INFECTION AND IMMUNITY, Apr. 1988, p. 977–983
0019-9567/88/040977-07$02.00/0

Vol. 56, No. 4

A Pleiotropic Iron-Uptake Mutant of *Neisseria meningitidis* Lacks a 70-Kilodalton Iron-Regulated Protein

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Received 4 November 1987/Accepted 22 December 1987

We isolated an iron-uptake mutant of *Neisseria meningitidis* M986-NCV-1 that was severely limited in the ability to use several sources of iron in the form of Fe^{3+}. This mutant, FAM11, grew poorly or not at all with human transferrin (TF) or lactoferrin (LF) as the sole iron source in a defined medium but grew as well as wild-type meningococci with hemin or hemoglobin. Uptake of ^{55}Fe bound to TF, LF, dicitrate complexes, aerobactin, or nitrilotriacetate was reduced to 0 to 4% of the wild-type level. FAM11 did not produce an iron-repressible outer membrane protein (FeRP) of 70 kilodaltons (kDa) found in membranes of iron-stressed M986-NCV-1. Western blot (immunoblot) analysis using rabbit antiserum against this protein revealed that at least 17 of 18 meningococcal and 10 of 14 gonococcal strains produced an FeRP of ca. 70 kDa. The 70-kDa FeRP was shown to be surface exposed by radioimmunoprecipitation with human immune sera. These data suggest that the 70-kDa FeRP is somehow involved in Fe uptake from TF and LF. However, we were unable to transform the iron-uptake phenotype from FAM11 into wild-type meningococci to confirm this. Revertants of FAM11 that grew with TF and LF did not regain the ability to make the 70-kDa FeRP but also did not completely regain the Fe-uptake phenotype of M986-NCV-1.

The ability to obtain iron (Fe) from the infected host is an important determinant in infection caused by many bacterial pathogens (30). The work of Holbein and co-workers with experimentally infected mice suggested that this is also true for *Neisseria meningitidis* (14–17), since the availability of transferrin (TF)-bound Fe was correlated with the progress of meningococcemia in these mice (14–16). Mice pretreated with an injection of iron dextran or Fe-TF developed a lethal septicemia when subsequently injected with meningococci, whereas mice injected with bacteria alone were only transiently bacteremic (14). This transient meningococcemia coincided with the kinetics of the hypoferremic response in these infected mice (14), suggesting that the ability to use Fe-TF for growth was critical to the ability of *N. meningitidis* to multiply in the bloodstream (14). Although *N. meningitidis* is capable of using exogenous aerobactin for growth (33), the organism does not produce siderophores (1, 28), as do many other bacteria. Instead, meningococci remove Fe directly from host Fe sources such as Fe-TF or Fe-lactoferrin (Fe-LF) (1, 28).

We have used the bactericidal action of streptonigrin (SNG) to isolate meningococcal Fe-uptake mutants (7a). Our strategy was based on the observations of Yeowell and White (34), who showed that the bactericidal effect of SNG on *Escherichia coli* depended on intracellular Fe levels. Bacteria with low intracellular Fe levels were relatively resistant to SNG, whereas Fe-replete organisms were rapidly killed by the drug (34). We found that SNG killing of meningococci was similarly affected by intracellular Fe (7a), and SNG could be used to enrich for meningococcal Fe-uptake mutants (7a). A culture of actively growing bacteria treated with SNG in the presence of a particular Fe source (such as Fe-TF) yielded survivors enriched for mutants incapable of using that Fe source (7a). Here we present an analysis of a meningococcal iron-uptake mutant which is impaired in the ability to use nonheme Fe for growth.

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MATERIALS AND METHODS

Bacteria, growth conditions, and isolation of mutant. *N. meningitidis* M986-NCV-1, a nonencapsulated derivative of strain M986 (10), was used in this study; M986 is serogroup B, serotype 2a. Meningococci were routinely maintained on GCB agar (Difco Laboratories), containing Kellogg supplements I and II (18), at 37°C in a 5% CO_2 atmosphere. To assess growth with a specific Fe source, we used an iron-depleted defined medium, designated CDM, which was previously described (32). Glassware was washed with acid to remove contaminating Fe (25). CDM agar plates were prepared by the addition of sterile molten agarose to a final concentration of 1%. To examine the kinetics of growth in liquid culture, meningococci were grown overnight on CDM agar, suspended in CDM broth, and grown to the mid-to-late log phase. These cells were then diluted into prewarmed CDM supplemented with an Fe source of interest. Growth was monitored by optical density by using a Klett-Summerson colorimeter with a green filter.

Strain FAM11 was isolated from an ethyl methanesulfonate-mutagenized culture of M986-NCV-1 after SNG enrichment and in plate assays was unable to use Fe-TF for growth. The details of ethyl methanesulfonate mutagenesis and SNG enrichment have been described (7a). Briefly, meningococci treated with ethyl methanesulfonate were grown to the late log phase in a medium containing human Fe-TF as the sole source of Fe. SNG (final concentration, 1 μg/ml) was then added to the culture to kill wild-type Fe-replete meningococci; FAM11 was identified as a survivor which did not use Fe-TF for growth in plate assays.

Iron sources. Human TF and LF were obtained from Sigma Chemical Co. and were at least 98% pure according to the supplier. Preparation of Fe-loaded forms of these proteins has previously been described (24, 25). Aerobactin (provided by S. E. H. West) was purified by the method of Bindereif (A. Bindereif, M. S. thesis, University of California, Berkeley, 1980) and made 50% saturated with ^{55}Fe. ^{55}Fe-nitrilotriacetate (NTA) complexes were prepared by

adding a twofold molar excess of sodium NTA to $^{55}FeCl_3$. Determination of Fe uptake from ^{55}Fe-labeled Fe sources was performed as previously described (Dyer et al., in press). $^{55}FeCl_3$ was from New England Nuclear Corp.; the specific activity of the ^{55}Fe was 235 cpm/pmol.

Heme was prepared fresh daily in 10 mM NaOH. Human hemoglobin (Hb) A (Sigma) was also prepared fresh daily, in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4). In some experiments, the growth medium was supplemented with 10% heat-inactivated (56°C for 30 min) human serum obtained from a pool of volunteers with no known overt disease caused by *N. meningitidis* or *Neisseria gonorrhoeae*. Protein concentration was measured by the method of Bradford (5), using the Bio-Rad protein assay, and Fe concentration was estimated by using the ferrozine reagent (29).

Analysis of membrane proteins. Sarcosyl-insoluble outer membranes were isolated and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (31). Western blots (immunoblots) were performed as described by Black et al. (4), using rabbit antiserum prepared against the 70-kilodalton (kDa) iron-repressible outer membrane protein (FeRP) purified by preparative SDS-PAGE (4). Colony immunoblots were used in some experiments to detect production of the 70-kDa FeRP in meningococcal colonies. To do this, colony lifts were made by placing a Millipore HATF nitrocellulose filter (diameter, 47 mm) over meningococcal colonies growing on CDM agar. After 30 min., the nitrocellulose was placed colony side up on Whatman 3MM filter paper soaked in 0.1 M $NaHCO_3$–1% SDS–100 μg of lysozyme per ml. The cells were lysed with $CHCl_3$ vapors at room temperature for 30 min; the colony lift was then placed onto a second filter soaked with 0.1 M $NaHCO_3$–0.1% SDS and baked for 1 h at 68°C. The nitrocellulose filter was then probed with rabbit anti-70-kDa FeRP serum as described for Western blots (4). This heat and detergent treatment was necessary to denature the antigen sufficiently to react with the rabbit antiserum (data not shown).

Radioimmunoprecipitation. Log-phase, Fe-stressed meningococci were concentrated by centrifugation and suspended in modified Dulbecco PBS (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 7.35 mM KH_2PO_4, 0.25 mM $CaCl_2$, 1.0 mM $MgCl_2$ [pH 7.4]). The cells were then surface labeled by radioiodination with 500 μCi of carrier-free $Na^{125}I$ (Amersham Corp.), using Iodobeads (Pierce Chemical Co.) as described by Markwell (22). The cells were washed three times with PBS by centrifugation in an Eppendorf Microfuge. The radiolabeled cells were then used in radioimmunoprecipitation essentially as described by Hansen et al. (13). Radioiodinated cells (approximately 10^7 cpm) were added to an Eppendorf tube containing 400 μl of PBS with 5% fetal calf serum. This mixture was incubated for 10 min at room temperature, followed by the addition of a 50- to 100-μl sample of normal human serum or serum obtained from an individual convalescing from disseminated neisserial infection. The radioiodinated cells and antiserum were then incubated at 4°C for 90 min with gentle mixing. The cells were pelleted and washed once with 1 ml of cold PBS to remove unbound antibody and suspended in 1 ml of triple detergent buffer (TD buffer) (0.1% SDS, 0.2% sodium deoxycholate, 1.0% Triton X-100, 10 mM Tris hydrochloride [pH 7.8]); the cells were lysed at 37°C for 1 h with occasional vigorous mixing to disperse clumps of cells. The lysate was then centrifuged for 30 min in a Microfuge at room temperature. The supernatant was carefully removed, and a portion was saved for SDS-PAGE analysis. A 100-μl portion of IgGSorb (The Enzyme Center, Inc., Malden, Mass.) in TD buffer was added to the remainder and mixed gently for 10 min. Before use, the IgGSorb was incubated with lysed nonradioactive meningococci to block nonspecific binding of meningococcal antigens to the immunosorbent.

After adsorbing antigen-antibody complexes, the IgGSorb was washed by centrifugation six times in 1.0 ml of TD buffer. The final pellet was suspended in 100 μl of SDS-PAGE solubilizing buffer and boiled for 5 min, and the IgGSorb was removed by centrifugation. The supernatant, containing immunoprecipitated radiolabeled meningococcal cell surface antigens, was electrophoresed on a 10 or 15% SDS-polyacrylamide gel; radioiodinated proteins were identified by autoradiography of the dried gel.

Genetic procedures. Transforming DNA was isolated from meningococci as described by Marmur (23), and meningococci were transformed as described for *N. gonorrhoeae* by Sarubbi et al. (27). Streptomycin-resistant transformants were selected on GCB plates containing 100 μg of antibiotic per ml.

RESULTS

Growth kinetics. In preliminary plate assays, FAM11 did not grow with Fe-TF or Fe-LF as the sole source of Fe in CDM agar. In liquid CDM, strain M986-NCV-1 grew rapidly and to a high final cell density with Fe-TF as the sole source of Fe (Fig. 1), whereas FAM11 grew poorly (Fig. 1). The Fe-TF concentration in this experiment was 30 μM and the Fe-binding capacity was 25% saturated, which approximates physiological conditions (30). Similarly, FAM11 was unable to grow when CDM was supplemented with 13 μM Fe-LF (5% saturated) or 10% heat-inactivated normal human serum (data not shown), although these conditions supported rapid and extensive growth of M986-NCV-1. However, FAM11 grew as well as the parent strain with heme as the sole source of Fe in CDM (Fig. 2); similar results were obtained with Hb (data not shown), indicating that heme and Hb utilization by FAM11 was unimpaired, even though growth with Fe-TF and Fe-LF was dramatically reduced.

^{55}Fe uptake. The data for ^{55}Fe uptake are presented in Table 1. Whereas strain M986-NCV-1 was capable of rapidly internalizing large amounts of ^{55}Fe bound to TF in an energy-dependent manner (Table 1), we were unable to detect any net ^{55}Fe accumulation by FAM11 from TF above the ^{55}Fe bound to KCN-poisoned cells, which do not internalize Fe (2, 28). Similarly, we were unable to detect the uptake of ^{55}Fe by FAM11 when the Fe was bound to LF, under conditions in which the uptake of ^{55}Fe from 5%-saturated LF by M986-NCV-1 was detectable. We used 5%-saturated LF in these experiments to approximate the low level of Fe found on LF in vivo (9). Using higher concentrations of ^{55}Fe bound to 13 μM LF increased the amount of ^{55}Fe internalized by M986-NCV-1 from LF, but uptake of ^{55}Fe from LF by FAM11 was still undetectable (data not shown).

Meningococci also internalize ^{55}Fe from dicitrate complexes (2); citrate is not internalized (2), similar to what occurs in the citrate-inducible Fe-uptake system in *E. coli* (11). However, unlike uptake by *E. coli* (11), citrate-dependent Fe uptake by meningococci does not depend on induction by citrate (2). We found that FAM11 internalized only about 2% of the amount of ^{55}Fe taken up by M986-NCV-1 from 1 μM Fe-dicitrate (Table 1). Increasing the concentration of ^{55}Fe-dicitrate to 10 μM yielded similar results (data

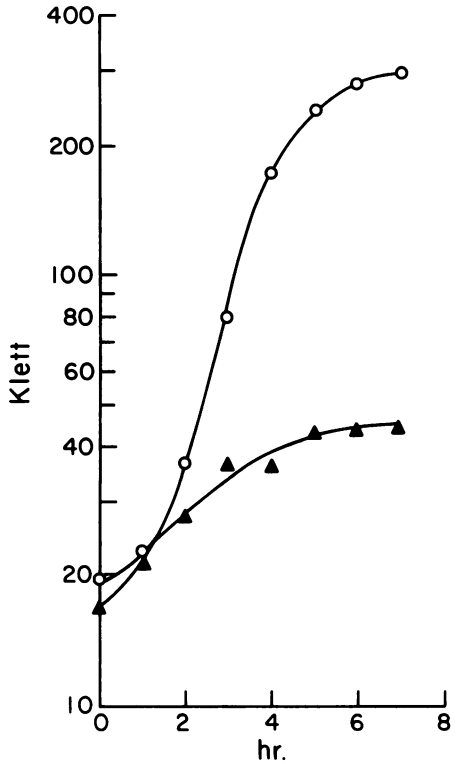

FIG. 1. Inability of FAM11 to grow with human Fe-TF. Log-phase M986-NCV-1 (○) or FAM11 (▲) cells were diluted into CDM supplemented with 30 μM human Fe-TF (25% saturated), and growth was monitored by optical density.

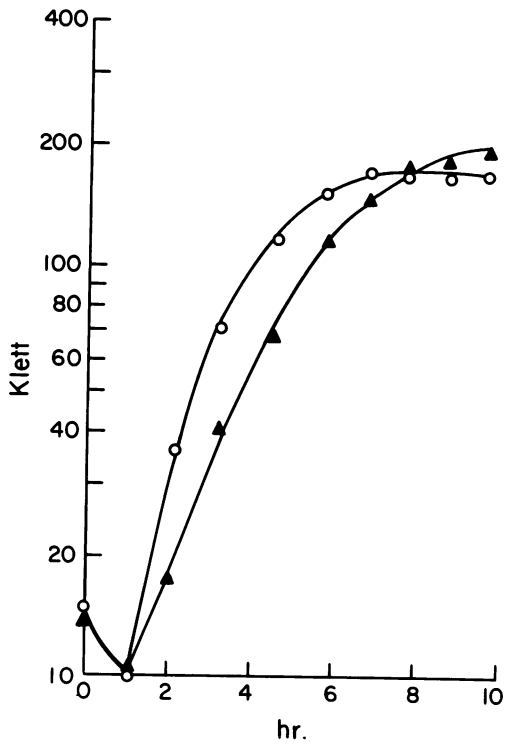

FIG. 2. Growth kinetics of M986-NCV-1 (○) and FAM11 (▲) with 5 μM heme in CDM. The slight reduction in the doubling time of log-phase FAM11 in this experiment is not significant. In other experiments, the growth of FAM11 equaled or exceeded that of M986-NCV-1, suggesting that the growth of FAM11 with heme is equivalent to that of the wild type.

not shown). This residual level of ^{55}Fe uptake by FAM11 was not inhibited by NTA (Table 1), indicating that the low-level Fe uptake by FAM11 was not via a low-affinity NTA-inhibitable uptake system, like that found in *E. coli* (11). Since meningococci have been shown to use the dihydroxamate siderophore aerobactin for growth (33), we determined the amount of ^{55}Fe taken up by M986-NCV-1 and FAM11 from this Fe source. FAM11 internalized approximately 1% of the amount of ^{55}Fe from aerobactin taken up by M986-NCV-1. Taken together, the Fe-uptake data suggested that FAM11 was impaired in the ability to take up ^{55}Fe from any Fe^{3+} source that we examined. Since ^{55}Fe-heme is not commercially available, we did not determine how efficiently FAM11 internalizes ^{55}Fe from this source. However, the wild-type growth of FAM11 with heme (Fig. 2) and Hb (data not shown) suggests that heme Fe utilization is unimpaired.

FAM11 lacks a 70-kDa FeRP. In many bacteria, Fe-regulated outer membrane proteins (OMPs) are often involved as OM components of high-affinity Fe-uptake systems (26). Thus, we prepared OM from FAM11 grown in CDM or CDM plus 100 μM $Fe(NO_3)_3$ and compared the OMP profiles of the mutant with the OMPs of wild-type meningococci grown similarly (Fig. 3). The OM of strain M986-NCV-1 grown under Fe-limiting conditions contained FeRPs of 37, 70, 75, and ca. 105 kDa (Fig. 3, arrows). In contrast, membranes of FAM11 lacked the prominent 70-kDa FeRP found in M986-NCV-1; additionally, the remaining FeRPs were expressed constitutively rather than regulated by Fe limitation. When reacted with rabbit antiserum directed against the 70-kDa FeRP (4), whole-cell lysates of FAM11 did not possess any immunoreactive material as assessed by either Western blot or colony immunoblot (data not shown). Thus, FAM11 did not produce detectable 70-kDa FeRP.

Recently, van Putten (Proc. 5th Int. Pathog. Neisseria Conf., abstr. V-131, 1986) used chemostat cultures to show that certain meningococcal OMPs are synthesized in response to lowered growth rate; such proteins were termed GSPs (growth-rate-sensitive proteins). Since Fe starvation also slows meningococcal growth, we wondered whether the

TABLE 1. ^{55}Fe uptake by M986-NCV-1 and FAM11

Strain	^{55}Fe uptake (pmol/10^8 CFU per 30 min) from iron source:				
	Fe-TF (30 μM, 26% saturated)	Fe-LF (13 μM, 5% saturated)	Fe-dicitrate (1 μM Fe)	Fe-aerobactin (10 μM Fe)	Fe-NTA (1 μM Fe)
M986-NCV-1	439.7	19.2	508.6	1,614.5	149.6
FAM11	0	0	9.8 (1.9%)[a]	21.3 (1.3%)	6.2 (4.1%)

[a] Percentage of uptake by M986-NCV-1.

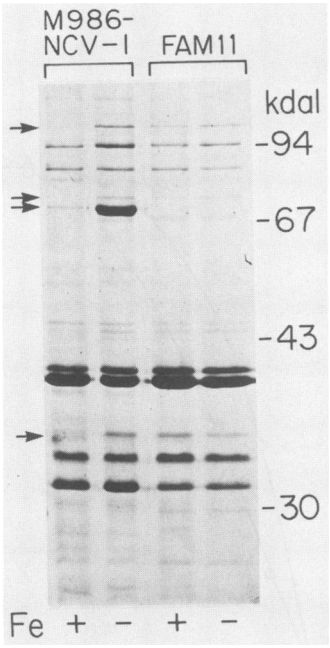

FIG. 3. SDS-PAGE of meningococcal OMPs. M986-NCV-1 and FAM11 were grown in CDM or CDM supplemented with 100 μM $Fe(NO_3)_3$, and sarcosyl-insoluble OMs were prepared, electrophoresed on a 10% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue (31). FeRPs produced by M986-NCV-1 are identified by the arrows. The FeRPs were constitutively produced by FAM11, except for the 70-kDa FeRP, which was missing. The migration of molecular mass standards is shown on the right. kdal, Kilodaltons.

70-kDa FeRP is regulated not by Fe stress but by changes in growth rate. Consequently, we slowed the growth of M986-NCV-1 by manipulating pH, temperature, or aeration and saw no appreciable effect on the synthesis of the 70-kDa protein. A typical result is presented in Fig. 4, which shows the growth of M986-NCV-1 in CDM at 37°C in a 5% CO_2 atmosphere when the organism was incubated either in static cultures (low aeration) or shaken vigorously at 250 to 300 rpm (high aeration). In rapidly shaken cultures, CDM supplemented with 100 μM $Fe(NO_3)_3$ supported rapid meningococcal growth and meningococci grew to a much greater final optical density than in CDM. Without vigorous aeration, M986-NCV-1 grew at essentially the same rate in CDM or CDM plus 100 μM $Fe(NO_3)_3$. When the relative amounts of the 70-kDa protein were assessed by Western blot, only those cultures deprived of Fe produced measurable amounts of the protein (Fig. 4, inset, and data not shown). Similar results were obtained with meningococci grown at pH 6.6 or 30°C (data not shown). Thus, Fe deprivation but not slow growth resulted in production of the 70-kDa FeRP.

We attempted to transfer the Fe-uptake deficit of FAM11 back into wild-type meningococci to determine whether the inability to produce the 70-kDa FeRP was responsible for the Fe-uptake phenotype. Initial transformations using M986-NCV-1 or the encapsulated parent, M986 (obtained from C. Frasch), as the recipient indicated that neither strain was competent for transformation. We were unable to select competent derivatives of these strains. Subsequently, we used a second strain, FAM18, as a recipient in these genetic crosses. *N. meningitidis* FAM18 is a serogroup C, serotype 2a strain that we have used in other experiments (7a); it makes a 70-kDa FeRP that cross-reacts immunologically with the 70-kDa FeRP of M986-NCV-1 (data not shown). Since SNG may be mutagenic in the neisseriae (7a) we chose to do this transformation by congression rather than after SNG enrichment. We first isolated a spontaneous Str^r mutant of FAM11; after transforming FAM18 for Str^r by using saturating concentrations of DNA (1 μg/ml), 2,019 transformants were transferred individually with toothpicks to GCB, CDM-TF, and CDM-LF plates to identify recipients that had received the TF^- phenotype. Although Str^r transformants of FAM18 were obtained at a frequency of ca. 10^{-4}, we did not identify any transformant that had lost the ability to use Fe-TF or Fe-LF for growth. Thus, we were not able to determine whether the loss of the 70-kDa FeRP was directly responsible for the Fe-uptake phenotype of FAM11.

We also looked for revertants of FAM11 which were able to use Fe-TF and Fe-LF for growth to determine whether restoration of the ability to use these Fe sources coincided with renewed expression of the 70-kDa FeRP. This was done by plating FAM11 onto CDM plates containing 5 μM apo-TF or apo-LF; revertant colonies able to use these Fe sources appeared at a frequency of 3.4×10^{-6} (on TF plates) or 3.9×10^{-6} (on LF plates). We screened 40 TF^+ revertants of FAM11 and found that each had regained the ability to use Fe-LF for growth; similarly, each of 40 tested LF^+ revertants grew with Fe-TF as the sole Fe source. None of these revertants, however, appeared to synthesize the 70-kDa FeRP, as assayed by colony immunoblot (data not shown). We also probed colony blots of FAM11 grown on CDM agar in an attempt to identify directly spontaneous revertants that had regained the ability to synthesize the 70-kDa FeRP. This

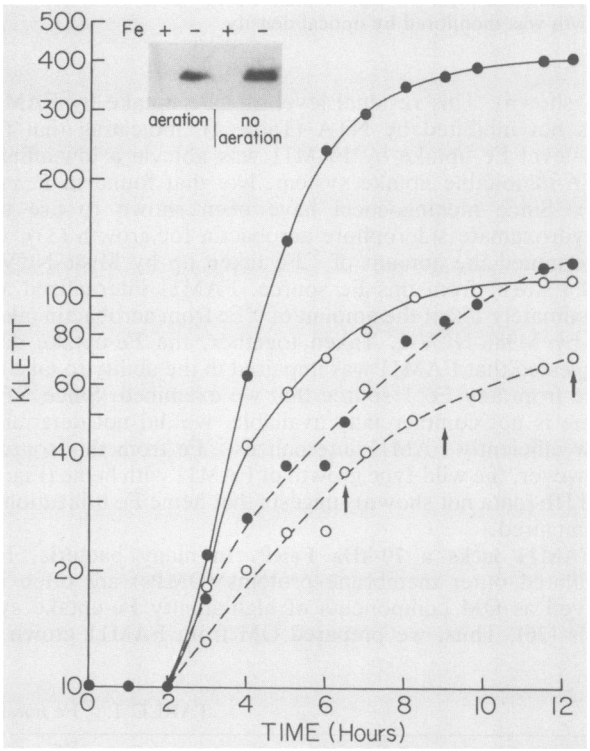

FIG. 4. Growth conditions and production of the 70-kDa FeRP. Strain M986-NCV-1 was grown in CDM (- - - -) or CMD plus 100 μM $Fe(NO_3)_3$ (—) with vigorous aeration (●) or in static culture (○). At the times indicated by the arrows, cells were removed from each culture and assayed for production of the 70-kDa FeRP by Western blot. The inset shows the results of a Western blot of cells taken at 6.5 h; later times gave similar results.

also was unsuccessful in identifying revertants of FAM11 that produced the 70-kDa FeRP. Thus, we were unable to associate directly the ability to produce the 70-kDa FeRP with the ability to use TF and LF as Fe sources.

Closer examination of selected TF^+ and LF^+ revertants, however, suggested that these strains did not regain wild-type growth kinetics with protein-bound Fe. The kinetics of growth in CDM supplemented with Fe-TF (Fig. 5) indicated that FAM63 (a TF^+ LF^+ revertant of FAM11) did not grow as efficiently as strain M986-NCV-1 with 30 μM Fe-TF (25% saturated); similar results were obtained with CDM supplemented with Fe-LF or inorganic Fe (data not shown). With each Fe source, FAM63 demonstrated at least a 1-h lag before growth commenced, and cultures of FAM63 did not reach the same final optical density as that of M986-NCV-1 cultures; log-phase doubling times of the revertant and M986-NCV-1 were identical, however. Similar results were obtained with three other independently isolated revertants (data not shown). We next compared the ^{55}Fe uptake of FAM63 to that of M986-NCV-1. FAM63 internalized 37% of the ^{55}Fe taken up by M986-NCV-1 from Fe-TF in 30 min. ^{55}Fe uptake from LF and dicitrate complexes was also reduced (65 and 81%, respectively, of the wild-type level at 30 min). This reduction in ^{55}Fe uptake and the altered growth kinetics of FAM63 (Fig. 5) suggested that this and other revertants had not regained wild-type ability to use Fe-TF and Fe-LF for growth; these revertants may constitute extragenic suppressors of the original TF^-/LF^- mutation in FAM11. We also examined the OMP profile of four revertants and found them to be identical. In each instance, no new proteins were found in the OM, however, FeRPs that were constitutively produced in the OM of FAM11 (Fig. 3) regained the Fe regulation observed in M986-NCV-1 (data not shown).

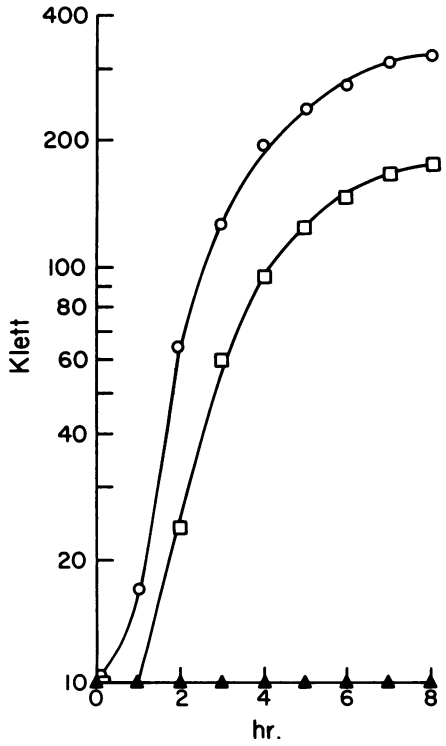

FIG. 5. Growth of TF^+ revertant FAM63 with human Fe-TF. Log-phase M986-NCV-1 (○), FAM63 (□), and FAM11 (▲) cells were diluted into CDM containing 30 μM Fe-TF (25% saturated). Although FAM63 grew well with Fe-TF, it did not display wild-type kinetics. The growth of FAM63 with Fe-LF or Fe-dicitrate was similarly reduced compared with that of M986-NCV-1.

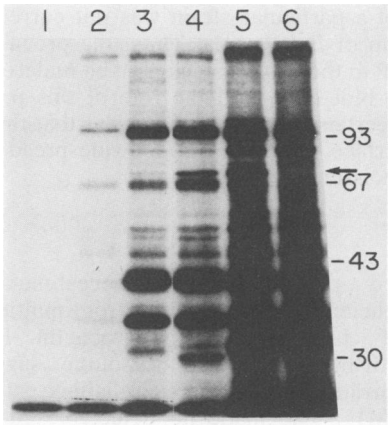

FIG. 6. Radioimmunoprecipitations of meningococcal OMPs with human convalescent-phase sera. Strain M986-NCV-1 was radioiodinated after growth in CDM or CDM plus 100 μM $Fe(NO_3)_3$; radioimmunoprecipitations were then performed as described in the text, the immunoprecipitates were electrophoresed on a 10% SDS-polyacrylamide gel, and an autoradiogram was made of the dried gel. The migration of molecular mass markers (masses indicated in kilodaltons) is shown on the right. Lanes: 1, PBS control; 2, normal human serum control; 3, convalescent-phase serum from a patient with meningococcemia; 4, convalescent-phase serum from a patient recovering from disseminated gonococcal infection; 5 and 6, radioiodination profile of Fe-stressed (lane 5) and Fe-replete (lane 6) strain M986-NCV-1.

The 70-kDa FeRP is a common, surface-exposed antigen. It was shown that the 70-kDa FeRP elicits antibody in infected humans (4). We used convalescent-phase sera from patients recovering from meningococcal or gonococcal disease to examine the surface exposure of the 70-kDa FeRP by radioimmunoprecipitation (Fig. 6). Radioiodination of whole cells of M986-NCV-1 resulted in incorporation of ^{125}I into the 70-kDa protein (Fig. 6, lane 5, arrow), but this may not directly indicate surface exposure (21). However, convalescent-phase serum from a patient recovering from disseminated gonococcal infection (Fig. 6, lane 4) or from meningococcal infection (Fig. 6, lane 3) immunoprecipitated the 70-kDa FeRP; the amounts of immunoprecipitable 70-kDa FeRP varied but were always greater than for a PBS or normal human serum control (Fig. 6, lanes 1 and 2, respectively). These results suggest that the protein is surface exposed and further indicate that the protein is cross-reactive between meningococcal and gonococcal strains. We previously showed that each of four tested meningococcal strains produced a 70-kDa FeRP that cross-reacted with antiserum directed against the protein made by *N. meningitidis* FAM20 (4). Using Western blots, we found that an additional 17 of 18 meningococcal strains and 10 of 14 gonococcal strains produced an FeRP of approximately 70 kDa (data not shown). Recently, one of us (W.M.) has observed that convalescent-phase sera from two patients recovering from disseminated gonococcal infection contained immunoglobulin G antibodies against the 70-kDa FeRP, although the gonococcal strains isolated from these patients did not produce the 70-kDa FeRP when Fe stressed in vitro. All tested meningococci and gonococci used TF for growth in vitro (25); all tested meningococci and 60% of gonococcal strains used LF for growth (24). The iron-uptake

phenotype of a particular strain was not correlated with a specific group of FeRPs (31). Thus, the prevalence of the 70-kDa FeRP in these pathogens may be underestimated by the Western blot data, and the role of this protein in Fe uptake is uncertain. However, it is clear that this antigen is not only surface exposed but is widespread within the pathogenic neisseriae.

DISCUSSION

We isolated a pleiotropic meningococcal mutant that was markedly deficient in uptake of Fe from multiple sources, including TF, LF, citrate, and aerobactin. It did grow normally with heme as a sole Fe source, suggesting that heme Fe utilization occurs through distinct pathways. The mutant, FAM11, also made no detectable 70-kDa FeRP, suggesting that the 70-kDa protein may be involved in uptake or utilization of Fe from TF, LF, citrate, or aerobactin. The observation that the 70-kDa FeRP is surface exposed is consistent with this possibility.

Our inability to transform the mutant phenotype into wild-type competent meningococci prevented more definite conclusions about the relationship between the 70-kDa FeRP and Fe uptake. It is not clear why we were unable to transform the Fe-uptake-deficient phenotype from FAM11 into FAM18. Using similar techniques, we were able to transfer an unrelated mutation that results in specific loss of the ability to use TF as an Fe source into FAM18 (7a); in the latter instance, the donor and recipient were isogenic, whereas in the present study the donor (FAM11) was serogroup B and the recipient (FAM18) was serogroup C. Thus, it is possible that genetic differences between strains prevented efficient transformation in the present experiments. It also is possible that FAM11 contains multiple unlinked mutations, which would markedly impair the ability to transform the Fe-uptake-deficient phenotype from FAM11 into another strain. This is consistent with the observation that TF^+ and LF^+ revertants of FAM11 did not regain wild-type Fe-uptake ability. There could be other explanations as well. Our results are compatible with the hypothesis that the 70-kDa FeRP is involved in some manner in Fe^{3+} uptake from diverse sources, but better evidence is required, preferably from introduction of a specific mutation of the cloned structural gene for the 70-kDa FeRP.

The complicated phenotype of FAM11 is similar in some respects to that of *tonB* mutants of *E. coli* (12). *tonB* function is required in *E. coli* to provide energy for high-affinity Fe^{3+} uptake from diverse sources, as well as for the uptake of vitamin B_{12} (12). We used a cloned *E. coli tonB* gene to probe genomic digests of meningococcal or gonococcal DNA and found no evident homology (E. Rosenberg, unpublished data) under conditions of low stringency which had been previously used to detect a functional gonococcal analog of the *E. coli fhuB* gene (32).

The biochemical defects in FAM11 that prevented the use of Fe^{3+} bound to TF, LF, citrate, or aerobactin are uncertain. One possibility is the loss of an Fe reductase active on extracellular Fe. This is consistent with the observation that FAM11 appears to grow normally with heme or Hb as the sole source of Fe; reduction of Fe bound in a porphyrin ring occurs readily, but the Fe atom remains bound to the heme moiety (20). The reduction of Fe^{3+} to Fe^{2+} is known to favor the release of Fe from TF (19). *Listeria monocytogenes* releases Fe from TF by a reductive mechanism in the absence of a siderophore (6). Both M986-NCV-1 and FAM11 reduce appreciable amounts of extracellular Fe^{3+}-dicitrate (data not shown); since meningococci contain an OM cytochrome oxidase (7), this is probably not surprising. The relationship of this OM cytochrome oxidase to Fe uptake is not clear. However, A. E. Lefaou and S. A. Morse (Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D-20, p. 75) have found evidence for both cytoplasmic and membrane-bound Fe^{3+} reductases in gonococci and meningococci; they kindly examined both M986-NCV-1 and FAM11 for each of these Fe reductases and found no significant differences between wild-type and mutant meningococci (data not shown).

The suggestion that a surface-exposed iron-regulated protein may be critically involved in Fe uptake from TF and LF is provocative. TF and LF are thought to be the primary Fe sources for both meningococci and gonococci in vivo (8, 14, 16, 24, 25). This antigen is common to many of the pathogenic neisseriae that we examined, although not all strains of *N. gonorrhoeae* and *N. meningitidis* expressed a protein which reacted with our rabbit antiserum. Further studies are necessary to demonstrate whether this protein is involved in Fe uptake by *N. meningitidis* and *N. gonorrhoeae*.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI23357 (to D.W.D.), AI15036 (to P.F.S.), and AI07001 (to W.M.) from the National Institute of Allergy and Infectious Diseases and training grant GM07092 (to S.A.T.) from the National Institute of General Medical Sciences.

Special thanks are given to Lynn Brooks for preparing the manuscript, to Susan West for the gift of purified gonococcal membranes, and to Nicholas Carbonetti and David Trees for helpful suggestions.

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