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Iron Acquisition by *Haemophilus influenzae*

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The mechanisms for acquisition of iron by *Haemophilus influenzae* and their role in pathogenesis are not known. Heme and nonheme sources of iron were evaluated for their effect on growth of type b and nontypable strains of *H. influenzae* in an iron-restricted, defined medium. All 13 strains acquired iron from heme, hemoglobin, hemoglobin-haptoglobin, and heme-hemopexin. Among nonheme sources of protein-bound iron, growth of *H. influenzae* was enhanced by partially saturated human transferrin but not by lactoferrin or ferritin. Purified ferrienterochelin and ferridesferrioxamine failed to provide iron to *H. influenzae*, and the supernatants of *H. influenzae* E1a grown in iron-restricted medium failed to enhance iron-restricted growth of siderophore-dependent strains of *Escherichia coli*, *Salmonella typhimurium*, and *Arthrobacter terregens*. Marked alterations in the profile of outer membrane proteins of *H. influenzae* were observed when the level of free iron was varied between 1 μM and 1 mM. Catechols were not detected in the supernatants of strain E1a; however, iron-related hydroxamate production was detected by two biochemical assays. We conclude that the sources of iron for *H. influenzae* are diverse. The significance of hydroxamate production and iron-related outer membrane proteins to *H. influenzae* iron acquisition is not yet clear.

Haemophilus influenzae is the most frequent cause of bacterial meningitis in the United States, with age-related incidence peaking within the first year of age; approximately 10,000 infants are affected annually (14). Other invasive infections due to *H. influenzae* include bacteremia, pneumonia, epiglottitis, and arthritis (16).

Greater than 95% of invasive isolates of *H. influenzae* are encapsulated with the type b polysaccharide (38); and production of the type b capsule is known to contribute to *H. influenzae* virulence (31). Research in several laboratories is currently focused on the contribution of additional factors, including lipopolysaccharide (19) and immunoglobulin A protease (13), to the virulence of *H. influenzae* type b.

High-affinity iron acquisition by pathogenic microorganisms may play a role in the development of infections in mammalian hosts, due to the severely limited availability of iron in vivo. Excess iron contributes to virulence of many bacterial pathogens in animal infection models (6), and possession of specific iron uptake mechanisms has been associated with invasiveness (21). Thus, iron acquisition systems may help to define virulent populations of bacterial species. Additionally, certain iron-regulated outer membrane proteins (OMP) are immunogenic (5, 17); the utility of these OMP as vaccine candidates has not been defined.

It has been reported (18) that *H. influenzae* can acquire iron bound to human transferrin by an unknown mechanism. The experiments reported here were designed to provide further definition of clinically relevant sources of iron available to *H. influenzae* and to characterize alterations in *H. influenzae* type b OMP associated with the extracellular iron concentration.

MATERIALS AND METHODS

Organisms and growth conditions. *H. influenzae* E1a is a streptomycin-resistant, stably encapsulated type b strain isolated from cerebrospinal fluid (37). The other clinical type b and nontypable isolates used in these experiments have been described previously (35). *Escherichia coli* LG1315 (39), *Shigella boydii* 1392 (20), and *Salmonella typhimurium* LT-2 *enbl* (30) were provided by Shelley Payne, University of Texas, Austin.

Storage and growth of *H. influenzae* in supplemented brain heart infusion have been described (35). For defined growth of *H. influenzae*, *Haemophilus* defined medium (HDM; 35) or modified HDM (mHDM) was used. mHDM is identical to HDM except that oxaloacetic acid is removed, phosphate is reduced to 0.03%, and the medium is buffered with 0.1 M Tris hydrochloride to pH 7.2. All of these modifications are intended to remove sources of iron-binding activity from HDM and to prevent removal of iron by mHDM from the iron-binding dye Crome Azurol S (33). Agar was added to 1.5% for the plate assays. Protoporphyrin IX (2 μg/ml) was provided as a precursor for heme synthesis; after digestion with perchloric acid (7), protoporphyrin IX (lot 13F-0598; Sigma Chemical Co., St. Louis, Mo.) contained ≤1% iron contamination. When added, iron was supplied as $Fe(NO_3)_3$. Contaminating iron was made unavailable by the addition of deferrated (32) ethylenediamine-di(*o*-hydroxy)phenylacetic acid (EDDA) to 20 μM. Glassware for growth in media containing defined concentrations of iron was acid rinsed; the media contained $\leq 5 \times 10^{-7}$ M iron before iron supplementation. Liquid media were inoculated as described by Herrington and Sparling (18), with minor modification. Growth was monitored with a Klett-Summerson apparatus.

Iron-binding compounds. Partially heme-saturated human serum hemopexin, human serum haptoglobin, and human serum albumin were purified and prepared as described previously (35). Human serum transferrin (Sigma) was deferrated by dialysis against two changes of 0.1 M sodium citrate–acetate (pH 4.5), followed by dialysis against T buffer (20 mM sodium bicarbonate in 40 mM Tris hydrochloride [pH 7.4]) (1). Transferrin was saturated to approximately 33% by the addition of a calculated quantity of $Fe(NO_3)_3$ in a solution containing a 10-fold molar excess of sodium citrate, followed by dialysis against T buffer to remove iron not specifically bound to transferrin. The percent saturation

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was confirmed by comparison of the A_{470} of the complex with a standard curve (15). Purified human lactoferrin was kindly provided by Richard Rest (Hahnemann Medical Center, Philadelphia, Pa.); after deferration by dialysis against 0.1 M sodium citrate–acetate (pH 2.3), human lactoferrin was made 33% iron saturated as described above for transferrin. Purified human hepatic ferritin (Sigma) was used without further processing. Purified enterochelin was a gift from Patricia Worsham (University of Illinois, Urbana), and desferrioxamine was obtained from CIBA-GEIGY Corp. (Summit, N.J.); both siderophores were used in their iron-saturated form.

Bioassays. HDM agar containing EDDA was used to determine which iron sources satisfy the total cellular iron requirements of *H. influenzae*. Approximately 10^7 CFU of an *H. influenzae* strain were homogeneously spread onto HDM agar containing 20 μM EDDA. Wells (6 mm) were cut into the agar, and potential iron sources were added to the wells. Each well contained 0.04 μg of iron if the iron source was a heme-containing compound or an iron-binding protein; all other wells contained 10 μg of iron. After incubation for 48 h at 37°C in 5% CO_2, the bacterial density of the lawn was visually graded +1 to +4. A growth index was calculated from the growth zone diameter and the graded bacterial density as defined by Archibald and DeVoe (2); the mean of duplicate assays was recorded. Each plate had a well containing 10 μg of iron as $Fe(NO_3)_3$; the coefficient of variation for the growth index of these wells was 10.7%. No growth was detected around wells containing <1 μg of iron as $Fe(NO_3)_3$.

Cell-free supernatants of *H. influenzae* grown in iron-limited HDM were screened for the presence of analogs of enterochelin and aerobactin by their ability to provide iron to organisms requiring defined siderophores in iron-limited media. Approximately 10^7 CFU of the indicator strains *S. boydii* 1392 and *S. typhimurium* LT2 *enbI* were homogeneously spread onto Tris-minimal medium (34) without glucose containing 0.5% succinic acid and 150 μM EDDA. Positive controls consisted of supernatants of *E. coli* LG1315 (aerobactin) and *E. coli* AN102 (enterochelin). *Arthrobacter terregens* ATCC 13345 was grown on nutrient agar containing desferrioxamine and was used as an indicator strain on nutrient agar.

Analytical methods. Iron concentrations were determined with tripyridyl-*s*-triazine (8) by using a 10-cm path length for increased sensitivity. The assay of Arnow (3) was used to screen for the presence of catecholates with 3,4-dihydroxybenzoic acid as a standard. Hydroxamates were detected and quantitated as described by Csaky (10) with a standard curve of hydroxylamine between 0.1 and 1.0 μg/ml; the assay was linear within this range. Results from the Csaky assay on experimental material were expressed as hydroxylamine equivalents. The presence of hydroxamates in experimental material was confirmed by the method of Atkin and Neilands (4). OMP were isolated as described previously (35). Protein concentrations were determined by the method of Lowry et al. (22) with bovine serum albumin as a standard. OMP were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 10% separating gel (36) and were visualized by the silver staining method of Wray et al. (42).

RESULTS

Iron-restricted growth of *H. influenzae*. Iron contamination of individual reagents necessary to the composition of HDM, a complex defined medium, precluded primary preparation of an iron-restricted growth medium. Treatment of HDM with Chelex-100 ion-exchange resin (Bio-Rad Laboratories, Richmond, Calif.) resulted in a medium which inhibited growth of *H. influenzae* type b despite reconstitution with ions known to be bound by the resin. For attaining iron-restricted conditions for growth of *H. influenzae*, contaminating iron in HDM was chelated with 20 μM EDDA. Growth limitation, defined as a slower rate of increase and a lower peak in bacterial density as statistically compared with growth in iron-replete medium, was achieved under these conditions (data not shown). We confirmed that EDDA is not toxic to *H. influenzae*, as determined by growth of *H. influenzae* in the presence of iron-saturated EDDA at 10 times the concentration that limits growth in iron-deplete conditions. In the absence of EDDA, no variation in growth rate was observed among cultures grown in mHDM supplemented with $Fe(NO_3)_3$ at concentrations between 10^{-6} and 10^{-3} M. Minimal growth of *H. influenzae* was visible on HDM agar containing 20 μM EDDA unless an additional source of iron was provided, and this formed the basis for evaluating the sources of iron available to *H. influenzae*. None of the compounds which were tested inhibited this minimal growth around the wells.

Acquisition of iron from heme proteins. Previous experiments performed in this laboratory (35) demonstrated that *H. influenzae* grown in medium without iron starvation can acquire essential porphyrin from a variety of serum sources, including hemoglobin, heme-hemopexin, hemoglobin-haptoglobin, and heme-albumin. The total cellular iron requirements of *H. influenzae* could be provided by these heme sources, and *H. influenzae* was capable of acquiring iron from these heme proteins as efficiently as from free heme (Table 1).

Acquisition of iron from nonheme iron-binding proteins. Experiments with the iron-binding proteins transferrin and lactoferrin confirmed a previous report (18) that *H. influenzae* could utilize partially saturated transferrin (Table 1) but not lactoferrin as the sole source of available iron. The strains of *H. influenzae* tested in these experiments were unable to acquire iron from ferritin.

Acquisition of iron from microbial siderophores. Some microorganisms, including *E. coli* (26) and *S. typhimurium* (23), possess the ability to remove iron from siderophores produced by other species. *H. influenzae* strains were therefore tested in iron-limiting conditions for the ability to utilize saturated ferric enterochelin and ferric desferrioxamine (Desferal) as sole iron sources. Neither of these microbial siderophores could provide iron to *H. influenzae*, even when the quantity of iron in the well was 250 times greater than the quantity of transferrin-bound iron which allowed growth. The inability of *H. influenzae* to acquire iron from ferric desferrioxamine has been noted by Williams and Brown (41), who used the siderophore to chelate iron in *Haemophilus* growth media.

Production of soluble siderophores by *H. influenzae* E1a. Supernatants from iron-limited cultures of *H. influenzae* E1a failed to provide iron to siderophore-dependent strains of *E. coli*, *S. typhimurium*, and *A. terregens*. Catechols and hydroxamates were undetectable in the supernatants, as determined by the spectrophotometric assays of Arnow (3) and Csaky (10), respectively.

In contrast to supernatants from iron-depleted cultures, supernatants from cultures of *H. influenzae* E1a grown in HDM containing ≥10 μM iron were strongly positive in the Csaky assay, indicating the production of large quantities of

TABLE 1. Sources of iron for *H. influenzae*

Strain[a]	Source[b]	Growth index[c]							
		Hm	Hm-SA	Hm-HX	Hb	Hb-HP	Trans	Imfer	Fe
E1a	CSF	++	++	++	++	++	++	0	++
C65	CSF	++	++	++	++	+	+	+	+
C148	CSF	++	++	++	++	++	+	±	+
R152	CSF	++	++	++	++	+	++	±	++
R211	NP	+	++	++	++	++	++	+	++
R212	NP	+	++	++	++	++	++	±	++
R213	NP	++	++	++	++	++	+	+	+
R214	NP	++	++	++	++	++	++	+	++
C120	NP	++	++	+	++	++	+	±	++
C132	Eye	++	++	+	++	++	++	±	+
C134	RT	++	++	++	++	++	++	±	+
C136	NP	++	++	+	++	+	+	+	+

[a] Strains are type b, except C120, C132, C134, and C136, which are nontypable.
[b] CSF, cerebrospinal fluid; NP, nasopharyngeal; RT, respiratory tract.
[c] Growth index (GI) was calculated as described in the text and graded as follows ±, $0 < GI \leq 100$; +, $100 < GI \leq 300$; ++, $GI > 300$. Hm, Heme; SA, human serum albumin; HX, hemopexin; Hb, hemoglobin; HP, haptoglobin; Imfer, imferon; Trans, ferritransferrin; Fe, $Fe(NO_3)_3$.

hydroxamates by these cultures. These supernatants were also positive in the iron-binding assay of Atkin and Neilands (4), which is also specific to hydroxamates. Figure 1 shows the production of hydroxamates in the supernatants of cultures of *H. influenzae* E1a grown in mHDM containing various concentrations of iron. Differences in growth rate in mHDM containing various iron concentrations were not detected. After 6 and 24 h, hydroxamate production was maximal in mHDM containing 10^{-4} M Fe(III). Along with *H. influenzae* E1a, two additional *H. influenzae* type b strains and one nontypable strain produced hydroxamates in mHDM containing 10^{-4} M iron (data not shown). Greater than 90% of the hydroxamate compounds remained in the supernatant after centrifugation at 100,000 × *g* for 1 h. Whether the hydroxamate(s) produced by *H. influenzae* provides iron to siderophore-dependent microbial species has not been evaluated.

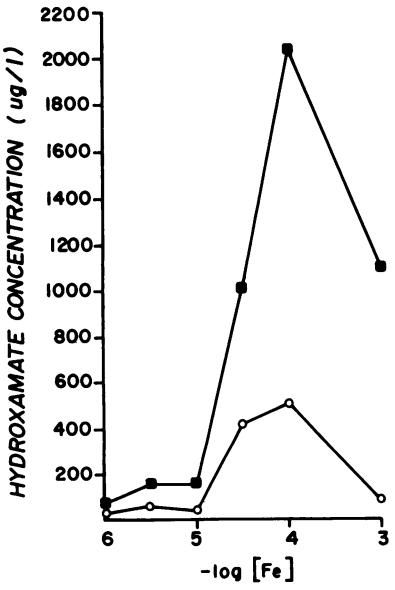

FIG. 1. Production of hydroxamates by *H. influenzae* E1a. Cell-free supernatants were tested after growth of *H. influenzae* E1a in mHDM containing graded concentrations of $Fe(NO_3)_3$ for 6 h (○) and 24 h (■). Data represent the means of two separate experiments.

Iron regulation of *H. influenzae* E1a OMP. We observed distinctive changes in OMP patterns of *H. influenzae* E1a grown in defined medium when the concentration of iron was varied (Fig. 2). Our results support those of others (18), who reported increased expression of 90- to 95-kilodalton (kDa) OMP in iron-limited cultures of *H. influenzae*. In addition to these high-molecular-weight molecules, we also observed increased expression of a 77-kDa OMP by *H. influenzae* E1a grown in low iron concentrations. Two OMPs, estimated at 65 and 24 kDa, became more abundant as the iron concentration was increased. Finally, we noted two OMPs of 83 and 74 kDa which were expressed only when the iron concentration was increased to 1 mM. Of these iron-related OMP, only the 83-, 74-, and 65-kDa proteins were evident after growth of *H. influenzae* E1a in supplemented brain heart infusion broth (data not shown).

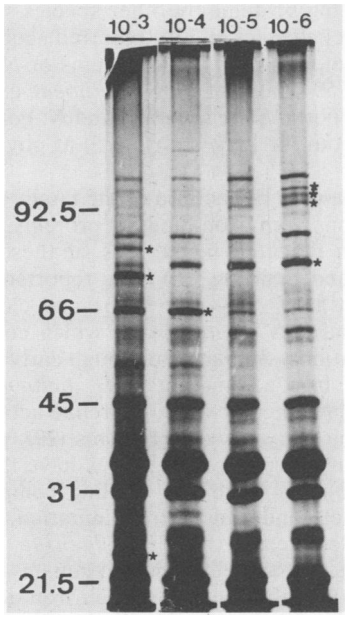

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OMP of *H. influenzae* E1a grown in mHDM containing various concentrations of $Fe(NO_3)_3$. Astericks indicate iron-related OMP in each lane which revealed maximum production. Molecular sizes of standards, in kilodaltons, are indicated to the left.

DISCUSSION

The mechanisms by which *H. influenzae* acquires iron and the significance of iron acquisition to *H. influenzae* virulence remain poorly understood. The results of our in vitro investigations suggest that multiple sources of iron are potentially available to *H. influenzae* in vivo.

H. influenzae exhibits an absolute requirement for protoporphyrin IX, which is presumably provided in vivo as heme. Studying the kinetics of active transport of doubly radiolabeled heme by *H. influenzae* type b, Coulton and Pang (9) demonstrated that both iron and the poryphyrin ring were taken up at the same rate, suggesting that *H. influenzae* could utilize heme as an iron source. Our investigations have revealed that heme can provide the total cellular iron requirements of *H. influenzae* and that *H. influenzae* can acquire heme from a variety of sources, some of which may be significant in vivo. Haptoglobin forms a stable association with hemoglobin, and the addition of haptoglobin suppresses acquisition of hemoglobin iron by *E. coli* as well as hemoglobin-driven *E. coli* peritonitis in rats (12). *H. influenzae* can acquire both iron (this study) and porphyrin (35) from hemoglobin-haptoglobin complexes, and this may enhance its survival in blood. Although knowledge of the role of the heme-binding serum proteins hemopexin and albumin in host defenses against microbial invasion is limited, the ability of *H. influenzae* type b to remove heme iron from heme-hemopexin and heme-albumin suggests that sequestration of heme by these proteins would provide an inadequate host defense against *H. influenzae* invasion. In contrast, neither heme-hemopexin nor heme-albumin provides essential iron to *Neisseria gonorrhoeae* or to *Neisseria mengingitidis* (11), although both pathogens utilize heme iron from hemoglobin.

Our in vitro investigations confirm a previous report (18) that *H. influenzae* can acquire iron from ferri-transferrin. None of the *H. influenzae* strains we examined utilized ferri-lactoferrin or ferritin as sole iron sources. It is interesting to compare these results with reports of iron acquisition from iron-binding proteins by other species-specific human pathogens. Mickelson et al. (24) reported acquisition of iron from ferri-lactoferrin by all of 15 strains of *N. meningitidis* and by 53% of 59 strains of *N. gonorrhoeae* examined. Like *H. influenzae*, both *N. meningitidis* and *N. gonorrhoeae* can acquire iron from ferri-transferrin (25) at physiologic levels of saturation.

Despite the ability of bacteria of the family *Neisseriaceae* to remove iron from iron-binding proteins, siderophores have not been detected in cultures of these bacteria, although West and Sparling (40) have reported utilization of ferric aerobactin by *N. gonorrhoeae* and cloning of a genomic fragment of *N. gonorrhoeae* which complements *E. coli fhuB* mutations. Similarly, although early investigations of the role of iron acquisition in *H. influenzae* virulence suggested the presence of siderophorelike activity in culture supernatants of iron-starved organisms (29), previous studies (18) and our current investigations have failed to reveal the production of soluble iron-binding compounds by *H. influenzae* under conditions of iron limitation. It is therefore of considerable interest that *H. influenzae* E1a produces large quantities of extracellular hydroxamates during growth in high iron concentrations. Although iron-dependent production of hydroxamate compounds has been reported for certain nonpathogenic bacteria (27, 28), hydroxamate synthesis by these bacteria is repressed at the level of added iron (100 μM) which we found to be optimal for hydroxamate production by *H. influenzae* E1a. This relationship between iron concentration and bacterial hydroxamate production has not been previously reported; bacterial hydroxamates have been previously associated with iron acquisition during iron-limited growth. It is possible that the trigger for hydroxamate production is complex and that our defined culture conditions do not accurately reflect the availability of iron in vivo. It is also possible that hydroxamates do not function in acquisition of iron by *H. influenzae* but serve some other purpose, such as sequestration of iron from proteins or protection from iron-mediated oxidative damage. The structure of *H. influenzae* hydroxamates, the conditions by which their synthesis is regulated, and their possible significance in vivo are currently under investigation.

Our data demonstrate that the expression of OMP is altered over wide ranges of iron concentration. In addition to the minor iron-repressible, high-molecular-weight OMP noted by Herrington and Sparling (18), major alterations in the pattern of OMP were detected at higher iron concentrations. Expression of two OMP of 65 and 24 kDa was roughly parallel to production of hydroxamates by *H. influenzae* E1a. The role of iron-related OMP in *H. influenzae* iron metabolism and the immunogenicity of these OMP should be further investigated.

H. influenzae acquires iron from a variety of heme and nonheme sources. Therefore, iron sequestration in human blood may not be an adequate host defense against this pathogen, and the molecular mechanisms for iron acquisition by *H. influenzae* may function as virulence factors. The production of hydroxamate compounds at very high iron concentrations is an intriguing phenotype not previously reported in bacteria; current investigations will focus on elucidating the role of hydroxamates and of the iron-related OMP in the pathogenesis of invasive disease due to *H. influenzae*.

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