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Energy-Independent Uptake of Iron from Citrate by Isolated Outer Membranes of *Neisseria meningitidis*

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Cyanide-poisoned *Neisseria meningitidis* SD1C cells rapidly took up ^{55}Fe from iron-citrate complexes during the first 2 min, after which no further iron was accumulated. [^{14}C]citrate was not taken up concomitantly with ^{55}Fe by these cells. The ^{55}Fe taken up by the poisoned cells was found in the membrane fraction after cells were broken; 70% of the radioactivity was distributed in the outer membrane, and 30% was in the inner membrane. Isolated outer membranes from iron-starved cells were as capable of iron uptake from citrate as intact cells were. As with whole cells, [^{14}C]citrate was not taken up by isolated outer membranes. A polyacrylamide gel electrophoresis analysis of the proteins from citrate-dialyzed outer membranes after the uptake of ^{55}Fe revealed that the radioactivity was associated with a major band of 36,500 molecular weight.

The acquisition of iron from vertebrate hosts has been associated with the ability of a variety of pathogenic microbes to establish and maintain infections (35). Iron is an essential nutrient for nearly all bacteria (26, 27), including meningococci (2). Therefore, the mechanism of iron acquisition from hosts is an important consideration among the factors involved in meningococcal pathogenicity.

In a recent report from this laboratory (4) 20 strains of meningococci were analyzed for their ability to obtain iron from a variety of sources. Among the 20 strains, eight different serogroups were represented; there were 5 nasopharyngeal isolates and 11 isolates from patients with acute disease, and the 50% lethal doses for mice ranged from $>10^8$ to <2 colony-forming units. The results were essentially the same for all of the strains tested; i.e., all meningococci tested had similar iron-acquiring abilities irrespective of serogroup, source of isolation, and virulence for mice. The meningococcal strains exhibited high-affinity iron-acquiring capabilities and showed a preference for phosphate ester- or carboxylic acid-bound iron. Such functional siderophores made iron available to the meningococci from sources which otherwise would be unavailable (e.g., ferritin, mucin, $FePO_4$). The only siderophore-like compound which was found to function as a siderophore for meningococci was salicylhydroxamate, which facilitated the removal of usable iron from ferritin, hog gastric mucin, and $FePO_4$ (4).

Other recent evidence has shown that meningococci can use human transferrin-bound iron as a sole source of iron for growth (3). In that study, the organism was unable to obtain the transferrin-bound iron if the transferrin was separated from the culture by a dialysis membrane. On the other hand, the iron on the transferrin was readily available if the organism came into direct contact with the transferrin molecules.

In this report we present evidence that iron is removed from citrate by an energy-independent mechanism without the uptake of citrate itself. Moreover, isolated outer membranes are as capable of iron uptake from citrate as whole cells are, and such bound iron is associated with a major outer membrane protein peak on polyacrylamide gels after electrophoresis.

MATERIALS AND METHODS

The smooth colonial variant (11) of group B *Neisseria meningitidis* SD1C was used throughout this study. The procedures for routine maintenance of stock cultures and checks for strain purity have been described elsewhere (10). The formulation for the *Neisseria* defined medium (NDM) used was described previously (2). Cells from frozen (−70°C) working slant cultures on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) were cultured as a lawn on NDM agar containing added iron (final concentration, 50 ng of ferrous ammonium sulfate per ml) for 13 h (37°C, 5% CO_2 in air, high humidity). Cells were harvested from the plates and suspended in NDM broth containing 50 ng of ferrous ammonium sulfate per ml (final concentration) to a final optical density at 600 nm of 0.3 (model 250 spectrophotometer; Gilford Instrument Laboratories, Ontario, Canada). A 75-ml volume of this suspension was the inoculum for 1,425 ml of NDM. The final culture medium contained less than 10 ng of Fe per ml as contaminating iron before inoculation. Cultures in this iron-limited medium (Fig. 1) were incubated at 37°C, in 5% CO_2 in air until division in

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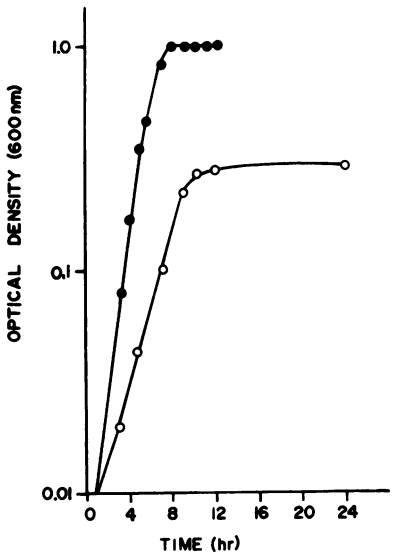

FIG. 1. *Iron-limited growth of N. meningitidis SD1C in NDM. Symbols: ○, low-iron NDM (<6 ng of Fe per ml); ●, high-iron NDM (200 ng of Fe per ml).*

the culture ceased due to iron limitation (optical density at 600 nm, 0.3 to 0.4).

Cells were harvested from the medium by centrifugation at 6,000 × *g* for 10 min, the supernatant fluids were discarded, and the pellets were suspended in 20 ml of cold (4°C) morpholinepropanesulfonic acid (MOPS) buffer (Sigma Chemical Co., St. Louis, Mo.), pH 7.5. Cells were broken by extrusion from a precooled French pressure cell (12,000 to 15,000 lb/in^2). "Cell debris" and unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant solution was centrifuged at 70,000 × *g* for 1 h with a Beckman L5-65 ultracentrifuge and a 60Ti rotor. The pellet containing cell envelopes (inner and outer membranes) was resuspended in 20 ml of cold MOPS buffer (pH 7.5), and the suspension was again centrifuged at 70,000 × *g* for 1 h. Washed membranes were suspended in 9 ml of cold MOPS; 1 ml of a 10% solution of sodium *N*-lauroyl sarcosinate (ICN Pharmaceuticals, Inc., Plainview, N.Y.) was added to the membrane suspension to solubilize selectively the inner (cytoplasmic) membranes, as described by Filip et al. (14). This suspension was incubated for 30 min at 37°C, and the insoluble outer membranes were harvested selectively by centrifugation at 70,000 × *g* for 1 h. The pellets containing outer membranes were washed once by suspension in and centrifugation from MOPS buffer (pH 7.5). Isolated outer membranes were dialyzed (4°C) against MOPS buffer for 16 h.

When ^{55}Fe was used to trace iron uptake, cells were grown as described above, and either whole cells or isolated outer membranes were exposed to 3.6 μM $^{55}FeCl_3$ (0.147 μCi/μmol) in 36 μM sodium citrate. When [^{14}C]citrate was used as a radioactive tracer, its specific activity was 2.5 μCi/μmol. Ferric ions and citrate at close to equimolar concentrations tend to form $[Fe(OH)]_n$ particles (molecular weight, 2 × 10^5) coated with citrate (5). At a ratio of iron to citrate of 1:10, iron is in a stable dicitrate complex (5). When membranes from whole cells were loaded with iron, stationary-phase cells in their iron-depleted growth medium were treated with 2 mM KCN for 15 min before the addition of ^{55}Fe-(di)citrate complexes at the concentrations and specific activities described above. This procedure permits energy-independent saturation of the iron uptake system, while at the same time preventing the energy-dependent uptake (incorporation) of iron by meningococci (2). Cells were harvested after 5 min in the solution containing ^{55}Fe-dicitrate complexes by centrifugation at 10,000 × *g* for 10 min; these cells washed twice by resuspension in and centrifugation from a solution containing 0.05 M MOPS (pH 7.5), 360 μM sodium citrate, and 2 mM KCN. The 5-min incubation was sufficient to permit complete saturation of the energy-independent iron uptake sites (3). After KCN-poisoned cells were exposed to Fe-dicitrate complexes, their outer membranes were isolated as described above, and they were dialyzed for 16 h against 0.05 M MOPS (pH 7.5)–2 mM KCN–360 μM sodium citrate. Whole cells loaded with ^{55}Fe-dicitrate complexes in the presence of KCN released only a negligible amount of iron to the supernatant fluid as a result of cell breakage by extrusion from a French pressure cell.

The protocol for testing the ability of isolated outer membranes to remove iron from citrate was much the same as the protocol described above, except that outer membranes were isolated first (as described above) and then treated with KCN and exposed to FE-dicitrate complexes in MOPS buffer and KCN as described above. These isolated outer membranes were then dialyzed as described above.

The energy-independent uptake of iron from citrate was measured in whole cells by a filtration method. In these experiments 8 ml of an iron-starved, stationary-phase culture was treated in the growth medium with 5 mM KCN for 15 min before the addition of the Fe-dicitrate complex solution (final concentrations, 3.6 μM Fe^{3+} and 36 μM sodium citrate). In each instance radioactive $^{55}FeCl_3$ or [^{14}C]citrate or both were used at the specific activities stated above. At intervals 0.5-ml samples of the cell suspensions were added to 0.5-ml portions of a solution containing nonradioactive $FeCl_3$ (72 μM) and citrate (720 μM) in 0.05 M MOPS buffer (pH 7.5). From these diluted cell suspensions triplicate 0.2-ml portions were deposited onto 0.45-μm filters (25 mm; Millipore Corp., Bedford, Mass.) prewashed with sterile NDM, and the cells on the filters were washed with 5 ml of sterile NDM.

Polyacrylamide disc gel electrophoresis of solubilized outer membranes was carried out essentially by the method of Lugtenberg et al. (23). Outer membranes were solubilized in 2% sodium dodecyl sulfate (SDS) at 95°C for 5 min. A 10% acrylamide gel was used with 0.025 M tris(hydroxymethyl)aminomethane (Trizma base; pH 8.3; Sigma Chemical Co.) and 0.19 M glycine in 0.1% SDS. A sample containing 20 μg of protein was applied to each gel. Other modifications to the procedure of Lugtenberg et al. (23) were the substitution of sucrose (16%) for glycerol and a bromophenol blue concentration of 0.05 instead of 0.001%. Electrophoresis was initiated at 12 mA/gel and main-

tained at a constant voltage of 40 V throughout the run.

Protein molecular weight standards for SDS-polyacrylamide gel electrophoresis were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Proteins in gels were stained with Coomassie blue as described elsewhere (33). Stained gels were scanned with a gel scanner attachment on a Gilford model 250 spectrophotometer at 420 nm. Protein concentrations in samples were estimated by the method of Lowry et al. (21), using bovine serum albumin as the standard. Radioactivities of samples were determined after 16 h of incubation in 8 ml of PCS scintillant (Amersham Corp., Arlington Heights, Ill.) containing 50 μg each of Desferal (Ciba-Geigy Co., Montreal, Canada) and *o*-phenanthroline (Sigma Chemical Co.). Samples were counted with a Packard Tri-Carb model 3375 liquid scintillation spectrophotometer. Polyacrylamide gels were sliced (1 mm) with a Gilson Aliquogel fractionator (Mandell Scientific, Ltd., Montreal, Canada).

RESULTS

Other studies from this laboratory have shown that iron-starved, cyanide-poisoned meningococci rapidly take up iron from citrate by an energy-independent saturable mechanism (F. S. Archibald, C. Simonson, and I. W. DeVoe, submitted for publication). As a functioning respiratory chain was not required for this event, it was of interest to determine whether isolated outer membranes were capable of removing the iron from Fe^{3+}-dicitrate complexes. The possibility existed that the citrate-chelated iron complexes might themselves be lipid soluble and partition between the aqueous phase of the medium and the lipid phase of the meningococcal cell membranes, whereas neither the metal nor the citrate alone would be expected to enter a nonpolar phase.

If the iron-chelator complexes did in fact partition into the lipid phase of the meningococcal membranes, extensive dialysis in an aqueous system could fail to remove all of the complexes, and this would lead to the erroneous conclusion that the iron had been taken from citrate by some iron uptake mechanism in the membranes. To distinguish between specific removal of iron from dicitrate complexes and the partitioning of the entire chelate complexes into the lipid phase, we performed experiments with both [^{14}C]citrate and ^{55}Fe.

In the first experiment, iron-starved cells grown in NDM were treated with KCN before the addition of either ^{55}Fe-dicitrate complexes, Fe^{3+}-di[^{14}C]citrate complexes, or [^{14}C]citrate alone. Cells were deposited onto filters at varying times and washed, and the levels of radioactivity retained on the filters were determined by liquid scintillation counting. Figure 2 shows that the maximum uptake of ^{55}Fe occurred by 2 min. On the basis of this uptake (640 pmol of Fe^{3+} per 10^9 cells), we could predict that if citrate had been taken up and retained as the Fe^{3+}-dicitrate complexes, radioactivity equivalent to 1,280 pmol of [^{14}C]citrate per 10^9 cells should have been retained by the cells after 2 min. On the contrary, citrate was not taken up in concert with iron, nor was it retained by cells when [^{14}C]citrate alone was added.

Next, iron-starved, KCN-poisoned cells were exposed to ^{55}Fe-di-[^{14}C]citrate complexes as described above, and cell envelopes (containing inner and outer membranes) were prepared. Isolation of the outer membranes was achieved by the selective solubilization of the inner membranes with 1% sodium lauroyl sarcosinate. Table 1 shows that of the ^{55}Fe retained by the isolated envelopes, 70% was localized in the outer membranes, whereas the retention of [^{14}C]citrate was essentially zero in both types of membranes.

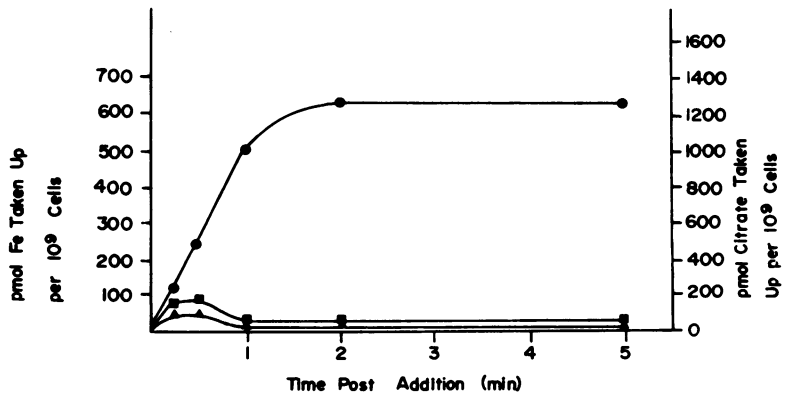

FIG. 2. *Uptake of labeled iron from citrate by iron-starved whole cells of N. meningitidis when they were incubated in solutions containing ^{55}Fe-dicitrate complexes (●), Fe-di-[^{14}C]citrate complexes (■), and [^{14}C]citrate (▲).*

TABLE 1. *Uptake of ^{55}Fe from Fe-dicitrate complexes by cell membranes of azide-poisoned N. meningitidis*

Membrane fraction[a]	Uptake (cpm/mg of protein)[b]	
	^{55}Fe	[^{14}C]citrate
Total envelope	9.0×10^6 (100)[c]	<20
Outer membrane	6.4×10^6 (70)	<20
Inner membrane	2.7×10^6 (30)	<20

[a] Envelopes were isolated from whole cells after high-pressure extrusion from a French pressure cell. Inner membranes were solubilized selectively in 1% sodium lauroyl sarcosinate (see text).

[b] Radioactive $^{55}FeCl_3$ and sodium [^{14}C]citrate, which were added at a ratio of 10:1, had specific activities of 0.15 and 2.5 μCi/μmol, respectively.

[c] Numbers in parentheses are percentages.

TABLE 2. *Effect of SDS on the retention of ^{55}Fe in isolated outer membranes from iron-starved N. meningitidis cells*

Treatment	^{55}Fe citrate added to isolated outer membranes[a]	
	cpm/mg of protein[b]	Relative % of Fe retained[b]
No SDS	5.8×10^6	100
SDS (25°C)	5.10×10^6	88
SDS (100°C)	5.08×10^6	88

[a] Outer membranes isolated by the sodium lauroyl sarcocinate method were exposed to ^{55}Fe-dicitrate complexes as described in the text.

[b] Radioactive counts were determined after a 16-h dialysis in citrate, as described in the text.

The question remained as to whether isolated outer membranes themselves were capable of removing iron from citrate. Outer membranes from iron-starved cells were isolated as described above and exposed to ^{55}Fe-dicitrate complexes. Table 2 shows that intact cells or cytoplasmic membranes were not required for iron uptake from citrate. Moreover, although citrate acted as a functional siderophore for iron, it was not itself taken up (Fig. 1 and Table 1).

The possibility that the ^{55}Fe in the outer membranes was associated with a specific protein or complex of proteins was tested. It was shown previously that the energy-independent iron uptake capability of meningococci was destroyed by heating the cells at 60°C for 5 min (Archibald et al., submitted for publication), which suggested a protein-mediated mechanism. We investigated whether the ^{55}Fe was associated with a protein in the outer membranes by determining whether the metal remained associated with its binding sites during solubilization of the membranes in SDS followed by electrophoresis. For the complete dissociation of proteins from lipid complexes, the procedure of Lugtenburg et al. (23) calls for heating of the membranes in a detergent solution. The retention of labeled iron by the membranes was determined after a 16-h dialysis to equilibrium against excess citrate (with KCN) in MOPS buffer. The excess citrate not only ensured against nonspecific binding of released iron to the dialysis bag or to noniron proteins within the bag, but also ensured that the label was bound firmly at the time when the SDS-solubilized preparation was subjected to SDS-polyacrylamide gel electrophoresis.

The solubilized, radioactive outer membranes were subjected to SDS-polyacrylamide gel electrophoresis, and the gels were stained with Coomassie blue and scanned at 520 nm (Fig. 3). The peak of radioactivity in gel slices was associated only with the major 36,500-dalton peak on the gels. Our recent experience suggests strongly that this major peak may very well be a complex of proteins rather than a single protein (S. Wulkan and I. W. DeVoe, unpublished data). We do not know the nature of the iron binding within this peak or to which specific polypeptide iron is bound. (The slight discrepancy between the widths of the 36,500-dalton protein bands on Coomassie blue-stained gel scans and the widths of the radioactive bands in the sliced gels was due to the degree of destaining in stained gels rather than a diffusion of the radioactive iron.) Cells grown and maintained in an excess of nonradioactive iron took up no ^{55}Fe from ^{55}Fe-dicitrate complexes by the energy-independent mechanism, presumably because the iron-binding sites on the cell surfaces were at or near saturation (Simonson and DeVoe unpublished data). The outer membranes of meningococci elaborated two major outer membrane proteins (molecular weights, 84,500 and 69,000) that were induced when cells were starved for iron. Although these proteins may well be involved in iron uptake, neither was associated with the ^{55}Fe in the gels. Such proteins may be involved in the removal of iron from transferrin or other iron complexes, but their role, if any, in iron metabolism is as yet unknown.

DISCUSSION

We have presented evidence that isolated outer membranes of meningococci are capable of energy-independent removal of iron from Fe^{3+}-dicitrate complexes. Previous reports from this laboratory have shown that meningococci have a predilection for iron chelated to functional siderophores, such as the organic acids citrate, isocitrate, pyruvate, and nitrolotriacetate, as well as pyrophosphate (4). Moreover, iron was readily obtainable by meningococci from human transferrin (3), the major source of iron in serum.

Our finding (4) that citrate is a part of an iron

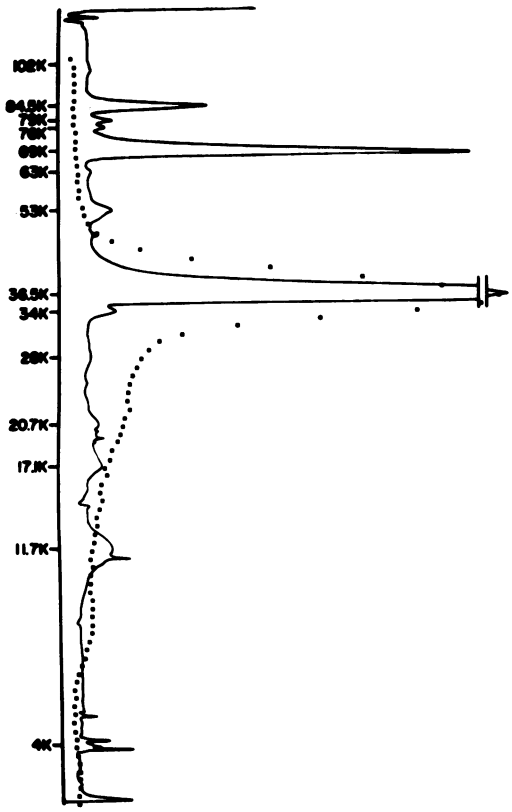

FIG. 3. *SDS-polyacrylamide gel electrophoresis of major outer membrane proteins from N. meningitidis grown in low-iron NDM (<6 ng of Fe per ml). Scan (520 nm) of Coomassie blue-stained gel of proteins (solid line) and ^{55}Fe radioactive counts of sliced gels (dotted line). The radioactivity scale ranges from 0 on the bottom line to 16,900 cpm in the gel slice with the highest activity. 102K, 102,000 daltons.*

acquisition mechanism is not unique among bacteria. Citrate as a functional siderophore of iron has been reported also for *Escherichia coli* (15), *Corynebacterium diphtheriae* (20), *Neurospora crassa* (38), *Microbacterium lacticum* (1), and *Micrococcus lysodeikticus* (26). No doubt more extensive screenings will reveal many other microorganisms capable of iron uptake from citrate. It has been suggested that citrate may have been a primordial iron transporter (30). The ability of meningococci to use carboxylic acid or phosphate ester compounds, which are present in relatively high quantities in body fluids, as functional siderophores would give these organisms an obvious advantage over organisms having a mechanism requiring the synthesis of usable concentrations of secreted siderophores.

The liberation of ferritin and heme proteins during severe meningococcemia with its characteristic coagulopathy could provide a ready source of iron, provided organic acids or phosphate esters are present. The mean normal levels of citrate and pyrophosphate in human serum are 120 and 200 μM, respectively (12). Therefore, it seems likely that during conditions involving tissue trauma or destruction, meningococci would have little trouble obtaining iron. The use of functional siderophores, such as citrate and pyrophosphate, could be especially useful if transferrin iron became limiting for growth of the meningococci in the circulation of a host. Hosts respond rapidly to invasion by microbes by making serum iron less accessible (35). During infection, transferrin iron is thought to be removed by the lactoferrin excreted by neutrophils and, subsequently, the iron is sequestered in the liver (33).

The results which we have presented here and elsewhere (4) support the hypothesis that meningococci have a siderophore-free high-affinity iron-acquiring mechanism in their outer membranes which is capable of removing iron from its citrate complex. The evidence for such a mechanism is strengthened by the complete inability of either isolated outer membranes or KCN-poisoned whole cells to take up the functional siderophore citrate from Fe-citrate complexes concomitantly. *Salmonella typhimurium* LT-2 enb-7 and *Arthrobacter terregens* ATCC 13345 do not produce soluble siderophores and are incapable of obtaining iron from Fe-citrate complexes (3), but they can use a number of siderophores produced by other organisms (8, 13, 22). The low-affinity iron uptake system of *E. coli* (15) has been defined by its inability to take iron from nitrilotriacetate, a particularly useful complex for meningococci (4). Moreover, the citrate-dependent high-affinity iron uptake system of *E. coli* is inducible when cells are grown in the presence of citrate (15). The ability of meningococci to use citrate-bound iron is not affected by the presence of citrate in the growth medium (Simonson and DeVoe, unpublished data); i.e., the iron-citrate mechanism is a constitutive property in this bacterium.

A conclusion from the work of DeVoe and Archibald (2–4) was that meningococci did not produce siderophores detectable by any of the many methods employed during their study. Moreover, none of the 20 strains tested (4) was able to use the iron from or compete with the common microbial siderophores deferrioxamine B (Desferal), ferrichrome, and enterochelin or the siderophore precursors or precursor-like compounds. The one exception to this was salicylhydroxamate, which was able to facilitate iron uptake in meningococci. This observation takes on significance in view of the recent finding of Yancey and Finkelstein (Abstr. Annu. Meet.

Am. Soc. Microbiol. 1980, B83, p. 31) that pathogenic neisseriae produce siderophores that are hydroxamate derivatives.

The association which we found between ^{55}Fe and a major outer membrane protein peak cannot be taken a priori as evidence that such a protein(s) is directly responsible for removal of iron from citrate. Such an event could involve a multistep process, with more than one protein participating.

The induction of proteins of 84,500 and 69,000 daltons by iron starvation is not a unique finding. In *Neisseria gonorrhoeae* outer membrane proteins with similar molecular weights appeared in gel profiles after cells were starved for iron by adding deferrioxamine B to the growth medium (28). A similar pattern was observed in outer membrane protein profiles from iron-starved fluorescent pseudomonads (25). Iron starvation also leads to the appearance of several high-molecular-weight proteins in outer membranes of enteric bacteria (6, 7, 16, 18, 19, 24, 29).

The major consideration regarding iron acquisition mechanisms of pathogenic microbes, including meningococci, is the role that such mechanisms might play in the ability of organisms to acquire iron from their hosts during infection. *E. coli* strain LG1315 was virulent for mice only when this bacterium was infected with the ColV plasmid, which gives this organism the ability to acquire iron at a high efficiency (31, 32, 36, 37). In this instance, this high-efficiency mechanism for iron retrieval appears to be the determining factor in the virulence of the organism. In most instances the ability to obtain host iron is probably only one of a number of factors of equal importance required for the establishment of a population in a host.

The meningococci can use the iron from human transferrin as a sole source of iron (3). Such iron is apparently obtained by direct contact of the bacterium with the transferrin. Iron could not be obtained from transferrin separated from the meningococci by a dialysis membrane. Recently, Calver et al. (9) and Holbein et al. (17) have shown that sustained infections by meningococci in mice are dependent upon exogenous iron administered to animals. Subsequently, Holbein (16a) obtained mouse infections with virulent meningococci without added exogenous iron. In these experiments high concentrations of bacteria remained in the blood only as long as transferrin-iron complex levels were maintained; i.e., as transferrin-iron complexes disappeared, the microorganisms were no longer able to grow and were cleared from the circulation.

In summary, meningococci possess siderophore-free mechanisms for removal of iron from citrate, pyrophosphate, and other functional siderophores (4), as well as human transferrin (3). From the evidence discussed above for the synthesis of hydroxamate siderophores, it appears that meningococci are capable of iron acquisition in the variety of environments in the human body in which they grow.

ACKNOWLEDGMENT

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