CHARACTERIZATION OF A NUCLEOTIDE BINDING DOMAIN ASSOCIATED WITH *NEISSERIAL* IRON TRANSPORT

by

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ABSTRACT

The ability for the pathogen *Neisseria gonorrhoeae* to sequester iron from sources such as transferrin from the human host plays an important role in initiating infection. The *Neisserial fbpABC* operon encodes an ABC (ATP binding cassette) transporter proposed to function in transporting iron at the periplasm-to-cytosol level. The highly conserved ATP binding domain of these transporters typically utilizes the energy of ATP hydrolysis to pump substrates across the membrane against a concentration gradient. The goal of my project is to show that FbpC functions as a nucleotide binding domain for this iron transport system. First, the *N. gonorrhoeae fbpC* gene was successfully amplified and cloned into the pET28a expression vector. The resulting fusion protein ($FbpC_{(his6)}$) of approximately 40kDa was overexpressed in *Escherichia coli* HMS174(DE3) cells and purified to near homogeneity by nickel-chelate affinity followed by anion-exchange chromatography. Isolated $FbpC_{(his6)}$ has intrinsic ATPase activity uncoupled from the iron transport process and displays a specific activity of approximately 0.5 μmol/min/mg, similar to that determined for the distantly related nucleotide binding domains HisP and MalK in their purified forms. An FbpC mutant, E164D, designed to be defective in ATP hydrolysis was produced, purified, and found to contain a ten-fold reduction in specific activity as compared to the wild-type. Purified $FbpC_{(his6)}$ was also covalently modified by 8-azido-[γ^{32}P]ATP, and this interaction was shown to be specific by preincubation of reactions with unlabeled ATP. In conclusion, FbpC is a functional nucleotide binding domain capable of powering the iron transporter.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ATP	Adenosine 5' triphosphate
bp	Base pair(s)
CAPS	(3-[Cyclohexylamino]-1-propanesulfonic acid)
CBR	Coomassie Blue R
CFTR	Cystic fibrosis transmembrane conductance regulator
CHES	2-[N-Cyclohexylamino]ethane-sulfonic acid
C-terminal	Carboxyl terminal
Da	Dalton
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
Fbp	Ferric binding protein
FPLC	Fast Protein Liquid Chromatography
Hb	Hemoglobin
Hp-Hb	Haptoglobin-hemoglobin
IM	Inner membrane
IPTG	Isopropyl-β-D-Thiogalactopyranoside
kDa	Kilodaltons

K_{sol}	Solubility equilibrium constant
LB	Luria-Bertani (media)
Lbp	Lactoferrin binding protein
Lf	Lactoferrin
MES	(2-[N-Morpholino]ethanesulfonic acid}
MOPSNa	(3-[N-Morpholino]propanesulfonic acid), sodium salt
N.A.P.S. Unit	Nucleic-Acid and Protein Service Unit
N_3-ATP	Azido-adenosine triphosphate
NBD	Nucleotide binding domain
Ni-NTA	Nickel-nitrilo-triacetic acid
N-terminal	Amino-terminal
NTHI	Non-typeable *Haemophilus influenzae*
O.D.	Optical density
OM	Outer membrane
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pi	Inorganic phosphate
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
Taq	*Thermus aquaticus*
Tbp	Transferrin binding protein
Tf	Transferrin

TM	Transmembrane
Tris	Tris(hydroxymethyl)aminomethane
UV	Utraviolet
v/v	Volume-to-volume ratio
w/v	Weight-to-volume ratio

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1. INTRODUCTION

1.1 Importance of iron in microorganisms

Iron is an essential nutrient for most microorganisms, including pathogenic bacteria. It is an integral part of many metabolic enzymes and participates in a number of key metabolic functions such as cell respiration and DNA synthesis. Examples of important proteins that contain iron include electron-transfer proteins such as cytochromes and iron-sulfur proteins, as well as hydroperoxidases, ribonucleotide reductases and iron-activated enzymes. The ability of iron to exist in aqueous solution as Fe (II) or Fe (III), or as inorganic or organic ferrous or ferric complexes also makes it an important cofactor for a variety of biochemical reactions.

Even though iron is the fourth most abundant in the earth's crust, its extremely low solubility (K_{sol} Fe $(OH)_3 \sim 10^{-38}$) (Neilands et al., 1987) under physiological conditions, especially around neutral pH in aqueous solution, makes iron limiting in the living environment of many microorganisms. The concentration of free, uncomplexed Fe (III) in aqueous solution is less than 10^{-17} M; this presents a challenge for the survival of microorganisms.

Iron deprivation leads to reduced growth rates in many bacteria (Archibald and DeVoe, 1978), important morphological changes and alterations in the compositions of proteins that contain iron or whose syntheses are regulated by iron (Criado et al., 1993). Early studies by Schade and Caroline (1946) established the bacteriostatic property of serum in *Shigella dysenteriae* that can be reversed by the addition of iron (Schade and

Caroline, 1944; Schade and Caroline, 1946). Other investigators have also demonstrated the importance of iron availability in microbial infection (Payne and Lawlor, 1990). Iron enhanced lethality of *N. meningitidis* and *N. gonorrhoeae* has been demonstrated in mice and chicken embryos, respectively (Holbein, 1980; Holbein, 1981; Payne and Finkelstein, 1975). Table 1 shows enhancement of virulence or lethality of different pathogens in animal models by the addition of exogenous iron.

Table 1. Enhancement of virulence or lethality of different pathogens in animal models by the addition of exogenous iron (Payne and Lawlor, 1990).

Pathogen	**Animal Model**
Bacillus anthracis	Mouse
Campylobacter jejuni	11-Day chick embryo
Clostridium perfringens	Guinea pig
Escherichia coli	Guinea pig
Klebsiella pneumoniae	Rat, mouse
Listeria monocytogenes	Mouse
Mycobacterium tuberculosis	Mouse
Neisseria gonorrhoeae	11-Day chick embryo
Neisseria meningitidis	Mouse
Pasteurella multocida	Guinea pig
Pseudomonas aeruginosa	Rabbit
Salmonella typhimurium	Mouse
Staphylococcus aureus	Mouse
Vibrio cholerae	Mouse
Vibrio vulnificus	Mouse
Yersinia enterocolitica	Mouse
Yersinia pestis	Mouse

1.2 Sources of iron for different pathogenic bacteria

In the infection process, pathogenic bacteria must be able to acquire sufficient iron from the environment to establish colonies in the host. Depending on where they grow in the host, different pathogens have developed a variety of strategies to obtain the

required iron and transport it into the cytoplasm (Payne, 1993; Payne and Lawlor, 1990; Schryvers and Stojiljkovic, 1999). Many bacteria produce and secrete small molecular-weight siderophores to their extracellular environment to satisfy their iron requirement (Neilands, 1995; Neilands et al., 1987; Payne and Lawlor, 1990).

Siderophores are small molecule (500 – 1000 Da), nonproteinaceous organic Fe (III) chelators secreted by many bacteria, including *Escherichia coli*, *Salmonella, Shigella* and *Vibrio* Species (Neilands et al., 1987). These ferric carriers have very high affinity for ferric iron and are usually categorized based on their ligands as either hydroxamate or catechol siderophores. These siderophores, together with their corresponding receptors on the microbial surface, enable transport of iron into the cell (Neilands, 1982). Iron acquisition with the use of siderophores has the advantage that specific sources of iron are not restricted, thereby allowing microorganisms to grow in a variety of hosts and environments (Mietzner et al., 1998). *E. coli*, for example, has developed multiple siderophore-driven systems that reflect the physiological flexibility to use different iron sources in different environments. The disadvantage of siderophore-mediated iron transport is the high energy cost of continually synthesizing new apo-siderophores and maintaining specific uptake components (Mietzner et al., 1998).

Therefore, some other bacteria, especially those that have a narrow host range, possess special transport systems to mobilize and take up iron directly from the host environment (Cornelissen et al., 1993; Cornelissen and Sparling, 1994; Criado et al., 1993; Schryvers et al., 1998). A representative example is the *Neisseria* spp. These

bacteria express specific surface receptor proteins that interact with a variety of iron-containing proteins and compounds, including extracellular host iron-binding proteins such as transferrin and lactoferrin, and heme-containing proteins such as haemoglobin (Hb) and haptoglobin-hemoglobin (Hp-Hb) (Genco and Desai, 1996; Gray-Owen and Schryvers, 1996). Receptor-mediated iron acquisition in *Neisseria* will be discussed further in Section 1.4.

1.3 Pathogenic Neisseria

The two most clinically significant species of the genus *Neisseria* are *N. gonorrhoeae* and *N. meningitidis*, which cause the diseases gonorrhea and meningitis (inflammation of the membranes covering the central nervous system), respectively. Both microorganisms are non-spore-forming, non-motile Gram-negative diplococci which inhabit human mucosal surfaces (Genco and Desai, 1996). In this study, only *N. gonorrhoeae* was used, and will be discussed in detail below.

N. gonorrhoeae strains infect the mucosal surfaces of urogenital sites and the oro- and nasopharynx, causing symptomatic or asymptomatic infection. Despite the availability of excellent treatment, gonorrhea is one of the most widespread human infectious diseases in the United States and worldwide (Brock et al., 1994). This prevalence of infections could be attributed to several factors, including the lack of acquired immunity, widespread use of oral contraceptives, and the frequent occurrence of subclinical infections (Brock et al., 1994). The increase in antibiotic resistance of *N.*

gonorrhoeae (Gorwitz et al., 1993; Lind, 1997) further raised concern and interest to study the pathogenesis of this bacterium.

1.4 Receptor-mediated iron acquisition in *Neisseria gonorrhoeae*

The ability for the pathogen *N. gonorrhoeae* to sequester its iron requirement from the human host plays an important role in establishing infection and causing disease. Within the mucosal surfaces that *N. gonorrhoeae* inhabit, the majority of iron is bound to lactoferrin (Genco and Desai, 1996); hemin is also present at mucosal sites as a result of the desquamation of epithelial cells. Unlike many bacteria, *N. gonorrhoeae* does not produce siderophores; however, the ability to use some siderophores has been observed (Finkelstein and Yancey, 1981; Yancey and Finkelstein, 1981a; Yancey and Finkelstein, 1981b). Instead, the gonococci express on their outer membrane surface an array of receptors that interact specifically with human iron-binding proteins such as lactoferrin (Mickelsen et al., 1982), transferrin (McKenna et al., 1988), hemoglobin (Archibald and DeVoe, 1980) and hemin (Mickelsen and Sparling, 1981; Yancey and Finkelstein, 1981a).

Cornelissen et. al. (1992) identified and isolated two iron-repressible gonococcal transferrin receptors, Tbp1 and Tbp2, both shown to be homologous to the TonB dependent class of outer membrane receptors in gram-negative bacteria (Cornelissen et al., 1992). Homologous receptors were then found to bind to lactoferrin (Pettersson et al., 1994), hemoglobin (Stojiljkovic et al., 1995), and haptoglobin-hemoglobin complex (Stojiljkovic et al., 1996) for the closely related *N. meningitidis*. In addition, several open

reading frames that share homology with siderophore receptors have been revealed recently (Beucher and Sparling, 1995; Carson et al., 1999; Klee et al., 2000), suggesting that *Neisseria* can also utilize exogenous siderophores provided by neighboring microbes. These receptor-mediated iron uptake systems typically function in a two-stage process, first removing iron from the host iron-binding proteins into the periplasm, then transporting the metal into the cytoplasm (Figure 1). In the case of siderophore-mediated iron transport, the whole siderophore is taken up into the cell via a similar mechanism (Schryvers and Stojiljkovic, 1999). The transferrin-mediated iron transport process is discussed below.

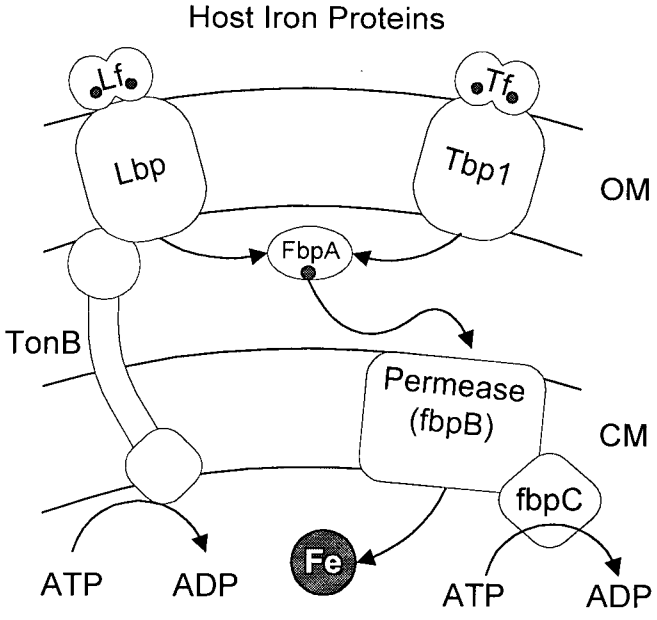

Figure 1. Schematic diagram of receptor-mediated iron uptake in *Neisseria.* Tbp1 and Lbp are shown as outer membrane (OM) proteins and initiate the first step of iron uptake by binding to the host iron proteins, transferrin (Tf) and lactoferrin (Lf). The second stage of transferrin-mediated iron-uptake involves the transfer of iron from the periplasmic binding protein (FbpA) into the inside of the cell via a membrane bound permease (FbpB). See text for detailed description. Adapted from (Cornelissen and Sparling, 1994).

1.4.1 Extracellular-to-periplasm

The outer membrane receptor consists of two transferrin binding proteins, Tbp1 and Tbp2, of which Tbp1 is essential for growth of gonococci on transferrin *in vitro* (Cornelissen et al., 1992). Once bound to the receptors, iron is removed from transferrin in an energy-dependent process and transferred to a periplasmic protein, FbpA (Fbp, for ferric binding protein). Energy required for this transport process is proposed to be transferred from the cytoplasm to the outer membrane receptor via a TonB-dependent process (Cornelissen et al., 1997; Gray-Owen and Schryvers, 1996).

FbpA is a 34 kDa protein, the first soluble component identified from the *Neisseria* iron uptake system (Mietzner et al., 1986; Mietzner et al., 1984). It is iron regulated by the *fur* locus (Berish et al., 1993; Desai et al., 1996; Karkhoff-Schweizer et al., 1994; Thomas and Sparling, 1994) and is compartmentalized to the periplasmic space of the pathogen (Ames, 1986; Chen et al., 1993; Mietzner et al., 1987; Mietzner et al., 1984). Expressed by all strains of *N. gonorrhoeae* and *N. meningitidis* and highly conserved between species, FbpA functions as the periplasmic binding component of a high-affinity active transport system of iron from human transferrin and other non-heme iron carriers (Chen et al., 1993; Khun et al., 1998). The gene was cloned and the nucleotide sequence determined (Berish et al., 1990). Recombinant FbpA can be expressed in *E. coli* and purified in large quantities. FbpA binds reversibly a single molecule of ferric iron with a high affinity approaching that of transferrin (Chen et al., 1993; Crichton, 1990). Iron coordination of the homologous periplasmic iron binding protein, HitA, is through four protein ligands (from the side chains of two tyrosines, a

histidine and a glutamate) and a phosphate anion (Bruns et al., 1997). Pulse chase experiments demonstrate transient association of transferrin-bound iron with FbpA (Chen et al., 1993) and suggest that iron is deposited from transferrin to FbpA in the periplasmic space.

1.4.2 Periplasm-to-cytosol

The second step of *Neisserial* iron acquisition, the metal transport from the periplasm across the cytoplasmic membrane, is of equal importance. Studies on other periplasmic transport systems (Adhikari et al., 1995; Angerer et al., 1990; Davidson and Nikaido, 1991; Kerppola et al., 1991) led to the generalization that three components are employed in these types of transporters involved in active transport of many growth-essential nutrients: 1) a periplasmic binding protein, that binds the substrate to be transported and thus initiates the periplasmic transport process; 2) a cytoplasmic permease, through which the substrate is transported across the membrane, and 3) a nucleotide binding domain, which provides the energy for the transport process through the hydrolysis of ATP.

Adhikari *et al.* (1996) determined the nucleotide sequences of two open-reading frames (ORFs), termed *fbpB* and *fbpC*, downstream of the *fbpA* gene in *Neisseria gonorrhoeae* and proposed that these two ORFs correspond to genes for the cytoplasmic permease and nucleotide-binding domain respectively for the periplasmic transport of iron. Altogether the *fbpABC* operon composes an iron transport system that functions at the periplasm-to-cytosol level (Figure 2).

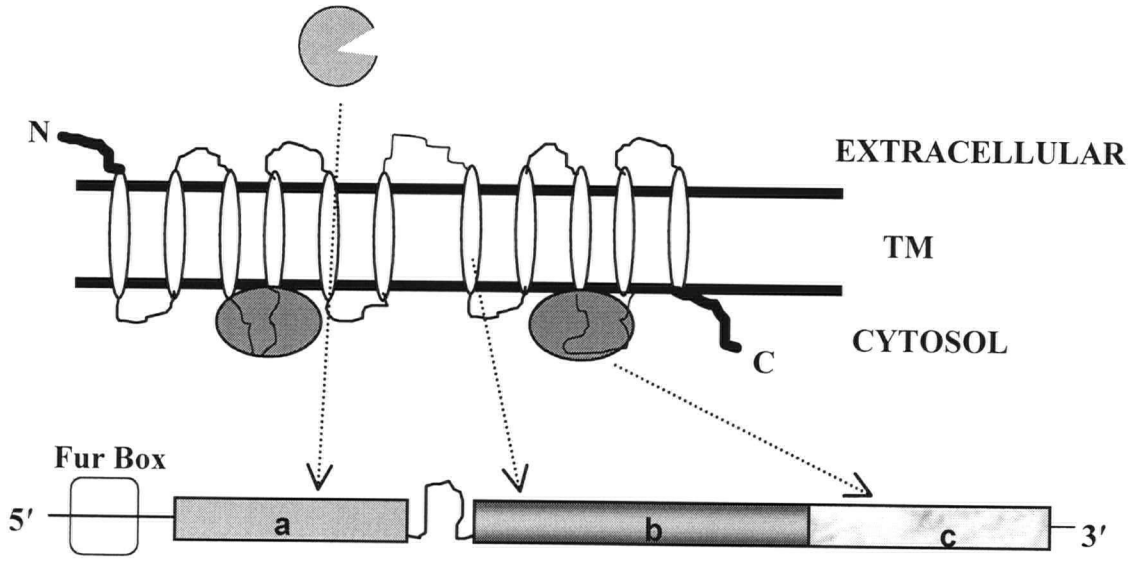

Figure 2. Schematic diagram of the Fe(III) periplasm-to-cytosol iron transporter encoded by the *fbpABC* operon. The operon is iron-regulated by the *fur* locus. The first gene (a) encodes the periplasmic binding protein, FbpA, followed by a stem-loop structure preceding the permease-encoding gene, *fbpB* (b). The nucleotide binding domain is encoded by *fbpC*, the last gene in the operon (c).

FbpB is a highly hydrophobic protein consisting of 511 amino acids, 62% of which are hydrophobic. FbpB has an estimated molecular weight of 56 kDa, and contains an EAA loop typical for cytoplasmic permeases in bacterial uptake systems. The toxicity from the expression of *fbpABC* operon to *E. coli* was found to be due to the expression of FbpB, even when only a partial gene product was produced (Adhikari et al., 1996). Because of these properties, little is known about this protein. FbpC, on the other hand, could be readily expressed ((Adhikari et al., 1996), this study). This proposed nucleotide binding domain (NBD), of 352 amino acids, contains 50.5% hydrophobic residues. It contains conserved motifs with other members of NBDs in ABC transporters, such as the Walker A, Walker B, and the helical and linker regions in between (Adhikari et al., 1996).

The involvement of the *fbpABC* operon in periplasm-to-cytosol transport of iron has been demonstrated, as the addition of a plasmid containing the *fbpABC* operon can enable siderophore-deficient *aroB E. coli* strains to grow on nutrient agar containing an inhibitory concentration of 2,2'-dipyridyl, an iron chelator (Adhikari et al., 1996). However, the mechanism by which the three components of the transport system work together is yet to be described.

1.5 Studies on homologous transport systems

Fe(III) periplasm-to-cytosol transporters are expressed by *N. gonorrhoeae* and *N. meningitidis* as well as other pathogens (Table 2). Two homologous iron transport systems are encoded by the *hitABC* operon in *H. influenzae*, the etiologic agent of otitis media and meningitis (Murphy and Apicella, 1987), and by the *sfuABC* operon in *Serratia marcescens*, an important nosocomial pathogen (Hejazi and Falkiner, 1997). All three operons were shown to confer the ability of siderophore-deficient *E. coli* strains to grow on nutrient agar containing dipyridyl, suggesting roles in non-heme iron acquisition (Adhikari et al., 1996; Sanders et al., 1994; Zimmermann et al., 1989). At the genetic level, *fbpABC* shares approximately 60% amino acid identity with the *hitABC* and 40% identity with *sfuABC* (Mietzner et al., 1998).

Table 2. List of Fe(III) periplasm-to-cytosol transporters

Pathogen	Genetic locus	Reference
Neisseria gonorrhoeae	*FbpABC*	(Adhikari et al., 1996)
Neisseria meningitidis	*FbpABC*	(Khun et al., 1998)
Haemophilus influenzae	*HitABC*	(Sanders et al., 1994)
Serratia marcescens	*SfuABC*	(Angerer et al., 1990; Angerer et al., 1992)
Yersinia enterocolitica	*YfuABC*	(Saken et al., 2000)
Actinobacillus pleuropneumoniae	*AfuABC*	(Chin et al., 1996)

Comparing the *fbp*, *hit*, and *sfu* operons reveals several common features including gene organizational similarity and some biochemical and functional properties. Studies on the importance for pathogenesis of the periplasmic binding proteins FbpA and HitA in the corresponding pathogens were hindered by the inability to construct mutants by allelic exchange, probably because the proteins are growth-essential (Chen et al., 1993; Sanders et al., 1994). Studies on the NBDs of Fe(III) periplasm-to-cytosol transporters will be described in more detail in section 1.8.

Together, these bacterial iron uptake systems belong to the superfamily of ABC (ABC, for ATP-binding cassette) transporters, as discussed below.

1.6 ABC transporters: general characteristics, functions and examples

The ABC transporter superfamily (also called traffic ATPases) consists of a very diverse group of integral membrane proteins involved in the ATP-dependent transport of different solutes across biological membranes (Higgins, 1992; Schneider and Hunke,

1998). These proteins share a conserved nucleotide-binding domain (Higgins, 1992; Holland and Blight, 1999) and are ubiquitous in both eukaryotes and prokaryotes. They can typically be divided into subfamilies based on the type of substrates as well as the direction of transport across membranes. Despite the prominence of these ABC transporters in all biological systems, the molecular mechanisms are not well characterized.

In bacteria, many ABC transporters function in the uptake of amino acids, sugars, peptides and ions. These transporters have low capacity but high affinity systems, can accumulate substrate against very large concentration gradients (>10,000-fold) and are most appropriate for a scavenging role (Higgins, 1992). The focus of the current study, the *Neisserial* periplasmic iron transport system (FbpABC), also belongs to this subfamily. On the other hand, ABC exporters are involved in the export of hydrophobic drugs, toxins (colicin, haemolysin), capsule polysaccharide, proteases, peptides, ions and heavy metals (Fath and Kolter, 1993; Higgins, 1992; Holland and Blight, 1999; Schneider and Hunke, 1998). These do not have a ligand-binding protein, and the ligand presumably interacts with the membrane domain initially. A well-studied example is the signal peptide-independent export of the 107 kDa HlyA hemolysin polypeptide of *E. coli* (Fath and Kolter, 1993; Koronakis et al., 1995; Koronakis et al., 1993). Although the vast majority of ABC transporters are involved with membrane transport events, a few apparently atypical ABC proteins serve alternative function, such as the UvrA protein which is a cytoplasmic enzyme involved in DNA repair (Doolittle et al., 1986).

1.6.1 Prokaryotic members

Biochemically and genetically, the two best characterized examples of prokaryotic ABC transporters are the histidine permease in *S. typhimurium* and periplasmic maltose transport system in *E. coli*. Both systems belong to the subfamily of bacterial nutrient uptake systems. The histidine permease complex consists of HisJ, the periplasmic binding protein, HisM and HisQ, the transmembrane proteins, and two ATP-binding HisP subunits. Similarly, the *E. coli* maltose transporter complex, $MalEFGK_2$, is made up of the MalE, the periplasmic binding subunits, MalF and MalG, the integral membrane components, and MalK, the ATP-binding protein.

Both periplasmic binding proteins function to confer high affinity to the corresponding ligand transport processes (histidine and maltose). Purified recombinant HisP and MalK were shown to exhibit a spontaneous ATPase activity in the absence of integral membrane components (Morbach et al., 1993; Nikaido et al., 1997; Walter et al., 1992); however, in both permease complexes ($HisQMP_2$ and $MalFGK_2$) reconstituted in proteoliposomes, ATP hydrolysis and ligand translocation depend greatly on the presence of the liganded periplasmic binding protein (Davidson and Nikaido, 1991; Liu et al., 1997).

1.6.2 Eukaryotic members

Many eukaryotic members of the ABC transporter superfamily are of biomedical relevance. For example, P-glycoprotein pumps hydrophobic drugs out of cells in an

ATP-dependent manner, and its over-expression is frequently associated with multidrug resistance especially important in cancer cells (Germann et al., 1993; Gottesman and Pastan, 1993). Mutations in the nucleotide binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) protein, a unique human epithelial chloride channel that regulates the rate of movement of Cl^- ions across epithelia, lead to the genetic disease cystic fibrosis (Collins, 1992). More examples include TAP1 and TAP2 peptide transporters associated with the major histocompatibility complex (MHC) Class I antigen (Meyer et al., 1994), as well as ABCR, the rod photoreceptor-specific ABC transporter whose mutations at varying sites are responsible for Stargardt disease, an early onset macular degeneration (Ahn et al., 2000; Allikmets et al., 1997; Azarian and Travis, 1997).

1.7 ABC transporters: components

An ABC transporter is typically composed of four parts: two integral membrane domains, each of which spans the membrane approximately six times, and two ATP-hydrolyzing domains (Doige and Ames, 1993; Higgins, 1992). In prokaryotes, the different domains are often individually expressed as separate polypeptides translated from the same operon, such as the histidine permease of *S. typhimurium* (Higgins et al., 1982). However, in many eukaryotic systems, the domains are fused into one large, multifunctional polypeptide, an example of which is the human multidrug resistance P-glycoprotein.

Bacterial periplasmic solute transporters are frequently equipped with an additional periplasmic protein component that has the following potential functions: 1) scavenging molecules with high affinity for the subsequent transport; 2) communicating its state of occupancy to the membrane components; 3) triggering the transport/ATPase cycle; and 4) delivering its ligand to the membrane components (Doige and Ames, 1993). Figure 3 shows different domain organizations of ABC transporters.

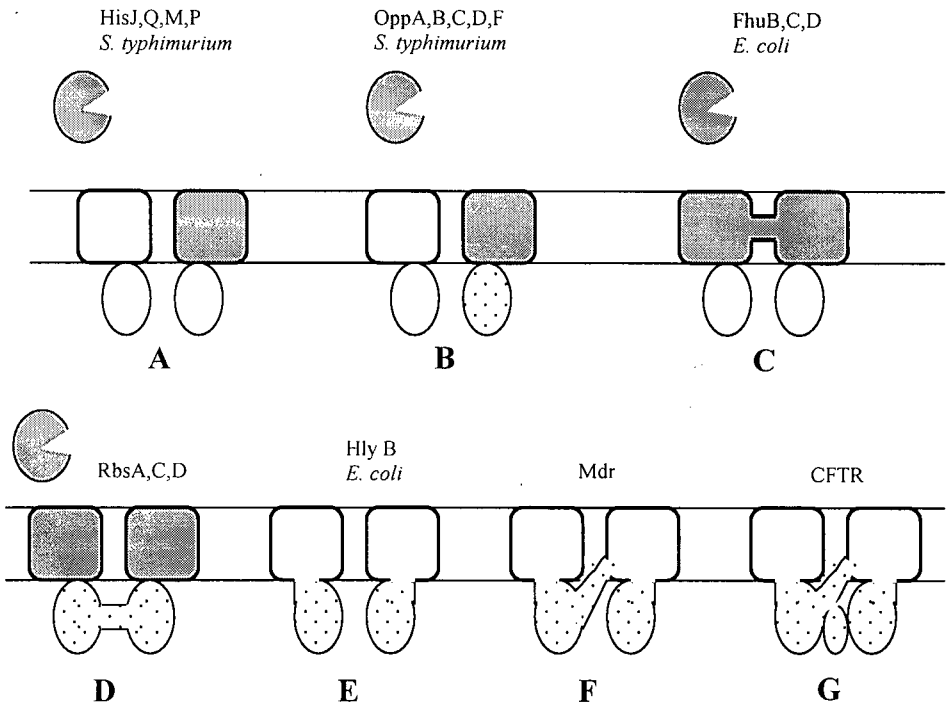

Figure 3. Different domain organizations of ABC transporters. The basic system has four subunits, two membrane domains and two ATPase subunits. Certain transporters have additional domains, e.g. the periplasmic binding protein in prokaryotic importers (A – D). Examples A – E represent prokaryotic systems; examples F and G are eukaryotic transporters. Adapted from (Higgins, 1992; Holland and Blight, 1999).

1.7.1 Integral membrane components

The transmembrane domains of ABC transporters are highly hydrophobic, consisting of multiple α-helical segments that span the membrane. Although the majority of transporters are predicted to form six membrane-spanning segments per domain, the number of transmembrane segments can vary from three to eleven (Holland and Blight, 1999). Sequence similarity between different permeases is relatively limited, but structural similarity is extensive based on computer-assisted topology studies, especially in their carboxy-terminal ends (Kerppola and Ames, 1992). A common motif in all membrane-bound permeases that function in bacterial import systems is a conserved EAA loop located in the large hydrophilic cytoplasmic loop between the third and fourth membrane spanning segments of the minimum structure (Doige and Ames, 1993). Both transmembrane proteins forming the heterodimers reveal such a feature, which is unique to bacterial importers and is apparently neither in eukaryote membrane domains of ABC transporters nor in the *E. coli* HlyB-transporter nor in any ABC-dependent export systems in *B. subtilis*.

The binding of liganded periplasmic protein to specific sites on the permease probably triggers a sequence of events that leads to ATP hydrolysis. This initiates the release of the receptor-bound substrate and the opening of a specific pore in the complex through which the substrate is translocated (Liu et al., 1999; Nikaido and Ames, 1999).

1.7.2 The ATP-hydrolyzing subunits

The ATP-binding domains are the most conserved and are most characteristic of ABC transporters, with two short sequence motifs in their primary structure, called the Walker A and Walker B motifs (Walker et al., 1982) (Figure 4). The known nucleotide binding domains share considerable sequence identity, varying between 30 to 50% depending on the transporters being compared. The Walker A motif (GXS/TGXGKS/TS/T) corresponds to the phosphate-binding loop (P-loop), a glycine-rich loop that is followed by an uncapped α-helix. The invariant lysine within this motif is crucial for the binding of β- and γ-phosphates of the nucleotide substrate (Schneider and Hunke, 1998). The Walker B site (hhhD, where h stands for hydrophobic) is also directly involved in the binding and hydrolysis of ATP (Jones and George, 1999; Schneider and Hunke, 1998). Preceding this site is a highly conserved sequence motif, the linker peptide, which has the following consensus sequence LSGGQQ/R/KQR. Mutations in this region abolished ATP hydrolysis (Schneider and Hunke, 1998), suggesting an essential role of the linker peptide in the transport process.

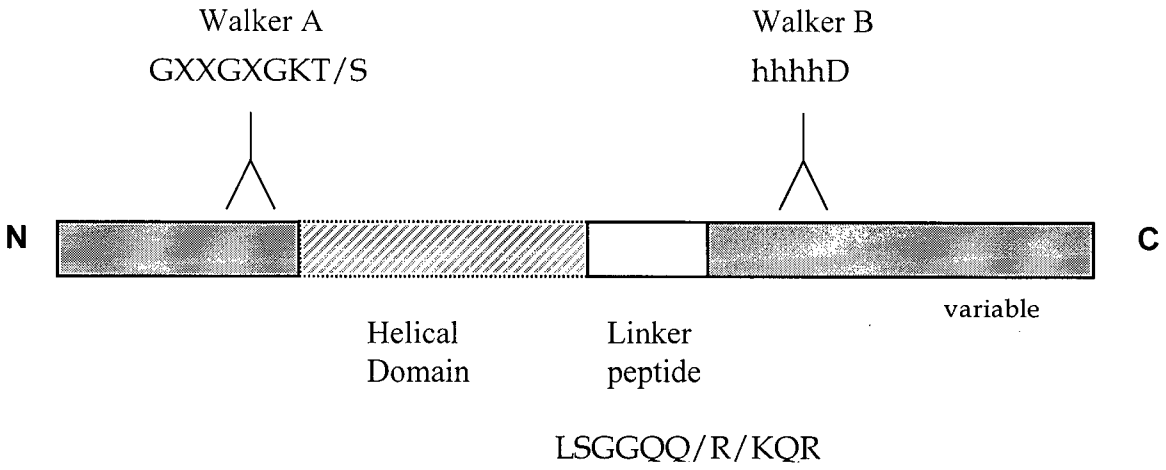

Figure 4. Linear representation of a prototype ABC domain. The highly conserved motifs, including the Walker A, Walker B and the Linker peptide, are marked.

The ATP-hydrolyzing subunit of ABC transporters provides the energy for the transport process and has been studied for over 30 years (Jones and George, 1999). The high-resolution crystal structure of HisP, the nucleotide binding domain of the *S. typhimurium* histidine permease complex (Hung et al., 1998), led to a proposed mechanism by which the NBD couples the energy of ATP hydrolysis to ligand substrate transport across the membrane.

The HisP structure (Hung et al., 1998) reveals an overall shape of an "L" with two thick arms (arm I and arm II). Arm I is a domain with an $\alpha+\beta$ structure and contains the ATP binding pocket formed by the Walker A residues and the Walker B aspartate (D178) which is involved in the coordination of the catalytic Mg^{2+} ion (Jones and George, 1999). Arm II is predominantly composed of α helices and appears to be embedded in the membrane by interacting with the integral subunits, HisQ and HisM. Many mutations

located on Arm II result in constitutive ATPase activity and a loosely assembled $HisQMP_2$ complex, suggesting that interaction with the integral subunits of Arm II is responsible for regulation of the ATPase activity (Liu et al., 1999).

Based on the HisP structure, as well as other biochemical and genetic evidence (Kerppola et al., 1991; Liu and Ames, 1998; Liu et al., 1999; Mimura et al., 1990; Nikaido and Ames, 1999), a model of ATP hydrolysis and ligand translocation in *Salmonella* histidine permease system was proposed (Figure 5). Briefly, ATP binds to one of the HisP subunits in the tight membrane-bound complex $HisQMP_2$, causing the HisP dimer to disengage from the integral components HisQM and also increasing the affinity of the second ATP to bind. Interaction of the liganded HisJ (receptor) with the integral subunits cause conformational change and further disengagement of HisP. ATP hydrolysis occurs and leads to the opening of the translocation pathway and the release of the histidine ligand. Finally, after the release of ADP and Pi, the HisP dimer is reengaged and the system resumes its resting stage.

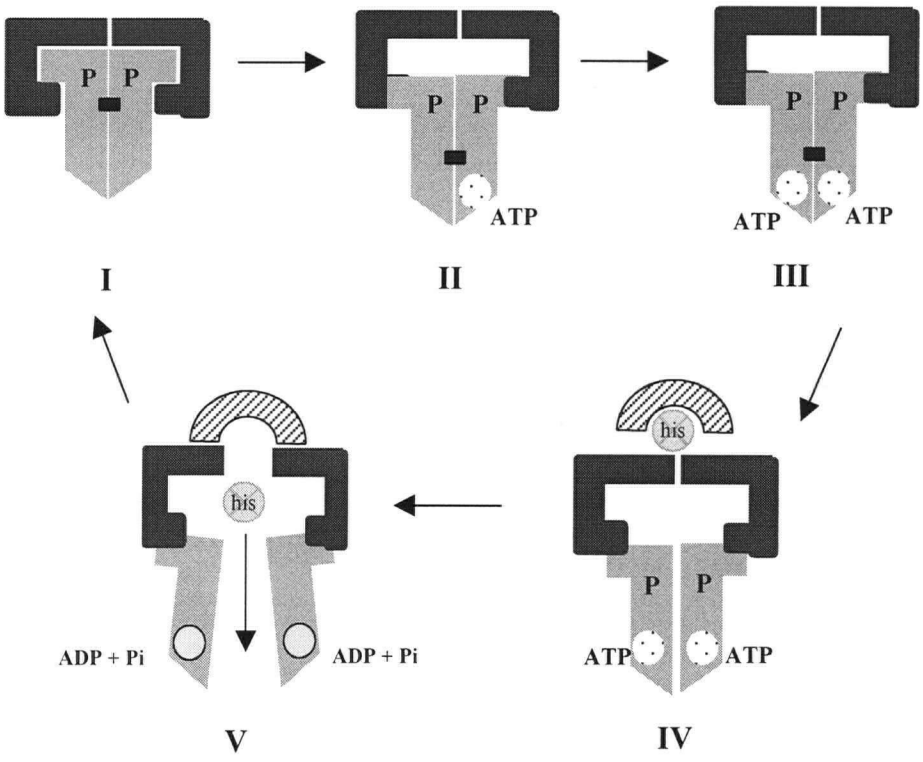

Figure 5. Proposed model representing ATP hydrolysis and ligand translocation by wild type $HisQMP_2$. HisQ and HisM are represented in dark gray; HisP is shown in light gray; ATP is represented by dotted circle, while ADP + Pi is indicated by white circle. See text for detailed description of the ATP hydrolysis cycle. Adapted from (Liu et al., 1999; Nikaido and Ames, 1999).

1.8 FbpC and *Neisserial* iron transport

Unlike the more distantly-related HisP of the histidine permease and MalK of the maltose transporter in *S. typhimurium*, the bacterial iron transport systems have not been well characterized. The involvement of these systems to function in periplasm-to-cytosol transport of iron has been demonstrated, however. Siderophore-deficient *E. coli* strains unable to utilize many iron sources can be complemented by the addition of a plasmid containing the *hitABC* (Adhikari et al., 1995), *sfuABC* (Zimmermann et al., 1989), or

fbpABC operons (Adhikari et al., 1996). These experiments provided solid evidence indicating the importance of these operons in iron transport.

An elegant experiment by Sanders *et. al.* showed that insertional inactivation of the *hitC* gene produced an isogenic nontypeable *H. influenzae* (NTHI) strain unable to utilize iron bound to transferrin or iron chelates. Interestingly, reconstitution of the wild-type genotype by replacing the mutated *hitC* gene with the wild-type allele by allelic exchange created a new strain that was able to utilize all of these iron sources (Sanders et al., 1994), indicating the critical role of HitC in the iron transport process. Khun et. al. (2000) recently showed the presence of *fbpAB* and *fbpBC* transcripts by RT-PCR using an *fbpC*-sequence-specific oligonucleotide, suggesting that the *fbpABC* locus in *N. meningitidis* is transcribed as a single contiguous mRNA (Khun et al., 2000). This result contradicts previous reports that failed to detect *fbpC* transcripts in RNA samples from *N. gonorrhoeae* cultures (Adhikari et al., 1996; Sebastian and Genco, 1999). However, the difference in results may be due to several factors, including quality of the total *Nesserial* RNA preparation used in the RT-PCR procedure (Bowler et al., 1999), differences in primer design and the PCR amplification protocol (Khun et al., 2000). Detection of *fbpBC* transcripts implies a functional role for FbpB and FbpC in *Neisseria meningitidis* periplasmic iron transport. A potential stemloop structure after the *fbpA* gene may function to stabilize mRNA, which may explain the higher expression level of FbpA in comparison to those of FbpB and FbpC.

Genetic conservation of the *fbpABC* operon also suggests that the above observation applies to *N. gonorrhoeae*. However, an *N. gonorrhoeae fbpC* mutant (by insertional inactivation) was previously shown to be capable of growth with transferrin as the sole exogenous iron source (Sebastian and Genco, 1999), conflicting the evidence obtained in the *H. influenzae* iron transport model. Whether FbpC is essential for transferrin-mediated periplasm-to-cytosol transport of iron remains controversial.

1.9 Objective of the present study

Biochemical characterization of FbpC is crucial for understanding better how energy can be coupled to translocate iron across the *Neisserial* cytoplasmic membrane. The goal of this study was to show biochemically that FbpC binds ATP and has ATPase activity in the absence of the first two gene products in the *fbpABC* operon. An FbpC mutant was created with E164 replaced by an aspartate residue, and the mutant ATP-hydrolyzing activity was compared with that of the wild-type FbpC. The functional characteristics of FbpC were also compared with the more extensively characterized HisP and MalK systems.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical supplies and media

All chemicals were purchased from Fisher Scientific, Sigma Chemical Co, or Boehringer Mannheim unless otherwise specified. Restriction endonucleases and IPTG were obtained from Gibco-BRL, and Vent DNA polymerase and T4 DNA Ligase were from New England Biolabs. Taq polymerase and buffer were kindly provided by Dr. Ross MacGillivray (University of British Columbia). Acrylamide and other electrophoresis reagents were obtained from Bio-Rad laboratories. Nickel-NTA agarose resin was purchased from Qiagen, Inc. Bacterial media components were purchased from Difco laboratories. LB broth with appropriate antibiotics was used for overnight inoculation of bacteria; 2xYT media (with the modification of 10g of NaCl per liter of culture) were used for bacterial protein expression. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml.

2.1.2 Bacterial strains and plasmids

Table 3 lists the bacterial strains and plasmid constructs used in the study. *Escherichia coli* strain DH5α was used as the host strain in genetic manipulations. HMS174(DE3) *E. coli* strain was used for protein overexpression. Bacterial stocks were stored at –80°C in LB medium containing 15% glycerol. pBluescript® II SK- and pET28a vectors were obtained from Stratagene and Novagen Inc., respectively.

Table 3. Bacterial strains and plasmid constructs used in this study

Strain, or plasmid	Relevant characteristic(s)	Reference
Strains		
N. gonorrhoeae (clinical strain, Q3,21)	Genomic DNA for PCR amplification of *fbpC* fragment	This study
E. coli DH5α	General host for cloning *fbpC* and plasmid propagation	Novagen
E. coli HMS174(DE3)	Strain for high-level expression of *fbpC* cloned into pET28a vector containing bacteriophage T7 promoter	Novagen
Plasmids		
pBluescript® II SK-	*E. coli* cloning vector , Amp^r	Stratagene
pET28a	*E. coli* expression vector, *Kan*^{r}	Novagen
pBSfbpC3	*fbpC* fragment ligated into *Hin*dIII and *Xho*I of pBSSK(-) , Amp^r	This study
pEfbpC3	*fbpC* fragment ligated into *Nde*I and *Xho*I of pET28a, Kan^r	This study
pBSMutC3	*fbpC* fragment with amino acid change E164D ligated into *Hin*dIII and *Xho*I of pBSSK(-), Amp^r	This study
pEMutC3	*fbpC* fragment with amino acid change E164D ligated into *Nde*I and *Xho*I of pET28a, Kan^r	This study

2.2 Methods

2.2.1 PCR amplification of the fbpC gene

The *fbpC* gene was amplified from the genomic DNA of a clinical isolate (Q3, 21) of *N. gonorrhoeae* (Provincial Laboratory, BC Center for Disease Control,

Vancouver, BC) by PCR using Taq polymerase. Synthetic oligonucleotide primers for the PCR reactions were designed with the aid of the program Oligo and prepared by the UBC Nucleic Acid and Protein Service Unit (N.A.P.S.) with an Applied Biosystems 394-08 or 380A DNA synthesizer. Restriction enzyme sites, *Hin*dIII, *Nde*I, and *Xho*I, as well as stop codons, were encoded within the primer sequences for cloning purposes. All primers are described in Table 4.

A PCR reaction mixture was prepared by adding together the following components to a 0.5 ml thin-walled microcentrifuge tube: 1 μl of both forward and reverse primers (10 μM), 2 μl of 10 mM dNTPs, 5 μl of genomic DNA (prepared by the InstaGene Matrix method, Bio-Rad, Inc.), 5 μl of Buffer E (10X PCR reaction buffer composed of 0.67 M Tris-HCl pH 9.0, 0.11 M ammonium sulfate, 0.1 M 2-mercaptoethanol), 1 μl Taq polymerase. The PCR was carried out using a Perkin Elmer DNA thermal Cycler with the following conditions: denaturation at 94°C for 45 seconds; annealing at 58°C for 45 seconds; extension at 72°C for 1 minute; 30 cycles.

Table 4. List of primers used in construction of vectors and sequencing

Primers	DNA sequence
FbpCf1	5' - ATG AAG CTT CAT ATG ACC GCC GCC CTG CA - 3'
	*Hin*dIII *Nde*I
FbpCr1	5' - CAT CTC GAG TCA GAG GGT ATT TCC GGG GAA GAA - 3'
	*Xho*I stop
T3 *	5' - ATT AAC CCT CAC TAA AGG GA - 3'
T7 *	5' - TAA TAC GAC TCA CTA TAG GG - 3'
FbpC-t1	5' – CAT CTC GAG TCA TCG GTA CAA TTC GTG AGG GCT TG – 3'

* Standard primers for DNA sequence analysis

2.2.2 Cloning and DNA sequence analysis of the fbpC gene

Construction of pBluescript cloning vector. PCR products were mixed with DNA loading buffer (Sambrook et al., 1989) and electrophoresed on a 1% agarose gel using the Bio-Rad mini-Sub cell GT apparatus. The bands were visualized and photographed on a UV transilluminator after staining with ethidium bromide for approximately 10 minutes. The fragments of interest were excised from the gel and subjected to purification by the QIAquick Gel Extraction Kit (Qiagen, Inc.).

The purified PCR product *fbpC* was digested with restriction enzymes *Hin*dIII and *Xho*I (Gibco-BRL) for 3 hours in React2 buffer (Gibco-BRL) at 37°C. The cut fragment was then purified by gel electrophoresis as before and then cloned into

pBluescript® II SK- vector at the *Hin*dIII and *Xho*I sites by overnight ligation at 16°C, resulting in the pBSfbpC3 plasmid.

Competent cell preparation. An overnight bacterial culture (500 µl, DH5α for cloning and HMS174(DE3) for protein expression) was inoculated into 500 ml of Luria-Bertani (LB) broth and incubated at 37°C in a shaking incubator until an $O.D._{600}$ of approximately 0.55 was reached. Cells were then transferred to two sterile centrifuge bottles, cooled on ice for 30 minutes, and were centrifuged at 2,000 g for 15 minutes in a GSA rotor at 4°C. The supernatant was then decanted, and each bottle of cells was resuspended gently in 20 ml cold sterile RF1 solution (100 mM RbCl, 10 mM $CaCl_2$, 30 mM potassium acetate, 50 mM $MgCl_2$, 15% glycerol, pH 5.8). The bacterial suspensions were then incubated on ice for 15 minutes and then centrifuged at 1,500 g for 15 minutes at 4°C. Each cell pellet was resuspended with 3.5 ml cold sterile RF2 solution (75 mM $CaCl_2$, 10 mM RbCl, 10 mM MOPSNa, 15% glycerol, pH 6.8). The cells were cooled on ice for 15 minutes, aliquoted (150 µl), flash-frozen in a dry ice/ethanol bath, and stored at –80°C.

Transformation and isolation of plasmids. Frozen competent cells were thawed on ice. DNA solutions (7.5 µl of ligation products) were added to the cells, and the mixture was cooled on ice for 30 minutes, then heat shocked at 37°C for 90 seconds. LB broth (200 µl) was added to the mixture followed by a 45-minute incubation with shaking at 37°C. Different quantities of bacterial suspension were then plated on LB agar plates with appropriate antibiotic and incubated overnight.

Crude plasmid purification from bacterial cells to screen for successful transformation was performed by the alkaline lysis method as described previously

(Sambrook et al., 1989). Isolated plasmids were then analyzed by restriction enzyme digestion to confirm the presence of the desired insert. Sequencing grade plasmids were then prepared using the QIAprep Miniprep Kit (Qiagen, Inc.) as described by the manufacturer.

DNA sequence analysis. The *fbpC* fragment in pBluescript®II SK- was sequenced using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) with an Applied Biosystems model 377 automated sequencer at the UBC N.A.P.S. Unit. The primers used for DNA sequencing are shown in Table 4.

Cloning of fbpC into expression vector. The *fbpC* insert was excised from pBSfbpC3 and introduced into the pET28a expression vector (Novagen, Inc.) at the *Nde*I site at the 5' end and *Xho*I site at the 3' end resulting in the plasmid construct pEfbpC3. The pET28a expression vector was chosen because it codes for an N-terminal histidine tag as well as an optional C-terminal His-tag which allows for simplified protein purification. It also contains a T7 promoter and a *lac* operator sequence to regulate expression of the inserted gene (*fbpC*).

2.2.3 Site directed mutagenesis

Mutagenesis procedure. An FbpC mutant, E164D, was created using a PCR-based method (Nelson and Long, 1989). Synthetic oligonucleotides used for mutagenesis were prepared by UBC N.A.P.S. (Nucleic Acid and Protein Service) Unit and are listed in Table 5. The overall scheme of the procedure is illustrated in Figure 6. The first step results in the production of a PCR product defined by the mutagenizing oligonucleotide (5' primer: FbpC-Mut1) and the flanking oligonucleotide (3' primer: Primer B). The

vector pBSfbpC3 was used as a template. A 50 µl PCR reaction mixture was prepared containing the following components in a 0.5 ml thin-walled microcentrifuge tube: 1 µl of both 5' and 3' primers (10 µM final concentration), 2 µl of 10 mM dNTP, 1 µl of template (pBSfbpC3), 5 µl of 10x Buffer E, 1 µl Taq polymerase. Thirty cycles of PCR amplification was carried out using the Mastercycler® gradient 5331 (Eppendorf Scientific, Inc., N.Y.) using the following conditions: denaturing at 94 °C for 45 seconds, annealing at 58 °C for 45 seconds, extension at 72 °C for 1 minute. The 0.7 kb PCR product was then purified using the QIAquick kit (Qiagen, Inc.), and 5 µl was used in the second step of PCR mutagenesis to prime a single round of replication. The same template (pBSfbpC3), dNTPs, buffer and Taq polymerase were used in the condition as follows: denaturation at 94 °C for 2 minutes; annealing at 50 °C for 2 minutes; and extension at 72 °C for 2 minutes. Primers C and D (100 pmol – see Table 5) were added to the reaction tube, followed by 30 cycles of PCR amplification using the following program setting: denaturation at 94 °C for 30 seconds; annealing at 50 °C for 30 seconds; extension at 72 °C for 45 seconds. The final product was cloned into the pBluescript vector at restriction sites *Hin*dIII and *Xho*I, forming a new construct pBSMutC3. The ligation mixture was transformed, and the resulting colonies were screened for positive mutants.

Table 5. Primers designed for the construction of E164D FbpC mutant by PCR-based site directed mutagenesis (Nelson and Long, 1989)

Primers	DNA sequence
FbpC-Mut1	5' - TGTTGGACGACCCCTTCAGC - 3'
Primer B	5' - GGAGTACTAGTAACCCTGGCCCCAGTCACGACGTTGTAAA - 3'
Primer C	5' - CAGGAAACAGCTATGACCAT - 3'
Primer D	5' - GGAGTACTAGTAACCCTGGC - 3'

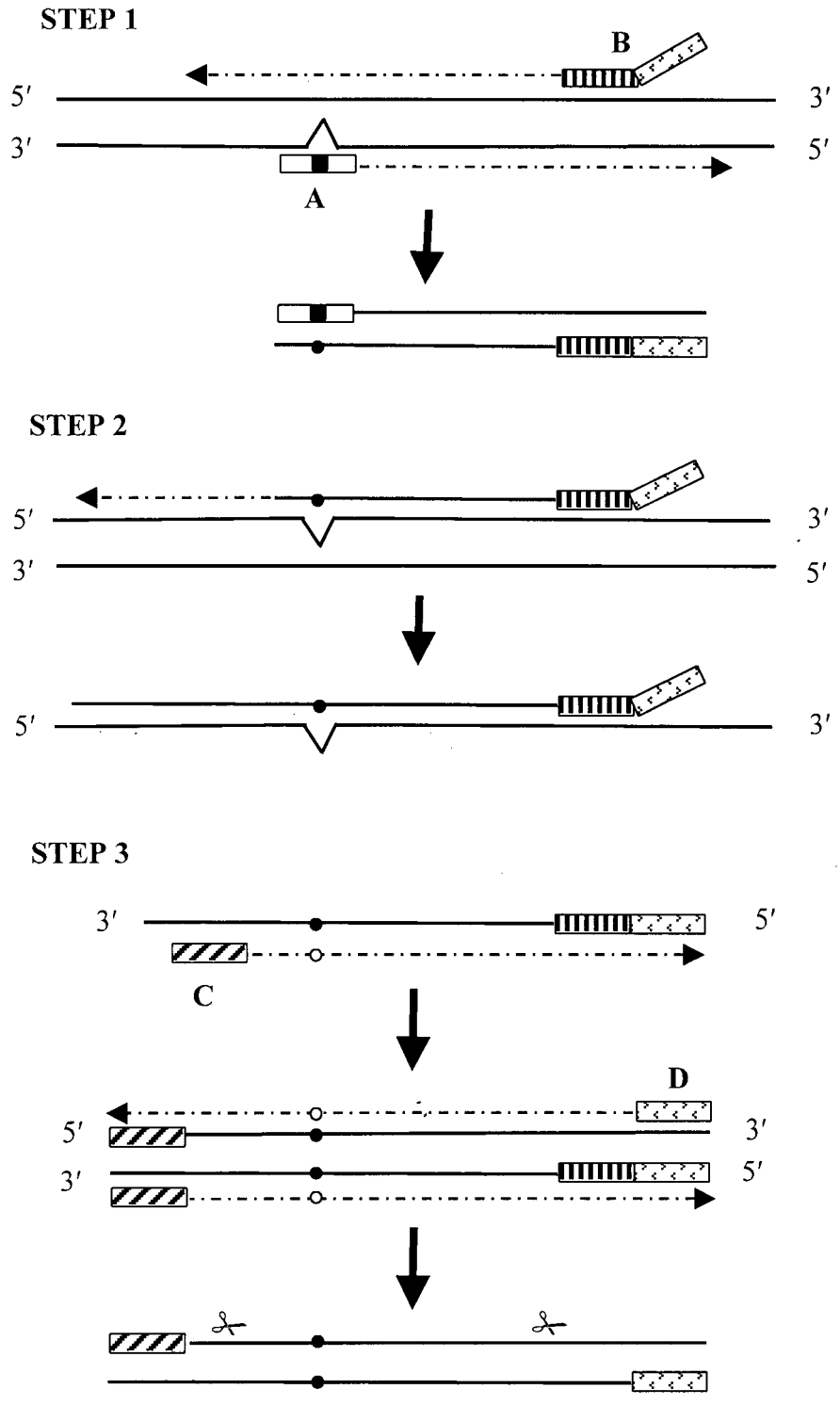

Figure 6. Schematic illustration of PCR-based site-directed mutagenesis.

Screening for mutants. A positive mutant clone was confirmed by DNA sequence analysis using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.). The primer used for sequencing mutants (FbpC-t1) is described in Table 4 and is a 3' internal primer located at 662 bp downstream of the start codon of *fbpC* (170 bp downstream of the altered site). The entire sequence of the mutant *fbpC* was also confirmed by sequencing at N.A.P.S. Unit.

2.2.4 Overexpression of FbpC

A single colony of *E. coli* strain HMS174 (DE3) containing pEfbpC3 was inoculated into 10 ml of LB broth with 25 μg/ml kanamycin and incubated with shaking at 30 °C overnight. The culture was then diluted 1:200 into fresh 2xYT media (modified with the addition of 5g of NaCl per liter) and incubated at 30°C with shaking until an OD_{600} of 0.9 to 1.0 was reached. Expression of the FbpC fusion protein was induced with 0.5 mM IPTG for 2 to 2.5 hours at 30 °C. The cells were then harvested by cooling for 5 minutes on ice followed by centrifugation at 5,000 g for 25-30 minutes. The cell pellet was frozen and stored at -80 °C until needed.

2.2.5 Purification of FbpC

Nickel-chelate affinity chromatography. Cells were defrosted and resuspended in 40 ml of cold buffer A (50 mM Tris-HCl pH 8.0, 15% glycerol, 0.4 M NaCl). ATP was added to the cell resuspension to a final concentration of 5 mM, and the cells were

then lysed using a French press. After centrifugation at 5,500 g for 30 minutes, the supernatant was applied to a column containing 10 ml of a slurry of Ni-NTA resin (Qiagen, Inc.) previously equilibrated with cold binding buffer. The column was washed with (i) 40 ml of buffer B (50 mM Tris-HCl pH 8.0, 15% glycerol, 0.4 M NaCl, 4 mM ATP), (ii) 25 ml of buffer B containing 20 mM imidazole, and (iii) 30 ml of buffer B containing 50 mM imidazole. Subsequently, FbpC was eluted with 30 ml of buffer B supplemented with 250 mM imidazole. All fractions were collected, kept on ice, and analyzed by SDS-PAGE. EDTA was added to the FbpC fraction to a final concentration of 1 mM immediately following elution, and the fraction was dialyzed overnight against the following buffer: 40 mM Tris-HCl pH 7.9, 1 mM EDTA, 20% glycerol, 75 mM NaCl, 1 mM DTT, 2 mM ATP.

High-Q ion-exchange chromatography. The 5-ml high-Q Econo-Pac cartridge (Bio-Rad) (prepared for use as instructed by the manufacturer) was connected to a Pharmacia FPLC system and washed with 20 ml of degassed deionized distilled water, followed by 20 ml of degassed high salt buffer D (20 mM Tris-HCl pH 7.9, 20% glycerol, 1 M NaCl, 1 mM EDTA) at a flow rate of 2 ml/min before each use. The cartridge was then equilibrated with degassed low salt buffer C (20 mM Tris-HCl pH 7.9, 20% glycerol, 75 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 to 2 mM ATP). The 10 ml FbpC dialysis fraction from the nickel affinity chromatography was loaded onto the cartridge at 1 ml/min using a peristaltic pump. The FbpC protein was present in the flow through fraction (10 ml) and was kept on ice. The cartridge was washed with (i) 10 ml of low salt buffer C, (ii) 10 ml of buffer F (Buffer C and Buffer D in ratio of 1:1), and (iii) 10 ml high salt buffer D. The cartridge was cleaned as described by the manufacturer

and stored in 20% (v/v) ethanol solution at 4 °C. Fractions were analyzed by 10% SDS-PAGE. Pure FbpC (from flow through fraction) was immediately aliquoted (500 µl), flash-frozen in dry-ice/ethanol bath and stored at –80 °C.

2.2.6 Protein characterization

Bradford Standard assay. Protein samples were diluted to an appropriate concentration and quantified by the Bradford method (Bradford, 1976) using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) as described by the manufacturer.

SDS PAGE. Samples were heated at 95 °C in 5x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% β-mercaptoethanol, 0.025% (v/v) bromophenol blue) for 5 minutes. After denaturation, samples were resolved on 10% polyacrylamide gels.

N-terminal Amino Acid Sequence Analysis. Purified FbpC protein was electrophoresed on a 10% SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane in CAPS electroblotting buffer (10 mM CAPS in 10% methanol) at 50 V (100-170 mA) for 30 minutes. Subsequently, protein was visualized by staining with Coomassie Brilliant Blue R solution (0.025% (w/v) CBR 250 in 40% (v/v) methanol). Bands corresponding to FbpC were excised, pooled, and subjected to sequence analysis using the Perkin Elmer ABI 476A automated sequencer at UBC N.A.P.S. Unit. The first 10 amino acids were found to be identical to those predicted from the *fbpC* gene sequence except that the amino terminal methionine was cleaved off.

2.2.7 ATPase activity assay

ATPase assays were performed essentially as described previously (Nikaido et al., 1997) with a few minor modifications. Purified $FbpC_{(his6)}$ (10 μg, final concentration: 45 μM, 320 μl total reaction volume) in assay buffer (100 mM Tris-HCl, pH 8.0, 40 mM NaCl, 4 mM ATP, 20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT) was equilibrated at 37°C for 3 minutes. The reaction was started by the addition of $MgCl_2$ (final concentration: 4 mM). Samples (75 μl) were taken every 2.5 minutes and added to microcentrifuge tubes containing 75 μl of 12% SDS to stop the reaction. A control reaction was performed in parallel without the addition of $MgCl_2$. The amount of inorganic phosphate liberated from each reaction was measured by a colorimetric assay (Chifflet et al., 1988) using Na_2HPO_4 as a standard. The standards result in a linear calibration curve with a range of 1 - 30 nmol of inorganic phosphate. The procedure for phosphate determination is briefly described as follows. Solution P1 (12% SDS, 150 μl) was added to the sample (150 μl) and vortexed, followed by the addition of 300 μl of solution P2 (1:1 ratio of 6% ascorbic acid in 1 N HCl (freshly prepared prior to each use) and 1% ammonium molybdate). All tubes were incubated for 7-10 minutes before 562.5 μl of solution P3 (2% sodium citrate, 2% sodium metaarsenite, 2% acetic acid) was added. The mixtures were further incubated at 37 °C for 10 minutes, and the absorbance at 850 nm was read with a Cary 50 UV-*vis* spectrophotometer.

Similar assays were performed to determine the dependence of FbpC ATPase

activity on pH, FbpC concentration, ATP, and $MgCl_2$, with the exception that the total reaction volume was 90 µl only, and that only the 10-minute time point (75 µl) was taken. The procedure for each experiment is detailed in appropriate figure legends.

2.2.8 Photoaffinity labeling with [γ-^{32}P]N_3ATP

[γ-^{32}P]N_3ATP was purchased from ICN Biomedical Inc. and was supplied with a specific activity of 19.8 Ci/mmol. The labeling procedure was as follows: purified $FbpC_{(his6)}$ (1.5 µg) were diluted in photoaffinity labeling buffer (40 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 50 mM NaCl, 5 mM $CaCl_2$, 7.5% glycerol) to a final volume of 75 µl, placed in a 24-well glass plate, and kept on ice. 2.0 µCi of [γ-^{32}P]-N_3ATP was mixed with unlabeled N_3ATP to a final concentration of 100 µM and added to the well. The reaction mixture was irradiated with a handheld short wave (254 nm) UV lamp (Spectronics Corporation, N.Y.) placed at a 3 cm distance for two 1-minute intervals, with 1-minute cooling in between. The reaction was stopped by the addition of 4 mM dithiothreitol and protein sample loading buffer. Following photolabeling, the samples were heated in a boiling water bath for 5 minutes prior to electrophoresis. One third of the sample volume (30 µl) was subject to SDS-PAGE analysis. Autoradiography was performed using Kodak Bio Max film and an intensifying screen at –80°C after transfer of the proteins onto nitrocellulose. Quantitative studies were performed with a phosphorimager (PSI-MAC) and the program ImageQuant (Molecular Dynamics, Inc.).

3. RESULTS

3.1 Cloning and construction of $FbpC_{(his6)}$ expression vector

Genomic DNA of a clinical strain of *Neisseria gonorrhoeae* was isolated in the Provincial Laboratory at the BC Center for Disease Control (Vancouver, BC) using InstaGene matrix (Bio-Rad). The *fbpC* gene was amplified by PCR, generating a DNA fragment of 1059 nucleotides that encode *fbpC* flanked by restriction sites for *Hin*dIII and *Nde*I at the 5' end and a stop codon and a *Xho*I restriction site at the 3' end. Cloning the amplified *fbpC* into the pBluescript and the pET28a expression vectors produced stable genetic constructs pBSfbpC3 and pEfbpC3, respectively. After ligation and transformation into *E. coli* strain DH5α, positive clones containing the *fbpC* insert were confirmed by restriction digestion. Plasmids were prepared using the QIAprep Miniprep kit (Qiagen, Inc.) and successfully transformed into *E. coli* expression strain HMS174(DE3) which carries an IPTG-inducible chromosomal T7 RNA polymerase gene.

DNA sequence analysis of *N. gonorrhoeae fbpC* was performed using standard T7 and T3 primers designed for the pBluescript vector (Table 4) to confirm the correct amplification of *fbpC* from the *N. gonorrhoeae* genome. When the DNA sequence for the amplified *fbpC* fragment was compared to the available *Neisseria gonorrhoeae* complete genome sequence of Strain FA 1090 (Roe et al., 2000), it was found to be identical except at nucleotide position 567 (A instead of C), which represents a silent mutation (Figure 7).

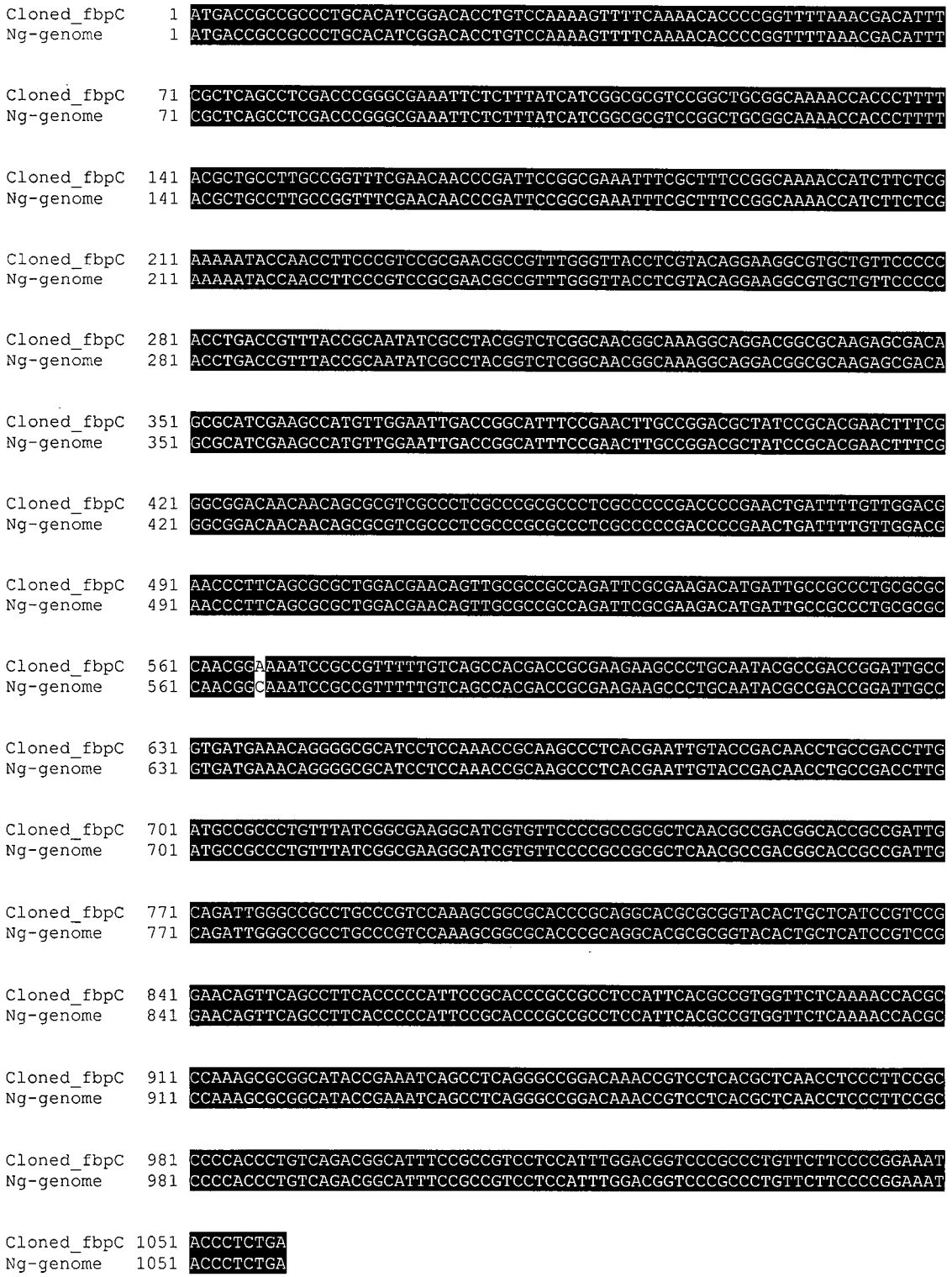

Figure 7. Sequence alignment of PCR-amplified *fbpC* gene (this study) and data from *N. gonorrhoeae* genome sequencing project (Strain FA1090) at the University of Oklahoma (Roe et al., 2000).

3.2 Overexpression and Purification of $FbpC_{(his6)}$

$FbpC_{(his6)}$ was successfully overexpressed in HMS174(DE3) cells containing the plasmid pEfbpC3 upon induction by IPTG to a final concentration of 0.5 mM for 2.5 hours (Figure 8). The cells were grown at 30°C at all times to optimize the amount of soluble fbpC protein. The amount of overexpressed protein increases with respect to time of induction; however, a greater fraction of cells was susceptible to lysis if induction was allowed for more than 3 hours. The overproduced protein had an apparent molecular weight of approximately 40 kDa as judged by SDS-PAGE, corresponding well with the predicted molecular weight of fusion FbpC with an N-terminal histidine tag. N-terminal sequence analysis of the first 10 amino acids also confirmed the identity of the expressed FbpC.

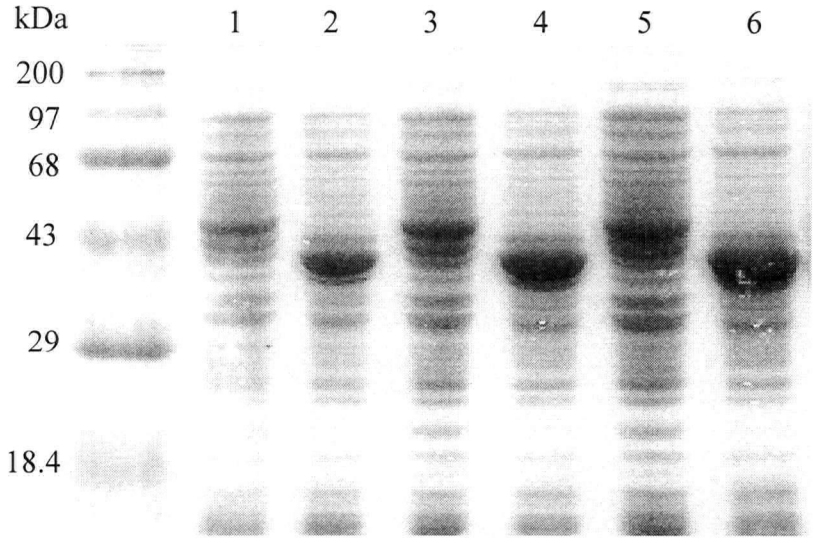

Figure 8. Expression profile of $FbpC_{(his6)}$. SDS-PAGE showing $FbpC_{(his6)}$ overproduction. HMS174 (DE3) cells containing the pEfbpC3 plasmid were grown under conditions as described in the Materials and Methods section. Lanes 1, 3, 5 represent total proteins in cells growing in non-inducing conditions at 30°C for 2 hours (*lane 1)*, 4 hours (*lane 3)*, and 16 hours (*lane 5)*. Lanes 2, 4, 6 represent total proteins of cells induced with 0.5 mM IPTG for 2 hours (*lane 2)*, 4 hours (*lane 4)*, and 16 hours (*lane 6)* respectively.

Harvested cells were disrupted by passage through a French press twice. After centrifugation, the cell pellet was found to contain a large amount of insoluble FbpC aggregates. However, due to the difficulty encountered in refolding functional protein after denaturing purification by the use of 6 M urea, a native purification procedure was used to purify soluble, recombinant FbpC protein in the clear lysate. Nickel-affinity column chromatography was used as the first step of purification, taking advantage of the metal-binding properties of the histidine tail of fusion FbpC. Figure 9 shows the result of a typical purification procedure from a 2-liter preparation. Most of the overproduced $FbpC_{(his6)}$ remains in the pellet fraction after cell lysis by French press. The majority of $FbpC_{(his6)}$ is released from the column upon addition of elution buffer (lane 9, Figure 9a). The presence of ATP, NaCl, and glycerol is critical to maintain the bulk of recombinant FbpC in solution during the entire purification procedure before the elution step.

The FbpC preparation after elution from the nickel column is not sufficiently pure. The fraction was immediately dialyzed overnight against buffer containing 5 mM ATP and 20% glycerol and further purified using the high Q cartridge on an FPLC system (Pharmacia). $FbpC_{(his6)}$ was present in the flow-through fraction (lane 2, Figure 9b), and is over 95% pure as judged by SDS-PAGE. The yield of a typical 2-liter preparation is around 1.3 mg of pure $FbpC_{(his6)}$.

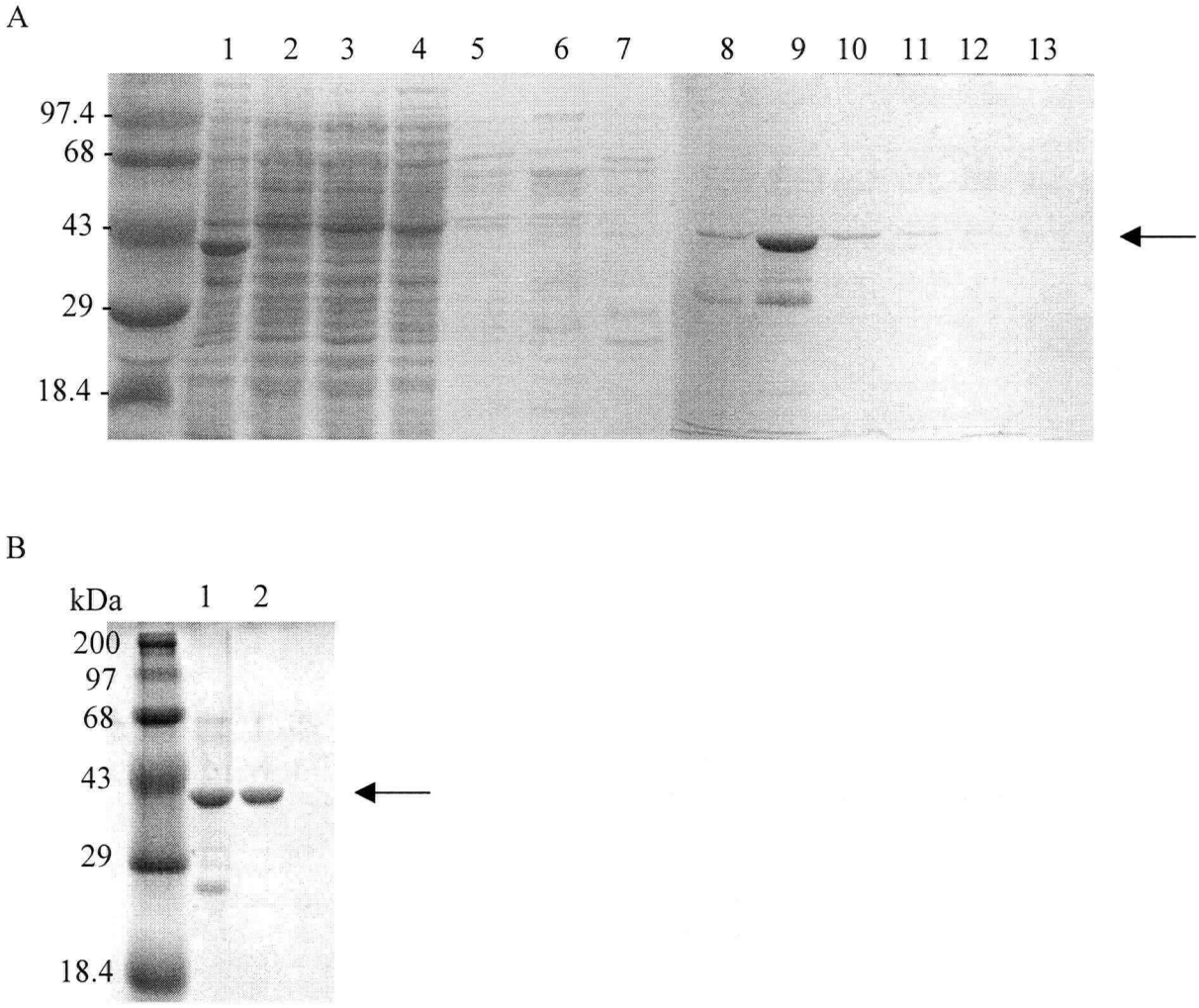

Figure 9. Purification of $FbpC_{(his6)}$. ***A***. SDS-PAGE of fractions from FbpC purification by nickel-affinity chromatography. Molecular weight markers in kDa are shown on the left. *Lane 1*, total cell proteins after 2 hours of induction at 30°C*; lane 2*, supernatant fraction after cell lysis by French Press; *lane 3*, column flow-through; *lanes 4-7,* wash fractions; *lanes 8-12,* 250 mM imidazole eluate; *lane 13,* 500 mM imidazole eluate. ***B.*** Further purification of $FbpC_{(his6)}$ by passage of proteins obtained from nickel column (lane 9 in *A.*) through a high-Q cartridge. *Lane 1,* proteins before high Q; *lane 2,* flow-through fraction. FbpC comes off at flow-through. $FbpC_{(his6)}$ is indicated by an arrow.

3.3 Properties of $FbpC_{(his6)}$

$FbpC_{(his6)}$ has a high content of hydrophobic amino acid residues (50%) (Adhikari et al., 1996) and is difficult to solubilize even before purification. Different non-ionic detergents, including Triton X-100, Tween-20, MEGA-8, and LDAO, were ineffective in increasing the solubility of $FbpC_{(his6)}$ (data not shown). Lauroylsarkosine (0.5%) or SDS completely solubilize but denature the protein, making recovery of functional $FbpC_{(his6)}$ extremely difficult. The addition of ATP to at least 2 mM throughout the whole purification procedure was found to be essential in maintaining $FbpC_{(his6)}$ solubility, as the final yield of FbpC is reduced by more than 50% if ATP is omitted before the nickel column purification step and thereafter. It is possible that ATP interacts with $FbpC_{(his6)}$ in such a way as to stabilize the protein.

Pure $FbpC_{(his6)}$ had a tendency to form a white precipitate within 24 hours at 0°C. Varying salt, concentration of salt, pH, and additives like DTT did not have a positive effect in maintaining $FbpC_{(his6)}$ solubility. Previous experiments involving the purification of $HisP_{(his6)}$ indicated the importance of 20% glycerol and 5 mM ATP in maintaining $HisP_{(his6)}$ solubility over 2 mg/ml (Nikaido et al., 1997). In this study, I estimate that at least 30-40% of the purified $FbpC_{(his6)}$ precipitates within 2 weeks at 4°C in a buffer containing 20% glycerol and 2 mM ATP. Increasing the glycerol concentration to 50% significantly improves the solubility of pure $FbpC_{(his6)}$, but renders the purified protein harder to work with. Attempts to concentrate $FbpC_{(his6)}$ by both Amicon concentrators and Ultrafree-4 centrifugal filter units (Millipore, Inc.) yielded precipitates and were unsuccessful. Therefore, final, pure $FbpC_{(his6)}$ was immediately aliquoted and stored at –80°C in 20%

glycerol and 2 mM ATP. EDTA, a divalent cation chelator, was also added to 1 mM to avoid hydrolysis of ATP.

3.4 ATPase activity of $FbpC_{(his6)}$

Isolated nucleotide binding domains of more extensively characterized bacterial ABC transporters, such as HisP and MalK, were demonstrated previously to display ATP hydrolysis activity. Purified $FbpC_{(his6)}$ was therefore first characterized by its ability to hydrolyze ATP into ADP and inorganic phosphate. Figure 10a shows linear ATPase activity of $FbpC_{(his6)}$ with respect to time in the presence of Mg^{++}. This activity is linear up to at least 10 minutes, and is dependent on the Mg^{++} cation with the maximal activity displayed when the concentration of Mg^{++} is between 1 to 2 mM (Figure 10b). The calculated specific activity of $FbpC_{(his6)}$ is 0.5 ± 0.1 μmol/min/mg.

ATP hydrolysis by $FbpC_{(his6)}$ activity was also monitored by varying pH values from 6.0 to 10.2, and the pH optimum was found to be slightly alkaline, from pH 7.5 to 8.0 (Figure 10c). Glycerol was essential for maintaining $FbpC_{(his6)}$ in solution, and it was added throughout the entire purification procedure up to a concentration of 20% (v/v). However, it was not known if the presence of glycerol would inhibit $FbpC_{(his6)}$ activity. This was found to be true for HisP (Nikaido et al., 1997) where glycerol above 7.5% inhibits the activity. In the case of $FbpC_{(his6)}$, however, the presence of glycerol up to 20% actually stimulated the rate of ATP hydrolysis (Figure 10d) with the activity dropping off at higher glycerol concentration possibly due to disruption of hydrophobic interactions (Nikaido et al., 1997).

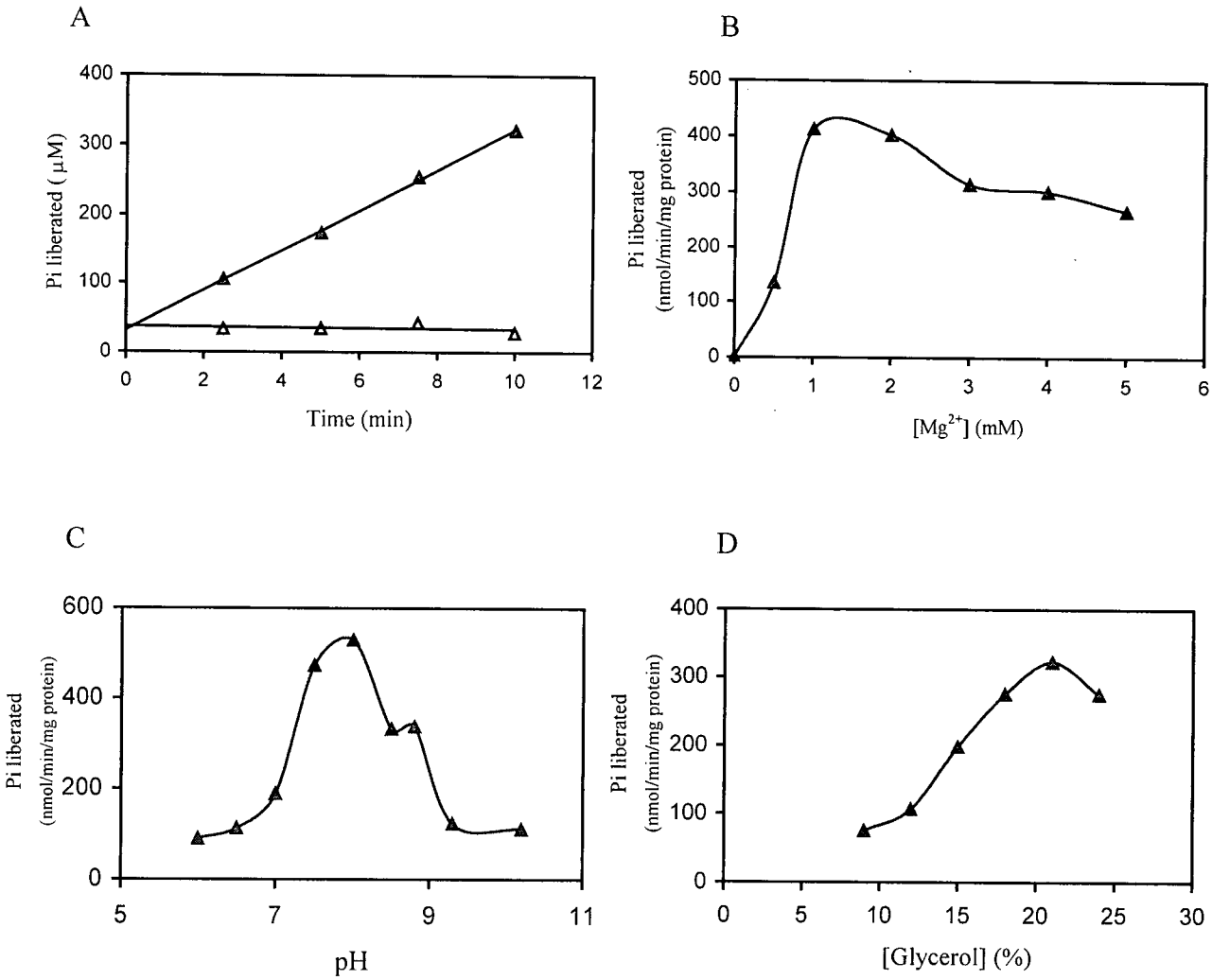

Figure 10. Properties of the ATPase activity of $FbpC_{(his6)}$. ***A***, Linearity of ATP hydrolyzing activity with respect to time. The assay was performed as described in "Materials and Methods" in the presence (▲) or absence (△) of $MgCl_2$. The concentration of $FbpC_{(his6)}$ is 60 μg/ml. ***B***, Mg^{2+} dependence of $FbpC_{(his6)}$ activity. The assay was performed as described with Mg^{2+} added to the concentration indicated on the *abscissa*. The reaction was allowed to proceed for 10 minutes. The *ordinate* represents specific ATPase activity. ***C***. pH dependence of $FbpC_{(his6)}$ activity. The $FbpC_{(his6)}$ solution was diluted in assay buffers of various pHs and the ATPase activity measured after 10 minutes of assay time. The buffers used are as follows: MES/Na, 6.0-6.5; MOPS/Na, 7.0; Tris/Cl, 7.5-8.8; CHES, 9.3; CAPS, 10.2. ***D***, Glycerol dependence of $FbpC_{(his6)}$ activity. $FbpC_{(his6)}$ solution containing 20% glycerol (v/v) was diluted in assay buffers with various amounts of glycerol such that the final concentration of glycerol is that indicated on the *abscissa*. The *ordinate* represents the ATPase specific activity. In panels ***B*** **-*D***, background was corrected using control assays with no $MgCl_2$ added.

3.5 Site-directed mutagenesis and properties of the E164D mutant

To ensure that the ATPase activity displayed by the purified $FbpC_{(his6)}$ preparation was not due to contaminating ATPases, PCR-based mutagenesis was performed to create the FbpC mutant E164D. Based on previous structural and mutational analyses of the HisP in the *Salmonella* histidine transport system (Hung et al., 1998; Shyamala et al., 1991), this mutant is hypothesized to be defective in hydrolysis but would have little effect in ATP binding. This feature is desirable in the characterization of $FbpC_{(his6)}$ because the presence of ATP is absolutely essential for the purification process.

The plasmid construct containing the point mutation in the *fbpC* gene was successfully cloned into the pBluescript vector and subcloned into the pET28a expression vector. The presence of the mutation was confirmed by DNA sequence analysis. The mutant construct (pEfbpCMut3) was transformed into the HMS174(DE3) *E. coli* strain, overexpressed and purified using identical protocol as the wild-type. Figure 11 shows the SDS-PAGE result of a typical E164D mutant purification. The E164D mutant expression profile is comparable to that of the wild-type; however, the final yield is lower, giving approximately 0.3 mg purified protein from a typical 2-liter preparation. The low yield may be due to lower solubility and weaker ATP binding of the E164D mutant as compared to the wild-type. When the E164D mutant was characterized by the ATPase activity assay, the mutant was found to have a specific activity of around 0.047 μmol/min/mg, representing a greater than 10-fold reduction in specific activity compared to the wild-type FbpC.

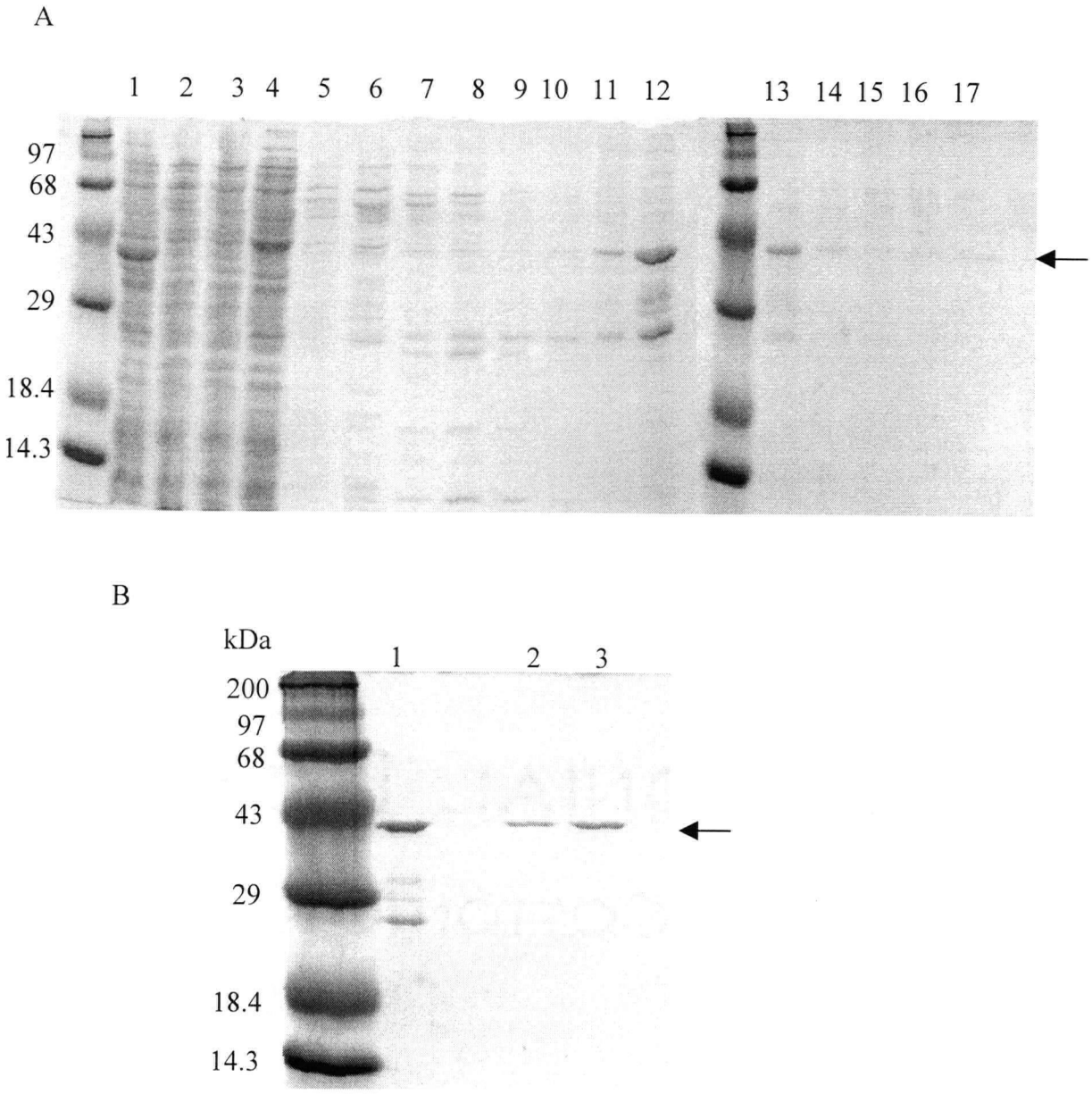

Figure 11. Purification of E164D FbpC$_{(his6)}$. ***A***. SDS-PAGE of fractions from E164D purification by nickel-affinity chromatography. Molecular weight markers in kDa are shown on the left. *Lane 1*, total cell proteins after 2 hours of induction by 0.5 mM IPTG at 30°C; *lane 2*, supernatant fraction after cell lysis by French Press; *lane 3*, column flow-through; *lanes 4-9,* wash fractions; *lanes 10-15,* 250 mM imidazole eluate; *lanes 16-17,* 500 mM imidazole eluate. ***B.*** Further purification of FbpC $_{(his6)}$ by passage of proteins obtained from nickel column through a high-Q cartridge. *Lane 1,* proteins before high Q; *lanes 2-3,* flow-through fraction. E164D FbpC $_{(his6)}$ is indicated by an arrow.

3.6 Binding of [γ-^{32}P]N_3ATP to purified $FbpC_{(his6)}$

To test the ability of $FbpC_{(his6)}$ to bind ATP, a photoaffinity ATP analog, 8-azido-[γ^{32}P]ATP, was used to label the purified $FbpC_{(his6)}$ protein. The protein was resolved on a 10% SDS-PAGE after UV irradiation, transferred to nitrocellulose and analyzed with autoradiography. The photoaffinity conditions were optimized by varying the FbpC concentration (0.5 μg to 1.5 μg per reaction), the amount of 8-azido-[γ^{32}P]ATP added (0.5 μCi to 2.0 μCi), and the time length of UV irradiation (two or three 1-minute irradiation with 1-minute cooling period). The optimal condition was determined as described in the Materials and Methods section. Purified $FbpC_{(his6)}$ was found to bind [γ-^{32}P]N_3ATP under different conditions (Figure 12a). The protein band was absent in the control experiment where UV irradiation was omitted. Preincubation with 5 mM unlabeled ATP led to reduced intensity in the FbpC band suggesting the specific binding of ATP to FbpC. Figure 12b shows that photoaffinity labeling of FbpC was reduced as the concentration of unlabeled ATP was increased.

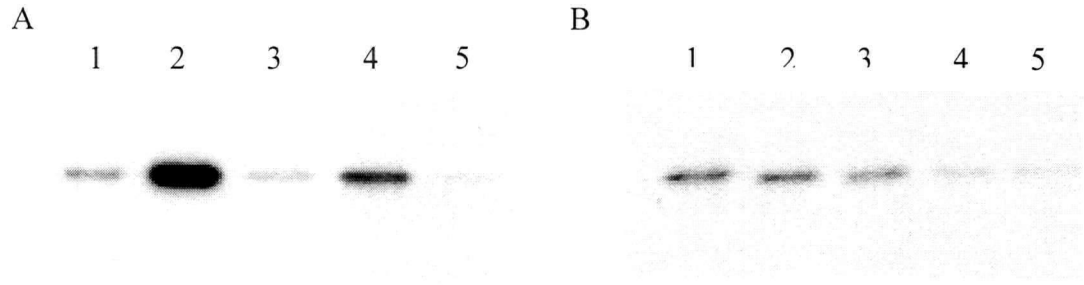

Figure 12. An autoradiogram showing binding of 8-azido-[γ-^{32}P]-ATP to $FbpC_{(his6)}$. ***A.*** Purified $FbpC_{(his6)}$ (1.5 μg) was diluted in photoaffinity labeling buffer (40 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 50 mM NaCl, 5 mM $CaCl_2$,7.5% glycerol) and incubated with 8-azido-[γ-^{32}P]-ATP in the absence of (*lanes* 1-2) and in the presence of (*lanes* 3-4) 5 mM unlabeled ATP. Labeled azido-ATP used per reaction was as follows: *lane 1.* 0.5 μCi; *lane 2.* 2.0 μCi; *lane 3.* 1.0 μCi; *lane 4.* 2.0 μCi. *lane 5.* control experiment with the UV irradiation step omitted. ***B.*** Preincubation of photoaffinity labeling reactions with different concentrations of unlabeled ATP prior to UV irradiation. *Lane 1.* 0 mM; *lane 2.* 1 mM; *lane 3.* 2 mM; *lane 4.* 5 mM; *lane 5.* 10 mM. Approximately 1.5 μCi labeled azido-ATP was used in each reaction.

4. DISCUSSION

4.1 Cloning and sequence analysis of *fbpC*

All bacterial ATP-binding cassette (ABC) importers share a conserved nucleotide binding motif, shown to provide energy for the transport of many growth essential nutrients through the hydrolysis of ATP (Higgins, 1992). The cloning of the *fbpC* gene from the genome into the pBluescript® II SK⁻ and the expression pET28a vectors was successful. DNA sequence analysis of the *fbpC* clone, when aligned with the *N. gonorrhoeae* complete genome sequence of FA 1090 (Roe et al., 2000), was found to be identical except at one nucleotide position that represents a silent mutation (Figure 7). Comparison of the cloned *fbpC* with the published *N. gonorrhoeae fbpC* sequence by Adhikari *et. al.* (1996) revealed a difference of 16 base pairs (Adhikari et al., 1996), including a frame shift in a region between WalkerA and WalkerB (Figure 13). Since this region is highly conserved among NBDs, the frame shift present in the published *fbpC* but not in the genome sequences of *N. gonorrhoeae* and *N. meningitidis* or in the nucleotide binding domain of a closely related Fe (III) periplasm-to-cytosol transporter, *hitC*, suggests that there is some error in the published DNA sequence of *fbpC*.

The FbpC protein shares 51% amino acid identity with HitC of the *hitABC* operon in *H. influenzae* and 40% identity with SfuC of the *sfuABC* operon in *S. marcescens,* both are nucleotide binding subunits of closely related Fe (III) periplasmic transporters (Figure 13) (Mietzner et al., 1998). FbpC is compared to HisP (29kDa) and MalK (40 kDa), the nucleotide binding subunits of the *S. typhimurium* histidine permease and maltose transporter by multiple sequence alignment (Figure 14). The region flanked by Walker A

and Walker B motifs display considerable sequence similarity, sharing approximately 38% identity. However, HisP lacks a sequence of approximately 100 amino acids at the C-terminus, the function of which remains unclear in FbpC.

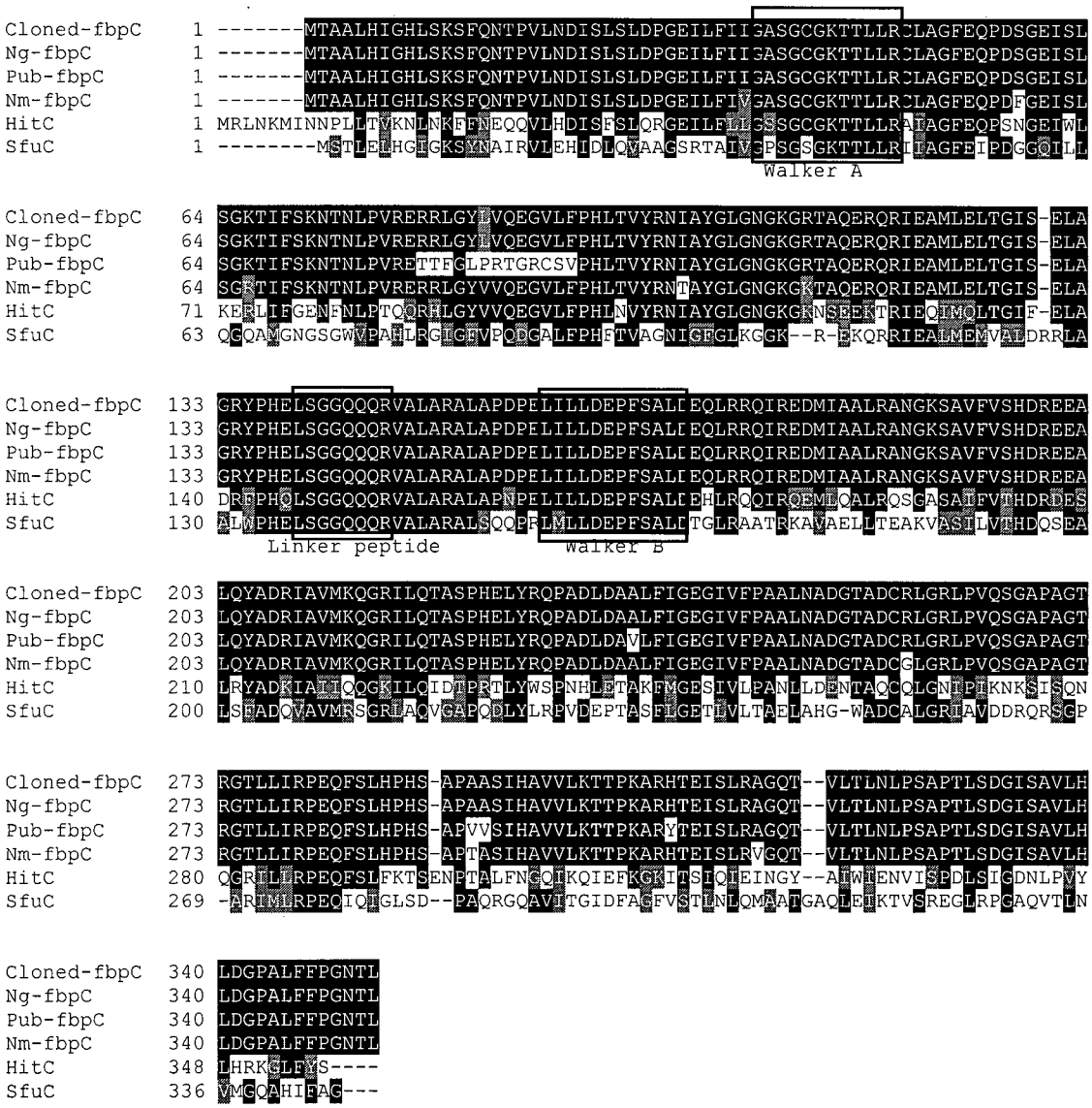

Figure 13. Amino acid sequence comparison of FbpC sequences with HitC and SfuC. Four FbpC sequences are used: (1) *N. gonorrhoeae* FbpC sequence from this study (Cloned-fbpC); (2) *N. gonorrhoeae* FbpC genome sequence (Roe et al., 2000); (3) Previously published *N. gonorrhoeae* FbpC sequence (Adhikari et al., 1996); (4) *N. meningitidis* FbpC genome sequence (Parkhill et al., 2000). The highly conserved Walker A, Walker B and the Linker peptide are indicated.

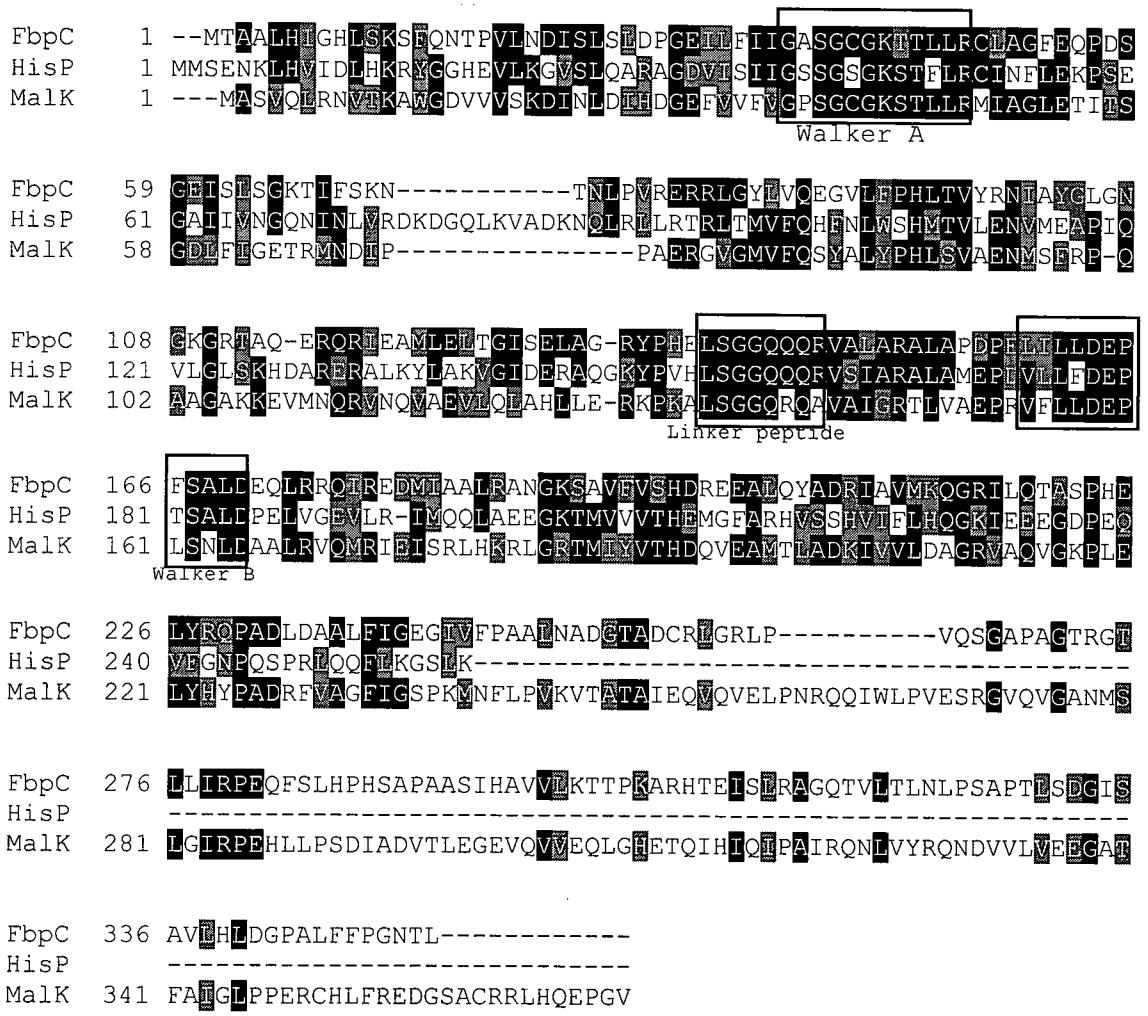

Figure 14. Comparison of FbpC amino acid sequence with HisP and MalK. The FbpC sequence used is that obtained from this study. The highly conserved Walker A, Walker B and the Linker peptide are indicated.

4.2 Production and purification of $FbpC_{(his6)}$ and the E164D mutant

The *fbpC* gene is likely not transcribed in large quantities in *N. gonorrhoeae*; therefore, FbpC was expressed and purified in a recombinant system. The high content of the hydrophobic residues in FbpC (Adhikari et al., 1996) may explain the relatively low solubility of the overproduced FbpC fusion proteins. The first few attempts of native FbpC purification did not yield protein fractions clearly visible on Coomassie blue-stained SDS-PAGE. Denaturing purification of $FbpC_{(his6)}$ using urea was attempted, and resulted in a much greater yield (data not shown); however, recovering native proteins was unsuccessful. Urea was removed by drop-wise, continuous dilution by buffer containing 0.1% Tween-20, 50 mM NaCl and 5% glycerol. Most of the FbpC protein precipitated at approximately 0.6 M urea. The remaining renatured protein did not show ATP-hydrolyzing activity, possibly due to improper folding following removal of the denaturant.

A native $FbpC_{(his6)}$ purification procedure was optimized. Purification of $FbpC_{(his6)}$ to near homogeneity was achieved by a two-step procedure, by nickel-chelate affinity chromatography followed by ion-exchange chromatography (Figure 9). Keeping a 0.4 M NaCl concentration in the initial purification step and the addition of 2 mM ATP and 20% glycerol throughout the purification procedures are both critical to maintain the protein in solution. The difficulty encountered in solubilizing $FbpC_{(his6)}$ is consistent with the previous findings that both MalK and HisP were prone to aggregation (Nikaido et al., 1997; Walter et al., 1992). Overproduced MalK was sequestered in inclusion bodies and purified by denaturation-renaturation procedures that included solubilizing inclusion bodies with urea and purifying the protein by red agarose chromatography (Walter et al., 1992). Soluble

HisP also showed a requirement for 5 mM ATP and 20% glycerol, both added throughout the purification procedure to obtain sufficient quantities for characterization (Nikaido et al., 1997).

Purification by nickel-affinity chromatography does not yield sufficiently pure $FbpC_{(his6)}$ (Figure 9). The problem of co-purification of targeted protein with non-histidine tagged host proteins was solved by the addition of an extra purification step: anion exchange chromatography. In contrast, the purification of HisP yielded proteins greater than 95% pure by a single metal (cobalt-based) affinity chromatography step (Nikaido et al., 1997). The extra purification step for $FbpC_{(his6)}$ is required possibly because of the lower initial concentration of soluble overexpressed $FbpC_{(his6)}$, leading to a higher level of non-specific binding of host proteins on the column. The difference in the properties of the two proteins and of the interaction between host cell proteins to the column may also be a contributing factor. In any case, the goal of removing contaminant proteins from $FbpC_{(his6)}$ after passage through the high-Q cartridge was achieved.

The maximum yield of pure $FbpC_{(his6)}$ was 0.6 - 0.7 mg per liter of culture. In comparison, purification of $HisP_{(his6)}$ yielded approximately 2 mg of pure protein per liter of culture (Nikaido et al., 1997). It should also be noted that attempts to concentrate pure $FbpC_{(his6)}$ to greater than 1 mg/ml had not been successful, which made characterization of the protein difficult because larger volumes had to be used. The additional 100 amino acids at the C-terminus that contain many hydrophobic residues may increase the tendency for $FbpC_{(his6)}$ to aggregate, effectively reducing the solubility.

The recently resolved HisP crystal structure showed that residue Glu^{179} in HisP is responsible for forming a water bridged hydrogen bond with the γ-phosphate of the bound ATP at the active site (Hung et al., 1998). The E179D mutant of HisP eliminated transport activity but allowed ATP binding (Shyamala et al., 1991), suggesting that this residue is only necessary for ATP hydrolysis but does not affect greatly nucleotide binding. The corresponding residue in FbpC is E164. The construction of such a mutant is desirable because the presence of ATP is absolutely essential for solubility in the current purification process, possibly due to the stabilization effect provided by the interaction between ATP and $FbpC_{(his6)}$.

I constructed an FbpC mutant E164D and compared its activity with the wild-type. The E164D $FbpC_{(his6)}$ preparation by this identical purification step also yielded proteins that contain similar ATPase activity as the wild-type, but the addition of the second ion-exchange chromatography step produced a E164D $FbpC_{(his6)}$ preparation that contained at least a ten-fold reduction in specific activity. This observation of reduced activity in the E164D mutant was reproducible, suggesting that the activity observed from the wild-type (after the two-step purification procedure) is due to the pure $FbpC_{(his6)}$ protein and not some impurities from the preparation.

4.3 Nucleotide binding and ATP hydrolyzing activity of $FbpC_{(his6)}$

The purified wild-type $FbpC_{(his6)}$ protein could be photolabeled with N_3ATP, indicating a suitable nucleotide binding site. The interaction was specific as preincubation with increasing ATP concentration washed out the labeling (Figure 12). The Michaelis-Menten constant of other traffic ATPases for ATP varies widely, with high K_m values mostly in the range of 50 to several thousand μM (Holland and Blight, 1999). Additional binding and activity assays are required in order for the binding constant of $FbpC_{(his6)}$ (for ATP) to be determined accurately, but preliminary experiments indicated that the K_m is in the millimolar range (data not shown), consistent with the idea that in general traffic ATPases have relatively low affinity for ATP (Holland and Blight, 1999).

FbpC displays ATP-hydrolyzing activity with a specific activity of approximately 0.5 μmol/min/mg, comparable to that determined for MalK of the *S. typhimurium* maltose transporter (0.7-1.3 μmol/min/mg) (Morbach et al., 1993; Schneider and Hunke, 1998) and HisP of the histidine permease in *S. typhimurium* (0.5 μmol/min/mg) (Nikaido et al., 1997). General characteristics of the enzyme, such as Mg^{2+} and pH dependence are in agreement with previous findings of isolated MalK (Morbach et al., 1993) and HisP (Nikaido et al., 1997). Glycerol was found to exert a positive effect on the activity of $FbpC_{(his6)}$. This finding is different from that observed for HisP, where glycerol concentration greater than 10% (v/v) resulted in a decrease in ATP hydrolysis. It should be noted, however, that the activity values obtained and the general characteristics observed should be treated with caution because isolated ATP-binding domains may exhibit different properties in the

absence of the integral permease subunits and the periplasmic binding component in a reconstituted complex.

The ATPase activity of HisP was studied in both purified forms (0.5 μmol/min/mg) and in a $HisQMP_2$ reconstituted liposome complexes (0.37 μmol/min/mg in the presence of liganded HisJ, the periplasmic binding component). Observed differences in the properties of HisP in the two forms include a lack of cooperativity for ATP in isolated HisP, and a stimulatory effect of HisJ on ATPase activity only in the reconstituted complex (Liu et al., 1997) and not in isolated HisP (Nikaido et al., 1997). Different HisP mutants were characterized to investigate how different amino acid residues contributed to ATP binding or hydrolysis, or to the interaction between the nucleotide binding domain and the membrane permease components. The mutational analyses have further established the functional importance of conserved motifs such as the Walker A and Walker B regions for ATP binding, and the signature motif (also called the linker region) and the helical region for coupling of ATP hydrolysis to transport (Schneider and Hunke, 1998).

4.4 Conclusion

The goal of this study was to work towards showing that FbpC is a nucleotide binding domain for the periplasm-to-cytosol transport of iron in *Neisseria gonorrhoeae*. A biochemical approach was taken to characterize the recombinant FbpC protein. Amplification and cloning of the *N. gonorrhoeae fbpC* gene into the pET28a expression vector produced a stable genetic construct, pEfbpC3. $FbpC_{(his6)}$ was successfully overexpressed in HMS174(DE3) cells containing the pEfbpC3 plasmid and purified by

nickel-chelate affinity chromatography and anion-exchange chromatography. The purified fusion protein was enzymatically active, as it has the ability to hydrolyze ATP and to interact with 8-azido-[γ^{32}P]ATP. The specific activity of FbpC as an isolated ATPase is 0.5 μmol/min/mg.

4.5 Future directions

Establishing $FbpC_{(his6)}$ as a functional ATPase biochemically was an important initial characterization of FbpC. To further establish the physiological relevance of the ATPase activity of $FbpC_{(his6)}$, the activity should be compared to that of the reconstituted FbpABC complex. Mutational analyses of FbpC will also help identify its interaction with the permease subunit (FbpB) and provide further insight into the mechanism of energy coupling to the rest of the transport system. Given the diversity of pathogenic bacteria that express ABC transporter operons that take up iron at the periplasm-to-cytosol level and their amino acid sequence similarity, I am convinced that FbpC functions as an ATP-binding subunit in *Neisserial* iron transport.

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