transferrin as an iron source (60- and 55-min generation times, respectively) were identical to those of cultures containing a 10- to 20-fold excess of $Fe(NO_3)_3$. When strain FA19 was grown in lactoferrin (2.0 μM available iron concentration), the generation time was 55 min, the same as for cultures with 50 μM Desferal and 100 μM $Fe(NO_3)_3$. Iron-starved cells of strain F62 did not grow in medium containing lactoferrin as a sole iron source. For both strains, reduced growth rates (100- to

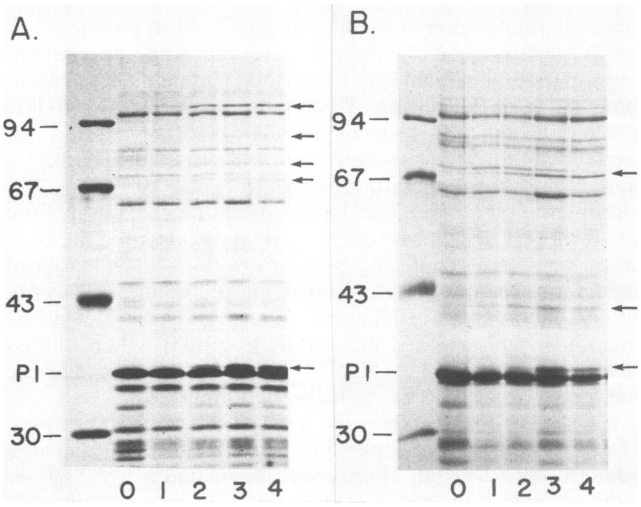


FIG. 1. Response of *N. gonorrhoeae* strains FA19 and F62 to iron limitation. Crude membranes were prepared 0, 1, 2, 3, and 4 h after dilution of a log-phase culture into GC broth containing 50 μM Desferal. (A) Strain FA19; (B) strain F62. 10% SDS-polyacrylamide gel stained with Coomassie brilliant blue R-250, 20 μg of protein per lane. Arrows indicate proteins induced as a result of iron limitation. Molecular weight markers ($\times 10^3$) are given on the left side of the gels. Protein I (8), the gonococcal 34 to 38K major outer membrane protein which is antigenically variable between strains, is labeled PI.

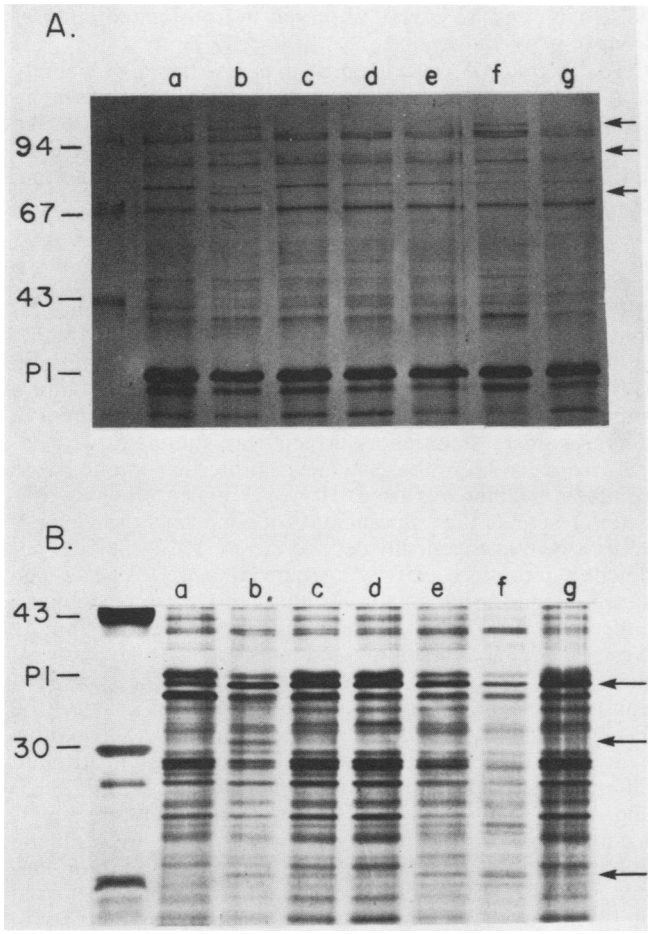


FIG. 2. Response of *N. gonorrhoeae* strain FA19 to specific iron sources. (A) 10% SDS-polyacrylamide gel stained with Coomassie brilliant blue R-250, 20 μg of protein per lane. (B) 15% SDS-polyacrylamide gel stained with silver, 5 μg of protein per lane. Crude membranes were prepared from late-log-phase cultures grown in GC broth containing 50 μM Desferal (lane b), or 50 μM Desferal supplemented with 100 μM $Fe(NO_3)_3$ (lane a); 10 μM transferrin, 9% saturated with Fe (lane c); 5 μM transferrin, 25% saturated with Fe (lane d); 10 μM lactoferrin, 10% saturated with Fe (lane e); 10 μM hemoglobin (lane f); or 10 μM hemin (lane g). Arrows indicate iron-repressible proteins. Molecular weight markers ($\times 10^3$) are given on the left side of the gel. PI denotes the major outer membrane protein of the gonococcus.

140-min generation times) were observed in medium containing hemin or hemoglobin as the iron source.

Differential expression of the iron-repressible proteins was observed in F62 and FA19, depending on the particular source of iron provided. With strain FA19, a 36K protein which comigrated with protein I on the 10% gel (Fig. 2A) but not on the 15% gel (Fig. 2B) was present when transferrin and lactoferrin were the sole iron sources (lanes c, d, and e). A 19.5K protein was also present in cells grown with lactoferrin as the sole iron source. When hemoglobin was the iron source (Fig. 2A and B, lane f), additional iron-repressible proteins of 104K, 88K, and 19.5K were present; with hemin as the iron source (lane g), 36K and 19.5K proteins were expressed. A 31K protein was observed in this and one other preparation of Desferal-treated cells from FA19 (Fig. 2B, lane b); in other preparations it may have been obscured by comigration with a 31K protein II variant. This protein was not expressed with any of the tested iron sources (Fig. 2B, lanes c to g).

Similar results were obtained with strain F62 (Fig. 3). When transferrin, 25% iron saturated, was supplied as the sole iron source (lane d), 41K and 36K iron-repressible proteins were expressed. Lowering the iron saturation of transferrin to 9% (lane c) resulted in expression of the 70K protein, but in much smaller quantities than observed in Desferal media without supplemental iron (e.g., iron-starved cells, lane b). With lactoferrin, hemoglobin, or hemin as the sole iron source (lanes e, f, and g), we observed expression of 70K, 41K, 36K, and 19.5K iron-repressible proteins. An

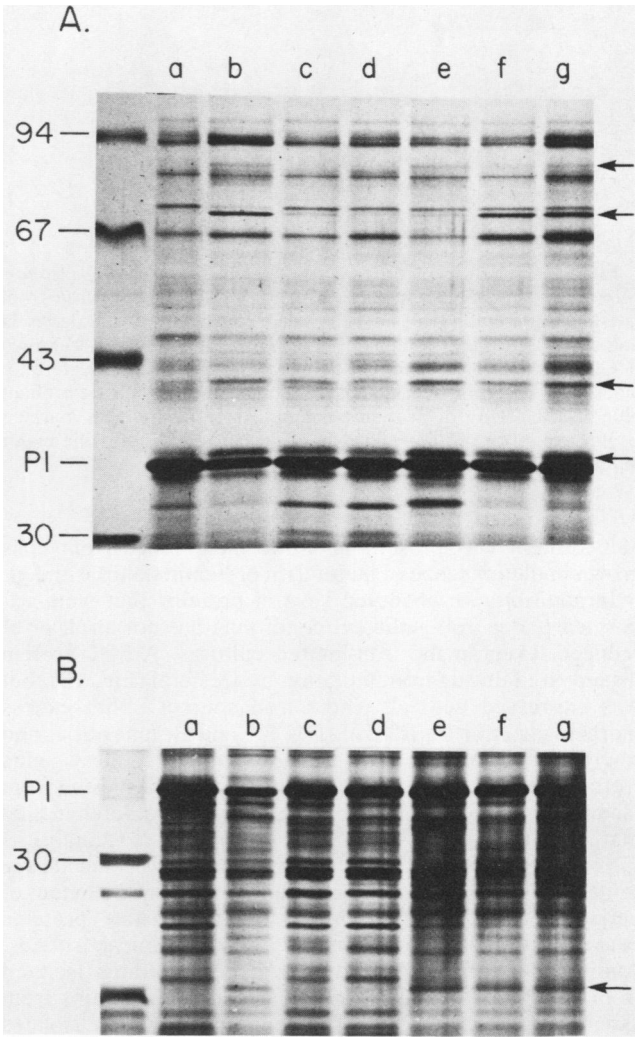


FIG. 3. Response of *N. gonorrhoeae* strain F62 to specific iron sources. (A) 10% SDS-polyacrylamide gel stained with Coomassie brilliant blue R-250, 20 μg of protein per lane. (B) 15% SDS-polyacrylamide gel stained with silver, 5 μg of protein per lane. Crude membranes were prepared from late-log-phase cultures grown in GC broth containing 50 μM Desferal (lane b), or 50 μM Desferal supplemented with 100 μM $Fe(NO_3)_3$ (lane a); 10 μM transferrin, 9% saturated with Fe (lane c); 5 μM transferrin, 25% saturated with Fe (lane d); 10 μM lactoferrin, 30% saturated with Fe (lane e); 10 μM hemoglobin (lane f); or 10 μM hemin (lane g). Arrows indicate iron-repressible proteins. Molecular weight markers ($\times 10^3$) are given on the left side of the gels. PI denotes the major outer membrane protein of the gonococcus.

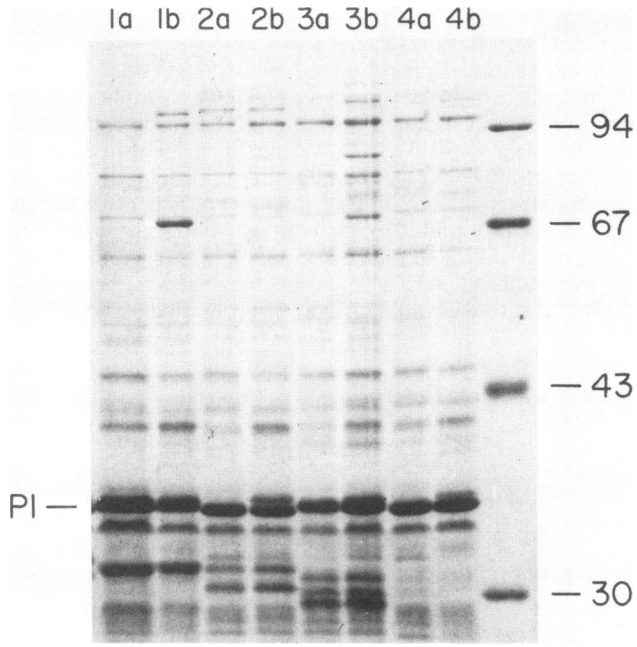


FIG. 4. Response of four recent clinical isolates of *N. gonorrhoeae* to iron limitation. Crude membranes were prepared from late-log-phase cultures of FA1126 (lanes 1a and 1b), JB118 (lanes 2a and 2b), JB103 (lanes 3a and 3b), and JB135 (lanes 4a and 4b) grown in GC broth, a lanes, or in GC broth containing 50 μM Desferal, b lanes. 10% SDS-polyacrylamide gel stained with Coomassie brilliant blue R-250, 20 μg of protein per lane. Molecular weight markers ($\times 10^3$) are given on the right side of the gel. PI denotes the major outer membrane protein of the gonococcus.

88K protein was present in crude membranes from cells grown in the presence of lactoferrin or hemin (lanes e and g).

In addition, we observed several proteins that were expressed under iron-sufficient conditions but not at all or at reduced levels in the iron-limited cultures. A 71K protein disappeared during iron limitation by Desferal (Fig. 1B), but was expressed with all tested iron sources. With excess inorganic iron or transferrin (Fig. 3A and B, lanes a, c, and d) proteins of 33,000 and 20,000 apparent molecular weights were present, but were less apparent in cells harvested from media containing Desferal or Desferal plus lactoferrin, hemin, or hemoglobin (Fig. 3A and B, lanes b, e, f, and g).

**Variability between strains in expression of membrane proteins in response to iron limitation.** To assess the extent of variability in expression of the iron-repressible proteins between strains, we examined 12 recent clinical isolates. Among the urogenital isolates were four strains isolated from symptomatic males and females and two strains from asymptomatic males. The disseminated infection isolates included five strains from either blood or joint fluid and one strain from the cervix of a patient with disseminated disease. A variety of auxotypes, including the arginine-hypoxanthine-uracil type, and various protein I serotypes (30) were included among these strains. Protein profiles from four representative strains are shown in Fig. 4. A particular pattern was not associated with auxotype, serotype, or type of infection. Expression of proteins in the range of 70K to >100K varied among the different isolates. A 104K and a 70K protein were observed in 75 and 50% of the isolates, respectively. Proteins of 36K and 19.5K were seen in all 12 isolates. Less prominently expressed proteins of about 38K, 40K, 55K, and 62K were observed in iron-limited cultures from some of the strains.

To determine if a specific protein is associated with ability to remove iron from lactoferrin, we compared crude membrane preparations from the isogenic strains FA6028 and F62, which could and could not remove iron from lactoferrin, respectively. No significant difference in protein composition was seen (data not shown).

## DISCUSSION

There are conflicting reports of siderophore production by pathogenic *Neisseria* species. Payne and Finkelstein (25) first reported production of a siderophore by disseminating strains of the gonococcus. Later, Yancey and Finkelstein (34) isolated from concentrated (30- to 100-fold) culture supernatants of both gonococci and meningococci a dihydroxamate siderophore which was stimulatory for *A. flavescens* JG-9. For these studies, a proteose peptone-based complex medium was used. However, when other investigators tested culture supernatants of gonococci or meningococci grown in chemically defined media (1, 24) they did not detect siderophore activity. Archibald and DeVoe (1) assayed unconcentrated and concentrated (20×) meningococcal culture supernatants (continuous culture in iron-limiting *Neisseria*-defined medium) and ethyl acetate extracts of these supernatants for siderophore activity. They obtained uniformly negative results with a variety of assays: the Csáky test (4) for hydroxamate siderophores, the Arnow assay (2) for phenolate siderophores, ferric iron absorption difference spectra, and the *S. typhimurium* LT-2 *enb7* (15) and *A. terregens* (26) bioassays. In addition, culture supernatants from iron-starved cells were unable to stimulate growth of the homologous meningococcal strain in iron-limited medium (1). With the Csaky and Arnow tests, Norrod and Williams (24) did not detect any siderophore activity in spent culture supernatants of an *N. gonorrhoeae* strain grown in a chemically defined medium.

Yancey and Finkelstein (34) detected 1.7 to 4.0 nM Desferal equivalent concentrations of "gonobactin" in culture supernatants. It is questionable whether this quantity of siderophore could solubilize sufficient iron to support growth of the gonococcus. For *S. typhimurium* LT-2, the minimal concentration of the hydroxamate siderophore ferrichrome required to support growth is 0.1 μM (20). Since hydroxamate siderophores solubilize on a molar basis an equivalent amount of iron and most microorganisms require 0.1 to 2.0 μM iron for growth (31), gonobactin would have to be recycled to provide this amount of iron. Whether gonobactin is recycled was not established. Aerobactin is recycled (3); however, other siderophores are either chemically modified or degraded (7, 13).

Additional evidence which argues against siderophore production by the gonococcus was obtained by Mickelsen and Sparling (19). They found that gonococcal strains F62 and FA19 were unable to grow in a chemically defined medium containing Desferal and excess $Fe(NO_3)_3$ but lacking weak chelators such as pyrophosphate, citrate, or oxalacetate. Addition of exogenous iron-solubilizing compounds (citrate, pyrophosphate, oxalacetate, nitrilotriacetate, or transferrin) stimulated iron-dependent growth. Addition of these iron chelators should be unnecessary if the gonococcus produces sufficient quantities of its own iron-solubilizing compound. Thus, it is unlikely that the gonococcus produces a siderophore which is not detected by these bioassays or the standard chemical assays for a hydroxamate or phenolate siderophore. Absence of siderophore

production would distinguish gonococcal iron acquisition from the systems used by *E. coli* and related organisms.

We have demonstrated that a siderophore(s) is present in uninoculated culture medium and that spent culture supernatants from *N. gonorrhoeae* strains F62 and FA19 do not contain any additional quantities of these siderophores. With the *A. flavescens* assay, we detected quantities of siderophore similar to those reported by Yancey and Finkelstein (<0.5 to 3 versus 1.7 to 4 nM Desferal equivalent concentration) (34). The siderophore in the culture medium is probably a hydroxamate, based on its ability to stimulate growth of *A. flavescens* JG-9. Yancey and Finkelstein (33) reported that the gonococcus can utilize the dihydroxamate siderophores aerobactin, arthrobactin, and schizokinen. We have since confirmed the ability of the gonococcus to use aerobactin as an iron source (unpublished data). If similar compounds contribute to the siderophore content of uninoculated media, their use by the gonococcus could explain the lower concentrations of siderophore activity detected in spent culture supernatants (Table 1).

A possible source for this activity is siderophore production by contaminating microorganisms during the manufacturing of Difco Proteose Peptone no. 3. Sciortino and Finkelstein have isolated small amounts of a low-molecular-weight iron-binding compound from Difco Proteose Peptone no. 3 that stimulated the growth of *Neisseria* species in low-iron media and in the presence of either deferricon-albumin or EDDHA [ethylenediamine-di(*o*-hydroxyphenyl acetic acid)]. This factor was chromatographically similar to the host-associated iron transfer factor described by Jones et al. (9) and appeared to be related, but not identical, to gonobactin and "meningobactin" described earlier by Yancey and Finkelstein (5, 6, 33, 34; C. V. Sciortino and R. A. Finkelstein, personal communication).

Outer membrane proteins whose synthesis is induced specifically as a result of iron limitation are integral components of microbial iron acquisition mechanisms described for *E. coli* and *S. typhimurium* (20, 22). In this report, we have confirmed the earlier work of Norqvist et al. (23) in which they demonstrated that the gonococcus synthesizes several outer membrane proteins of 70,000 to 100,000 apparent molecular weight in response to iron limitation. The addition of iron to the growth media repressed synthesis of these proteins. In response to iron starvation, we observed the expression of additional proteins of lower apparent molecular weights. Proteins of 36K and 19.5K were expressed by all strains examined. A 70K protein was prominently expressed by 50% of the strains. Additional proteins (38K, 40K, 41K, 55K, and 62K) were expressed by some strains. These proteins appeared to be expressed in smaller quantities than the 104K, 70K, and 36K proteins when the intensity and size of the Coomassie blue-stained bands on SDS-PAGE gels were compared. We examined crude membrane preparations which are comprised of both inner and outer membranes and, therefore, cannot state the exact cellular location of the iron-repressible proteins.

Small quantities of the 36K protein were observed under iron-sufficient conditions, but the quantity of this protein apparently increased in the iron-starved cultures. Its consistent expression by different strains and under all conditions of iron limitation suggests that it may play a central role in iron acquisition by the gonococcus. Mietzner et al. (T. Mietzner, G. Luginbuhl, and S. A. Morse, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, D13, p. 53) observed an iron-repressible 37K protein on silver-stained 9.5 to 12% gradient SDS-PAGE gels containing 70 mM NaCl in the separating gel. Peptide mapping indicated that this 37K protein was distinct from protein I (T. A. Mietzner, G. Luginbuhl, E. Sandström, and S. A. Morse, personal communication). This protein and the 36K protein we detected may be the same.

In *E. coli*, synthesis of a 90K inner membrane protein was inhibited by iron limitation; this protein was postulated to be an iron storage protein (11). In iron-sufficient cultures, we observed several gonococcal proteins which were not present in membranes from iron-limited cultures. The significance of these observations has yet to be determined.

Expression of the iron-repressible proteins was highly variable among strains. In addition, various combinations of the iron-repressible proteins characteristic for a specific strain were expressed depending on the particular iron source. When the gonococcus was supplied with readily used iron sources such as transferrin or lactoferrin, a specific subset of these proteins was expressed. However, in media containing either hemin or hemoglobin, which are poorly utilized iron sources, we observed expression of all of the iron-repressible proteins characteristic of the strain studied. These results suggest that expression of the iron-repressible proteins may not be strictly coordinately regulated. Differences in the size of the intracellular iron pool, depending on the available source of iron, may be responsible for differences in expression of membrane proteins. Confirmation of this hypothesis will require careful measurement of intracellular iron pools during balanced growth, which was not attempted in this study. Variability of membrane protein expression depending on the source of iron is compatible with the idea that there are multiple iron acquisition systems, specific for particular iron sources, but considerable further evidence will be required to confirm this hypothesis.

The role of these iron-repressible proteins in iron acquisition, if any, has not been determined. We are hopeful that genetic approaches to this problem will facilitate our understanding of the regulation of iron-repressible proteins and their possible role(s) in utilization of specific iron sources. Since gonococci appear to scavenge iron from their environment in the absence of a specific gonococcal siderophore, understanding how gonococci acquire iron may provide novel insights into microbial mechanisms for iron utilization.

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## ADDENDUM IN PROOF

Mietzner et al. (**T. A. Mietzner, G. H. Louginbuhl, E. Sandstrom, and S. A. Morse**, Infect. Immun. **45**:410–416, 1984) have recently published their observation of a 37,000-dalton iron-regulated protein from *N. gonorrhoeae*.

## LITERATURE CITED

1. **Archibald, F. S., and I. W. DeVoe.** 1980. Iron acquisition by *Neisseria meningitidis* in vitro. Infect. Immun. **27**:322–334.
2. **Arnow, L. E.** 1937. Colorimetric determination of the compo-

nents of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531–537.
3. **Braun, V., C. Brazel-Faisst, and R. Schneider.** 1984. Growth stimulation of *Escherichia coli* in serum by iron (III) aerobactin. Recycling of aerobactin. FEMS Microbiol. Lett. **21**:99–103.
4. **Csáky, T. Z.** 1948. On the estimation of bound hydroxylamine in biological materials. Acta Chem. Scand. **2**:450–454.
5. **Finkelstein, R. A., C. V. Sciortino, and M. A. McIntosh.** 1983. Role of iron in microbe-host interactions. Rev. Infect. Dis. **5**:S759–S777.
6. **Finkelstein, R. A., and R. J. Yancey.** 1981. Effect of siderophores on virulence of *Neisseria gonorrhoeae*. Infect. Immun. **32**:609–613.
7. **Hartman, A., and V. Braun.** 1980. Iron transport in *Escherichia coli*: uptake and modification of ferrichrome. J. Bacteriol. **143**:246–255.
8. **Johnston, K. H., K. K. Holmes, and E. C. Gotschlich.** 1976. The serological classification of *Neisseria gonorrhoeae*. I. Isolation of the outer membrane complex responsible for serotypic specificity. J. Exp. Med. **143**:741–758.
9. **Jones, R. L., C. M. Peterson, R. W. Grady, and A. Cerami.** 1980. Low molecular weight iron-binding factor from mammalian tissue that potentiates bacterial growth. J. Exp. Med. **151**:418–428.
10. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85**:1274–1279.
11. **Klebba, P. E., M. A. McIntosh, and J. B. Neilands.** 1982. Kinetics of biosynthesis of iron-regulated membrane proteins in *Escherichia coli*. J. Bacteriol. **149**:880–888.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Langman, L., I. G. Young, G. E. Frost, H. Rosenburg, and F. Gibson.** 1972. Enterochelin system of iron transport in *Escherichia coli*: mutations affecting ferric-enterochelin esterase. J. Bacteriol. **112**:1142–1149.
14. **LaScolea, L. J., Jr., and F. E. Young.** 1974. Development of a defined minimal medium for the growth of *Neisseria gonorrhoeae*. Appl. Microbiol. **28**:70–76.
15. **Luckey, M., J. R. Pollack, R. Wayne, B. N. Ames, and J. B. Neilands.** 1972. Iron uptake in *Salmonella typhimurium*: utilization of exogenous siderochromes as iron carriers. J. Bacteriol. **111**:731–738.
16. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**:206–210.
17. **Mazurier, J., and G. Spik.** 1980. Comparative study of the iron-binding properties of human transferrins. I. Complete and sequential iron saturation and desaturation of the lactotransferrin. Biochim. Biophys. Acta **629**:399–408.
18. **Mickelsen, P. A., E. Blackman, and P. F. Sparling.** 1982. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from lactoferrin. Infect. Immun. **35**:915–920.
19. **Mickelsen, P. A., and P. F. Sparling.** 1981. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from transferrin and iron compounds. Infect. Immun. **33**:555–564.
20. **Neilands, J. B.** 1981. Iron absorption and transport in microorganisms. Annu. Rev. Nutr. **1**:27–46.
21. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50**:715–731.
22. **Neilands, J. B.** 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. **36**:285–309.
23. **Norqvist, A., J. Davies, L. Norlander, and S. Normark.** 1978. The effect of iron starvation on the outer membrane protein composition of *Neisseria gonorrhoeae*. FEMS Microbiol. Lett. **4**:71–75.
24. **Norrod, P., and R. P. Williams.** 1978. Growth of *Neisseria gonorrhoeae* in media deficient in iron without detection of siderophores. Curr. Microbiol. **1**:281–284.
25. **Payne, S. M., and R. A. Finkelstein.** 1978. The critical role of iron in host-bacterial interactions. J. Clin. Invest. **61**:1428–1440.
26. **Reich, C. V., and J. H. Hanks.** 1964. Use of *Arthrobacter terregens* for bioassay of mycobactin. J. Bacteriol. **87**:1317–1320.
27. **Schleif, R. F., and P. C. Wensink.** 1981. Practical methods in molecular biology. Springer-Verlag, New York.
28. **Sparling, P. F.** 1966. Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J. Bacteriol. **92**:1364–1371.
29. **Stookey, L. L.** 1970. Ferrozine—a new spectrophotometric reagent for iron. Anal. Chem. **42**:779–781.
30. **Tam, M. R., T. M. Buchanan, E. G. Sandström, K. K. Holmes, J. S. Knapp, A. W. Siadak, and R. C. Nowinski.** 1982. Serological classification of *Neisseria gonorrhoeae* with monoclonal antibodies. Infect. Immun. **36**:1042–1053.
31. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
32. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**:197–203.
33. **Yancey, R. J., and R. A. Finkelstein.** 1981. Assimilation of iron by pathogenic *Neisseria* spp. Infect. Immun. **32**:592–599.
34. **Yancey, R. J., and R. A. Finkelstein.** 1981. Siderophore production by pathogenic *Neisseria* spp. Infect. Immun. **32**:600–608.