Evidence of a Novel Gene from *Aeromonas hydrophila* Encoding a Putative Siderophore Receptor Involved in Bacterial Growth and Survival

Shaw-Wei D. Tsen

Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD U.S.A.

Abstract: The pathogenic bacterium *Aeromonas hydrophila* has been shown to exclusively utilize a ligand exchange mechanism for siderophore-mediated iron uptake, with a single nonspecific siderophore receptor facilitating iron exchange. However, the genes involved in this process, including the gene encoding the nonspecific receptor, are unknown. Here we identify and characterize a novel gene, *nsr1*, from *A. hydrophila* that encodes a putative protein with high homology and significant predicted structural similarities to the FhuA protein and other known ferric-siderophore receptors. This protein appears to localize on the cell membrane and is likely to be the receptor involved in the ligand exchange siderophore-mediated iron uptake mechanism of *A. hydrophila.* It is expected that this information may lead to the development of new antibiotics targeting either *nsr1* or its gene product for use in controlling *A. hydrophila* infection.

Keywords: ferric iron, iron uptake, bacterial virulence, pathogenicity, infection

Introduction

Iron is necessary for many of the critical biochemical processes in bacterial growth and metabolism. Although iron is a relatively abundant element, its bioavailability is severely limited due to its low solubility (Apostol et al. 2005). To overcome this problem, bacteria secrete low molecular weight Fe(III)-chelating compounds known as siderophores to assist in iron acquisition. These siderophores form complexes with extracellular iron and are generally transported into the bacterial periplasm via membrane transport proteins (Stintzi et al. 2000). The ability of bacteria to scavenge iron sources from the environment has been shown to be a significant factor in bacterial survival, as well as in bacterial pathogenicity (Payne and Finkelstein, 1978). Therefore, siderophore-mediated iron acquisition systems play a central role in the progression of bacterial infection (Stintzi et al. 2000; Wooldridge and Williams, 1993).

Recently, members of the genus *Aeromonas* have drawn increased interest as human pathogens (Janda and Abbott, 1998). In particular, the bacterium *Aeromonas hydrophila* has been reported to cause a multitude of human diseases, including wound infections, septicemia, and diarrhea (Agger et al. 1985). Iron transport in *A. hydrophila* has been shown to occur via a single membrane-bound siderophore receptor that is able to recognize a broad range of siderophores, utilizing a ligand exchange uptake mechanism (Stintzi et al. 2000). However, the genes involved in this iron uptake mechanism have not been reported or characterized to date.

In the current paper we identify and characterize a novel gene from *A. hydrophila*, *nsr1*, encoding a putative membrane receptor protein likely involved in siderophore-mediated iron uptake. The gene product exhibits a high degree of homology to the FhuA protein, which encodes an outer membrane-associated ferric-siderophore receptor in many bacteria (Coulton et al. 1986). Further investigation into the structure and function of the protein product of *nsr1* may elucidate the iron acquisition mechanism of *A. hydrophila* and may provide insight into the pathogenesis of *A. hydrophila* infection in humans.

Materials and Methods

Data mining

The TIGR database (www.tigr.org) was mined for genomic data, and the novel gene sequence was derived from the unfinished genome of *A. hydrophila*, contig. 1047085923793 (5'-GGCCTTCTGT…

Correspondence: Shaw-Wei D. Tsen, Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD, U.S.A., Email: tsen@jhu.edu.

GTTGCGAGC-3'), by selection of the sequence encoding the longest observed translated open reading frame within the contig (SIXFRAME program, Biology Workbench, San Diego, CA, http://workbench.sdsc.edu). This yielded a 2106-bp DNA sequence encoding the putative protein (Fig. 1).

Sequence/product analysis

Database comparisons to known nucleotide sequences and proteins were made by using the rapid sequence database query programs BLASTP, BLASTN, and TBLASTN (Genbank, National Center for Biotechnology Information, Bethesda, MD, http://www.ncbi.nlm.nih.gov/BLAST). Protein crystal structure prediction and hydropathy plots were carried out using publicly available online programs including the 3D-JIGSAW Protein Comparative Modeling Server (http://www.bmm.icnet.uk/servers/3djigsaw), the ExPASy proteomics server (www.expasy.org), and Biology Workbench.

Results and Discussion

DNA sequence analysis of *nsr1*

The nucleotide sequence of *nsr1* was compared to known DNA sequences in the Genbank database with BLASTN (Genbank, NCBI) and no significant similarities were found. This is not surprising, given the generally high mutation rate in bacteria and the evolutionary time span of iron uptake mechanisms which were crucial for survival in the earliest known organisms. The nucleotide sequence of *nsr1* contains a high GC content (61.8%) characteristic of gene-containing genomic regions (NASTATS, Biology Workbench).

Characterization of the *nsr1* gene product

The DNA sequence of *nsr1* was translated and found to contain an open reading frame of 702 amino acids encoding the putative protein (SIXFRAME program, Biology Workbench). The molecular mass of the resulting protein was predicted to be ~77.4 kDa (ExPASy, Swiss Institute of Bioinformatics, Canada).

A Kyte-Doolittle hydropathy plot indicates the presence of numerous potential membrane-spanning regions in the predicted polypeptide, suggesting that the putative protein may be localized on the cell membrane (Kyte and Doolittle, 1982) (Fig. 2). This is supported by the high percentage of hydrophobic residues (%LVIFM = 23.8%) present in the sequence (SAPS program, Biology Workbench). Individual amino acid composition of the polypeptide includes a high percentage of glycine (9.5%) and leucine (9.6%) residues (AASTATS program, Biology Workbench), and further analysis of the polypeptide revealed alternating glycine-rich and leucine-rich regions. This observed pattern of alternating hydrophilic and hydrophobic regions is also seen in other bacterial ferric-siderophore receptors (Newton et al. 1997); hydrophilic regions constitute surface loops and may facilitate siderophore binding in the extracellular compartment, while hydrophobic regions anchor the protein to the cell membrane. The protein is predicted to be slightly negatively charged in general (%KR—ED = −3.4%); however, no charge clusters were predicted (SAPS program, Biology Workbench). Protein pI was estimated to be ~4.94, in agreement with the environment necessary for the acidification process and removal of iron within the barrel of the receptor in a ligand exchange iron uptake mechanism (Stintzi et al. 2000).

The homology of the putative protein to currently known proteins was characterized with BLASTP (Genbank, NCBI). The FhuA protein and similar iron uptake proteins from various bacteria were obtained and aligned using the algorithm of Thompson et al. (Thompson et al. 1994) (CLUSTALW program, Biology Workbench). In the multiple sequence alignment (Fig. 3), the conserved residues are distributed along the entire length of the polypeptide, suggesting a strong evolutionary pressure to preserve amino acids at specific positions for structural and/or functional reasons. Ferric-siderophore receptors in general are characterized by evolutionarily conserved surface loops and anti-parallel β-barrels; the former is required for interaction with iron-loaded siderophores in the extracellular compartment, and the latter constitutes the iron removal acidification site within the receptor (Stintzi et al. 2000; Newton et al. 1997).

The predicted crystal structure of the putative protein (Fig. 4) was modeled using the 3DJIGSAW Protein Comparative Modeling Server (Bates et al. 2001; Bates and Sternberg, 1999; Contreras-Moreira and Bates, 2002) and contains an

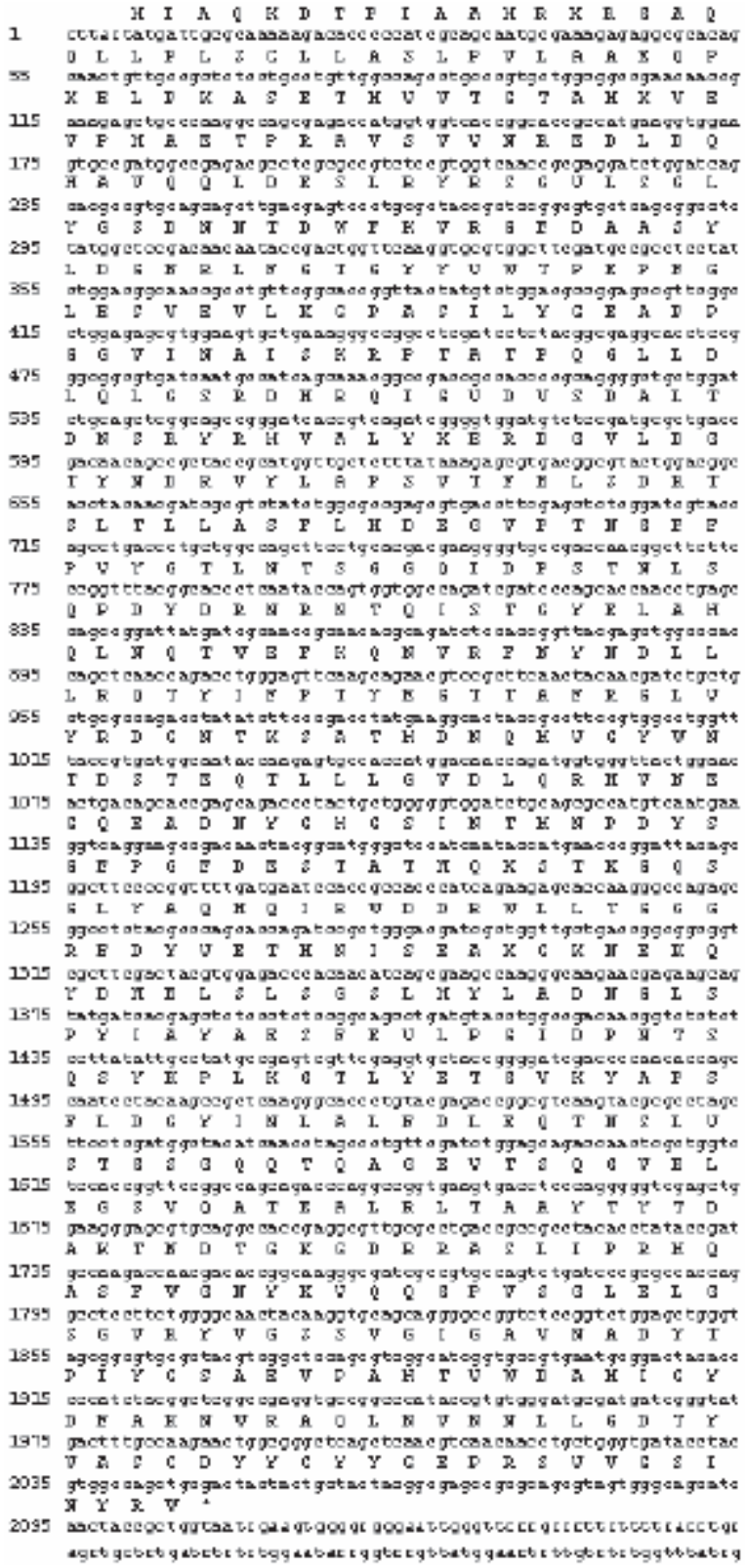

Figure 1. DNA sequence of *nsr1 and* its translated amino acid sequence. Only those nucleotides encoding the predicted polypeptide are numbered.

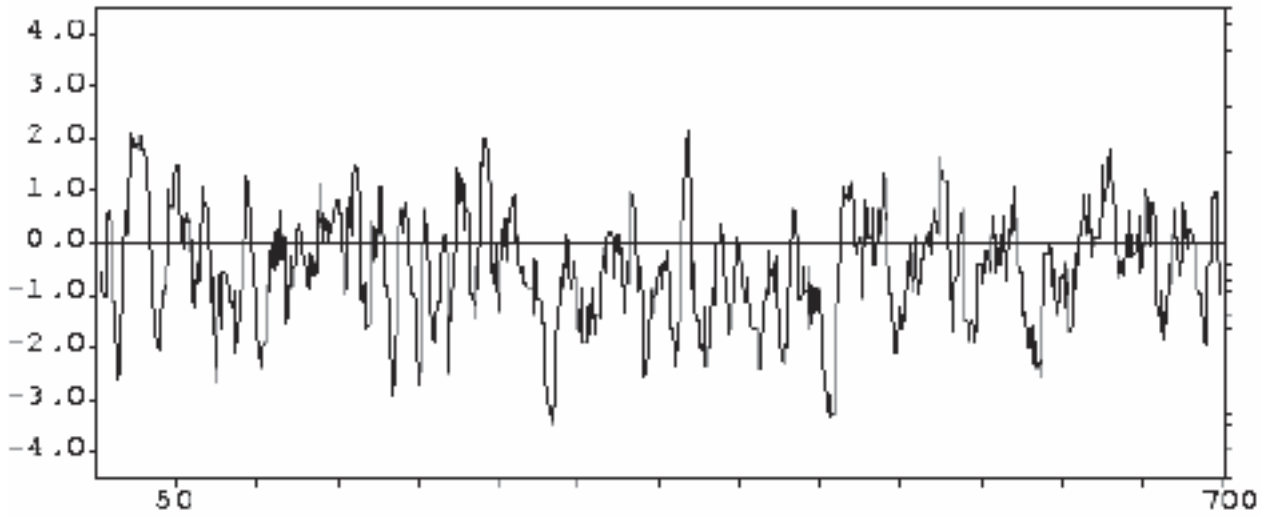

Figure 2. Hydropathy plot of the predicted polypeptide of *nsr1*, obtained using the Kyte-Doolittle algorithm and hydropathy values (Kyte and Doolittle, 1982). Alternating regions of hydrophilicity and hydrophobicity are observed.

anti-parallel β-barrel and a globular domain that forms surface loops and folds down into the β-barrel—two features thought to be common to all ferric-siderophore receptors (Stintzi et al. 2000). The interior of the β-barrel represents the site where iron is removed from iron-loaded siderophores, and the N-terminal domain predominantly consists of surface loops that appear to be hydrophilic in nature and that may facilitate binding of hydrophilic ferric-siderophores to the receptor. *A. hydrophila* has been shown to acquire iron via a ligand exchange model (Stintzi et al. 2000), in which a siderophore is initially bound to the receptor as a siderophore-receptor complex. When a second, iron-loaded siderophore is in close proximity of the receptor, iron is likely removed from the second siderophore via a pH gradient within the barrel of the receptor (Stintzi et al. 2000). This results in a protonation/deprotonation reaction, and the iron is donated to the initial siderophore which translocates from the membrane to the periplasmic space. The second siderophore then binds to the receptor, replacing the initial siderophore. In the predicted structure of the *nsr1* gene product, the interior of the protein is enclosed by β-sheets and is likely to be a suitable environment for local acidification. The hydrophilic regions probably extend into the extracellular space to interact with ferric-siderophores, as is seen with many bacterial siderophore uptake proteins such as FepA, the enterobactin receptor expressed by *E. coli* (Newton et al. 1997).

Bacteria frequently possess feedback systems that upregulate or downregulate the expression of certain iron uptake proteins, including the energy transducing protein TonB, in response to environmental iron levels (Beddek et al. 2004). However, expression of the *fhuA* gene has been shown to be unaffected by iron conditions (Mikael et al. 2003). Based upon the similarities between the protein products of *nsr1* and *fhuA*, it is possible that expression of *nsr1* may also be unaffected in the presence or absence of iron. In *A. hydrophila*, and in other bacteria, iron-dependent regulation of iron uptake occurs at the level of siderophore biosynthesis rather than at the level of siderophore receptor production (Stintzi et al. 2000; Venturi et al. 1995). Therefore it appears to be advantageous for *A. hydrophila* to express *nsr1* constitutively to maintain a constant means of iron acquisition. Further studies are warranted to clarify the iron-dependent (and potentially iron-independent) mechanisms by which *nsr1* is regulated.

The presence of a unique *nsr1*-driven siderophore system has implications for the development of drugs to control *A. hydrophila*. Since the utilization of specific siderophore systems are often confined to certain bacteria, and because siderophores often play a critical role in bacterial growth, survival and virulence, targeting siderophore-mediated iron uptake is an attractive approach to antibacterial drug development. Siderophore-antibiotic conjugates, called sideromycins, have been shown to be highly effective at entering bacteria by exploiting natural

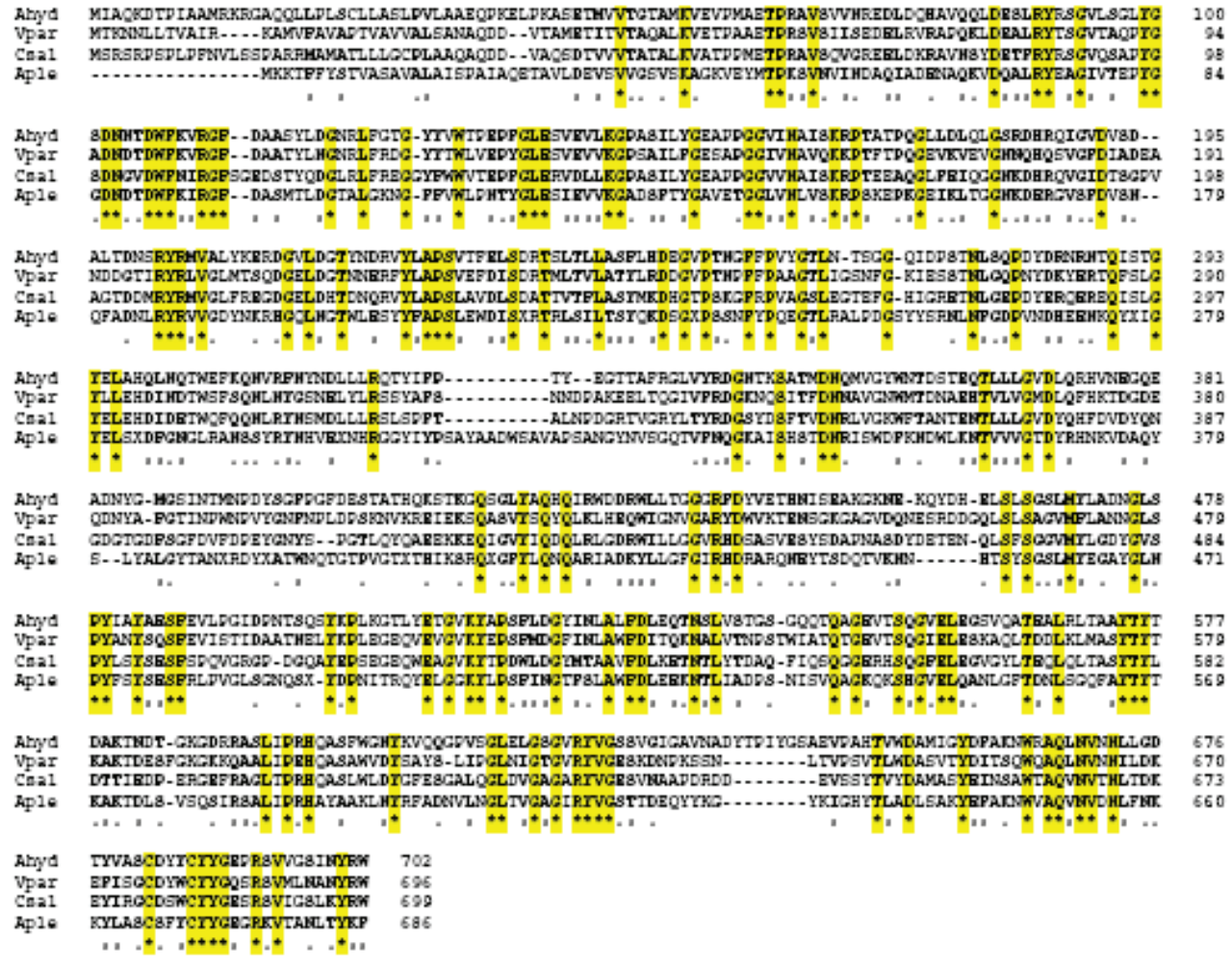

Figure 3. Amino acid sequence alignment of the putative *A. hydrophila* siderophore receptor (here denoted Ahyd) with known ferric-siderophore receptors from various bacteria: Vpar, *Vibrio parahaemolyticus* ferrichrome receptor FhuA (Genbank BAD06905); Csal, *Chromohalobacter salexigens* TonB-dependent siderophore receptor (Genbank YP_573101); and Aple, *Actinobacillus pleuropneumoniae* ferric hydroxamate receptor FhuA (Genbank DQ249800). Single fully conserved residues (*) are highlighted, and strong conserved groups (:) and weak conserved groups (.) are indicated.

siderophore uptake mechanisms to traverse bacterial cell membranes (for a review, see Miethke and Marahiel, 2007). This could provide a method for specific and efficient drug targeting to *A. hydrophila*. On the other hand, drugs that inhibit expression of *nsr1* or interfere with siderophore pathways involving *nsr1* could conceivably suppress bacterial multiplication by blocking iron metabolism. These and other strategies may provide a basis for therapeutic intervention for the control of *A. hydrophila* infection.

Conclusions

The iron uptake strategy of *A. hydrophila* has previously been described as a ligand exchange mechanism utilizing a single nonspecific siderophore receptor (Stintzi et al. 2000). Here we report the identification of a novel gene from *A. hydrophila* potentially encoding the abovementioned ligand exchange siderophore receptor. The putative protein product of *nsr1* bears high amino acid sequence identity and similarity to several known siderophore receptors. In addition, the predicted secondary structure of the protein features two distinct functional domains that are thought to be common to all ferric-siderophore receptors (Stintzi et al. 2000). Thus *nsr1* likely encodes a receptor protein involved in siderophore-mediated iron transport. As *A. hydrophila* has been shown to exclusively utilize a single ferric-siderophore receptor for iron transport, the gene product of *nsr1* is an excellent candidate for the receptor. Further investigation and molecular characterization of the protein product of *nsr1* may

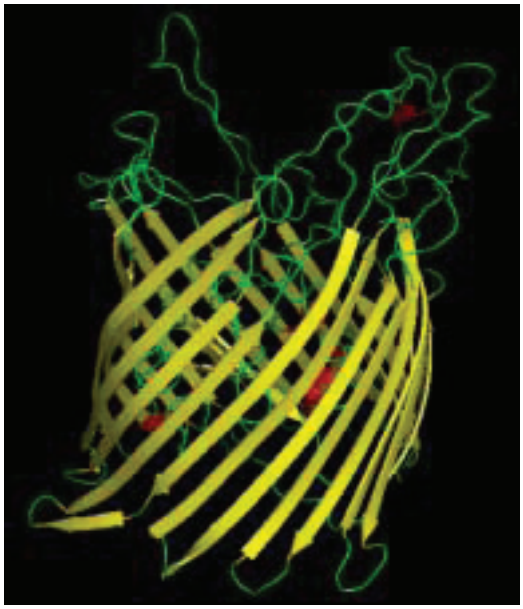

Figure 4. Predicted crystal structure of the gene product of *nsr1* (Bates et al. 2001; Bates and Sternberg, 1999; Contreras-Moreira and Bates, 2002) (3D-JIGSAW Protein Comparative Modeling Server, UK). The structure bears striking similarity to all other currently known ferric siderophore receptors. The putative protein is comprised of an antiparallel β-barrel enclosing the iron removal site within the receptor, as well as an N-terminal globular domain that forms surface loops and folds down into the interior of the β-barrel. Protein is colored by secondary structure (red helices, yellow sheets, green loops).

contribute to the elucidation of the iron uptake mechanism of *A. hydrophila* and may lead to the development of new antibiotics targeting either *nsr1* or its product for use in controlling *A. hydrophila* infection.

Acknowledgements

The author would like to thank Nicholas Fitzkee for his important contributions to the implementation of the computer programs used in this study and for his helpful and informative discussions.

References

Agger, W.A., McCormick, J.D. and Gurwith, M.J. 1985. Clinical and microbiological features of *Aeromonas hydrophila*-associated diarrhea. *J. Clin. Microbiol.*, 21:909–13.

Apostol, M., Baret, P., Serratrice, G., Desbrières, J., Putaux, J.L., Stèbè, M.J., Expert, D. and Pierre, J.L. 2005. Self-assembly of an amphiphilic iron(III) chelator: mimicking iron acquisition in marine bacteria. *Angew. Chem.*, 117:2636–8.

Bates, P.A., Kelley, L.A., MacCallum, R.M. and Sternberg, M.J.E. 2001. Enhancement of protein modeling by human intervention in applying the automatic programs 3D-JIGSAW and 3DPSSM. *Proteins: Structure, Function and Genetics*, (Suppl 5):39–46.

Bates, P.A. and Sternberg, M.J.E. 1999. Model building by comparison at CASP3: using expert knowledge and computer automation. *Proteins: Structure, Function and Genetics*, (Suppl 3):47–54.

Beddek, A.J., Sheehan, B.J., Bossé, J.T., Rycroft, A.N., Kroll, J.S. and Langford, P.R. 2004. Two TonB systems in *Actinobacillus pleuropneumoniae*: their roles in iron acquisition and virulence. *Infect. Immun.*, 72:701–8.

Contreras-Moreira, B. and Bates, P.A. 2002. Domain fishing: a first step in protein comparative modeling. *Bioinformatics*, 18:1141–2.

Coulton, J.W., Mason, P., Cameron, D.R., Carmel, G., Jean, R. and Rode, H.N. 1986. Protein fusions of β-galactosidase to the ferrichrome-iron receptor of *Escherichia coli*. *J. Bacteriol.*, 165:181–92.

Janda, J.M. and Abbott, S.L. 1998. Evolving concepts regarding the genus Aeromonas: an expanding Panorama of species, disease presentations, and unanswered questions. *Clin. Infect. Dis.*, 2:332–44.

Kyte, J. and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.*, 157:105–32.

Miethke, M. and Marahiel, M.A. 2007. Siderophore-based iron acquisition and pathogen control. *Microbiol. Mol. Biol. Rev.*, 71:413–51.

Mikael, L.G., Srikumar, R., Coulton, J.W. and Jacques, M. 2003. *fhuA* of *Actinobacillus pleuropneumoniae* encodes a ferrichrome receptor but is not regulated by iron. *Infect. Immun.*, 71:2911–5.

Newton, S.M.C., Allen, J.S., Cao, Z., Qi, Z., Jian, X., Sprencel, C., Igo, J.D., Foster, S.B., Payne, M.A. and Klebba, P.E. 1997. Double mutagenesis of a positive charge cluster in the ligand binding site of the ferric enterobactin receptor, FepA. *Proc. Natl. Acad. Sci.*, 94:4560–5.

Payne, S.M. and Finkelstein, R.A. 1978. The critical role of iron in host-bacterial interactions. *J. Clin. Invest.*, 61:1428–40.

Stintzi, A., Barnes, C., Xu, J. and Raymond, K.N. 2000. Microbial iron transport via a siderophore shuttle: a membrane ion transport paradigm. *Proc. Natl. Acad. Sci.*, 97:10691–6.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, 22:4673–80.

Venturi, V., Ottevanger, C., Bracke, M. and Weisbeek, P. 1995. Iron regulation of siderophore biosynthesis and transport in *Pseudomonas putida* WCS358: involvement of a transcriptional activator and of the Fur protein. *Mol. Microbiol.*, 15:1081–93.

Wooldridge, K.G. and Williams, P.H. 1993. Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiol. Rev.*, 12:325.