Foreign signal peptides can constitute a barrier to functional expression of periplasmic proteins in *Haemophilus influenzae*

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To study the periplasmic branch of iron (ferric ion) uptake systems in Gram-negative bacteria, genetic reconstitution experiments were initiated in *Haemophilus influenzae* involving exchange of the periplasmic iron-binding protein. The expression of many of the heterologous periplasmic ferric-binding proteins (FbpAs) was quite limited. Transformation experiments with the *fbpA* gene from *Neisseria gonorrhoeae* yielded two colony sizes with different phenotypic characteristics. The small colonies contained the intact *N. gonorrhoeae* *fbpA* gene and were deficient in utilization of transferrin iron. The large colonies contained hybrid *H. influenzae/N. gonorrhoeae* *fbpA* genes, were proficient in transferrin iron utilization and had enhanced levels of expression of FbpA. These hybrid genes included several that encoded the mature *N. gonorrhoeae* FbpA with the *H. influenzae* signal peptide. To more fully evaluate the effect of foreign signal peptides, a series of hybrid genes were prepared that exchanged the signal peptides from *H. influenzae* FbpA, *N. gonorrhoeae* FbpA and the TEM-1 β-lactamase. The presence of the *H. influenzae* leader was required for functional expression of FbpAs and was shown to dramatically increase the level of β-lactamase activity.

INTRODUCTION

The eukaryotic host provides an iron-limited environment for potential bacterial pathogens by virtue of iron sequestration by the host iron-binding glycoproteins transferrin (Tf) and lactoferrin (Ratledge & Dover, 2000). Host-specific Gram-negative pathogens from the *Neisseriaceae* and *Pasteurellaceae* possess surface receptors that specifically bind the host Tf and facilitate removal and transport of iron across the outer membrane (Gray-Owen & Schryvers, 1996). The uptake process requires the presence of a functional TonB protein (Jarosik *et al*., 1995, 1996). Isogenic mutants lacking FbpA are defective in utilizing several different sources of ferric ion for growth (Khun *et al*., 1998; Kirby *et al*., 1997), suggesting that the FbpABC pathway represents a convergence point for non-siderophore-mediated outer-membrane ferric ion transport systems.

In view of its critical role in iron acquisition from transferrin, it is not surprising that FbpABC pathways have been identified in species that possess transferrin receptors such as *Neisseria gonorrhoeae* (Adhikari *et al*., 1996), *Haemophilus influenzae* (Adhikari *et al*., 1995), *Mannheimia* (Pasteurella) haemolytica (Kirby *et al*., 1998) and *Actinobacillus pleuropneumoniae* (Chin *et al*., 1996). Functional homologues of FbpABC are also found in species such as *Serratia marcescens* (Angerer *et al*., 1990) and *Yersinia* spp. (Gong *et al*., 2001) that lack the transferrin receptor-mediated uptake pathway. The role of the FbpABC pathway in these species is less clear, as they possess alternative means of transporting iron such as siderophore-mediated uptake systems.

Production of functional FbpA is dependent upon export into the periplasmic space, a process initiated by the presence of a leader (signal) peptide at the N-terminus of the nascent protein chain (Lipmann, 1973). Once the ferric ion has crossed the outer membrane it is transported into the cytoplasm by an ABC (ATP-binding cassette) (Doige & Ames, 1993) import system. This system comprises a periplasmic iron-binding protein, FbpA, and an inner-membrane complex consisting of FbpB, a predicted transmembrane channel-forming protein, and FbpC, a predicted ATPase (Adhikari *et al*., 1995, 1996). Isogenic...
membrane by binding to the translocation channel composed of SecYEG (Stathopoulos et al., 2000). The leader peptide associates with the SecYEG complex and is cleaved by the signal peptidase, LepB, located on the periplasmic face of the membrane prior to translocation of the remainder of the polypeptide chain (Danese & Silhavy, 1998).

Heterologous genetic exchange experiments were initiated in *H. influenzae* to probe the interaction between FbpA and other pathway components. In this study we demonstrate that the functional expression of foreign FbpAs was defective and that the defect was attributable to the leader peptide region of the foreign *fbpA* gene. A series of hybrid genes with foreign leader peptides were prepared and expressed in both the *Escherichia coli* and *H. influenzae* backgrounds to demonstrate that the defect in functional expression was directly attributable to the presence of the foreign leader peptide.

### METHODS

**Bacterial strains and growth media.** Bacterial strains used in this study are listed in Table 1. All strains were stored in 30% (v/v) glycerol at −70 °C. *Mannheimia haemolytica*, *Haemophilus influenzae*, *Actinobacillus pleuropneumoniae*, *Neisseria gonorrhoeae* and *Neisseria meningitidis* were streaked onto chocolate agar plates and incubated overnight at 37 °C in a 5% CO₂ atmosphere. *Yersina enterocolitica* was grown at 30 °C on Luria–Bertani (LB) medium (Gibco-BRL). *E. coli* was grown at 37 °C in LB medium. Nutrient broth (NB, Difco) agar supplemented with 5 g NaCl l⁻¹ and 200 μM 2,2’-dipiridyl (Sigma) was used for growth assays with a siderophore-deficient *entA* *E. coli* host strain (E573). *H. influenzae* strains were inoculated from chocolate plates into BNH [Brain Heart Infusion (BHI, Difco) medium supplemented with 3-3 μg NAD⁺ (Sigma) ml⁻¹ and 10 μg haemin (Sigma) ml⁻¹] broth or propagated onto BNH agar. For anaerobic growth of *H. influenzae*, agar plates were incubated in a sealed anaerobic jar with an Anaerocult A catalyst and an Anaerotest strip at 37 °C (Merck). When appropriate, antibiotics were added to media at the following concentrations: ampicillin, 150 μg ml⁻¹ for *E. coli* and 100 μg ml⁻¹ for *H. influenzae*, kanamycin, 40 μg ml⁻¹ for *E. coli* and 20 μg ml⁻¹ for *H. influenzae*.

### Table 1. Bacterial strains

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<th>Strain</th>
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<td>E157</td>
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<td>E1930</td>
<td>Serotype O:9, pYV⁺, clinical isolate</td>
<td>J. Boyd⁵</td>
</tr>
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*¹ S. Lundberg, Alberta Agriculture, Airdrie, Alberta, Canada; ² R. Kadner, University of Virginia, Charlottesville, VA, USA; ³ E. Hansen, University of Texas, Dallas, TX, USA; ⁴ T. A. Mietzner, University of Pittsburgh, Pittsburgh, PA, USA; ⁵ J. Boyd, National Research Council, Halifax, Nova Scotia, Canada.
Construction of hybrid fbpABC operons in *H. influenzae*. A gene replacement vector previously used for preparing an isogenic fbpA mutant was selected for replacement of the native *H. influenzae* fbpA gene with heterologous fbpA genes in *H. influenzae* (Kirby et al., 1997). The replacement vector, pUC41ΔS, contained flanking regions upstream of the *H. influenzae* fbpA gene, the intergenic region between *H. influenzae* fbpA and fbpB genes and a portion of the *H. influenzae* fbpB gene in order to facilitate homologous recombination. Based on published sequences, specific primers were designed to PCR amplify the fbpA genes from *N. gonorrhoeae* strain F62 (Adhikari et al., 1996), *Y. enterocolitica* strain 3196 (Gong et al., 2001), *M. haemolytica* strain H44 (Kirby et al., 1998) and *A. pleuropneumoniae* strain H49 (Chin et al., 1996) with flanking NsiI sites engineered on both the forward and reverse primers. The PCR products, which included the ribosome-binding site of the foreign gene, were subcloned into the Sse8387I site of pUC41ΔS and a kanamycin-resistance cassette was inserted into either the BamHI or EcoRV site immediately downstream of the foreign fbpA gene. The gene replacement vectors were linearized by EcoRI or ScaI digestion and the digestion mixtures were used to transform *H. influenzae* that was induced for competence by growth on M-IV medium (Barcak et al., 1991). Transformants were selected aerobically on BNH plates supplemented with the 20 μg kanamycin ml⁻¹.

Construction of hybrid fbpA and blaP genes with different signal sequences. A series of hybrid genes with foreign leader peptides were prepared as illustrated in Fig. 1. The amino acid sequences of the leader peptides are shown in part A, the first 20 residues of the mature protein of the hybrid genes are shown in part B and the nomenclature used for the hybrid proteins is illustrated in part C. The overall strategy was to amplify the mature coding sequence of *H. influenzae* FbpA, the *N. gonorrhoeae* FbpA and the TEM-1 β-lactamase and ligate them into vectors containing the leader sequences of *H. influenzae* fbpA, the *N. gonorrhoeae* fbpA genes or simply a start codon. For this set of experiments, the ColE1 origin of replication in the gene replacement vector was replaced by the lower copy number p15a origin. Vectors with the appropriate inserts were used as templates for inverse PCR reactions with the appropriate reverse primers (1013, 1090 or 1016) and ligated with the amplified mature coding sequences. The first codon of the mature FbpA protein that encoded an aspartate residue was also incorporated into the 5’ end of primers 1013 and 1090 so that an extra aspartate residue was added to the mature proteins. The correct orientation of the resulting plasmids was confirmed by colony PCR and sequencing was performed to confirm the junctional sequences. The selected plasmids were linearized and used to transform an *H. influenzae* strain with a chloramphenicol-resistance cassette replacing the fbpA gene (h306) essentially as described above.

**A. Leader Peptides**

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HIFA(L)
MQFKFKLTAALAPANSFAD
1090
NGFA(L)
MKTSIRVALLAALTAPAAD
BlaP(L)
MSIQHRVALIPFCAFCLPVFA
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**B. Mature Coding Regions**

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HIFA
DITYVNYQKKEAAAVAKFA...
1083
NGFA
DITYVNYQKKEAAAVAKFA...
1089
BlaP
HPETLKVKDADQLRAGVD...
1076
```

**C. Hybrid Proteins**

![Fig. 1. Schematic diagram of FbpA proteins and β-lactamase containing different leader peptides. Arrows represent the oligonucleotide primers used for the construction of hybrid proteins. Designations to the left of the schematics refer to nomenclature used for the hybrid proteins. HIFA, *H. influenzae* FbpA; NGFA, *N. gonorrhoeae* FbpA; BlaP, TEM-1 β-lactamase; WT, wild-type; (L), leader peptide; (AL), leaderless protein; M, methionine at start of mature coding region. *Indicates that an additional aspartic acid residue was included with the cloned leader peptide regions. Underlining indicates the additional junctional aspartic acid residue cloned into the leader peptide regions.](http://mic.sgmjournals.org)
Electrophoresis and Western immunoblotting. Samples of whole cells (approx. 1 x 10^8 cells per well) from cultures grown under iron-limited conditions in NB supplemented with 50 μM dipiridyl were separated on 12% SDS-PAGE gels and electrophoretically onto PVDF membrane (Millipore). The membrane was blocked with 0.5% (w/v) skim milk in TBS for 1 h and probed with rabbit polyclonal antisera prepared against the individual purified FbpA proteins at dilutions of between 1:500 and 1:2000. After 1 h incubation, the membrane was washed and incubated with a 1:3000 dilution of the secondary antibody (goat anti-rabbit IgG–horseradish peroxidase conjugate, Sigma). The blots were developed with a chloronaphthol/hydrogen peroxide substrate mixture (Bio-Rad HRP reagent).

Iron starvation of H. influenzae and anaerobic growth assays. For iron starvation of H. influenzae, colonies from a fresh cultured chocolate plate were used to inoculate prewarmed BNP [BHI, 3-32 mg NAD^+ ml^-1 and 20 μg PPIX (protoporphyrin IX, Sigma) ml^-1] broth. The culture was grown at 37°C with shaking to an OD_600 of 0-2 and was then supplemented with EDDHA to a final concentration of 40 μg ml^-1 (BNPE). The culture was grown for an additional 2 h, diluted to an OD_600 of 0-05 in BNPE broth, and 200 μl of this suspension was applied onto sterile cotton swabs onto BNE agar plates (BNPE minus PPIX).

Anaerobic growth assays were conducted as previously described (Kirby et al., 1997) to test the ability of H. influenzae strains to utilize different iron sources. Sterile solutions of fully iron-saturated human transferrin and human lactoferin (Sigma) were prepared at a stock concentration of 8 mg ml^-1 (100 μM) in 50 mM Tris. A stock solution of haemin at a concentration of 100 μM was also prepared and filter-sterilized. Twenty-five microlitres (equivalent to 250 μg) of the above iron supplements were applied onto sterile 0-25 inch (0-64 cm) concentration disks (Difco). The plates were incubated overnight anaerobically at 37°C and growth around the disk was evaluated.

Nitrocefin assay. Nitrocefin is a chromogenic cephalosporin that undergoes a distinctive colour change from yellow to red upon hydrolysis by β-lactamase (O’Callaghan et al., 1972). The level of TEM-1 β-lactamase production in different bacterial strains can be determined by measuring the steady-state rate of nitrocefin hydrolysis in the cell lysates. To prepare cell lysate, 1 ml of culture with an OD_600 of 1-0 was centrifuged at 14,000 r.p.m. for 1 min. The cell pellet was resuspended in 150 μl B-per detergent (Pierce) and incubated on ice for 1 min. The cell lysate was then vigorously vortexed for 30 s. The cell lysate was centrifuged at 14,000 r.p.m. for 1 min and the supernatant was removed for assay.

In a 1 ml cuvette, 470 μl phosphate buffer (pH 7-2), 20 μl cell extract and 10 μl nitrocefin (500 μg ml^-1) were mixed. The nitrocefin solution was prepared by dissolving nitrocefin powder in a few drops of DMSO, made up to the final volume with phosphate buffer, and stored in the dark at −20°C. After the cell lysate was mixed with the substrate, the increase in A_482 was followed for 1 or 2 min. For use as a blank, 490 μl buffer and 10 μl nitrocefin were mixed. The rate (ΔA_482 min^-1) was determined by plotting absorbance over time.

Bioinformatic analyses. The periplasmic binding proteins (PBPs) of the ABC transporters in E. coli and H. influenzae were selected from the ABCdb database (http://abcmdb.cnrsc-irccm.fr/ABCdb/presentation.html) and from a genomic transport analysis page that provided tabulations of possible ABC transporters annotated from complete genomes (http://www-biology.ucsd.edu/~msauer/transport/). The signal peptides were identified using SignalP V2.0.2b on the signalP server (http://www.cbs.dtu.dk/services/SignalP-2.0/), which also provided predictions of the length of the different regions of the signal peptide (n-, h- and c-regions). The net charges of the n-regions were assessed by determining the number of cationic amino acids.

**RESULTS**

Analysis of hybrid fbpABC operons in H. influenzae

Heterologous genetic exchange experiments were initiated in H. influenzae to evaluate the interaction between FbpA and other pathway components. The replacement of the native H. influenzae fbpA gene with foreign fbpA genes was achieved by homologous recombination, taking advantage of the efficiency of natural transformation in this species. Each foreign fbpA gene (including its upstream ribosome-binding site) was first cloned into the gene replacement vector, pUC4J1AS, between the upstream fbp promoter region and a partial fbpB gene. A kanamycin-resistance marker was introduced immediately downstream of the fbpA gene in the same orientation. The resulting plasmid was linearized by digestion of the vector backbone and used to transform wild-type H. influenzae. A positive control containing the H. influenzae fbpA gene and the kanamycin-resistance cassette was included to ensure that the insertion of the resistance marker did not abrogate expression of the downstream fbpB and fbpC genes. A negative control was prepared with a vector containing only the kanamycin-resistance determinant between the flanking regions. Kan^R transformants containing the hybrid operons were selected on BNI plates containing 20 μg kanamycin ml^-1. The recombinant strains were examined by PCR analysis and by sequencing using gene specific primers to confirm that the native H. influenzae fbpA gene was replaced by the corresponding foreign fbpA gene (data not shown).

To evaluate functional reconstitution of the FbpABC pathway, the recombinant H. influenzae strains were tested for their ability to acquire iron from human transferrin (hTF) and ferric citrate on an iron-limited medium (Kirby et al., 1997). Under these assay conditions none of the strains containing intact foreign fbpA genes were capable of using hTF as a source of iron for growth (data not shown), suggesting a defect in the pathway. The control strain with the H. influenzae fbpA gene and kanamycin-resistance cassette grew as well as the wild-type strain, indicating that the deficiency was not due to a polar effect on expression of the fbpBC genes. All of the strains grew on haemin as an iron source, indicating that the defect was specific to the FbpABC pathway.

To determine whether the impairment in transport was due to a deficiency in expression of the foreign FbpAs, the recombinant H. influenzae strains were tested for the expression of the foreign fbpA genes by Western blot analysis using species-specific antisera (Fig. 2, column I). Relative to the negative control strain (lane 1, H263), there was strong expression of the H. influenzae FbpA (Fig. 2A, lane 2), low-level expression of the N. gonorrhoeae FbpA (Fig. 2B, lane 2) and M. haemolytica FbpA (Fig. 2D, lane 2) but no detectable expression of the Y. enterocolitica FbpA (Fig. 2C, lane 2) or the A. pleuropneumoniae FbpA (Fig. 2E, lane 2). To evaluate whether the lack of expression of the
A more extensive analysis of the signal peptide preference was pursued by constructing a series of hybrid genes with different leader peptides and evaluating their export into the periplasm in *H. influenzae* and *E. coli*. The most convenient means of evaluating export was to test the function of periplasmic proteins that required periplasmic localization. The presence of intrinsic phosphatase activity in periplasmic extracts from the parent *H. influenzae* strain precluded the use of alkaline phosphatase. Thus β-lactamase and FbpA were selected for the study, as functional activities of these proteins could be readily assessed.

The leader peptides from the *H. influenzae* and *N. gonorrhoeae* FbpA genes were PCR amplified along with a control containing only a start codon and ligated to the mature FbpA leader sequence (Fig. 3). No alteration other than that of the crossover event was found after sequencing the entire gene. The results indicate that the mature *N. gonorrhoeae* FbpA protein is capable of replacing the *H. influenzae* FbpA in reconstitution of the iron acquisition pathway. These observations also indicate that the defect in functional expression of the intact *N. gonorrhoeae* fbpA gene in *H. influenzae* was due to the presence of the foreign leader peptide and thus there are probably deficiencies in its recognition by the export apparatus or signal peptidase.

**Analysis of hybrid fbpA genes**

Two colony sizes were obtained when vector containing the cloned *fbpA* gene from *N. gonorrhoeae* was used as the donor DNA. PCR analysis demonstrated that the small-colony transformants contained the complete *N. gonorrhoeae* *fbpA* gene whereas the large-colony transformants contained hybrid *Haemophilus/Neisseria fbpA* genes that resulted from recombinational events within the *fbpA* gene. The small-colony transformants were unable to use hTf as the sole iron source under iron-restricted conditions whereas the large-colony transformants were proficient in transport of iron from hTf (data not shown). To define the smallest portion of the *H. influenzae* gene required for conferring the large-colony phenotype, transformation experiments were repeated and large-colony transformants were screened by colony PCR. The selected large-colony transformants were tested for growth with hTf as the iron source and were shown to be positive (data not shown). Selected hybrid genes were amplified from the chromosome, subcloned and sequenced. Several large-colony transformants were obtained with hybrid genes that resulted from crossover events near the 5′ end of the gene that encoded the complete mature coding region of *N. gonorrhoeae* FbpA with the *H. influenzae* FbpA leader sequence (Fig. 3). No alteration other than that of the crossover event was found after sequencing the entire gene. The results indicate that the mature *N. gonorrhoeae* FbpA protein is capable of replacing the *H. influenzae* FbpA in reconstitution of the iron acquisition pathway. These observations also indicate that the defect in functional expression of the intact *N. gonorrhoeae* fbpA gene in *H. influenzae* was due to the presence of the foreign leader peptide and thus there are probably deficiencies in its recognition by the export apparatus or signal peptidase.

**Analysis of hybrid fbpA genes**

A more extensive analysis of the signal peptide preference was pursued by constructing a series of hybrid genes with different leader peptides and evaluating their export into the periplasm in *H. influenzae* and *E. coli*. The most convenient means of evaluating export was to test the function of periplasmic proteins that required periplasmic localization. The presence of intrinsic phosphatase activity in periplasmic extracts from the parent *H. influenzae* strain precluded the use of alkaline phosphatase. Thus β-lactamase and FbpA were selected for the study, as functional activities of these proteins could be readily assessed.

The leader peptides from the *H. influenzae* and *N. gonorrhoeae* FbpA genes were PCR amplified along with a control containing only a start codon and ligated to the mature coding sequences of the *H. influenzae* FbpA, *N. gonorrhoeae* FbpA and TEM-1 β-lactamase (Fig. 1). The hybrid genes were assembled in a vector flanked by the region from *H. influenzae* upstream of the *fbpA* gene and a kanamycin-resistance marker followed by a partial *fbpB* gene from *H. influenzae* to facilitate incorporation into the native *fbpA* locus in *H. influenzae* through homologous recombination. The recombinant plasmids were linearized by digestion with ScaI and used to transform the parent
H. influenzae

strain. In order to avoid the complication of unexpected crossover events within the fbpA locus, all transformations were performed with an H. influenzae strain that had a chloramphenicol-resistance cassette in place of the native H. influenzae fbpA gene (strain H306).

KanR/CmS colonies were screened to confirm the presence of the modified genes by PCR analysis.

The activity of the hybrid β-lactamase genes in E. coli could be tested directly with the p15a hybrid plasmids but an intact fbp pathway was required for evaluating the hybrid fbpA genes. Thus, the hybrid fbpA genes were subcloned into a complete fbpABC operon for assessment of function. The reconstitution of hybrid operons in E. coli was achieved by directional cloning with BamHI and SacII double digestion of the vector p15aHiUPFBC and the replacement vectors that harboured the hybrid genes. BamHI and SacII subfragments containing the hybrid fbpA genes and the H. influenzae fbpBC genes were ligated together and the resultant plasmids were transformed into an E. coli entA strain for expression and functional analysis.

The expression of the H. influenzae FbpA and the N. gonorrhoeae FbpA from genes containing different leader peptides in both the E. coli and H. influenzae backgrounds was analysed by Western blotting using polyclonal antisera (Figs 4 and 5). There was no detectable FbpA in recombinant strains that contained the leaderless fbpA genes regardless of the host species (lanes A4, B8, C4 in Fig. 4 and lanes A3, B8, C4 in Fig. 5), suggesting that protein expressed in the cytoplasm is misfolded and degraded. The hybrid gene constructed with the H. influenzae leader and mature coding sequence [HIFA(L)HIFA] differed from the wild-type H. influenzae fbpA gene (WT HIFA) only by having an extra aspartate residue at the beginning of the mature protein and both were shown to express at a similar level in E. coli (compare lanes A2 and A3, and C3 and C5 in Fig. 4). The most significant observation

![Fig. 3. Site of crossover for hybrid H. influenzae/N. gonorrhoeae fbpA genes. The DNA sequences of the H. influenzae, N. gonorrhoeae and two hybrid H. influenzae/N. gonorrhoeae fbpA genes with corresponding amino acid sequences are shown. The dashed lines represent the middle segment of the leader peptide region. A gap has been introduced between the leader peptide and mature protein sequences. The underline indicates the region of crossover between the H. influenzae and N. gonorrhoeae fbpA genes. The remainder of the sequence of the hybrid genes was identical to the N. gonorrhoeae fbpA gene.](image)

![Fig. 4. Western blot analysis of H. influenzae FbpA containing different leader peptides. Whole-cell preparations were subjected to SDS-PAGE, electrophoresed and then probed with rabbit antiserum directed against H. influenzae FbpA. The proteins being expressed are indicated by the nomenclature used in Fig. 1. M, molecular mass marker (40–7 kDa). A. Lanes 1–5, expression in E. coli DH5α strains containing replacement vectors. Lane 1, vector control is a strain containing the replacement vector without insert. B. Lanes 6–9, expression in H. influenzae recombinant strains. Lane 7 is strain H306, with a chloramphenicol-resistance cassette replacing the fbpA gene. C. Expression in E. coli entA strains containing hybrid FbpABC pathways. Lane 1 (negative control), is the strain with no plasmid and lane 2 (vector control) is a strain containing the vector without insert.](image)
The function of the hybrid FbpAs was evaluated in *E. coli* and *H. influenzae* based on their ability to mediate iron transport via the FbpABC pathway. In *E. coli* this involved growth studies using an *entA* mutant strain. The absence of siderophore production in the *entA* mutant makes it susceptible to iron restriction by the addition of the iron chelator dipyridyl. The growth restriction by dipyridyl can be overcome by expression of foreign *fbpABC* operons (Adhikari et al., 1995; Zimmermann et al., 1989). Thus the hybrid FbpAs were evaluated by monitoring growth of an *E. coli* *entA* mutant containing the hybrid operons on iron-restricted medium containing 150 μM dipyridyl. Only the hybrid FbpAs that lacked a leader peptide, and thus were not exported into the periplasmic space, were unable to support growth on media containing dipyridyl (Fig. 6). The foreign FbpAs were able to support growth when they contained either an *H. influenzae* or an *N. gonorrhoeae* leader peptide, indicating that there was efficient export into the periplasm. These results also indicate that there is sufficient similarity between the *H. influenzae* and *N. gonorrhoeae* FbpAs such that the latter can functionally interact with the *H. influenzae* FbpBC complex.

In *H. influenzae* the evaluation of the function of the hybrid FbpAs involved monitoring growth on iron-limited medium with hTf as the exogenous iron source, a process shown to be dependent on the presence of a functional FbpA (Kirby et al., 1997). As expected, strains expressing leaderless FbpAs that would be defective in export to the periplasm were deficient in growth with hTf (Fig. 6). In contrast, strains expressing hybrid FbpAs with an *H. influenzae* leader peptide were capable of growing on iron-restricted media in the presence of exogenous hTf, indicating that the mature FbpAs were exported to the periplasm. In addition, the growth of the strain expressing the HIFA(L)NGFA protein indicates that the *H. influenzae* FbpA is capable of functionally interacting with the rest of the iron acquisition pathway components from *H. influenzae*. Strains expressing FbpAs with the *Neisseria* leader peptide were incapable of growing on BNHE agar supplemented with hTf, suggesting that *H. influenzae* was not able to efficiently process and export proteins with a *Neisseria* leader peptide into the periplasm.

### Analyses of β-lactamase with different signal peptides

In the absence of available antiserum against β-lactamase, expression of hybrid TEM-1 β-lactamases was monitored functionally: by growth on ampicillin-containing agar and by assaying enzyme activity using the chromogenic substrate nitrocefin. Except when the leader peptide was absent, expression of all of the β-lactamases resulted in growth on LB agar with 150 μM ampicillin in *E. coli* and on BNF agar with 100 μM ampicillin in *H. influenzae* (Fig. 6B). Since it was probable that even a low level of functional β-lactamase expression was sufficient to confer ampicillin resistance upon the host, a more sensitive and quantitative measure of β-lactamase was required. Thus an

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**Fig. 5.** Western blot analysis of *N. gonorrhoeae* FbpA containing different leader peptides. Whole-cell preparations were subjected to SDS-PAGE, electroblotted and then probed with rabbit antiserum directed against *N. gonorrhoeae* FbpA. The proteins being expressed are indicated by the nomenclature used in Fig. 1. M, molecular mass marker (40-7 kDa). A. Lanes 1–4, expression in *E. coli* DH5αF’ strains containing replacement vectors. Lane 1, vector control is a strain containing the replacement vector without insert. B. Lanes 5–9, expression in *H. influenzae* recombinant strains. Lane 5 is strain H306, with a chloramphenicol-resistance cassette replacing the fbpA gene. Lane 7 is strain H292, which contains the hybrid *H. influenzae*/*N. gonorrhoeae* fbpA gene isolated as a large-colony variant. C. Expression in *E. coli* *entA* strains containing hybrid FbpABC pathways. Lane 1 (negative control), is the strain with no plasmid and lane 2 (vector control) is a strain containing the vector without insert.
enzyme assay using nitrocefin as a substrate was employed for quantitative analysis. The rate of nitrocefin hydrolysis was measured by the change of A482. No activity was detected for the strain of H. influenzae expressing b-lactamase lacking a leader peptide. The highest rate of nitrocefin hydrolysis was observed for the gene containing the H. influenzae leader peptide [HIFA(L)BlaP], which was more than 10-fold higher than that from the gene containing the N. gonorrhoeae leader peptide [NGFA(L)BlaP] and >180-fold higher than that of the wild-type b-lactamase gene (Fig. 6). These results indicate that even though the N. gonorrhoeae leader peptide and native b-lactamase leader peptide resulted in substantially reduced levels of export relative to the H. influenzae leader peptide, there was sufficient enzyme to support growth in the presence of 100 μM ampicillin.

**DISCUSSION**

Genetic exchange experiments were initiated in H. influenzae in order to evaluate the interaction of FbpA with other pathway components, with the expectation that the processing and export of foreign FbpAs would be readily accomplished. This expectation was based on the assumption that the export process was conserved amongst Gram-negative bacteria, a concept supported by the observation that high levels of expression of foreign periplasmic proteins can be readily achieved in E. coli, as exemplified by FbpAs (Bruns et al., 1997). However, in this study, the relative level of expression of several foreign FbpAs was substantially lower in H. influenzae than in E. coli (Fig. 2, compare lanes 2 and 5). Genetic exchange experiments with the N. gonorrhoeae fbpA gene revealed that recombinational events involving exchange of the leader (signal) peptide region (Fig. 3), restored expression levels of FbpA (Fig. 2B, compare lanes 2 and 3) and enabled the strain to grow on hTf as a sole iron source. A more extensive set of experiments (Figs 4–6) established that the presence of the leader peptide region from the N. gonorrhoeae fbpA gene, as well as from the TEM-1 b-lactamase gene, resulted in inefficient export of periplasmic proteins specifically in H. influenzae. To the best of our knowledge this is the first report of substantial variation in the specificity of the Sec-dependent export pathway in Gram-negative bacteria.

The lack of function of the leaderless FbpAs and b-lactamase in either host (Fig. 6) confirms that the leader peptides are
required to mediate the export of these proteins, as has been proposed for most extra- cytoplasmic proteins (Danese & Silhavy, 1998). Signal peptides consist of three regions: (i) a positively charged, hydrophilic, N-terminal region (n-region), (ii) a largely hydrophobic core region of 9–15 amino acids postulated to form an \( \alpha \)-helix (h-region), and (iii) a 4–6 amino acid C-terminal region (c-region). These regions of the signal peptide primarily interact with different components of the export apparatus and thus are implicated in different stages of the export process. In this study, we do not provide direct experimental evidence to determine what segments of the signal peptide are responsible for the recognition specificity by the \( H. \) influenzae secretion apparatus or to determine what stage of the export process is impaired. However, some insights might be gleaned from a comparison of the signal peptides from \( H. \) influenzae FbpA, \( N. \) gonorrhoeae FbpA and TEM-1 \( \beta \)-lactamase (Fig. 1) or a more extensive analysis of signal peptides from periplasmic proteins from \( H. \) influenzae and \( E. \) coli.

The n-region is important for recognition by SecA, the first step in the export process (Akita \textit{et al.}, 1990). The n-region of the leader peptides used in this study did vary (Fig. 1) and comparisons between leader peptides from \( H. \) influenzae and \( E. \) coli ABC transporter periplasmic binding proteins (PBPs) revealed some differences. A majority (82\%) of the signal peptides from the \( H. \) influenzae periplasmic proteins had two or more lysines at the N-terminus, and unlike the situation in \( E. \) coli, arginine was rarely present. There were no obvious differences in the \( \alpha \)-helical h-region, particularly between the \( H. \) influenzae and \( N. \) gonorrhoeae leader peptides (Fig. 1). Likewise, there were no obvious differences in the \(-3\) and \(-1\) positions of the leader (von Heijne, 1990) that are required for recognition by the signal peptidase. It is interesting to note that both the \( N. \) gonorrhoeae FbpA leader and the TEM-1 \( \beta \)-lactamase leader have a proline in the \(-4\) position (Fig. 1), implying that there may be structural differences in the junction between the transmembrane \( \alpha \)-helical h-region and the c-region segment (Barkocy-Gallagher \textit{et al.}, 1994).

The sequence comparisons suggest that the n-region or junctional regions of the leader peptides and their interaction with the SecA or leader peptidase components are most likely responsible for the observed barrier to functional expression of periplasmic proteins in \( H. \) influenzae. Thus it would be logical to target these regions and genes in subsequent studies designed to further probe the novel features of the \( H. \) influenzae export apparatus revealed in this study. The availability of a simple screening procedure (large vs small colony) and a high-efficiency natural transformation system should facilitate genetic approaches for probing the interactions between the leader peptide and export apparatus. Amino acid alignments of various signal peptidases reveal that the \( H. \) influenzae signal peptidase contains an extra 26 amino acids predicted to form an extended loop near to the S3 substrate-binding site (Paetzol \textit{et al.}, 1998) (data not shown) and thus genetic experiments should be included to evaluate the role of this region.

The specificity of the \( H. \) influenzae export apparatus constitutes a barrier to functional expression of foreign FbpAs (Fig. 6) since low levels of expression (Fig. 2B–E, lane 2) are not sufficient to mediate iron acquisition from transferrin. Although the levels of production of TEM-1 \( \beta \)-lactamase were also dramatically reduced (Fig. 6), very low levels of functional enzyme were sufficient to retain function, resistance to antibiotics in the medium. Thus it seems that specificity of the Sec-dependent pathway can constitute a barrier to functional expression of foreign periplasmic proteins in Gram-negative bacteria but depends upon the level of periplasmic protein required for function. Since the Sec-dependent pathway also constitutes the first phase in the export and assembly of many outer-membrane proteins and some secreted proteins, the specificity of this process may have much broader significance.

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REFERENCES


