

MOLECULAR AND GENETIC ANALYSIS OF IRON UPTAKE PROTEINS IN THE BRAZILIAN PURPURIC FEVER CLONE OF *HAEMOPHILUS INFLUENZAE* BIOGROUP AEGYPTIUS

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Received 8/17/98 Accepted 8/17/98

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1. ABSTRACT

Haemophilus influenzae biogroup aegyptius (*H. aegyptius*) is the etiological agent of Brazilian purpuric fever (BPF), a recently described pediatric disease that is often fatal. The vascular destruction that occurs in this disease is a distinctive trait, and little is known about the mechanism(s) of the overwhelming purpura fulminans that causes the high mortality associated with this pediatric infection. Iron is an essential micronutrient for nearly all living cells, and the mechanisms used by bacteria to acquire and internalize iron are often associated with virulence. Therefore, the focus of our studies is the molecular characterization of the iron uptake system used by *H. aegyptius*. Specifically, we are investigating the high-affinity transferrin binding proteins in the bacterial outer membrane, components of ABC transporter systems, and a possible regulatory mechanism for the genes encoding these proteins. A detailed understanding of the molecular nature of the regulatory genetic components and proteins involved in the acquisition of iron will broaden the knowledge of the pathogenesis of the disease caused by *H. aegyptius* and will also lead to a better understanding of the nature of other infections that affect the vascular system.

2. INTRODUCTION

Haemophilus aegyptius was first described by Koch in 1883 when he noted the presence of this microorganism in the eye secretions of Egyptian children suffering from conjunctivitis (1, 2). This pathogen, until recently, has been associated only with self-limiting cases of conjunctivitis. However, between October 14, 1984 and December 15, 1984 in Sao Paulo State, Brazil, ten fatal cases of Brazilian purpuric fever (BPF), a fulminant septicemic disease of young children, were reported (3). This disease is preceded by purulent conjunctivitis which resolves but is followed 10-14 days later by acute onset of

fever associated with systemic toxicity, petechiae, purpura, and vascular collapse (3, 4). Initial studies suggested that a single clone was responsible for these deaths in Sao Paulo State. However, after additional outbreaks in Australia (5) and other areas in Brazil, epidemiological evidence suggested that there was more than one BPF-causing clone of *H. aegyptius*. Genotypic and phenotypic factors initially thought to be responsible for virulence, such as a 24 MDa plasmid, were absent in clinical isolates from the later outbreaks (6, 7).

Many investigators have studied the evolutionary origin of this pathogenic clone of *H. aegyptius* at the biochemical, serological, and clinical levels (8). Pittman and Davis (9) proposed that the microorganism be classified as *H. aegyptius*, but others disagreed since *H. aegyptius* and *H. influenzae* share greater than 70% nucleotide sequence homology. Currently the microorganism is referred to as *H. influenzae* biogroup aegyptius (8). Although *H. influenzae* and *H. aegyptius* are a single genetic species, evidence showed that the Brazilian BPF isolates belong to a unique clone that is more closely related to *H. influenzae* serotype c strains than to classical *H. aegyptius* isolates (2, 8). Further analysis showed that isolates from the Australian cases exhibited none of the markers described for the BPF clone, representing a second clone (10). These findings suggest that although the BPF clone may harbor unique virulence determinants, BPF results from the action of virulence factors unrelated to markers of the original case-clone strains.

Little is known about the mechanisms of BPF pathogenesis, and the virulence determinants that *H. aegyptius* uses to cause this disease are currently not well described. It is believed that the pathogenesis of BPF is likely multi-factorial. Previous studies have shown that the

acquisition of iron is an important virulence factor for several bacterial species (11). To establish an infection, bacteria must survive within their host, and an essential nutrient that nearly all living cells, including bacteria, need for survival is iron. *H. aegyptius* initially invades the conjunctiva and then disseminates to the bloodstream, and during this disease progression, the microorganism must acquire this essential nutrient. In humans, most of the extracellular iron is complexed to the iron binding glycoproteins transferrin and lactoferrin, and intracellular iron is contained in complexes such as heme containing compounds and ferritin (11, 12, 13). This iron is therefore unavailable for use by the microorganism, and since the concentration of free iron is approximately 10^{-18} M (12), bacteria have evolved several different mechanisms to acquire iron from the iron-restricted environment found in the host.

A common and well defined mechanism for iron uptake in many bacteria involves the synthesis of low molecular weight high-affinity iron chelators called siderophores (14, 15). Once a bacterium secretes its siderophore into the external milieu, a ferric iron-siderophore complex is formed and the iron is internalized via a specific set of proteins in the outer membrane, periplasmic space, and inner membrane (11). *Haemophilus*, however does not synthesize its own siderophore (12). Instead, it binds iron-loaded transferrin directly and transports the iron into the cell (16, 17). Once the transferrin is bound, the microorganism is able to extract the iron from the host glycoprotein and transport the iron into the bacterial cell via a cascade of other transport proteins located in the periplasmic space and inner membrane (16, 17). Understanding this system is significant because the mechanisms that pathogenic bacteria use to acquire unavailable iron are recognized as important components of the bacterial virulence repertoire (11).

Several researchers are evaluating the role of iron acquisition as a virulence factor for both typeable and nontypeable *H. influenzae* (18, 19), and others are investigating whether the conserved transferrin binding proteins 1 and 2 (Tbp1 and Tbp2) in the bacterial outer membrane are good vaccine targets (18, 20). Gray-Owen *et al.* (18) cloned, sequenced, and characterized the genes encoding Tbp1 and Tbp2, *tbp1* and *tbp2*, respectively, in *H. influenzae* type b. They also showed that both proteins are located in the outer membrane and bind transferrin. Their model suggests that Tbp2, a lipoprotein, is anchored in the outer membrane and contains an "arm" that functions to bring the iron-loaded transferrin to the cell surface. This triggers a conformational change that allows Tbp1, a transmembrane protein, to internalize the iron (18). Loosmore *et al.* (20) cloned and characterized genes encoding the transferrin receptors from several typeable and nontypeable *H. influenzae* strains, and sequence analysis suggested that Tbp2, a conserved surface-exposed protein which plays an essential role in iron acquisition and hence survival of the microorganism, is a likely candidate for a vaccine antigen. Work from the laboratory of E. J. Hansen (19, 21, 22, 23) has also contributed greatly to the

elucidation of the mechanisms involved in iron acquisition by *H. influenzae*. They showed that the membrane-spanning protein TonB (19, 23) and inner membrane proteins ExbB and ExbD (22) are also involved in iron uptake. In addition, they identified a locus, *hitABC*, which encodes products similar to the SfuABC proteins of *Serratia marcescens* and functions in acquiring iron independently of transferrin, lactoferrin, and siderophores (24). Studies using an isogenic mutant that lacked a functional *hitC* showed that this system is essential for iron acquisition by nontypeable *H. influenzae* (21). Furthermore, one open reading frame (ORF) in this locus, *hitA*, is 69% identical to the periplasmic ferric binding protein A (FbpA), which is also involved in the acquisition of iron by *Neisseria gonorrhoeae* (25).

The expression of genes that encode the components of iron acquisition systems is regulated by the iron concentration of the growth medium. Iron is also an important cellular signal in the regulation of the expression of genes encoding a variety of metabolic, biosynthetic, and pathogenic functions (26, 27). Genes regulated in response to changes in iron concentration constitute the iron stimulon, and within this group are genes that are regulated by the universal ferric uptake repressor (Fur). These Fur-regulated genes constitute the Fur regulon. Fur functions as a dimer by binding ferrous iron and subsequently binding to specific DNA sequences called Fur boxes, which are located in the promoter region of Fur-regulated genes (28). The long term objective of our work is to ascertain whether the iron acquisition components of *H. aegyptius* are significant factors in the fatal invasive disease Brazilian purpuric fever. Our approach for evaluating this research interest entails the use of isogenic mutants to help elucidate bacterial virulence determinants and better characterize the pathogenesis of infection. In our studies we have identified homologs to the iron acquisition proteins described in both typeable and nontypeable *H. influenzae* and have characterized at the molecular level the genes encoding these proteins (manuscript in preparation). In addition to the actual components of the iron uptake system, we are also investigating how the genes encoding these proteins are regulated.

3. EXPERIMENTAL FINDINGS

3.1. Siderophore-independent iron uptake

To initiate studies on the genetic and molecular characterization of the iron acquisition mechanisms of the BPF clone of *H. aegyptius*, the first objective was to evaluate potential iron sources used by BPF-causing strains of *H. aegyptius* and to determine whether the strains were able to grow under iron-limitation. To ascertain this, several strains of the BPF clone were cultured in brain heart infusion supplemented with heme and NAD (sBHI). The iron chelator ethylene-di-(*o*-hydroxyphenyl) acetic acid (EDDHA) and a greater concentration of heme were added the sBHI to achieve iron-limiting and iron-replete growth conditions, respectively. Liquid cultures of the BPF prototype strain F3031 reached cell densities similar to cultures of *H. influenzae* strain Eagan, a well characterized type b strain that grows well in iron-limiting conditions,

when cultured under these conditions (16). Therefore, since F3031 is a nontypeable strain and nontypeables are able to use protoporphyrin IX (PPIX) as an iron source (21), the ability of F3031 to acquire iron from the medium when PPIX was substituted for heme was investigated.

The results showed that *H. aegyptius* grew readily on BHI-NAD agar containing free iron and PPIX in place of heme. This ability to grow in the presence of limiting amounts of heme suggests that the BPF clone may express a siderophore-independent iron uptake system similar to the ones encoded by the *sfuABC*, *hitABC*, *fbpABC*, and *afuABC* (Ap) *afuABC* (Aa) genes in *S. marcescens* (24), *H. influenzae* (21), *N. meningitidis* (29), *Actinobacillus pleuropneumoniae* (30), and *A. actinomycetemcomitans* (31, 32), respectively. The significance of this is that both the Hit and Fbp ABC transporter systems are essential in the acquisition of iron by nontypeable *H. influenzae* (21, 33) and *N. meningitidis* (34), respectively. Thus, the potential presence of a HitA-like protein in BPF strains was examined. Western blot analysis with a polyclonal antiserum against the gonococcal ferric binding protein FbpA (25) showed that F3031 has a FbpA (or HitA) homolog that localizes to the periplasmic space and displays a molecular weight of 38 kDa. This size is 2 kDa smaller than that reported for *H. influenzae* strain Eagan and could be due to a different amino acid composition that alters the electrophoretic mobility or a shorter ORF. Additional western blot analysis with a *H. influenzae* panel, which consisted of both typeable and nontypeable strains, showed that the typeable strains tested had a 40 kDa HitA homolog; whereas, the same homolog in *H. aegyptius* F3031 and the nontypeable strain TN106 was 38 kDa.

The results from this immunoblot with the gonococcal antiserum against FbpA prompted us to further evaluate the similarity between TN106, the strain in which the *hitABC* system was identified and characterized (21), and the BPF clone. Sanders *et al.* showed by Southern blot analysis that the genes encoding the HitABC iron transport system in TN106 are present in one contiguous fragment on the chromosomal DNA (21). The same arrangement of the *hitABC* genes was found in F3031. Since no antisera are currently available for HitB and HitC, we used reverse transcription polymerase chain reaction (RT-PCR) and northern blot analysis to determine whether the genes encoding these proteins were expressed. Results from northern hybridizations of F3031 RNA with a PCR-amplified *hitB* probe from *H. influenzae* Rd suggest that this gene is expressed in *H. aegyptius*. This is supported by the fact that both *hitB* and *hitC* are also expressed in *H. influenzae* Rd.

Initial review of the Rd genome suggested that the *hitC* homolog was present in a separate DNA fragment from *hitA* and *hitB* (35). That is, the *hitC* homolog was originally assigned to ORF HI1474, which now is listed as being 58.1% homologous to an ABC transporter ATP-binding protein from *Helicobacter pylori*. This Rd gene is located independently of *hitA* and *hitB* and is flanked by *modD*, a gene involved in molybdenum uptake and *nifC*, a

gene involved in nitrogen fixation (35). Other genes located in this region on the Rd chromosome encode a homolog of the inner membrane ATPase involved in the internalization of iron by *Escherichia coli* (*fepC*), a hemin permease from *Yersinia enterocolitica* (*hemU*), a putative iron chelating ABC transporter periplasmic protein from *H. pylori* (*ceuE*), and a transcriptional regulatory protein from *Pseudomonas aeruginosa*. Even though these genes encode ABC transporter function and resemble genes involved in the acquisition of iron, the significance of these open reading frames in regards to iron uptake by *Haemophilus* remains to be determined. Preliminary studies using chromosomal DNA from F3031 suggest that several of these homologs are also present in the BPF clone. Specifically, we identified a *modD* and HI1474 homolog using high stringency Southern blot analysis with PCR-amplified probes from *H. influenzae* Rd. Both genes are located within the same DNA fragment on the F3031 chromosome, and the size resembles that seen with Rd. These results allowed us to speculate whether these genes in F3031, as well as in Rd, function in the uptake of iron or of other ions such as molybdenum.

HitA is not the only iron-regulated protein found in the periplasm of *H. influenzae*. *H. influenzae* strain Eagan also contains a 31-kDa iron-regulated periplasmic protein (36) of unknown biological function. When we compared the N-terminal amino acid sequence of this protein with the GenBank database, we found that it has high homology to *fimA* and *yfeA* of *Streptococcus parasanguis* and *Y. pestis*, respectively (37, 38). It has been suggested that FimA is a multifunctional membrane protein involved in adhesion and transport of unknown substrates (37) while YfeA is involved in inorganic iron utilization and bacterial virulence (38). As a result of this homology analysis, we looked for the presence of a FimA-like protein in BPF strains using an antiserum against the 31-kDa protein. This antiserum was prepared by injecting a rabbit with the 31-kDa protein isolated from the periplasm of strain Eagan and purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution. Our experimental data confirm the presence of a FimA homolog in the periplasm of BPF cells that displays the same electrophoretic mobility as the protein present in the Eagan strain.

Computer analysis showed that the *fimA* homolog is also present in the genome of *H. influenzae* Rd (35). Western blot analysis using the GHIHK58 clone, which was generated during the genome sequencing and contains the *fimA* gene (35), showed that this gene is expressed when *E. coli* is the host strain. Additional analyses by northern blots and RT-PCR showed that Rd expresses *fimA*. This gene is located together with three other potential ORFs within a putative polycistronic operon. These ORFs show homology to the *E. coli* FecE dicitrate transport ATP-binding protein (HI0361), an *S. gordonii* hydrophobic membrane protein (HI0360), and a hypothetical *Synechocystis* protein (HI0359) (35). In addition, an updated version of the *H. influenzae* database at The Institute for Genomic Research (TIGR) lists ORFs HI0362, HI0361, HI0360, HI0359 as respective homologs

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of *yfeA*, *yfeB*, *yfeC*, *yfeD*, the genes which encode ABC transporter components for iron utilization in *Y. pestis* (38). We recently confirmed the polycistronic nature of this operon in *H. influenzae* Rd by RT-PCR using total RNA and primers that span the entire genetic region. In addition to the *FimA* homolog, western blots with whole cell lysates from *H. influenzae* Rd showed that *fecE*, the ORF immediately downstream of *fimA* is also expressed. In support of this, northern blot analysis showed that all four genes were expressed in Rd. Whether or not these proteins are important in the uptake of iron by the bacterial cell or are involved in the transport of other ions remains to be determined.

Based on nucleotide homologies, the aforementioned genes in *H. influenzae* Rd encode proteins that resemble ABC transporter components. Since this system in Rd has a potential use in the uptake of iron for *H. influenzae*, we first sought to determine whether the genes encoding these proteins were present in the BPF clone. Using Southern blot analysis under high stringency conditions, we found that *H. aegyptius* F3031 contains a contiguous DNA fragment homologous to the four ORFs in *H. influenzae* Rd. In addition, western blot analysis with whole cell lysates and periplasmic proteins from F3031 showed that both *FecE* and *FimA* homologs, respectively, are expressed. In support of this, RT-PCR showed that both *fimA* and *fecE* are expressed. In addition, preliminary results from northern blot analysis indicated that all four ORFs are expressed. The summation of these results strongly suggests that the BPF clone expresses these four genes that may be involved in iron acquisition via a siderophore-independent system different from the HitABC system previously described (21).

The significance of two uptake systems potentially involved in iron acquisition in *H. aegyptius* is currently under investigation. It is possible that one system is used to bring in iron from bound transferrin and the other is more important in the acquisition of iron from heme and heme-containing compounds. In addition, the fact that these iron uptake systems may be differentially expressed by the BPF clone throughout the course of the infection is an interesting aspect that remains to be elucidated.

3.2. Transferrin binding activity

Since *H. aegyptius* belongs to the Pasteurellaceae family, members of which obtain the essential nutrient iron via a direct interaction with host iron binding proteins such as transferrin and lactoferrin, the ability of the BPF clone to bind human transferrin was investigated. However, prior to assaying for activity, we first checked for the presence of Tbp homologs in the outer membrane of the BPF clone. Immunoblot analyses showed that the BPF clone expresses a 90-kDa Tbp1 homolog and a 80-kDa Tbp2 homolog, as detected in other *Haemophilus influenzae* strains such as Eagan and the nontypeable strain TN106 (18). These data support the hypothesis that the BPF clone acquires iron from the human host by binding iron-loaded transferrin. Using an *in vitro* dot enzyme assay, we found that three BPF-causing strains of *H. aegyptius* (F3029, F3031, and F3033) and the type b clinical isolate *H. influenzae* strain

Eagan, which grows under iron restricted conditions and assimilates iron by binding human transferrin via outer membrane proteins specific for this host glycoprotein (39), bound human transferrin when cultured under iron-limiting conditions. These data indicate that BPF-causing strains likely acquire iron from the human host by direct interaction with iron-loaded human transferrin. This information is supported by other recent studies in *Haemophilus* which also suggest that iron is acquired via the direct interaction between the bacterial cell surface and the host glycoprotein and the subsequent release of the iron moiety into the cell (40). The expression of this potentially important virulence factor and other components of this iron acquisition system was further investigated in the prototype strain F3031.

To characterize the regulation of transferrin binding in the BPF clone, cells were grown in sBHI containing either additional heme or FeCl_3 (iron-replete) and sBHI containing EDDHA (iron-limited). The transferrin binding assay showed that *H. influenzae* strain Eagan bound human transferrin only under iron-limiting conditions. Conversely, the transferrin binding ability of *H. aegyptius* strain F3031 was not repressed under iron-limiting conditions. The latter results were somewhat unexpected because it seemed that the microorganism would not need to expend the energy to bind transferrin if iron conditions were plentiful. Perhaps this is an artifact of the microorganism being grown *in vitro* as to opposed in the vascular endothelial cells found in the natural human host. Alternatively, maybe the BPF clone is able to acquire iron via several different mechanisms and binding of transferrin is not the primary source. Though it is obvious from *in vitro* dot enzyme assays that *H. aegyptius* binds human transferrin, the significance of this *in vivo* remains to be elucidated.

A puzzling aspect of the use of human transferrin as an iron source by *H. aegyptius* is that this microorganism and other members of the *Haemophilus* genera grow quite well in sBHI, which contains no transferrin. This leads one to believe that *Haemophilus* spp. are able to utilize multiple iron sources and transferrin may be only a minor one or that the true significance of iron acquisition from transferrin is masked by *in vitro* analyses. Furthermore, studies have shown that the ability of a bacterium to bind transferrin is very species specific (40). For example, using a dot enzyme assay, Schryvers *et al.* (41) showed that the following pathogens: *Pasteurella haemolytica*, *A. pleuropneumoniae*, and *N. meningitidis* had a strict specificity for bovine, porcine, and human transferrin, respectively. However, the constitutive expression of the *tbps* during iron-replete and iron-limiting conditions and the ability of BPF isolates to grow and cause disease in infant rats (42) may reflect the ability of this bacterium to obtain iron through alternative pathways. It is also possible that the binding of human transferrin plays an alternative role during the infection process. In this regard, it was recently reported that the ability of other bacteria, such as the periodontal pathogen *A. actinomycetemcomitans*, to enter human epithelial cells was inhibited by human transferrin (43). This observation suggests that transferrin

and its receptor on the surface of the host-target cells are involved in the molecular mechanism by which certain pathogens attach to and invade human cells.

3.3. Regulation of iron acquisition components in the BPF clone

In our studies on iron uptake in the BPF clone of *H. aegyptius*, we have identified several homologs to components of iron acquisition systems in microorganisms that acquire iron via siderophore-independent mechanisms, and throughout this work a common theme was present. We found that the iron regulation of gene expression in the BPF clone is an interesting and unique aspect of the biology of this poorly characterized microorganism. As previously described, F3031 is able to bind human transferrin, but unlike *H. influenzae* strain Eagan, this activity was not well repressed under iron-limiting conditions. In addition, immunoblot analysis with outer membrane preparations from F3031 showed that the expression of Tbp1 and Tbp2 are poorly regulated by the concentration of free iron in the sBHI medium. In fact, these two genes seemed to be constitutively expressed, regardless of the iron concentration of the culture medium. Furthermore, western analysis using proteins from the periplasmic fraction of strains F3031 and Eagan showed that the *H. aegyptius* HitA and FimA homologs are also produced almost independently of the iron content of the medium, while both of these proteins are well controlled by the iron concentration of the culture medium for strain Eagan. The more relaxed iron regulation in the expression of these BPF genes cannot be attributed to the culture conditions since electrophoretic analysis of membrane and periplasmic proteins obtained from cells cultured in the same conditions proved that this clone expresses iron-repressible genes. These results support our findings that the regulation of genes involved in iron metabolism in the BPF clone of *H. aegyptius* is quite different from the classical control mechanisms for iron acquisition systems seen in other microorganisms.

To help answer the regulatory question in *H. aegyptius*, we first sought to determine whether the BPF clone contained a Fur homolog. Cloning and DNA sequencing analysis confirmed the presence of a *fur* gene in the BPF clone that is highly related to the *E. coli* and *H. influenzae* Rd *fur* genes. Furthermore, immunoblot assays showed that this gene is expressed in the BPF clone. We attempted to confirm the repressor activity of Fur in *H. aegyptius* by isolating an isogenic *fur* mutant by either site-directed insertion mutagenesis or selection on sBHI containing MnCl₂, but both experimental approaches failed even after several attempts. Nevertheless, the fact that the cloned BPF *fur* gene restored iron regulation of gene expression when used in genetic complementation experiments with the RRJC1 *fur* mutant strain of *E. coli* (44) strongly suggests that the BPF Fur protein indeed has repressor activity. Thus, one explanation for the defective iron regulation of the *tbp1*, *tbp2*, *hitA*, and *fimA* homologs in the BPF clone is the total absence of or the presence of defective Fur boxes in the promoter region of these genes. Alternatively, the expression of these genes might be under the control of other regulatory elements expressed

only under unique growth conditions, such as those found in the infected host. The last possibility is supported by a report indicating that potentially novel regulatory mechanisms may be involved in the control of the expression of iron responsive genes in *Haemophilus* (45).

When similar analyses were conducted using noninvasive strains of *H. aegyptius*, no significant differences were observed between these strains and the invasive BPF clone of biogroup aegyptius. The noninvasive *H. aegyptius* isolates showed transferrin binding activity and contained the Tbp1 and Tbp2 homologs in the outer membrane fraction. The expression of these proteins and the transferrin binding activity was almost constitutive. In addition, the *H. aegyptius* noninvasive strains grew in sBHI containing 100 M EDDHA and expressed the FimA and HitA homologs. Therefore, based on *in vitro* analyses, the difference in virulence between the noninvasive isolates and invasive BPF strains of biogroup aegyptius is likely not due to the differences in the iron acquisition phenotypes of these two groups of strains.

Even though our preliminary results suggest that the components of the iron uptake system are not unique to the invasive BPF strains, we cannot confirm the actual significance of these proteins until isogenic mutants are generated. Unfortunately, however, the generation of isogenic mutants in the BPF clone is quite a difficult task which has strict requirements for competence development, transformation, and homologous recombination. In fact, only one published report, which describes studies with a *H. aegyptius* P1 deficient mutant, exists (46). Therefore, in the meantime, we have decided to investigate the expression of other genetic determinants that might be responsible for the unique invasive properties of the *H. aegyptius* strain isolated from BPF cases. To achieve this goal we are employing a relatively novel technique called subtraction genome hybridization to identify the genetic differences between the virulent and avirulent strains of *H. aegyptius*. Previous investigators have used this method successfully to identify genetic differences between virulent and avirulent strains of *Mycobacterium* as well as between *N. meningitidis* and *N. gonorrhoeae* (47, 48). In brief, a subtracted library of genomic sequences present in the virulent strain (F3031) but absent in the avirulent strain (F1947) was generated. This was achieved by ligating adaptors to *RsaI*-digested chromosomal DNA from F3031 and F1947 and PCR amplifying these DNAs using primers that annealed to the adaptor sequences (49). Following heat denaturation, primer annealing, and two hybridization reactions, the DNA sample remaining was enriched for F3031 specific sequences, which were theoretically present only in the F3031 invasive strain. Using this method we have cloned several putative unique genes from F3031 and are currently in the process of evaluating their significance.

4. FUTURE DIRECTIONS: *IN VITRO* ANALYSES

Detection of the iron acquisition components of *H. aegyptius* has prompted us to generate isogenic mutants so that we can further investigate whether the iron uptake

proteins are responsible for the unique invasive properties of the *H. aegyptius* strains isolated from BPF cases. To achieve this, we plan to use the isogenic mutants in a recently developed immortalized human microvascular endothelial cell line (HMEC-1) (50). This cell culture model is an effective assay for the study of BPF pathogenesis because the vascular endothelial cells of the host are the target for *H. aegyptius* during the purpura fulminans stage of this infection. Several investigators have used this HMEC-1 cell line to evaluate different aspects of BPF pathogenesis. For example, an *in vitro* assay using these cells was able to clearly differentiate between BPF-associated strains and nonBPF-associated strains of *H. aegyptius* (51, 52). All BPF-associated strains multiplied and produced a cytotoxic phenotype in the cells; whereas, nonBPF-associated strains did not. In addition, only BPF-associated strains were able to attach to and invade the endothelial cells. This HMEC-1 tissue culture model will be used to help elucidate the significance of transferrin binding by the BPF clone and to determine when the *hitABC* and *fimA/fecE* ABC transporters are expressed during infection. For example, if both of the ABC transporter systems are in fact used in the uptake of iron, it is possible that one system is expressed early in the infection when the microorganism is present in the conjunctiva, while the other is expressed during the septicemic stage of the infection. These studies will provide information regarding the role of these iron acquisition components and systems during infection.

In addition to the genes involved in iron uptake, isogenic mutants of the unique F3031 genes found via subtractive hybridization will be generated so that their role in virulence can be further evaluated using the HMEC-1 cell culture model. The significance of this tissue culture system for these studies is that by using isogenic mutants affected in functions encoded by unique genes we will be able to determine whether these genes and their products have a role in the vascular destruction and overwhelming purpura fulminans, which are distinctive traits of Brazilian purpuric fever.

5. PERSPECTIVE

Bacteria must acquire iron to successfully establish an infection within their host, and the actual process of acquiring iron is an important virulence factor for several bacterial species. In these studies we have identified several proteins in the BPF clone that are homologous to components of iron acquisition systems. The high-affinity transferrin receptors in the outer membrane are crucial components for the acquisition of iron by *Haemophilus*. The ABC transporter systems are also essential because they are used in the transport of the iron through the periplasmic space and inner membrane, which results in the subsequent release of the iron into the bacterial cytosol. The true significance of these findings, however, lies in the discernment of determining at what stages of infection these iron acquisition systems are expressed. With this knowledge and the use of isogenic mutants, the role of these proteins in BPF pathogenesis will be ascertained.

It is very likely, though, that genes, other than those encoding iron uptake proteins, are also involved in the pathogenesis of BPF. Using subtractive hybridization, we hope to identify several of these virulence genes that are unique to invasive strains of *H. aegyptius*. Information gained from these studies may also provide insight on the evolution of the invasive BPF-causing strains of *H. aegyptius* versus the noninvasive nonBPF-causing strains. For instance, whether or not the virulence genes present only in the invasive strains are part of a pathogenicity island is an aspect of this poorly characterized pathogen that remains to be examined.

6. ACKNOWLEDGMENTS

The experiments reported in this review that were conducted in the laboratory of L. A. A. were supported by Public Health Service grant AI37781 from the National Institutes of Health. We thank S. H. Goodgal, H. O. Smith, and D. J. Brenner for the following strains: *H. influenzae* Eagan, Rd, and BPF isolates. We are grateful to E. J. Hansen for providing *H. influenzae* strains DL42, DL63, and TN106. We also thank S. A. Morse for providing antisera against the gonococcal FbpA and S. M. Loosmore for the anti-rTbp1 and anti-rTbp2 sera.

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Key words: Brazilian purpuric fever, ABC transporter, Transferrin binding proteins, Fur, Iron

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