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Identification of a locus involved in the utilization of iron by *Actinobacillus pleuropneumoniae*

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Abstract

The cloned *afu* locus of *Actinobacillus pleuropneumoniae* restored the ability of an *Escherichia coli* K-12 mutant (*aroB*) to grow on iron-limited media. DNA sequence analysis of the fragment showed that there are three genes designated *afuA*, *afuB* and *afuC* (Actinobacillus ferric uptake) that encode products similar to the SfuABC proteins of *Serratia marcescens*, the HitABC proteins of *Haemophilus influenzae*, the FbpABC proteins of *Neisseria gonorrhoeae* and the YfuABC proteins of *Yersinia enterocolitica*. The three genes encode a periplasmic iron-binding protein (AfuA), a highly hydrophobic integral cytoplasmic membrane protein with two consensus permease motifs (AfuB) and one hydrophilic peripheral cytoplasmic membrane protein with Walker ATP-binding motifs (AfuC), respectively. This system has been shown to constitute a periplasmic binding protein-dependent iron transport system in these organisms. The *afuABC* operon is locating approximately 200 bp upstream of *apxIC* gene, but transcribed in opposite direction to the ApxI-toxin genes.

Keywords: *Actinobacillus pleuropneumoniae*; Iron-uptake operon; *afuABC*; ApxI

1. Introduction

The low concentration of free iron, an essential nutrient for bacteria, on mucous membranes and in tissues is one of the first lines of host defense against bacterial infection. The presence of iron-binding proteins in the body fluids, such as transferrin, lactoferrin, haem, haemoglobin, and ferritin further serves to maintain low free-Fe concentrations, inhibiting bacteria growth [1]. To sequester the limited iron from the host, bacteria have evolved several mechanisms, such as the secretion of siderophores and iron chelators which compete with lactoferrin and transferrin for iron. Iron-repressible outer membrane proteins (IROMP) that serve as receptors for iron-siderophore complexes are essential for iron uptake have been identified in many pathogenic bacteria [2], including *Actinobacillus pleuropneumoniae* [3].

A. pleuropneumoniae obtains iron from haem compounds [3] via the production hemolysins [4,5], and membrane-bound transferrin-specific receptors [6]. *A. pleuropneumoniae* probably binds the iron-loaded transferrin molecule to its surface and then, transports the iron from the transferrin into the cells. However, a mechanism for the transfer of iron from the transferrin to the bacterium has not been elucidated.

In this study, we reported the cloning and se-

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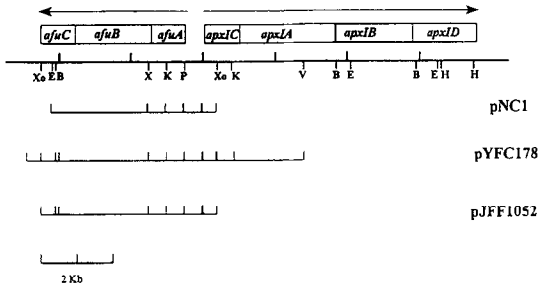

Fig. 1. Partial restriction enzyme map of *afu* and *apxI* operons of *A. pleuropneumoniae*. The inserted DNA from pNC1 and pJFF1052 were completely sequenced. E, *Eco*RI; V, *Eco*RV; B, *Bgl*II; K, *Kpn*I; H, *Hin*dIII; P, *Pst*I; X, *Xba*I; Xo, *Xho*I. Arrows indicate direction of expression.

quence analysis of an iron utilization system in *A. pleuronpneumoniae* that is very similar to the periplasmic-binding protein-dependent transport system in *Serratia marcescens* [7,8], *Haemophilus influenzae* [9,10], *Neisseria gonorrhoeae* [11,12] and *Yersia enterocolitica* (unpublished data). This locus, termed *afu* (**A**ctinobacillus **f**erric **u**ptake) is located upstream of *ApxICABD* gene cluster, but is transcribed in opposite direction to the hemolysin gene (*apxIC*). Complementation analysis showed that this locus could restore the ability of an *E. coli aroB* mutant to grow on iron-depleted medium.

2. Materials and methods

2.1. Bacterial strains, plasmids, growth conditions and DNA preparation

A. pleuropneumoniae serotype 1 to 12 reference strains described previously [13] were grown in brain–heart infusion broth (BHI, Difco Laboratories) supplemented with 0.1% NAD or on chocolate agar. The *E. coli* strains, TB1, *ara* Δ(*lac-proAB*)*rpsL*Φ80d*lacZ* ΔM15 *hsdR*17 (r^-m^+), H1443, *aro*D *ara*D139 Δ*lac*U169 *rpsL*150 *relA1 deoC1 ptsF*25 *rbsR thi flb*5301, and DH5α Φ80d*lac*-Z ΔM15 Δ(*lac*ZYA-argF)U169 *end*A1 *rec*A1*hsd*R17($r_k^-m_k^+$)*deo*R *thi*−1*sup*E44 γ^- *gyr*A96 *rel*A1 were grown in Luri-Bertani (LB) broth or LB agar. Antibiotics were used as appropriate for selection or maintenance of plasmids, ampicillin 40 μg/ml, and kanamycin 50 μg/ml. X-gal (5-bromo-indolyl-β-D-galactopyranoside) was added to agar plates to 40 μg/ml. Plasmids pYFC126 and pYFC127 contains *apxICA* genes and a segment upstream *apxIC* from serotype 1 and 5, respectively [14]. pNC1 contains *afuAB* and partial *afuC* genes. Plasmid pYFC178 contains *afuABC* genes subcloned from the phage clone, λyfc40 originating from *A. pleuropneumoniae* serotype 5 (Fig. 1). Plasmid pJFF1052 contains *afu*ABC in pBS (same direction to P_{lac}) (Fig. 1) and plasmid pSZ1 contains *sfuABC* in pBR322 [7].

2.2. DNA isolation and construction of a genomic and sub-genomic library of A. pleuropneumoniae DNA in λ-dash and screening

A. pleuropneumoniae genomic DNA from different serotypes was prepared as previously described [13]. A lamda-dash library was constructed by using the genomic DNA from a serotype 5 strain as previously described [5]. A subgenomic library from serotype 5 was also constructed using *Bgl*II and *Pst*I digested DNA fragments separated by agarose gel electrophoresis. The 4.5–6 kbp fragments were ligated into pHG165 digested with *Bam*HI and *Sal*I. The bacteriophage and subgenomic libraries were screened by hybridization using a probe (a 1033 bp *Pst*I–*Xho*I DNA fragment from pYFC126) contaning the partial *apxIC* gene and its upstream region from *A. pleuropneumoniae* serotype 5 [14].

Table 1
The conserved EAA motifs from the periplasmic permeases of *A. pleuropneumoniae*, *S. marcensens*, *H. influenzae*, *N. gonorrhoeae* and *Y. enterocolitica*

Protein[a]	Residue	Conserved sequence
AfuB (N)	303	EEASYTLRANRYQTFYNIIFP
SfuB (N)	167	EDVATSLGSRPPAVFFRVVLP
HitB (N)	148	EEVSISLGKSPVYTFWYAIFP
YfuB (N)	168	EDAAASLGSTPSAVFFHVVLP
FbpB (N)	153	EEVSLSLGKSRLQTFFSAILP
AfuB (C)	581	EASLSLKGSSLKTIWFIVFPL
SfuB (C)	428	ENVARSLGKSPAQAiWSTTLR
HitB (C)	410	EKVGGSLGRNPFYIFRTITLP
YfuB (C)	429	ENVARSLGKTPTQAIWSTTMR
FbpB (C)	426	EQVGATLGRGHFFIFRTLVLP

[a]The sequence were taken from the following sources: *afuB* (this study), *sfuB* [7], *fbp*b [12], *hit*b [9] and *yfuB* (unpublished data).

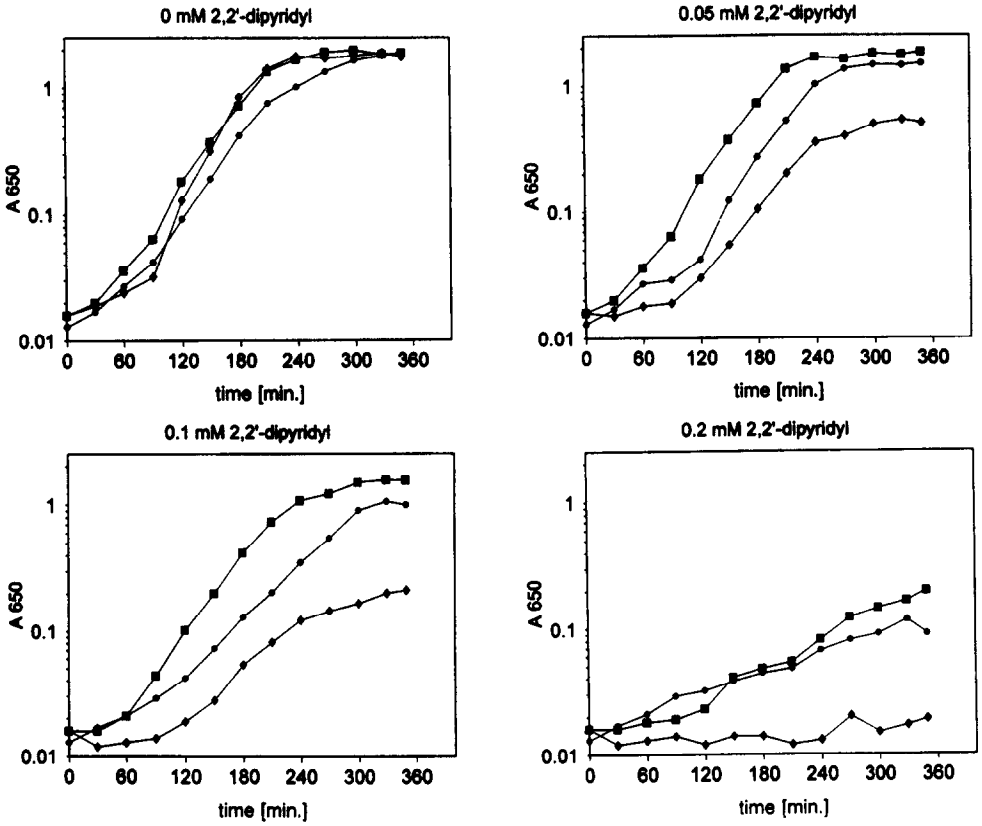

Fig. 2. Growth rates of the *aroB* mutant H1443 and the complemented mutant in LB containing different concentrations of 2,2′-dipyridyl. Growth was monitored with a spectrophotometer at A650. The cultures were inoculated to $A_{650} = 0.01$ with fresh precultures. ◆, H1443 (aroB); ●, H1443 [pJFF1052 (*afuA*$^+$*B*$^+$*C*$^+$)]; ■, H1443 [pSZ1 (*sfuA*$^+$*B*$^+$*C*$^+$)].

2.3. DNA sequencing and analysis

Plasmid DNA for cycle DNA sequencing was isolated with a mini-kit from Qiagen (Chatsworth, CA). The nucleotide sequence was determined by an automated fluorescence procedure based on the Sanger dideoxy chain termination method using a Taq Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystem, Inc.). DNA sequences were determined by using double-stranded DNA templates (pNC1, pYFC178 and pJFF1052). Oligonucleotide primers based on the DNA sequence in this study were prepared by the Analytical and Synthetic Facility, Cornell University. Both strands of the cloned DNA were completely sequenced.

2.4. Trans-complementation of E. coli aroB

E. coli H1443 (*aroB* mutant) transformed with plasmid pJFF1052 or with plasmid pSZ1 and *E. coli* C600 (*aroB*$^+$) was inoculated in LB broth or in LB broth supplemented with 0.05, 0.1, 0.2, or 0.4 mM 2,2′-dipyridyl (NBD) (Sigma, St Louis, MO) and the growth was monitored by measuring the absortion A_{650} in a spectrophotometer.

2.5. Southern blotting, hybridization

A *Pst*I and *Xba*I DNA fragment from pNC1 containing the *afuA* gene was isolated from agarose gel and labeled with [^{32}P]dATP by nick-translation. Filters were hybridized in 45% formamide, 5×SSC, 5×Denhardt's solution, and 100 μg/ml sheared calf

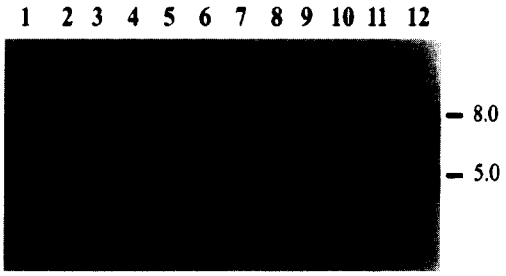

Fig. 3. Southern blotting analysis of 12 serotypes reference strains of *A. pleuropneumoniae*. The lanes are labeled according to the serotype number. The filter was hybrized and washed as described in Section 2. Molecular mass in kbp as indicated at the right.

thymus DNA for 12 h at 37°C. Filters were then washed twice with 2×SSC–0.1% SDS and twice with 0.2% SSC–0.1% SDS at room temperature [4,13]. The final wash was with 0.16% SSC–0.1% SDS at 37°C.

3. Results and discussion

3.1. Nucleotide sequences of the afuA, afuB and afuC genes

Plasmid pNC1 and phage clone λyfc40 were selected from subgenomic and λ-dash library, respectively. Plasmid pNC1 contains a 5.0 kb of the *A. pleuropneumoniae* serotype 5 chromosome, cloned into the *Sal*I and *Bam*HI sites of pHG165 [4,14]. λyfc40 was digested with *Sal*I and a 9 kb fragment was ligated into pBluescriptII- SK$^-$, to obtain pYFC178. Plasmid pJFF1052 contains a 5.8 kb chromosomal fragment of *A. pleuroneumoniae* serotype 1 (strain 4074) including the 5′-terminal part of the *apxIC* and a 5.4 kb segment upstream *apxIC* cloned into the *Xho*I site of pBluescriptII-SK$^-$ (Fig. 1). The sequences of both clones showed three open reading frames with high similarities to the *sfuABC* gene cluster of *S. marcescens* [7]. In analogy to *sfuABC*, the three reading frames were designated *afuA*, *afuB*, and *afuC*, respectively (Fig. 1). The DNA sequence of *afuABC* genes from *A. pleuropneumoniae* serotype 1 and 5 are identical.

The deduced amino acid sequence of AfuA deduced from the nucleotide sequence of *afuA* contains a typical signal sequence found in exported proteins. Cleavage of the signal peptide most likely occurs between the A and K residue at positions 27 and 28. AfuB is proposed to function as a cytoplasmic membrane permease and is composed of 663 amino acids, most of which are hydrophobic. Two sequences that match the consensus permease EAA motifs (EAA---G----------I-LP) are found (Table

Table 2
Comparison of non-polar membrane transport proteins containing sequences homologous to nucleotide-binding domains

Protein	Residue	Conserved sequence
AfuC (Ap)	136	ISGGQQQRVALARALVLK-PK------VLILDEPLSNLDANLRRSMREKIRE
SfuC (Sm)	136	LSGGQQQRVALARALSQQ-PR------LMLLDEPFSALDTGLRAATRKAVAE
HiTC (Hi)	146	LSGGQQQRVALARALAPN-PE------LILLDEPFSALDEHLRQQIRQEMLQ
YfuC (Ye)	136	ISGGQQQRVALARALGQR-PA------LMLLVEPFSTLDTALRASTRKAVAE
fBPc (Ng)	138	LSGGQQQRVALARALAPD-PE------LILLDEPFSALDEQLRRQIREDMIA
FecE	139	LSGGQRQRAFLAMVLAQNTP------VVLLDEPTTYLDINHQVDLMRLMGE
FhuC	141	LSGGERQRAWIAMLVAQDS-R------CLLLDEPTSALDIAHQVDVLSLVHR
BtuD	127	LSGGEWQRVRLAAVVLAITPQANPAGQLLLLDEPMNSLDVAQQSALDKILSA
HisP	154	LSGGQQQRVSIARAL-AMEPD------VLLFDEPTSALDPELVGEVLRIMQQ
MalK	134	LSGGQRQRVAIGRJLVA-EPS------VFLLDEPLSNLDAALRVQMRIEISR
PstB	152	LSGGQQQRLCIARGL-AIRPE------VLLLDEPCSALDPISTGRIEELITE
OppD1	165	FSGGQCQRIGIARAL-ILEPK------LIICDEPVSALDVSIQAQVVNLLQQ
OppD2	169	FSGGMRQRVMIAMALL-CRPK------LLIADEPTTALDVTVQAQIMTLLNE
OppD (St)	167	FSGGMRQRVMIAMALL-CRPK------LLIADEPTTALDVTVQAQIMTLLNE
RbsA (N)	144	LSIGDQQMVEIAKVLSF-ESK------VIIMDEPTSALTDTETESLFRVIRE

*Proteins were from *E. coli* unless otherwise indicated. A glycine–glutamine-rich sequence, LSGGQQQ (Linker peptide) is underlined. Ap, *A. pleuropneumoniae* (this study); Hi, *H. influenzae* [9]; Ng, *N. gonorrhoeae* [12]; Sm, *S. marcescens* [7]; St, *S. tryphimurium*; Ye, *Y. enterocolitica* (unpublished data). N, amino-terminal half of the polypeptide.

1). These two motifs are suggested to be located on cytoplasmic loops that interact with the ATP-binding protein [16,17]. AfuC shows strong similarities to the nucleotide-binding proteins of ABC (ATP Binding Cassette) transporters [18] (Table 2). A comparison of AfuA, AfuB and AfuC with homologous proteins are presented in Table 3. *A. pleuropneumoniae* together with other pathogenic bacteria possess a siderophore-independent mechanisms for iron sequestration ([7,10,12], this study). In *N. gonorrhoeae* and *N. meningitidis*, two proteins (Tbp1 and Tbp2) are responsible for binding transferrin to the cell surface [15]. Similarly, the genes for two transferrin binding proteins (Tbp1 and Tbp2) have been cloned and sequenced in *A. pleuropneumoniae* [6]. In *Neisseria* species, the iron can be removed from transferrin or lactoferrin to the periplasmic space, and carried by Fbp to transport the iron molecule into the cells [15]. It has also been suggested that the iron is diffusible through the *E. coli* porin to the periplasm that is independent of the transferrin receptor [10]. The mechansim of iron transport from porcine transferrin into *A. pleuropneumoniae* is unknown. However, the presence of *afu* operon homologs in *H. influenzae* [9,10], *S. marcescens* [7], and *N. gonorrhoeae* [11,12] suggests that the function of this operon may be involved in high-affinity iron acquisition from the host environment.

3.2. Complementation of the iron transport negative E. coli strain

To examine the function of the *A. pleuropneumoniae* transport genes in iron uptake, iron transport was studied in *E. coli aroB* mutant strain H1443 carrying cloned *afuABC* genes. As shown in Fig. 2, the *afuABC* genes confer the *E. coli aroB* mutant which is unable to synthesise enterochelin, to grow in iron-limiting medium. In medium supplemented with 0.05 mM or 0.1 mM 2,2′-dipyridyl, the strains containing the cloned *afuABC* genes or the *sfuABC* genes grow significantly faster and to a higher density than the non-complemented *aroB* mutant (Fig. 2). At 0.2 mM 2,2′-dipyridyl, growth of the *aroB* mutant was inhibited, but the complemented mutant was able to grow, albeit at a reduced growth rate. The growth rate of *E. coli* C600 (*aroB*$^+$) was unaffected at these concentrations, but was generally higher in this medium compared to H1443. Addition of 0.4 mM 2,2′-dipyridyl also inhibited growth of the complemented transformants of H1443, and also reduced the growth rate of the control strain *E. coli* C600 (*aroB*$^+$). Supplementation of medium containing 0.2 mM 2.2′-dipyridyl with 2 mM $Fe(SO)_4$ restored the growth rates of H1443 and the complemented H1443 strains. However, only partial restoration of the growth rates was observed in the medium contained 0.4 mM 2,2′-dipyridyl, supplemented with $Fe(SO)_4$. These results indicated that the importance of the functional *afu*ABC operon for iron acquisition by complementation of the siderophore-deficient *E. coli* H1443 to growth on dipyridyl-containing medium.

Table 3
Comparision of AfuA, AfuB and AfuC with homologous proteins

Organism	Gene	A(S/I)[a]	B(S/I)[a]	C(S/I)[a]
S. marcescens[b]	sfu	47.6/23.1	50.5/20.0	50.8/33.0
H. influenzae[b]	hit	45.5/25.0	52.1/23.9	56.2/33.5
Y. enterocolitica	yfu	45.7/21.5	49.7/20.0	55.4/31.9
N. gonorrhoeae[b]	*fbp*	45.6/22.8	51.4/22.9	55.6/32.2

[a]Percent similar/identical residues (S, similarity; I, identity). Percent similar residues assuming that the following amino acid pairs are equivalent; I and V, S and T, E and D, K and R, F and Y.
[b]The sequence were taken from the following sources: *afuABC* (this study), *sfuABC* (7), *fbp*(11,12), *hit* (9,10) and *yfuABC* (unpublished data).

3.3. Identification of the afuA gene by Southern blotting analysis

A *Pst*I and *Xba*I DNA fragment containing *afuA* was purified, labelled with [^{32}P]dATP, and used as a hybridization probe on genomic DNA of *A. pleuropneumoniae* serotypes. The results showed that the *afuA* DNA hybridized to one unique fragment in the DNA of the *A. pleuropneumoniae* serotypes 10 and 11 (8 kbp), 1, 5 and 9 (7.8 kbp), 3 (7.4 kbp) and 2, 4, 7, and 8 (5 kbp), but not to serotype 6 (Fig. 3).

In conclusion, the *afuABC* operon of *A. pleuropneumoniae* is sufficient to enable an *E. coli* K12(*aroB*) mutant to grow on iron-limited medium (4 mM dipyridyl). The three polypeptides deduced from the DNA sequence were similar to that of SfuABC [7], HitABC [9,10], FbpABC [11,12] and

YfuABC. Based on these data, we hypothesized that the AfuA, AfuB and AfuC polypeptides are involved the transport of ferric iron across the cytoplasmic membrane. An efficient iron acquisition system for these pathogenic bacteria may play an important role in the pathogensis of bacterial infection.

3.4. Nucleotide sequence accession number

The sequence of *afuABC–apxICA* genes from serotype 1 and 5 has been submitted to Genbank and assigned accession numbers U05042 and U04954, respectively.

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References

[1] Weinberg, E.D. (1978) Iron and infection. Microbiol. Rev. 42, 45–66.

[2] Otto, B.R., Verweij-van Vught, A.M.J.J. and Maclaren, D.M. (1992) Transferrins and heme-compounds as iron sources for pathogenic bacteria. Crit. Rev. Microbiol. 18, 217–233.

[3] Deneer, H.G., and Potter, A.A. (1989) Effect of iron restriction on the outer membrane proteins of *Actinobacillus (Haemophilus) pleuropneumoniae*. Infect. Immun. 57, 798–804.

[4] Chang, Y.F., Young, R. and Struck, D.K. (1989) Cloning and characterization of a hemolysin gene from *Actinobacillus (Haemophilus) pleuropneumoniae*. DNA 8, 635–646.

[5] Frey, J., Beck, M., Stucki, U. and Nicolet, J. (1993) Analysis of hemolysin operons in *Actinobacillus pleuropneumoniae*. Gene 123, 51–58.

[6] Gonzalez, G.C., Yu, R.-H., Eosteck, P.R., Jr. and Schryvers, A.B. (1995) Sequence, genetic analysis, and expression of *Actinobacillus pleuropneumoniae* transferrin receptor genes. Microbiology 141, 2405–2416.

[7] Angerer, A., Gaisser, S. and Braun, V. (1990) Nucleotide sequences of the *sfuA*, *sfuB*, and *sfuC* genes of *Serratia marcescens* suggest a periplasmic-binding protein-dependent iron transport mechanism. J. Bacteriol. 172, 572–578.

[8] Angerer, A., Klupp, B. and Braun, V. (1992) Iron transport systems of *Serratia marcescens*. J. Bacteriol. 174, 1378–1387.

[9] Sanders, J.D., Cope, L.D. and Hansen, E.J. (1994) Identification of a locus invlved in the utilization of iron by *Haemophilus influenzae*. Infect. Immun. 62, 4515–4525.

[10] Adhikari, P., Kirby, S.D., Nowalk, A.J., Veraldi, K.L., Schryvers, A.B. and Mietzner, T.A. (1995) Biochemical characterization of a *Haemophilus influenzae* periplasmic iron transport operon. J. Biol. Chem. 270, 25142–25149.

[11] Berish, S.A., Mietzner, T.A., Mayer, L.W., Genco, C.A., Holloway, B.P. and Morse, S.A. (1992) Molecular cloning and characterization of the structural gene for the major iron-regulated protein expressed by *Neisseria gonorrhoeae*. J. Exp. Med. 171, 1535–1546.

[12] Adhikari, P., Berish, S.A., Nowalk, A.J., Veraldi, K.L., Morse, S.A. and Mietzner, T.A. (1996). The *fbpABC* locus of *Neisseria gonorrhoeae* functions in the periplasm-to-cytosol transport of iron. J. Bacteriol. 178, 2145–2149.

[13] Chang, Y.F., Shi, J., Ma, D.P., Shin, S.J. and Lein, D.H. (1993) Molecular analysis of the *Actinobacillus pleuropneumoniae* RTX toxin-III gene cluster. DNA Cell Biol. 12, 351–362.

[14] Chang, Y.F., Wang, Y., Chin, N., Shin, S.J. and Lein, D.H. (1994) Expression and sequence analysis of an RTX-1 toxin determinant from *Actinobacillus pleuropneumoniae* serotype 5. Virulence mechanisms of bacterial pathogens, international symposium, June 6–8, 1994. Abstract 38. Iowa State University, Ames, IO.

[15] Chen, C.Y., Berish, S.A., Morse, S.A. and Mietzner, T.A. (1993) The ferric iron-binding protein of pathogenic *Nesseria* spp. functions as a periplasmic transport protein in iron acquisition from human transferrin. Mol. Microbiol. 10, 311–318.

[16] Saurin, W., Köster, W. and Dassa, E. (1994) Bacterial binding protein-dependent permeases: characterization of distinctive signatures for functionally related integral cytoplasmic membrane proteins. Mol. Microbiol. 12, 993–1004.

[17] Kerppola, R.E. and Ames, G.F.-L. (1992) Topology of the hydrophobic membrane-bound components of the histidine periplasmic permease. J. Biol. Chem. 267, 2329–2336.

[18] Higgins, C.F. (1992) ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. 8, 67–113.