Iron acquisition by *Actinobacillus suis*

by

Fariborz Bahrami

Microbiology Unit
Department of Natural Resource Sciences
Macdonald Campus, McGill University
Montréal, Québec, Canada

A thesis submitted to
McGill University
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

June 2005

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-21617-0
Our file Notre référence
ISBN: 978-0-494-21617-0

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

"There are no facts, only interpretations."

--**Friedrich Nietzsche**, from *Daybreak*

Abstract

Ph.D. **Fariborz Bahrami** **Microbiology**

Iron acquisition by *Actinobacillus suis*

Seven strains of *Actinobacillus suis* (ATCC 15557, B49, C84, H89-1173, H91-0380, SO4 and VSB 3714) were investigated with respect to iron acquisition from animal transferrins (Tfs) and haemoglobins (Hbs). Growth assays with porcine, bovine and human Tfs and Hbs revealed that all seven strains could use porcine (but not human or bovine) Tf and all three Hbs as iron sources. In solid phase binding assays, membranes derived from all strains exhibited strong binding of porcine Tf and each of the Hbs. Competition binding assays indicated that all three Hbs were bound by the same receptor(s). Affinity procedures allowed the isolation and identification of iron repressible Tf-binding (~100 kDa and ~63 kDa) and Hb-binding (~105 kDa) polypeptides from all strains. Nucleotide sequence analyses revealed that *A. suis* strains SO4 and C84 possess genes that encode homologues of the *Actinobacillus pleuropneumoniae* Tf-binding proteins, TbpA and TbpB, and Hb-binding protein, HgbA. In both strains, *tbpB* was located immediately upstream of *tbpA* and was shown to be preceded by *tonB*, *exbB* and *exbD* homologues; *hgbA* was shown to be preceded by a *hugZ* homologue. Putative promoter and Fur box sequences were located upstream of *tonB* and *hugZ* and RT-PCR revealed that the genes in each of these clusters (*tonB-exbB-exbD-tbpB-tbpA*; *hugZ-hgbA*) are co-transcribed and iron-repressible. The molecular masses of the predicted mature

TbpA, TbpB and HgbA proteins were calculated to be 104.3, 63.4 and 105.0 kDa, respectively, suggesting that the affinity-isolated, ~100 kDa and ~63 kDa Tf-binding polypeptides represent TbpA and TbpB, respectively, and that the ~105 kDa Hb-binding polypeptide represents HgbA. TbpB of *A. suis* was expressed in *Escherichia coli* and the recombinant TbpB (rTbpB) was identified by immunoblotting using swine sera raised against recombinant TbpB (*A. pleuropneumoniae*). It is envisaged that the acquisition of Tf- and Hb-bound iron by *A. suis* involves mechanisms analogous to those found in other members of the *Pasteurellaceae* and in particular, in *A. pleuropneumoniae*.

Résumé

Ph.D. **Fariborz Bahrami** **Microbiologie**

Acquisition du fer par *Actinobacillus suis*

Sept souches d'*Actinobacillus suis* (ATCC 15557, B49, C84, H89-1173, H91-0380, SO4 et VSB 3714) ont été examinées pour leur capacité à acquérir le fer de transferrines (Tfs) et d'hémoglobines (Hbs) d'origines animales. Les analyses de croissance avec les Tfs et les Hbs porcines, bovines et humaines ont démontré que les sept souches pouvaient utiliser la Tf porcine (mais pas les Tfs humaine et bovine) ainsi que les trois Hbs comme sources de fer. Dans des essais de liaison en phase solide, les membranes dérivées de toutes les souches ont exhibé des liaisons robustes à la Tf d'origine porcine et à chacune des Hbs. Des essais de liaison compétitive ont indiqué que les trois Hbs sont liées par le(s) même(s) récepteur(s). Des techniques d'affinité ont permis l'isolation et l'identification des polypeptides répressibles par le fer pouvant lier la Tf (~100 kDa et ~63 kDa) et l'Hb (~105 kDa) à partir de toutes les souches. Des analyses de séquençage nucléotidique ont montré que les souches SO4 et C84 d'*A. suis* possèdent des gènes homologues à *Actinobacillus pleuropneumoniae* qui codent pour des protéines pouvant lier la Tf, TbpA et TbpB, et l'Hb, HgbA. Il a été démontré que chez ces deux souches, *tbpB* est situé immédiatement en amont de *tbpA* et qu'il est précédé par des homologues de *tonB*, *exbB* et *exbD*; et que *hgbA* est précédé par un homologue de *hugZ*. De putatives séquences de promoteurs et boîtes Fur ont été situées en amont de *tonB* et de

hugZ et des RT-PCR ont démontré que les gènes présents dans chacun des groupes (*tonB-exbB-exbD-tbpB-tbpA*; *hugZ-hgbA*) sont co-transcrits et répressibles par le fer. Les poids moléculaires des protéines matures TbpA, TbpB et HgbA, déduites à partir des séquences des gènes *tbpA*, *tbpB* et *hgbA*, sont respectivement, 104.3, 63.4 et 105.0 kDa, suggérant que les polypeptides isolés par affinité, pouvant lier la Tf (~100 kDa et ~63 kDa) représentent TbpA et TbpB, respectivement, et le polypeptide pouvant lier l'Hb (~105 kDa) représente HgbA. TbpB d'*A. suis* a été exprimé dans *Escherichia coli* et le TbpB recombinant (rTbpB) a été identifié par immunobuvardage en utilisant des sérums porcins contre rTbpB (*A. pleuropneumoniae*). Ceci laisse envisager que l'acquisition du fer, liée à la Tf et à l'Hb par *A. suis* implique des mécanismes analogues à ceux retrouvés chez les autres membres de la famille des *Pasteurellaceae*, particulièrement chez *A. pleuropneumoniae*.

Contributions to knowledge

1. *A. suis* (seven strains) was shown to use porcine (but not human or bovine) Tf as an iron source for growth.

2. *A. suis* (seven strains) was shown to use human, bovine and porcine Hbs as iron sources for growth.

3. Acquisition of Tf-bound iron by *A. suis* (seven strains) was shown to involve two-component (TbpA/TbpB) iron-repressible receptors that are specific for porcine Tf.

4. Acquisition of Hb-bound iron by *A. suis* (seven strains) was shown to involve single-component (HgbA) iron-repressible receptors that can bind human, bovine and porcine Hbs.

5. Genes that encode TbpA (*tbpA*, 2808 bp), TbpB (*tbpB*, 1791 bp) and a TonB system (*tonB*, 741 bp; *exbB*, 669 bp; *exbD*, 411 bp) in two representative strains of *A. suis* (strains SO4 and C84) were identified and sequenced and putative promoter sequences and a putative Fur box were identified upstream of both *tonB* genes.

6. Genes that encode HgbA (*hgbA*, 2841 bp) and HugZ (*hugZ*, 522 bp) in two representative strains of *A. suis* (strains SO4 and C84) were identified and sequenced and putative promoter sequences and a putative Fur box were identified upstream of both *hugZ* genes.

7. Genes that encode a second TonB system (*exbB2*, 453 bp; *exbD2*, 390 bp; *tonB2*, 846 bp) in two representative strains of *A. suis* (strains SO4 and C84) were identified and sequenced.

8. The genes that comprise the *A. suis tonB-tbpA* cluster (*tonB-exbB-exbD-tbpB-tbpA*) were shown to be co-transcribed and iron-repressible.

9. The genes that comprise the *A. suis hugZ-hgbA* cluster were shown to be co-transcribed and iron-repressible.

10. It is suggested that the acquisition of Tf- and Hb-bound iron by *A. suis* involves receptor-mediated siderophore-independent mechanisms analogous to those found in other members of the *Pasteurellaceae*, and in particular, in *A. pleuropneumoniae.*

Acknowledgements

I would like to thank Andy Ekins, my former fellow graduate student, for showing me the ropes during the first two years of my studies and training in the laboratory. I am grateful to Dr. Brian T. Driscoll, my supervisory committee member and professor, for his constructive comments and his generous offerings of time and research equipment. I am thankful to Ms. Marlene Parkinson, Ms. Joanne Ten Eyck, Ms. Ann Gossage and especially, Ms. Marie Kubecki, for their administrative support throughout my postgraduate studies in the Department of Natural Resource Sciences.

I wish to extend my sincere gratitude to my supervisor, Dr. Donald F. Niven, for giving me an invaluable opportunity to gain knowledge and experience in his laboratory. His enthusiasm, guidance and meticulous approach in scientific methodology and communication were driving forces behind the compilation of this body of work. Our discussion topics, from bioenergetics and microbial pathogenesis, to Laphroaig and Bunnahabhain, will be remembered with pleasure.

This work was supported by the Canadian Research Network on Bacterial Pathogens of Swine (funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) and industrial partners) and by an NSERC Discovery Grant.

Table of contents

	Page
Abstract	i
Résumé	iii
Contributions to knowledge	v
Acknowledgements	vii
Table of contents	viii
List of abbreviations	xii
List of figures	xiii
List of tables	xvi
Contributions of authors	xvii
Chapter 1. Literature review	1
1.1 Introduction	1
1.2 *Actinobacillus suis*	1
1.3 Iron	6
1.3.1 The multifaceted roles of iron	6
1.3.2 Iron and redox stress	7
1.3.3 Sources of iron within the mammalian host	9
1.3.3.1 Indirect iron sources	9
1.3.3.1.1 Siderophores	9
1.3.3.1.2 Extracellular and membrane-bound ferric reductases	10
1.3.3.2 Indirect haem sources	11
1.3.3.2.1 Haemophores	11
1.3.3.3 Direct iron sources	13
1.3.3.3.1 Ferrous iron	13
1.3.3.3.2 Transferrins	14
1.3.3.3.3 Ferritins	16
1.3.3.4 Direct haem sources	16
1.3.3.4.1 Haem	16
1.3.3.4.2 Haemoglobin	17
1.3.3.4.3 Haemoglobin-haptoglobin	18
1.3.3.4.4 Haem-haemopexin	18

1.3.3.4.5 Albumin 19
1.3.3.4.6 Other haemoproteins 20

1.4 Iron acquisition by bacterial pathogens 20
1.4.1 TonB-dependent receptors 20
1.4.2 Siderophore-dependent iron acquisition 24
1.4.3 Contact-dependent iron acquisition 26
1.4.3.1 Transferrin- and lactoferrin-binding proteins 26
1.4.3.2 Haem- and haemoprotein-binding proteins 29
1.4.3.3 Removal and internalization of iron by TonB-dependent receptors 32

1.5 Regulation of iron uptake 32
1.5.1 Ferric uptake regulation protein 32
1.5.2 Recognition and binding of DNA by Fur 34

1.6 Transferrin and haemoglobin receptors as vaccine components 37

1.7 Aims of the research project 38

Chapter 2. Identification and characterization of transferrin receptor proteins and encoding genes 40

2.1 Abstract 40

2.2 Introduction 41

2.3 Materials and methods 43
2.3.1 Organisms 43
2.3.2 Transferrins and conjugates 43
2.3.3 Growth conditions and preparation of membranes 44
2.3.4 Transferrin-binding assays 45
2.3.5 Isolation and identification of transferrin-binding polypeptides 45
2.3.6 Amplification and sequencing of receptor and affiliated genes 46
2.3.7 Preparation of RNA and RT-PCR 51
2.3.8 Imaging 52

2.4 Results 53
2.4.1 Iron acquisition from transferrins 53
2.4.2 Binding of transferrins 53
2.4.3 Isolation and identification of transferrin-binding polypeptides 53
2.4.4 Identification and transcription of *tbpA*, *tbpB*, *exbB*, *exbD* and *tonB* homologues 69
2.4.5 Identification of *exbB2*, *exbD2* and *tonB2* homologues 73
2.4.6 Comparative analysis of the predicted TbpA and TbpB proteins 73

2.5 Discussion 77

Connecting statement 1 81

Chapter 3. Identification and characterization of a single-component haemoglobin receptor and encoding gene 82

3.1 Abstract 82

3.2 Introduction 83

3.3 Materials and methods 85
3.3.1 Organisms and storage conditions 85
3.3.2 Haemoglobins and conjugates 85
3.3.3 Growth conditions and preparation of membranes 85
3.3.4 Solid phase binding assays 86
3.3.5 Isolation and identification of haemoglobin-binding polypeptides 87
3.3.6 Amplification and sequencing of receptor and affiliated genes 88
3.3.7 RT-PCR 90
3.3.8 Imaging 91

3.4 Results 91
3.4.1 Acquisition of haemoglobin-bound iron and binding of haemoglobins by *A. suis* 91
3.4.2 Isolation and identification of haemoglobin-binding polypeptides 106
3.4.3 Identification and transcription of *hugZ* and *hgbA* homologues 106

3.5 Discussion 111

Connecting statement 2 118

Chapter 4. Cloning and expression of TbpB and HgbA 119

4.1 Abstract 119

4.2 Introduction 119

4.3 Materials and methods 121
4.3.1 Organisms and sera 121
4.3.2 Cloning of polynucleotides encoding TbpB and HgbA 121
4.3.3 Expression and purification of recombinant TbpB and HgbA 123
4.3.4 Immunoblotting of recombinant TbpB 124
4.3.5 Alignment 125
4.3.6 Imaging 125

4.4 Results 126
4.4.1 Cloning and expression of TbpB 126
4.4.2 Recognition of rTbpB (*A. suis*) by anti-rTbpB (*A. pleuropneumoniae*) 126
4.4.3 Cloning and expression of HgbA 130

4.5 Discussion 130

Chapter 5. Conclusions and general discussion 136

References 150

Appendix 1. GenBank entries 170
Appendix 1.1 *A. suis* strain SO4 *tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* genes, complete cds. GenBank accession number AY101604 170
Appendix 1.2 *A. suis* strain C84 *tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* genes, complete cds. GenBank accession number AY101605 173
Appendix 1.3 *A. suis* strain SO4 *exbB2*, *exbD2* and *tonB2* genes, complete cds. GenBank accession number AY838763 176
Appendix 1.4 *A. suis* strain C84 *exbB2*, *exbD2* and *tonB2* genes, complete cds. GenBank accession number AY838764 177
Appendix 1.5 *A. suis* strain SO4 *hugZ* and *hgbA* genes, complete cds. GenBank accession number AY335901 178
Appendix 1.6 *A. suis* strain C84 *hugZ* and *hgbA* genes, complete cds. GenBank accession number AY335902 180

Appendix 2. Biohazards certificate 182

Appendix 3. Copyright waivers 185

List of abbreviations

EDDA	Ethylenediamine di-*o*-hydroxyphenylacetic acid
Hb	Haemoglobin
His_6	Hexahistidine
HRP	Horseradish peroxidase
IPTG	Isopropyl-β-D-thiogalactopyranoside
Lf	Lactoferrin
LPS	Lipopolysaccharide
OM	Outer membranes
rHgbA	Recombinant haemoglobin-binding protein A
rTbpB	Recombinant transferrin-binding protein B
RT-PCR	Reverse transcription-PCR
SOD	Superoxide dismutase
Tbp	Transferrin-binding protein
TBS	Tris-buffered saline
Tf	Transferrin
TM	Total membranes
TTBS	TBS containing Tween 20
TY	Tryptone-yeast extract medium
TY/amp	TY containing 100 μg/ml ampicillin
TYE-H	HEPES-buffered tryptone-yeast extract medium

List of figures

Figure		Page
1.1	Acquisition of transferrin-bound iron and haem by *Pasteurellaceae*	33
1.2	Models for Fur-DNA interactions	36
2.1	Utilization of transferrin-bound iron by *A.* suis strain ATCC 15557	54
2.2	Utilization of transferrin-bound iron by *A.* suis strain B49	55
2.3	Utilization of transferrin-bound iron by *A.* suis strain C84	56
2.4	Utilization of transferrin-bound iron by *A.* suis strain H89-1173	57
2.5	Utilization of transferrin-bound iron by *A.* suis strain H91-0380	58
2.6	Utilization of transferrin-bound iron by *A.* suis strain SO4	59
2.7	Utilization of transferrin-bound iron by *A.* suis strain VSB 3714	60
2.8	Binding of transferrins by seven strains of *A. suis*	61
2.9	Identification of transferrin-binding polypeptides from *A. suis* strain ATCC 15557	62
2.10	Identification of transferrin-binding polypeptides from *A. suis* strain B49	63
2.11	Identification of transferrin-binding polypeptides from *A. suis* strain C84	64
2.12	Identification of transferrin-binding polypeptides from *A. suis* strain H89-1173	65
2.13	Identification of transferrin-binding polypeptides from *A. suis* strain H91-0380	66
2.14	Identification of transferrin-binding polypeptides from *A. suis* strain SO4	67
2.15	Identification of transferrin-binding polypeptides from *A. suis* strain VSB 3714	68

2.16 Recognition of the ~75-kDa polypeptide by streptavidin-HRP 70

2.17 Organization of *tbpA*, *tbpB*, *exbB*, *exbD* and *tonB* in *A. suis* strains SO4 and C84. 71

2.18 Amplification of *tonB-tbpA* gene clusters 72

2.19 RT-PCR with RNA isolated from *A. suis* strain C84 74

2.20 RT-PCR with RNA isolated from *A. suis* strain SO4 75

2.21 Amplification of *exbB2-tonB2* gene clusters 76

3.1 Utilization of haemoglobin-bound iron by *A.* suis strain ATCC 15557 92

3.2 Utilization of haemoglobin-bound iron by *A.* suis strain B49 93

3.3 Utilization of haemoglobin-bound iron by *A.* suis strain C84 94

3.4 Utilization of haemoglobin-bound iron by *A.* suis strain H89-1173 95

3.5 Utilization of haemoglobin-bound iron by *A.* suis strain H91-0380 96

3.6 Utilization of haemoglobin-bound iron by *A.* suis strain SO4 97

3.7 Utilization of haemoglobin-bound iron by *A.* suis strain VSB 3714 98

3.8 Binding of haemoglobins by *A. suis* strain ATCC 15557 99

3.9 Binding of haemoglobins by *A. suis* strain B49 100

3.10 Binding of haemoglobins by *A. suis* strain C84 101

3.11 Binding of haemoglobins by *A. suis* strain H89-1173 102

3.12 Binding of haemoglobins by *A. suis* strain H91-0380 103

3.13 Binding of haemoglobins by *A. suis* strain SO4 104

3.14 Binding of haemoglobins by *A. suis* strain VSB 3714 105

3.15 Identification of haemoglobin-binding polypeptides from *A. suis* strain SO4 107

3.16 Identification of Hb-binding polypeptides from six strains of *A. suis* 108

3.17 Organization of *hugZ* and *hgbA* in *A. suis* strains SO4 and C84 109

3.18 Amplification of *hugZ-hgbA* gene clusters 110

3.19 Transcription of *hugZ* and *hgbA* in *A. suis* strains C84 and SO4 112

3.20 Comparison of the 3'-end of *hugZ* and the *hugZ-hgbA* intergenic region in *A. suis* with the corresponding regions in *A. pleuropneumoniae* 116

4.1 Effect of IPTG on the expression of rTbpB 127

4.2 SDS-PAGE and immunoblotting of rTbpB 128

4.3 SDS-PAGE and immunoblotting of rTbpB and affinity-isolated polypeptides from *A. suis* strain SO4 129

4.4 Expression of rHgbA 131

4.5 Alignment of predicted TbpB precursors of *A. suis* and *A. pleuropneumoniae* 132

5.1 Dendrogram depicting amino acid sequence relatedness between the TbpA precursor of *A. suis* strain SO4 and representative TbpA precursors of other members of the *Pasteurellaceae* 139

5.2 Dendrogram depicting amino acid sequence relatedness between the TbpB precursor of *A. suis* strain SO4 and TbpB precursors of representative strains of *A. pleuropneumoniae* 141

5.3 Dendrogram depicting amino acid sequence relatedness between the TbpB precursor of *A. suis* strain SO4 and representative TbpB precursors of other members of the *Pasteurellaceae* 143

5.4 Dendrogram depicting amino acid sequence relatedness between the HgbA precursor of *A. suis* strain SO4 and representative HgbA precursors of other members of the *Pasteurellaceae* 144

5.5 Topological model of *A. suis* TbpA 145

5.6 Topological model of *A. suis* HgbA 147

List of tables

Table **Page**

2.1 Primers used in PCR and RT-PCR 47

3.1 Primers used in PCR and RT-PCR 89

4.1 Primers used in the construction of polynucleotides encoding rTbpB and rHgbA 122

5.1 Comparison of *A. pleuropneumoniae* and *A. suis* TbpB precursors 140

5.2 Putative extracellular loop sequences of *A. suis* TbpA 146

5.3 Putative extracellular loop sequences of *A. suis* strain SO4 HgbA 148

Contributions of authors

All of the experiments described in this Thesis were performed by myself, under the supervision of Dr. D.F. Niven. Dr. A. Ekins provided technical advice during some of the experiments described in Chapter 2. Manuscripts for publication were originally written by myself and subsequently, such manuscripts were edited and revised based on the comments of Dr. Niven.

Chapter 1. Literature review

1.1 Introduction

Actinobacillus suis is an important emerging pathogen of swine. With few exceptions, pathogenic bacteria must be able to obtain iron from host tissues in order to sustain their growth. Since iron is not readily available in animal hosts, pathogens have had to develop high-affinity mechanisms for its acquisition. In the last two decades, detailed investigations of iron acquisition processes, in conjunction with technological advances in areas such as molecular biology, bioinformatics and X-ray crystallography, have provided a wealth of information on bacterial iron uptake mechanisms. The results of such investigations, performed, initially, with Gram-negative bacteria such as *Escherichia coli*, have paved the way for a better understanding of homologous mechanisms in a variety of other microorganisms. The purpose of this chapter is to review the latest information available on *A. suis* and bacterial iron acquisition mechanisms. Special emphasis will be placed on the major components involved in iron acquisition by Gram-negative bacteria and in particular, by pathogenic members of the families *Pasteurellaceae* and *Neisseriaceae*.

1.2 *Actinobacillus suis*

A. suis, a member of the family *Pasteurellaceae*, is a Gram-negative, nonmotile, facultatively anaerobic bacterium. It is a coccobacillus, 0.5-3 μm long and about 0.8 μm in diameter, that forms greyish, adherent, circular, translucent colonies measuring 1-2 mm on blood agar within 24 h (Taylor, 1999). The generic name, *Actinobacillus*, was introduced in 1910 and by 1984, five species, including *A. suis*, were listed as members of the genus (Phillips, 1984). The number of species is now 18; however, based on molecular methods such as DNA hybridization and comparison of the 16S rRNA and translation initiation factor 2 (*infB*) genes, only a few, including *Actinobacillus arthritidis, Actinobacillus equuli, Actinobacillus hominis, Actinobacillus lignieresii, Actinobacillus pleuropneumoniae, A. suis* and *Actinobacillus ureae* are considered, with confidence, to belong to *Actinobacillus senso stricto* (Nørskov-Lauritsen *et al.*, 2004).

Members of the genus *Actinobacillus*, like other members of the *Pasteurellaceae*, are associated primarily with the mucosal membranes of the respiratory and genital tracts. Among them, only *A. pleuropneumoniae*, *A. suis* and *A. equuli* are frequently associated with serious diseases in animals, and only *A. pleuropneumoniae* is recognized as a primary pathogen. Based on phenotypic characteristics, *A. suis* can be confused with *A. equuli* and this is probably the reason for the reported isolation of *A. suis* from horses. *A. suis* can be differentiated from *A. equuli* by its ability to ferment (+)-L-arabinose and genuine *A. suis* has always been isolated from diseased pigs (Christensen and Bisgaard, 2004).

Early studies based on biotyping, restriction endonuclease fingerprinting and slide agglutination, suggested that the *A. suis* population structure was highly homogeneous. However, based on immunoblotting with crude lipopolysaccharide (LPS) preparations, it has been demonstrated that at least three different LPS O-chains (O1, O2 and O3) and two different cell surface capsular polysaccharides (K1 and K2) can be distinguished in representative strains of *A. suis* (Monteiro *et al.*, 2000). Immunoblot analyses of the O-antigen from a large number of *A. suis* isolates from both healthy and diseased pigs have revealed that serotype O2 strains are more likely to be associated with severe diseases than O1 strains (Slavić *et al.*, 2000*a*). A study using a total of 114 clinical, bacterin and slaughterhouse isolates of *A. suis* strains from various locations throughout Canada (Alberta, British Columbia, Ontario, Québec and Saskatchewan) and Kansas in the United States, indicated that O1/K1 and O2/K3 serotypes are the most prevalent among healthy and diseased swine. This study also revealed that swine antisera raised against *A. suis* cell surface antigens could recognize antigens of several other important pathogens including *A. pleuropneumoniae* sertoypes 1, 5 and 7, *Pasteurella multocida* and *Haemophilus parasuis*, with the strengths of the cross-reactions being highest with *A. pleuropneumoniae* and lowest with *H. parasuis*. Such broad cross-reactivities might account for the lack of success, to date, in development of a simple serodiagnostic test for *A. suis* (Slavić *et al.*, 2000*b*).

A. suis is likely to be present in the tonsils, nostrils, and oropharyngeal microbiota of healthy pigs of any age and in the vaginas of apparently healthy sows (Ross *et al.*, 1972; Taylor, 1999). The organism may change from a commensal to an opportunistic pathogen in response to an unknown signal(s). Infection probably begins with the inhalation of an aerosol containing the organisms. This may then be followed by the invasion of the upper respiratory tract mucosa. Infected emboli can spread from the mucosa into the tissues haematogenously where they adhere to the endothelia of the blood vessels or become trapped in smaller blood vessels (Rycroft and Garside, 2000). The pathology inflicted by the organism is often the result of septicaemia and gross lesions include haemorrhage and necrosis of the internal organs and/or skin. *A. suis* infection has also been associated with a wider range of clinical symptoms in older animals, including dyspnoea, cough, lameness, fever, weakness, wasting, abscesses, neurological illnesses, abortion, cyanosis and diffuse hyperaemia, indicating the ability of the organism to survive in a very wide range of environments within the host (Miniats *et al.*, 1989;

Sanford *et al.*, 1990; Yaeger, 1995). *A. suis* is also counted as a causative agent of pleuropneumonia-like respiratory diseases in older pigs (Taylor, 1999).

In the late 1980s, *A. suis* was often reported as a causative agent of an acute and rapidly fatal septicaemic disease in piglets in Europe and North America (Sanford, 1987). Later, *A. suis* infections were reported in grower/finisher pigs which were previously assumed to be resistant to this pathogen (Sanford *et al.*, 1990; Yaeger, 1996). In the 1990s, the increased use of modern swine management practices, such as segregated weaning, closed breeding and minimal disease herds, coincided with an escalation in the number of *A. suis* infection outbreaks in North America causing significant morbidity and mortality in pigs of all ages (Miniats *et al.*, 1989; MacInnes and Desrosiers, 1999; Rycroft and Garside, 2000). In the late 1990s, *A. suis* was isolated for the first time in Australia where it was associated with significant disease among preweaned pigs in high health status facilities. Interestingly, investigations revealed that the disease incidents in Australia, a country with highly restrictive animal import regulations, were not due to extraneous strains of *A. suis* but rather, were the result of virulence expression by previously undetected populations of *A. suis* among Australian pigs (Wilson and McOrist, 2000). Although modern swine production methods have helped in the eradication of traditional diseases such as enzootic pneumonia and swine dysentery, they have also inadvertently contributed to a population of young animals with decreased maternal antibodies to organisms acquired either from their mothers or from their environment. Such piglets are believed to be more susceptible to early colonizers such as *A. suis* (Wilson and McOrist, 2000). Furthermore, it would appear that in high health status facilities, older animals also suffer from deficiencies in specific and cross-reactive immunity. Such compromised immune systems, often manifested by lower levels of innate defences such as complement proteins, allow *A. suis* to express its full pathogenic potential (MacInnes and Desrosiers, 1999; Taylor, 1999).

The virulence factors of *A. suis* are not well defined; however, the few that have been identified have counterparts in *A. pleuropneumoniae*, the causative agent of swine pleuropneumonia, and other members of the *Pasteurellaceae*. In this context, homologues of the RTX genes and genes involved in capsule biosynthesis in *A. pleuropneumoniae* have been identified in *A. suis* (Burrows and Lo, 1992; Van Ostaaijen *et al.*, 1997). Furthermore, an antiserum prepared against *A. pleuropneumoniae* RTX (repeats in the structural toxins) proteins has been shown to detect cytotoxic proteins similar to ApxI and ApxII (*A. pleuropneumoniae* RTX toxins I and II, respectively) in *A. suis* (Burrows and Lo, 1992; Kamp *et al.*, 1994); immunoblocking of Apx toxins has revealed that *A. suis* does not produce other cytolytic compounds (Kamp *et al.*, 1994).

While vaccines for the prevention of *A. suis* disease do not appear to be available commercially, autogenous bacterins, suspensions of killed or attenuated bacteria, have been used successfully for disease control in herds that have experienced repeated outbreaks (Taylor, 1999). Notably, *A. suis* and *A. pleuropneumoniae* possess antigenically-related outer membrane proteins (MacInnes and Rosendal, 1987); while common antigens make serodiagnosis difficult, they could, potentially, prove useful for the formulation of improved vaccines against both pathogens. Due to the complex nature of swine pleuropneumonia and the existence of 15 different serotypes (Blackall *et al.*, 2002), attempts to formulate an efficient vaccine against *A. pleuropneumoniae* have so far proven to be challenging. Most commercial vaccines against *A. pleuropneumoniae* consist of heat-killed or formalin-treated whole cells. Although such vaccines can reduce mortality, they neither prevent initial infection nor provide cross-protection against all *A.*

pleuropneumoniae serotypes (Haesebrouck *et al.*, 2004). In contrast, when used in vaccine trials, subunit vaccines containing Apx toxins have been found to provide some cross-protection (Haesebrouck *et al.*, 1997) and vaccines containing Apx toxins and transferrin-binding proteins (Tbps) have been shown to confer enhanced protection in pigs against challenges with *A. pleuropneumoniae* (Van Overbeke *et al.*, 2001). It would appear, however, that the extent to which such vaccines can confer protection against *A. suis* remains to be established.

1.3 Iron

1.3.1 The multifaceted roles of iron

Iron is a transition metal and under physiological conditions exists mainly in two readily interchangeable redox states, namely, the reduced Fe^{2+} (ferrous) form and the oxidized Fe^{3+} (ferric) form. Exhibiting a wide range of redox potentials (–300 to +700 mV), depending on local environment and associated ligands, iron serves as a cofactor in many enzymes and electron-transfer proteins (Braun *et al.*, 1998). Iron is involved in a wide variety of biological activities including photosynthesis, methanogenesis, H_2 production and consumption, N_2-fixation, respiration, oxygen transport, gene regulation and DNA biosynthesis (Andrews *et al.*, 2003). Moreover, in multicellular organisms, iron is an essential component in oxygen transport proteins, such as haemoglobin (Hb), that are responsible for maintaining oxygen levels in the tissues.

Most known bacteria require iron ($\sim 10^{-7}$ M) for colonization and growth (Griffiths, 1987). However, a few organisms are known to be independent of iron. The lactobacilli were the first iron-independent organisms identified (Archibald, 1983). This independence of iron presumably explains the ability of the lactobacilli to sustain growth in iron-restricted environments such as milk (see section 1.3.3.3.2) (Weinberg, 1997; Andrews *et al.*, 2003). *Streptococcus suis*, a pathogen of swine and humans, seems to require little, if any iron (Niven *et al.*, 1999). Based on complete genome analyses, *Treponema pallidum*, the causative agent of syphilis, and *Borrelia burgdorferi*, the causative agent of Lyme disease, lack identifiable genes for iron-uptake related proteins and have no requirement for iron (Posey *et al.*, 1999; Posey and Gherardini, 2000). However, it is noteworthy that both of these organisms, *T. pallidum* and *B. burgdorferi*, have small genomes and are obligate intracellular parasites that rely on the iron-dependent metabolic processes of their hosts for their *in vivo* growth (Andrews *et al.*, 2003).

Despite its abundance in nature, the low concentration of free ferric iron in solution (~10^{-9} M; Chipperfield and Ratledge, 2000), under physiological conditions, makes it an extremely scarce commodity for bacteria, including pathogens. In addition, animals restrict the availability of iron (~10^{-18} M; Griffiths, 1987) in their tissues and secretions by transporting it through high-affinity iron-binding proteins. This primary line of defence against infection is known as the "iron blockade" (Rouault, 2004). Aside from restricting the availability of free iron to pathogenic bacteria, iron sequestration by specific proteins serves a second purpose by protecting the host against the toxicity of free iron (Kaplan, 2002; Hentze *et al.*, 2004).

1.3.2 Iron and redox stress

Bacteria need iron for their growth; however, they also need to be protected from iron toxicity caused by unrestricted interaction of iron with reactive oxygen species. Such reactive oxygen species originate from partially reduced derivatives of molecular oxygen that are produced as a natural consequence of aerobic metabolism (Fridovich, 1995). The mildly reactive one- and two-electron-reduction products, known as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), respectively, interact with free iron to generate highly reactive and extremely damaging hydroxyl radicals ($HO^\bullet$), as summarized below (Andrews *et al.*, 2003).

Iron reduction, $O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$ (1)

Fenton reaction, $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^\bullet$ (2)

Haber-Weiss reaction, (1) + (2):

$$O_2^- + H_2O_2 \xrightarrow{\text{Fe catalysis}} HO^\bullet + OH^- + O_2 \qquad (3)$$

Although the concentration of superoxide anion *in vivo* is too low (~10^{-10} M) to promote iron reduction (Eq. 1), it is sufficient to damage the exposed [4Fe-4S] clusters of members of the dehydratase-lyase family of enzymes including aconitase and α, β-dihydroxyacid dehydratase. This can cause the release of iron which in turn can take part in Fenton reactions and the production of hydroxyl radicals (Eq. 2). Hydroxyl radicals are capable of inflicting grave damage on biological molecules such as proteins, lipids and DNA (Keyer *et al.*, 1995; Schaible and Kaufmann, 2004). The enzyme superoxide dismutase (SOD) is present in almost all organisms and catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen. The hydrogen peroxide is subsequently eliminated by catalases and/or peroxidases (Fridovich, 1997). *E. coli* can produce a manganese SOD (MnSOD) and an iron SOD (FeSOD), encoded by *sodA* and *sodB*, respectively. Mutations in *sodA* and *sodB* give rise to organisms with high intracellular concentrations of superoxide anion and levels of DNA damage that can not be accounted for by the action of superoxide *per se* (Touati, 2000). It has been suggested that during iron-induced redox stress, flavins may be the *in vivo* mediators of iron reduction (Woodmansee and Imlay, 2002).

Many findings emphasize the importance of iron in redox stress. For instance, bacteria exhibit increased sensitivity toward redox stress following growth under iron-rich conditions (Repine *et al.*, 1981; Abdul-Tehrani *et al.*, 1999). Moreover, inactivation of the *fur* gene, which leads to deregulation of iron homeostasis (see section 1.5.1), increases sensitivity to redox stress. This phenomenon can be reversed by iron chelation, by blocking iron uptake through a *tonB* mutation (see section 1.4.1), or by increasing iron storage capacity through over-expression of the ferritin gene (*ftnA*) (see section 1.3.3.3.3) (Touati *et al.*, 1995; Touati, 2000). In effect, it is crucial for bacteria to regulate their uptake of iron (see section 1.5) in order to avoid lethal damage due to iron overload and the consequent redox stress.

1.3.3 Sources of iron within the mammalian host

1.3.3.1 Indirect iron sources

1.3.3.1.1 Siderophores

The production and secretion of low molecular mass (< 1 kDa), non-protein compounds, known as siderophores, are well-characterized mechanisms to circumvent iron deficiency by many but not all microorganisms. Siderophores chelate ferric iron with such a high affinity ($K_{\text{aff}} > 10^{30}$; Byers and Arceneaux, 1998) that they can extract it from most chemical and organic complexes, including ferritin, transferrin (Tf) and lactoferrin (Lf), but notably, not from haem (Ratledge and Dover, 2000), merely by equilibrium displacement. More than 500 different siderophores are known to be produced by bacteria but their basic structures are similar in that most consist of a peptide backbone that contains several non-protein amino acid analogues (Wandersman and Delepelaire, 2004). Being highly electronegative, siderophores bind ferric iron to form hexadentate octahedral complexes. Based on their iron-chelating groups, siderophores have been tentatively classified into three main chemical types, namely, hydroxamates (e.g. aerobactin and ferrichrome), α-hydroxycarboxylates (e.g. pyochelin) and catechols (e.g. enterobactin). *A. pleuropneumoniae* and *A. suis* are not known to produce endogenous siderophores but have been shown to be capable of using exogenous ferrichrome (Mikael *et al.*, 2003). Similar to antibiotic synthesis, siderophores are assembled by nonribosomal cytoplasmic peptide synthetases. The genes that encode such biosynthetic enzymes are iron-regulated and are often located alongside genes that are involved in siderophore uptake (Wandersman and Delepelaire, 2004).

1.3.3.1.2 Extracellular and membrane-bound ferric reductases

An important step in the incorporation of iron into enzymes and storage proteins in bacteria is reduction of ferric iron to ferrous iron either in the cytoplasm or on the inner side of the cytoplasmic membrane. Extracytoplasmic reductases that have the potential to provide the cell with easily assimilable ferrous iron have been reported for Gram-positive and Gram-negative bacteria including obligate and opportunistic intracellular pathogens such as *Listeria monocytogenes*, *E. coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* (Schroder *et al.*, 2003). However, none of these extracellular reductases has been cloned or purified although an extracellular ferric reductase has been recovered from the culture supernatant of *Mycobacterium paratuberculosis* (Hohmuth *et al.*, 1998).

Endogenous electron shuttles such as NAD(P), FAD and FMN are neither membrane permeable nor known to be secreted by any cell. Hence, it has been hypothesized that extracellular ferric reductases may only be physiologically functional if electron shuttles are provided to the bacteria, a situation that may occur in pathogenic bacteria internalized by their hosts (Schroder *et al.*, 2003). Aside from exogenous electron donors, soluble quinones, which can diffuse freely through the cell envelope, can render extracellular ferric reductases functional. A quinol compound which can reduce insoluble ferric oxides without any direct contact has been shown to be present in *Shewanella oneidensis* (formerly *Shewanella putrefaciens*) and to enable the organism to respire on iron as terminal electron acceptor (Newman and Kolter, 2000). The fate of the resulting ferrous iron, whether it is used as an iron source by *S. oneidensis* or by a symbiotic organism, is yet to be elucidated (Wandersman and Delepelaire, 2004). Similarly, the iron-reducing bacterium, *Geothrix fermentans*, is known to be able to reduce ferric oxides, embedded in inaccessible alginate beads, by secreting diffusible electron shuttling compounds. Thin-layer chromatographic analysis of culture filtrates has revealed that these diffusible electron-shuttling compounds have characteristics similar to those of water-soluble quinones (Nevin and Lovley, 2002).

1.3.3.2 Indirect haem sources

1.3.3.2.1 Haemophores

Haemophores are specialized extracellular proteins secreted by several Gram-negative bacteria. They can acquire haem from a variety of sources and shuttle it to a specific outer membrane receptor for internalization. The unloaded haemophores are then recycled. The best characterized haemophore is HasA which is known to be produced by *Serratia marcescens*, *P. aeruginosa*, *Pseudomonas fluorescens*, *Yersinia pestis*, and *Yersinia enterocolitica* (Wandersman and Delepelaire, 2004). HasA-type haemophores belong to a unique family of highly conserved proteins. The crystal structure of *S. marcescens* HasA indicates a globular protein with a fold and two faces (four α helices on one face and seven β-strands on the other). The haem-binding site, which consists of two loops, is located at the interface between the faces (Arnoux *et al.*, 1999) and does not appear to change conformation on haem binding (Genco and Dixon, 2001). HasA (19-kDa monomer) binds free haem with a stoichiometry of 1:1 and with high affinity (K_d of 10^{-11}M). HasA can also capture haem from a variety of haemoproteins including Hb, haemopexin, myoglobin, leghaemoglobin and haem-loaded albumin (Wandersman and Delepelaire, 2004). Interestingly, HasA does not appear to form stable complexes with such proteins. Rather, HasA would appear to remove the haem directly from the protein. Indeed, in solutions prepared with equimolar amounts of HasA and Hb, the haem is to be found largely in association with HasA (Létoffé *et al.*, 1999).

In most organisms, the genes that encode the proteins involved in haemophore-mediated iron acquisition are located on an operon and negatively regulated by Fur (see section 1.5) (Ghigo *et al.*, 1997). The *has* operon is also known to be positively regulated by a signalling system involving a bacterial extracytoplasmic function sigma factor, HasI, the activity of which is usually inhibited by its membrane-bound antisigma factor, HasS. Binding of haem-loaded haemophore to the outer membrane receptor, HasR, inactivates HasS and HasI can then participate in the transcription of the *has* operon. It would appear, therefore, that the operon is both activated and repressed by the availability of external haem. However, it is noted that positive regulation by the sigma and antisigma factors are more in line with fine-tuning by the external signal (Rossi *et al.*, 2003; Biville *et al.*, 2004).

Another haemophore system, the haem-haemopexin utilization or HxuA system, has been identified in *Haemophilus influenzae. H. influenzae* lacks the haem biosynthetic pathway and requires exogenous haem for aerobic growth. The genes coding for the HxuA-type haemophore system are found in a *hxuA-hxuB-hxuC* cluster encoding HxuA, HxuB and HxuC, respectively (Cope *et al.*, 1995). HxuA is a 100-kDa extracellular protein secreted by a signal peptide-dependent pathway that requires HxuB, a β-barrel, pore-forming accessory protein (Cope *et al.*, 1994). The HxuA-haem-haemopexin complex binds to the outer membrane receptor, HxuC. *H. influenzae* with mutated *hxuC* is incapable of haem-haemopexin utilization suggesting that HxuC is the only receptor for the HxuA-hemopexin complex (Cope *et al.*, 1995; Wandersman and Delepelaire, 2004). Emphasizing the uniqueness of HxuC receptors in *H. influenzae*, organisms with dysfunctional Hb-binding receptors, due to mutations in the *hgbA*, *hgbB* and *hgbC* genes, were shown to be dependent on HxuC for the uptake of free haem and haem bound to Hb (Cope *et al.*, 2001).

1.3.3.3 Direct iron sources

1.3.3.3.1 Ferrous iron

In aerobic environments at neutral pH, iron exists largely as Fe^{3+}. Under such conditions, ferric iron is insoluble and is not readily available to bacteria. Under anaerobic or reducing conditions, the highly soluble ferrous iron is the major form of iron and this form of iron can diffuse freely through the outer membrane porins of Gram-negative bacteria (Wandersman and Delepelaire, 2004). The ferrous iron is subsequently transported across the cytoplasmic membrane by an ATP-binding cassette (ABC) transporter which is conserved in many bacteria (Kammler *et al.*, 1993). An example of an environmental niche where ferrous iron is available is the mammalian gastrointestinal system. *Salmonella enterica* and *Helicobacter pylori* colonize the mammalian intestine and stomach, respectively, and obtain iron by means of ferrous iron utilization, or Feo, systems (Velayudhan *et al.*, 2000; Boyer *et al.*, 2002). A prototype of the Feo system was identified initially in *E. coli* where the iron-regulated *feoAB* genes encode a small (75 amino acids) protein, FeoA, of unknown function, and a putative membrane-bound ATPase, FeoB (Kammler *et al.*, 1993).

1.3.3.3.2 Transferrins

The Tf family of glycoproteins includes serum Tf, Lf, which is also known as lactotransferrin, and ovotransferrin. Tf and Lf have been investigated extensively in the context of iron acquisition by bacteria. Both are monomeric glycoproteins of ~ 80 kDa (670-690 amino acid residues) and exhibit 50-70% pair-wise sequence identity indicative of a common evolutionary progenitor. The amino acid sequences of the molecules contain a two-fold internal repeat, giving rise to two homologous lobes, the N- and C-lobes, with ~ 40% sequence identity, connected by a short hinge region. "Duplication" of the lobes is presumed to be the result of ancestral gene duplication events around 850 million years ago (Lambert *et al.*, 2005). Tf and Lf are similar in a number of functional properties. For instance, both can bind two ferric ions (one ion per lobe) very tightly but reversibly, in coordination with two carbonate ions, in a synergistic relationship. The C-lobe of Tf binds iron more tightly than the N-lobe and releases it more slowly (Aisen, 1998). The high affinities of Tf and Lf for iron (K_b 10^{22} M) are crucial for maintaining the concentration of iron below the precipitation point (10^{-18} M) of ferric hydroxides. The high affinities of Tf and Lf for iron also give these molecules bacteriostatic properties, as they significantly limit the availability of iron for bacterial growth in the tissues. Also, by sequestering iron in the tissues, Tf and Lf can inhibit the production of harmful free radicals (Baker *et al.*, 2002).

The differences in Tf and Lf can be summarized as follows. Tf transports iron primarily in the bloodstream and its concentration in serum is ~25 μM. Tf is only ~30% saturated with iron and hence, apo-Tf (without iron bound) is typically in excess of holo-Tf (with iron bound) (Cornelissen, 2003). The iron carried by Tf is transferred to mammalian cells via specific Tf receptors and receptor-mediated endocytosis (Octave *et al.*, 1983). In contrast, Lf is localized in exocrine secretions such as milk and mucosal secretions and in the secondary granules of neutrophils. Moreover, despite its iron-binding properties, Lf has not yet been clearly associated with iron delivery mechanisms (Baker *et al.*, 2002). Although specific Lf receptors have been identified on small intestinal mucosal surfaces in mammalian species such as humans, rhesus monkeys, pigs and mice, scientific experiments and clinical trials have yielded conflicting results in that Lf has been credited with the enhancement as well as the inhibition of iron delivery to intestinal cells (Ward and Conneely, 2004). The iron-withholding properties of Lf and Tf are also different in that Lf can retain iron at pH values as low as pH 3.5 whereas Tf begins losing iron at pH 6 (Mazurier and Spik, 1980). Finally, being a highly cationic molecule with a pI of ~9 and having high concentrations of positive charges on its surface, Lf is more capable of binding anionic molecules, such as DNA and heparin, than is Tf (van Berkel *et al.*, 1997).

1.3.3.3.3 Ferritins

Ferritins are cytoplasmic proteins that store iron. They provide iron to cells during periods of iron shortage and by sequestering iron, they can also protect cells from its subsequent toxic effects. Ferritins constitute a broad superfamily of shell-like, spherical (diameter ~ 120 Å) proteins; each ferritin molecule contains 24 subunits arranged to form a hollow sphere that can accommodate several thousand ferric ions (Carrondo, 2003). Although exogenous ferritin can be reduced by *L. monocytogenes* (Deneer *et al.*, 1995), there is no clear evidence that ferritins can be used as iron sources by bacteria (Wandersman and Delepelaire, 2004).

In *E. coli*, ferritin is the product of the *ftnA* gene and fulfils the classical iron storage role that was originally suggested for eukaryotic ferritins (Theil, 1987). Homologues of *E. coli* ferritin, providing protection against iron-mediated redox stress, have been identified in *Campylobacter jejuni* (Wai *et al.*, 1996), *H. pylori* (Waidner *et al.*, 2002) and *Neisseria gonorrhoeae* (Chen and Morse, 1999) and also in Gram-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis* (Morrissey *et al.*, 2004).

1.3.3.4 Direct haem sources

1.3.3.4.1 Haem

Haem and haemin are the reduced (ferrous) and oxidized (ferric) forms of iron protoporphyrin IX, respectively, and are major reservoirs of iron in mammals. Despite the difference in haem and haemin, the term haemin is used widely to indicate iron protoporphyrin IX in either oxidation state (Genco and Dixon, 2001). Haem acts as a prosthetic group in many enzymes; it can also function as a source of protoporphyrin for organisms such as *Enterococcus faecalis*, *Lactococcus lactis* and *H. influenzae* which are unable to synthesize the tetrapyrrol ring (Wandersman and Delepelaire, 2004). Haem is often associated with serum proteins such as haemopexin and albumin and is also known to bind to lipoproteins such as low-density and high-density lipoproteins (Camejo *et al.*, 1998; Miller and Shaklai, 1999).

1.3.3.4.2 Haemoglobin

Haemoglobin, the oxygen-transporting protein in red blood cells, has the potential to be a major source of haem for pathogenic bacteria. Hb is a tetrameric globular protein consisting of two α-subunits and two β-subunits (Wandersman and Delepelaire, 2004). The three-dimensional structure of Hb was solved by Perutz and colleagues (1960) using X-ray analysis. Each Hb subunit binds one molecule of haem. The iron in haem is coordinated to four nitrogen atoms in the porphyrin ring and in Hb, the iron is further coordinated to the imidazole of a histidine residue. Reflecting the oxidation state of the iron and the presence or absence of a sixth ligand to the haem, Hb is found in three major forms, oxy-Hb, deoxy-Hb and met-Hb. Oxy-Hb and deoxy-Hb contain ferrous iron and while oxygen serves as the sixth ligand in oxy-Hb, there is no sixth ligand in deoxy-Hb. In met-Hb, the iron is in the 3+ state and there is no binding of oxygen (Genco and Dixon, 2001).

The surface area of haem has been calculated to be ~800 $Å^2$. However, when embedded in Hb, the haem surface area exposed to solvent has been estimated to be ~120

Å^2 and ~170 Å^2 for the α and the β subunits, respectively (Stellwagen, 1978; Genco and Dixon 2001). Considering the limited exposure of haem within Hb, it has been suggested that binding of Hb by a receptor must rely to a great extent on the receptor recognizing the protein *per se* (Genco and Dixon, 2001) and in particular, conserved residues in the globin chains that surround the haem crevice (Lee, 1995). Receptors that can recognize both haem and Hb, however, remain problematic in that the determinants involved in the recognition of each or both of these ligands have yet to be identified (Genco and Dixon, 2001).

1.3.3.4.3 Haemoglobin-haptoglobin

Haptoglobin is a tetrameric glycoprotein; in the blood stream, it binds Hb, released as a result of haemolysis, with high affinity. The affinity of haptoglobin for Hb is so high that the complex remains intact until its degradation in the liver. Several bacteria, including *H. influenzae*, *N. gonorrhoeae* and *Neisseria meningitidis*, can use Hb-haptoglobin complexes as iron sources for growth (Stojiljkovic *et al.*, 1996; Lewis *et al.*, 1998; Morton *et al.*, 1999). In these organisms, the Hb-haptoglobin complexes are bound by receptors that can also bind free Hb (Ren *et al.*, 1998; Jin *et al.*, 1999; Rohde *et al.*, 2002)

1.3.3.4.4 Haem-Haemopexin

Free haem is potentially harmful to mammalian cells as it participates in the production of damaging oxygen species and it is also a potential source of iron for microbial pathogens. Protection against free haem is provided by haemopexin, an ~60-kDa glycoprotein (Muller-Eberhard, 1988). The crystal structure of haemopexin is solved and has revealed two similarly folded domains that tether haem by means of two histidine residues in a pocket dominated by aromatic and basic groups (Paoli *et al.*, 1999). The serum haemopexin level in humans is ~10^{-5} M. Haemopexin binds haem to yield an equimolar complex and exhibits an affinity for haem that is greater than that exhibited by Hb and much greater than that exhibited by albumin. Haem is released from haem-haemopexin complexes within hepatocytes and the apo-haemopexin (without haem) is then recycled (Genco and Dixon, 2001; Wandersman and Delepelaire, 2004). To date, the only bacterium known to use haem-haemopexin as a source of iron is *H. influenzae* (Cope *et al.*, 1998). Haem from haemopexin is captured by *H. influenzae* haemophores, as described in section 1.3.3.2.1.

1.3.3.4.5 Albumin

In humans, albumin, an ~68-kDa globular protein, is the most abundant plasma protein; it constitutes ~60% of the total serum protein content and can function as a haem carrier. The crystal structure of albumin has revealed three major domains and the binding of haem to one of these is characterized by a binding constant of 10^8 M^{-1} (Dockal *et al.*, 1999; Genco and Dixon, 2001). Although albumin can provide haem for bacterial growth under laboratory conditions, to date no bacterial albumin receptor has been reported. Since the affinity of albumin for haem is considered to be within the same range as that of bacterial surface receptors for haem, it is speculated that following the binding of haem-albumin by a haem/Hb receptor, the transfer of the haem to the receptor occurs passively (Wandersman and Delepelaire, 2004).

1.3.3.4.6 Other haemoproteins

Other haemoproteins found in nature, such as myoglobin, catalase and cytochrome *b*, can provide haem for bacterial growth under laboratory conditions; however, the existence of receptors specific for such haemoproteins has yet to be established. The lack of specific receptors tends to suggest that haem is the molecule that is recognized by the bacterial iron acquisition systems. However, some of these haemoproteins, such as myoglobin, bind haem with such high affinity that haem transfer from the haemoprotein to a bacterial haem receptor, merely by equilibrium displacement, can be ruled out (Wandersman and Delepelaire, 2004). Hence, it is speculated that haem is either stripped from the haemoprotein at the bacterial surface, by some unknown mechanism, or is released on degradation of the haemoprotein by bacterial extracellular proteases resembling, perhaps, the lysine- and arginine-specific cysteine proteinases, the gingipains, that are secreted by *Porphyromonas gingivalis* (O'Brien-Simpson *et al.*, 2003; Wandersman and Delepelaire, 2004). Indeed, extracellular proteases are known to be produced by many bacteria when they are grown under iron-restricted conditions (Wandersman and Delepelaire, 2004).

1.4 Iron acquisition by bacterial pathogens

1.4.1 TonB-dependent receptors

The outer membranes of Gram-negative bacteria function as selectively permeable barriers in that they restrict the passage of molecules with molecular masses greater than ~600 Da. Iron-and haem-containing proteins that are bound at the cell surface are unable to cross these membranes and the release of the iron and haem from these proteins and/or their transfer to the periplasm requires energy. This energy comes from the proton motive force that is generated across the cytoplasmic membranes of the organisms. Energy transduction involves a set of three proteins, TonB, ExbB and ExbD, referred to collectively as the TonB protein complex or, in short, the TonB system (Postle, 1993; Braun, 1995). The nomenclature of these proteins reflects the experimentation that eventually led to their association with energy transduction mechanisms involving the outer membrane of *E. coli*. TonB stands for T-one resistance and refers to the phenotype of a mutant strain of *E. coli* that was resistant to phage T1. In *tonB* mutants, the binding of phage T1 occurred normally but the energy-dependent uptake of the phage nucleic acid did not. Further investigations led to the sequencing of *tonB* (Postle and Good, 1983) and it was confirmed that the uptake of all ferric siderophores, vitamin B_{12} and group B colicins is absolutely dependent on TonB activity (Braun, 1995). Exb stands for excretion of colicin B inhibitor of the Ton complex and *exb* mutants of *E. coli* are resistant to colicin. Following the cloning and sequencing of the *exb* locus, two genes, namely *exbB* and *exbD*, were identified and shown to complement *exb* mutants (Eick-Helmerich and Braun, 1989).

TonB protein complexes have been shown to be involved in the active uptake of vitamin B_{12} (Buchanan *et al.*, 1999; Fanucci *et al.*, 2002) and in the active uptake of iron from siderophores (Nicholson and Beall, 1999), mammalian iron-carrier proteins (Jarosik *et al.*, 1995; Biswas *et al.*, 1997; Stojiljkovic and Srinivasan, 1997; Braun and Braun, 2002), and haem (Jarosik *et al.*, 1994; Torres and Payne, 1997). Many outer membrane receptors that interact with a TonB system contain a short conserved sequence, the "TonB box" (Pressler *et al.*, 1988), near the N-terminus. The TonB box consensus in *E. coli* is D/ETXXVXA, where X represents a hydrophobic amino acid (Fanucci *et al.*, 2003;

Peacock *et al.*, 2005). A C-terminal domain in TonB is involved in the binding of TonB to the TonB box (Peacock *et al.*, 2005). Since TonB-dependent transport is tolerant of mutations in the TonB box, it is believed that the interaction between the TonB protein and TonB box is guided by structural recognition rather than side-chain interactions.

The energy transducing protein, TonB, exists as a dimer and is anchored in the periplasm by the integral membrane proteins, ExbB and ExbD (Chang *et al.*, 2001). Cross-linking studies have shown that TonB, ExbB and ExbD form complexes *in vivo*, with stoichiometries of approximately 1:7:2 (Higgs *et al.*, 2002). TonB spans the periplasmic space and physically contacts outer membrane receptors (Chang *et al.*, 2001). ExbD is largely periplasmic with the N-terminal region embedded in the cytoplasmic membrane (Kampfenkel and Braun, 1992). ExbB, on the other hand, is believed to be located largely in the cytoplasm (Kampfenkel and Braun, 1993); a membrane-spanning segment crosses the cytoplasmic membrane three times and the N-terminus and a short loop are located in the periplasm (Saier, 2003). ExbB and ExbD are encoded by the *exbBD* operon and a mutation in either gene results in the same phenotype, that is, an ~90% loss in the energy transducing activity of the TonB system (Bradbeer, 1993; Skare *et al.*, 1993; Ahmer *et al.*, 1995; Braun *et al.*, 1996). It is believed that receptor-ligand interactions trigger a series of conformational changes in the TonB complex leading to ExbB/ExbD-mediated transfer of energy from the cytoplasmic membrane to TonB and the transfer of the substrate to the periplasm for eventual transport to the cytoplasm (Larsen *et al.*, 1997; Moeck *et al.*, 1997).

With the increasing volume of information on bacterial genes, it is becoming apparent that some bacteria, including *Vibrio cholerae* (Occhino *et al.*, 1998; Seliger *et al.*, 2001), *H. pylori* (Alm, *et al.*, 1999), *C. jejuni* (Parkhill *et al.*, 2000) and *P. aeruginosa*

(Zhao and Poole, 2000) have more than one TonB system. The advantage of having more than one TonB system, however, has not been clearly established (Beddek *et al.*, 2003). In *A. pleuropneumoniae*, the genes encoding the TonB system (*tonB*, *exbB* and *exbD*) are located upstream of the Tf receptor genes, *tbpA* and *tbpB*, and all five genes were shown to be co-transcribed and iron-regulated (Tonpitak *et al.*, 2000). Later, the genes encoding a second TonB system (*exbB2-exbD2-tonB2*), the TonB2 system, with no proximity to any gene related to iron uptake or metabolism, were identified in *A. pleuropneumoniae* and like the genes in the *tonB* cluster, these genes were shown to be co-transcribed and iron-regulated (Beddek *et al.*, 2003). Recently, the genes in the *tonB* and *tonB2* clusters of *Vibrio anguillarum* have also been shown to be regulated by iron (Stork *et al.*, 2004). Interestingly, the order of the genes in the *A. pleuropneumoniae tonB* gene cluster (*tonB-exbB-exbD*) resembles that found in non-*Pasteurellaceae* bacteria such as *N. meningitidis* (Stojiljkovic and Perkins-Balding, 2002) and *P. aeruginosa* (Zhao and Poole, 2000) while the order of the genes in the *tonB2* cluster (*exbB2-exbD2-tonB2*), and also the deduced amino acid sequence of TonB2, are more similar to those found in other members of the *Pasteurellaceae* such as *Haemophilus ducreyi* (Elkins *et al.*, 1998), *Mannheimia haemolytica* (Graham and Lo, 2002) and *H. influenzae* (GenBank accession number NC000907). These differences, and phylogenetic analyses, suggest that the TonB2 system is the system that is indigenous to *A. pleuropneumoniae* and that the TonB system, also known, now, as the TonB1 system (ExbB1, ExbD1 and TonB1; Beddek *et al.*, 2003), may have been acquired by means of horizontal gene transfer. While the TonB system may have been acquired by means of horizontal gene transfer, the presence of this system in all *A. pleuropneumoniae* serotypes suggests that such a transfer did not occur recently (Beddek *et al.*, 2003).

While studying the TonB system of *A. pleuropneumoniae*, Tonpitak and colleagues (2000) demonstrated that an in-frame deletion within the *exbB* gene negated the ability of the organism to use porcine Tf as an iron source indicating that ExbB is essential for iron uptake from Tf. Later, it was shown that the TonB2 system in *A. pleuropneumoniae* is also essential for the uptake of Tf-bound iron and it was suggested that elements of both *tonB* loci are essential for the acquisition of iron from porcine Tf (Beddek *et al.*, 2003). To what extent ExbB and TonB2 must function together and/or TonB and TonB2 must form the TonB-dimer that spans the periplasm are still matters for conjecture (Beddek *et al.*, 2003). Interestingly, the *A. pleuropneumoniae tonB2* locus has also been shown to be absolutely required for the acquisition of iron from haemin, Hb and ferrichrome, and TonB2 has been shown to be required for virulence (Beddek *et al.*, 2003). Other organisms for which virulence and the possession of *tonB* operons appear to be associated include *V. cholerae* (Seliger *et al.*, 2001), *Shigella dysenteriae* (Reeves *et al.*, 2000) and *Bordetella pertussis* (Pradel *et al.*, 2000).

1.4.2 Siderophore-dependent iron acquisition

Once synthesized, siderophores are secreted to the extracellular milieu by means of mechanisms that are not well-understood. Furrer and colleagues (2002), however, have recently added insights to this process in that they have identified a gene, within the enterobactin biosynthetic gene cluster of *E. coli*, that encodes a 43-kDa membrane protein that is highly homologous to members of the major superfamily of proton motive force-dependent efflux pumps. Once secreted, siderophores bind ferric iron and can remove iron from iron-binding proteins such as Tf and Lf. The resulting ferric siderophores are then bound by specific receptors on the cell envelope and are actively internalized by means of TonB-dependent mechanisms. Release of the iron from the ferric siderophores is mediated by ferric reductases that are located in the cytoplasm or on the cytoplasmic surface of the inner membrane; following the release of the iron, the unloaded siderophores are recycled (Ratledge and Dover, 2000).

The TonB-dependent receptors, FecA, FhuA and FepA, mediate the uptake of ferric citrate and the siderophores, ferrichrome and enterobactin, respectively, by *E. coli.* Resolution of the three-dimensional atomic structures of these receptors has revealed that they are structurally similar to porin proteins (Ferguson *et al.*, 1998; Locher *et al.*, 1998; Buchanan *et al.*, 1999; Ferguson *et al.*, 2002). Typically, these receptors/transport proteins form elliptical 22-β-sheet barrels (35 Å × 45 Å); the β-sheets are of variable length and can protrude from the membrane surface. On the periplasmic faces of these proteins, the β-barrels are connected by short stretches of amino acids and on the extracellular faces, long stretches of amino acids form extracellular loops. The first ~160 residues of the N-terminal domains of this class of proteins have a unique conformation. Instead of contributing to the β-barrel, the amino acids that make up the N-terminal domain fold to yield a globular domain, known as the "plug" or "cork" domain, that is held in place by numerous hydrogen bonds and polar contacts with the barrel interior. The plug or cork is assumed to completely occlude the inner orifice of the β-barrel and together with a cluster of extracellular loops, participates in the formation of a binding pocket to which ligands such as siderophores bind with high affinity (Ratledge and Dover 2000; Andrews *et al.*, 2003).

1.4.3 Contact-dependent iron acquisition

1.4.3.1 Transferrin- and lactoferrin-binding proteins

Bacteriostasis imposed by mammalian iron-carrying molecules is not complete in that many bacteria have evolved in such a way that they can use these same molecules as sources of iron. For example, pathogenic members of the *Neisseriaceae* and *Pasteurellaceae* are capable of binding specific mammalian Tfs and of using these proteins as iron sources for growth. While the pathogenic *Neisseriaceae* can also use Lf as source of iron, the *Pasteurellaceae* do not (Gray-Owen and Schryvers, 1996). Acquisition of Tf-bound iron involves bipartite receptor complexes composed of two outer membrane proteins referred to as Tf-binding proteins A and B (TbpA and TbpB, respectively; Gray-Owen and Schryvers, 1996; Cornelissen, 2003) or a single-component receptor (Ogunnariwo and Schryvers, 2001; Ekins and Niven, 2002) referred to now as TbpA2 (Ekins *et al.*, 2004*a*). Outer membrane receptors are also involved in the acquisition of Lf-bound iron but to date, all Lf receptors that have been identified have been shown to consist of two components, Lf-binding proteins A and B (LbpA and LbpB, respectively; Gray-Owen and Schryvers, 1996; Ekins *et al.*, 2004*b*). While TbpA and LbpA proteins have molecular masses of ~100 kDa, TbpB and LbpB proteins, typically, have molecular masses of ~65 kDa (Gray-Owen and Schryvers, 1996); the TbpA2 proteins that have been identified, have molecular masses of ~83 kDa (Ogunnariwo and Schryvers, 2001; Ekins and Niven, 2002; Ekins *et al.*, 2004*a*). While the Tf receptors of most organisms are reported to bind a single species of Tf, the receptor proteins of *M. haemolytica*, *P. multocida* and *Histophilus somni* are capable of binding ovine, bovine and caprine Tfs (Yu *et al.*, 1992; Yu and Schryvers, 1994; Ekins and Niven, 2001; Ekins *et al.*, 2004*a*). Lf receptors, on the other hand, appear to be present only in human pathogens and bind, specifically, human Lf (Mickelsen *et al.*, 1982; Campagnari *et al.*, 1994; Ekins *et al.*, 2004*b*). For additional information on Lf receptors, which is beyond the scope of this Thesis, the reader is directed to Gray-Owen and Schryvers (1996) and Ekins *et al.* (2004*b*).

In all organisms that produce a bipartite Tf receptor, with the exception of *Moraxella catarrhalis* (Myers *et al.*, 1998), the genes encoding TbpA and TbpB are arranged in an operon with *tbpB* upstream of *tbpA* (Legrain *et al.*, 1993; Gonzalez *et al.*, 1995; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997); in *M. catarrhalis*, *tbpA* is located upstream of *tbpB* and is separated from *tbpB* by an intervening ORF of unknown function (Myers *et al.*, 1998). Tbps are typically iron-repressible (Gray-Owen and Schryvers, 1996) and putative promoter and regulatory sequences believed to be involved in the regulation of gene expression have been located immediately upstream of *tbpBA* (Legrain *et al.*, 1993; Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997) and *tbpA2* (Ekins and Niven, 2002; Ekins *et al.*, 2004*a*). In *H. influenzae*, however, Tbps appear to be repressible by haem and/or iron (Morton *et al.*, 1993; Gray-Owen and Schryvers, 1995; Hasan *et al.*, 1997) and in at least two strains of *H. somni*, the expression of TbpA2 is also subject to a form of phase variation involving poly G tracts within *tbpA2* (Ekins and Niven, 2003; Ekins *et al.*, 2004*a*). Also, in *A. pleuropneumoniae* and *H. parasuis*,, *tbpBA* is preceded by *tonB-exbB-exbD* and it would appear that the transcription of all five genes is controlled by an iron-regulated promoter upstream of *tonB* (Tonpitak *et al.*, 2000; del Río *et al.*, 2005). Iron-regulated production of Tbps is presumed to involve homologues of the *E. coli* ferric uptake regulation (Fur) protein. When iron is plentiful, Fur-Fe^{2+} complexes bind at nucleotide sequences (Fur boxes) that overlap the promoter regions of iron-regulated genes thereby preventing their expression

(Escolar *et al.*, 1999; Hantke, 2001). Notably, putative Fur boxes have been identified in the promoter regions of several *tbp* genes (Legrain *et al.*, 1993; Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997; Ekins and Niven, 2002; Ekins *et al.*, 2004*a*).

Analysis of the predicted amino acid sequences of TbpA proteins has revealed homology between these proteins and TonB-dependent bacterial outer membrane transporters including some for which the crystal structures have been resolved. This homology has led to topological analyses and it is now beginning to appear that the TbpA proteins exist in the form of transmembrane β-barrels with extracellular loops serving as ligand-binding domains and the N-terminal regions serving as globular cork domains (Cornelissen *et al.*, 1992; Cornelissen, 2003, Oakhill *et al.*, 2005). The predicted amino acid sequences of TbpB proteins contain putative signal II cleavage sites; this suggests that the TbpB proteins are lipoproteins and indeed, this has been shown to be the case for the TbpBs of *N. gonorrhoeae* (Anderson *et al.*, 1994) and *A. pleuropneumoniae* (Gerlach *et al.*, 1992*a*; Gerlach *et al.*, 1992*b*). In effect, TbpBs appear to be surface proteins that are anchored in outer membranes by means of lipid moieties (Cornelissen, 2003). TbpBs, however, exhibit no particular homology with any other known outer membrane protein and additional structural details remain to be elucidated (Cornelissen, 2003).

An interesting difference between TbpA and TbpB is that unlike TbpA, TbpB can discriminate between apo-Tf and the ferrated (holo) form and binds, preferentially, holo-Tf. Also, it would appear that TbpB is not absolutely required for the acquisition of Tf-bound iron; while the acquisition of Tf-bound iron by mutants lacking TbpB is less efficient than it is in wild-type strains, it is not eliminated. It has been proposed that the ability of TbpB to discriminate between apo- and holo-Tf is important for the recognition and subsequent release of Tf by the receptor complex (Cornelissen, 2003).

1.4.3.2 Haem- and haemoprotein-binding proteins

Haem and haemoprotein receptors are loosely grouped into three subfamilies, the haem scavengers, produced by enteric organisms such as members of the genera *Yersinia*, *Escherichia* and *Shigella*, haemophore receptors, produced solely by a few Gram-negative bacteria, including some members of the genera *Serratia*, *Pseudomonas* and *Haemophilus*, and haem/Hb receptors, produced by a variety of organisms including several members of the families *Neisseriaceae* and *Pasteurellaceae* (Perkins-Balding *et al.*, 2003). The haem scavengers are haem/Hb receptors with wide substrate specificity and sharing more than 60% homology at the amino acid level (Bracken *et al.*, 1999; Wandersman and Stojiljkovic, 2000). As outlined above (section 1.3.3.2.1), haemophore receptors mediate the transfer of haem from haemophores to the bacterial periplasm (Cope *et al.*, 1994; Létoffé *et al.*, 1994, 1998). The haem/Hb receptors exhibit narrow substrate specificity and share 25%-50% homology at the amino acid level (Perkins-Balding *et al.*, 2003); these haem/Hb receptors are the focus of the remainder of this subsection with the emphasis being placed on Hbs as substrates.

Studies pertaining to the involvement of receptors in the acquisition of Hb-bound iron have been confined, largely, to the pathogenic neisseriae, *N. meningitidis* and *N. gonorrhoeae* (Lewis *et al.*, 1997; Chen *et al.*, 1998; Perkins-Balding *et al.*, 2004), the haemophili, *H. ducreyi* and *H. influenzae* (Elkins *et al.*, 1995; Ren *et al.*, 1998; Jin *et al.*, 1999; Morton *et al.*, 1999; Cope *et al.*, 2000), and most recently, *P. multocida* (Bosch *et al.*, 2002; Cox *et al.*, 2003) and the actinobacilli, *Actinobacillus actinomycetemcomitans* and *A. pleuropneumoniae* (Hayashida *et al.*, 2002; Srikumar *et al.*, 2004). Except for the bipartite HpuA (~40 kDa)/HpuB (~90 kDa) receptors produced by the pathogenic neisseriae (Schryvers and Stojiljkovic, 1999), all of the Hb receptors identified to date are single-component receptors with molecular masses ranging from ~90 kDa to ~120 kDa. Notably, while *N. meningitidis* can produce a single-component receptor (HmbR) in addition to HpuAB, a premature stop codon in *hmbR* of *N. gonorrhoeae* precludes its production by this organism (Chen *et al.*, 1996; Stojiljkovic *et al.*, 1996). Also, while *H. influenzae* and *P. multocida* are known to produce at least two distinct Hb receptors (Morton *et al.*, 1999; Cope *et al.*, 2000; Cox *et al.*, 2003), *H. ducreyi*, *A. actinomycetemcomitans* and *A. pleuropneumoniae* appear to produce only one (Elkins *et al.*, 1995; Hayashida *et al.*, 2002; Srikumar *et al.*, 2004). Growth assays with *H. ducreyi* and with *N. meningitidis* strains that produce only HmbR and only HpuAB, and competition binding assays with *H. ducreyi* and *H. influenzae*, have demonstrated that while HmbR and *H. influenzae* exhibit a preference for human Hb (Frangipane *et al.*, 1994; Stojiljkovic *et al.*, 1996), HpuAB and the *H. ducreyi* receptor, HgbA, exhibit no such specificity (Elkins, 1995; Stojiljkovic *et al.*, 1996). In the *Neisseria* spp., the genes encoding HpuA and HpuB are arranged in an operon with *hpuA* upstream of *hpuB* (Lewis *et al.*, 1997; Chen *et al.*, 1998) and in *P. multocida*, the gene that encodes the Hb-binding protein, HgbA, is on an operon that includes *Plesiomonas shigelloides hugX* and *hugZ* homologues, both upstream of *hgbA* (Bosch *et al.*, 2002). The Hb-binding proteins produced by the pathogenic neisseriae and by *P. multocida* (HgbA and HgbB) and *A. pleuropneumoniae* (HgbA), are iron-repressible and putative Fur boxes have been identified upstream of neisserial *hmbR* and *hpuA* (Stojiljkovic *et al.*, 1995; Lewis *et al.*, 1997; Chen *et al.*, 1998) and upstream of *A. pleuropneumoniae hgbA* (Srikumar *et al.*,

2004) and *P. multocida hugX* and *hgbB* (Bosch *et al.*, 2002; Cox *et al.*, 2003). Notably, HmbR and HpuAB are also subject to phase variation involving poly G tracts within *hmbR* and *hpuA* (Chen *et al.*, 1998; Lewis *et al.*, 1999; Richardson and Stojiljkovic, 1999). In contrast, the Hb-binding proteins of *H. influenzae* and *H. ducreyi* appear to be repressible by haem and/or iron (Frangipane *et al.*, 1994; Elkins, 1995, Hasan *et al.*, 1997). While putative Fur boxes have been identified immediately upstream of the genes encoding several of these proteins (Elkins *et al.*, 1995; Ren *et al.*, 1998; Jin *et al.*, 1999; Cope *et al.*, 2000), the role of Fur in their expression remains to be established. Interestingly, phase-variable expression of Hb-binding proteins would also appear to be common in strains of *H. influenzae* but in these organisms, phase variation involves strand slippage across CCAA repeats (Jin *et al.*, 1999; Ren *et al.*, 1999; Cope *et al.*, 2000).

Database searches based on the predicted amino acid sequences of several Hb-binding proteins have led to the conclusion that these proteins are homologues of TonB-dependent Tf, Lf and siderophore receptors of Gram-negative bacteria (Gray-Owen and Schryvers, 1996). Interestingly, topological modelling of *N. meningitidis* HmbR and *A. pleuropneumoniae* HgbA indicates that these receptors at least, are organized within outer membranes in much the same way as *N. meningitidis* TbpA (see Perkins-Balding *et al.*, 2003; Pawelek and Coulton, 2004; Oakhill *et al.*, 2005). Energy transduction, promoting receptor-mediated transfer of the haem from Hb to the periplasm, is assumed, once again, to involve homologues of the proteins TonB, ExbB and ExbD (Braun, 1995; Genco and Dixon, 2001).

1.4.3.3 Removal and internalization of iron by TonB-dependent receptors

Binding of an iron- or haem-containing protein, such as Tf or Hb, by a TonB-dependent receptor is believed to induce a conformational change in the bound protein reducing its affinity for its iron or haem. Concomitantly, energized TonB interacts with the receptor promoting the opening of a channel in the receptor and the active transport of the iron or haem across the outer membrane (Fig. 1.1) (Genco and Dixon, 2001; Cornelissen, 2003). Within the periplasm, iron, along with carbonate, is believed to be bound by ferric ion-binding protein A (FbpA). On binding these ions, FbpA assumes a closed conformation and an affinity for iron that drives the translocation of iron across the outer membrane (Shouldice *et al.*, 2004). FbpA then interacts with FbpB, the transmembrane component of the ABC system that transports the iron across the cytoplasmic membrane. Following the release of the iron to the FbpB channel, it is transported across the membrane powered by the ATPase, FbpC (Shouldice *et al.*, 2004). An analogous system (Fig. 1.1) is believed to be responsible for the transfer of haem from Hb to the cytoplasm of the cell (Ratledge and Dover, 2000). The release of iron from haem is believed to be mediated by haem oxygenases; haem oxygenases are known to be produced by a few pathogens including *Neisseria* spp. (Zhu *et al.*, 2000), *P. aeruginosa* (Ratliff *et al.*, 2001) and *Corynebacterium diphtheriae* (Wilks and Schmitt, 1998; Chu *et al.*, 1999).

1.5 Regulation of iron uptake

1.5.1 Ferric uptake regulation protein

Mutant strains of *S. typhimurium* (Ernst *et al.*, 1978) and *E. coli* (Hantke, 1981) in which proteins, whose expression was normally inhibited by iron, were expressed

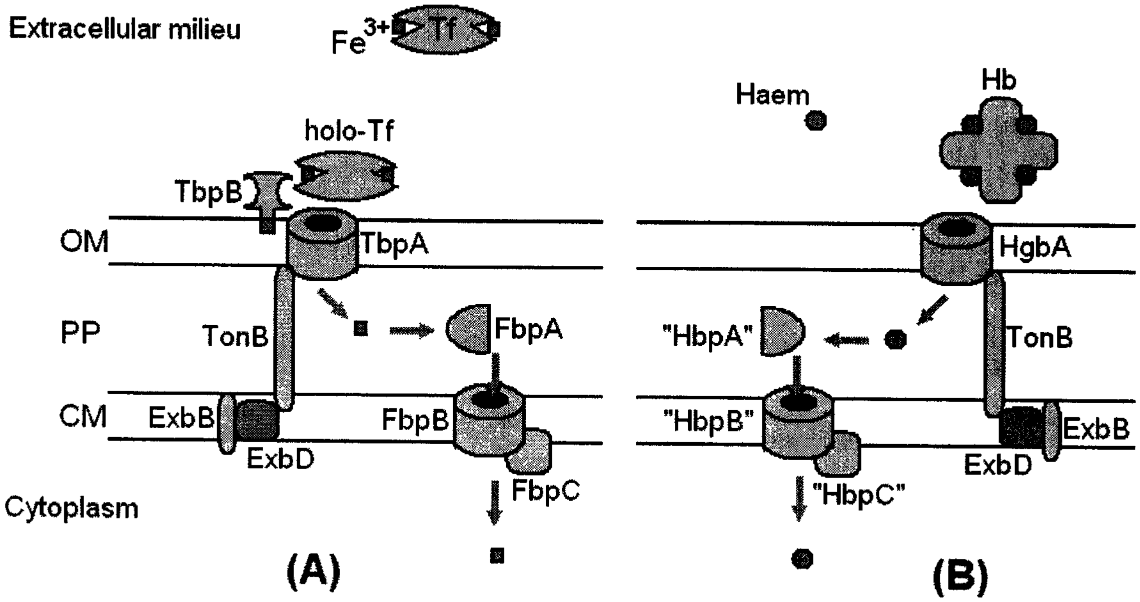


Fig. 1.1. Acquisition of Tf-bound iron (A) and haem (B) by *Pasteurellaceae*. The receptor components, TbpA, TbpB and HgbA are located in/on the outer membrane (OM). Iron from Tf (holo-Tf) and haem from Hb are transported to the periplasm (PP) where they are bound by the periplasmic binding proteins FbpA and "HbpA", respectively. The iron and haem are then transported across the cytoplasmic membrane (CM) via ABC transporters consisting of transmembrane proteins (FbpB and "HbpB", respectively) and ATPases (FbpC and "HbpC", respectively).

constitutively, were identified more than two decades ago. The implicated genes were termed *fur* (ferric uptake regulation) and using *E. coli*, *fur* was subsequently mapped and cloned (Hantke, 1984) and then sequenced (Schaffer *et al.*, 1985). The gene product, Fur, was subsequently purified and basic aspects of its regulatory properties were elucidated via *in vitro* experiments (Escolar *et al.*, 1999). It is now known that Fur is a 17-kDa polypeptide that forms homodimers in solution. While the C-terminal domain is capable of binding most first row divalent metal ions, with varying degrees of efficiency, the N-terminal domain is involved in DNA-binding (Stojiljkovic and Hantke, 1995). When iron is plentiful, ferrous iron binds to Fur resulting in conformational changes, dimerization and activation of the protein. Activated Fur physically attaches to specific palindromic regions within the promoters of iron-regulated genes (see section 1.5.2) and blocks the access of RNA-polymerase to the promoter areas. When iron is scarce, the ferrous iron-Fur complex is not formed and since RNA-polymerase may then gain easy access to appropriate promoter areas, transcription can begin (Ratledge and Dover, 2000). At least 90 *E. coli* genes are now known to be regulated by Fur and *fur* homologues are known to be present in a wide variety of bacteria (Hantke, 2001). Notably, recent studies with in-frame deletion mutants (Δ*fur*) have confirmed the Fur-dependent nature of Tbp expression in *A. pleuropneumoniae* (Jacobsen *et al.*, 2005).

1.5.2 Recognition and binding of DNA by Fur

DNA footprinting and comparative analysis of nucleotide sequences within the promoter regions of iron-regulated genes led to the identification of a Fur-binding sequence ("Fur box" or "Iron box") in the form of a 19-bp palindromic consensus (5'-GATAATGATAATCATTATC-3') (de Lorenzo *et al.*, 1987). This sequence was deemed capable of binding a single Fur dimer (de Lorenzo *et al.*, 1987) and as suggested in the classical model for Fur-DNA interaction (Fig.1.2A), each Fur monomer was deemed to bind to a 9-bp inverted repeat within the consensus sequence (Lavrrar and McIntosh, 2003). Since the initial identification of a Fur-binding sequence, closely-related Fur box sequences have been identified in the promoter regions of iron-regulated genes of many organisms, including Gram-positive bacteria (e.g. Helmann, 1997; Bsat and Helmann, 1999).

While studies with synthetic oligonucleotides demonstrated that the 19-bp consensus sequence was sufficient for Fur-mediated gene repression (Calderwood and Mekalanos, 1988), until recently, the means by which Fur, a small dimeric protein, could interact with an extended operator region such as a Fur box, remained to a great extent, only poorly understood. As an example of a typical conundrum, Fur appears to be a helix-turn-helix DNA-binding protein (Baichoo and Helmann, 2002; Pohl *et al.*, 2003); while most helix-turn-helix DNA-binding proteins interact with stretches of DNA containing ~12 bp (Harrison and Aggarwal, 1990), the Fur-DNA interaction, based on DNA footprinting, covers ~30 bp (Baichoo and Helmann, 2002).

An alternative interpretation of the means by which Fur recognizes the consensus sequence was provided by Escolar and colleagues (1999). They suggested a model, referred to as the 6-6-1-6 model (Fig. 1.2B; Baichoo and Helmann, 2002), in which the 19-bp Fur box consensus could be viewed as a head-to-head-to-tail repeat of a GATAAT hexamer (Escolar *et al.*, 1999). Based on studies with synthetic oligonucleotides, Escolar *et al.* (1998) proposed that the hexamer, NAT(A/T)AT (where N is any nucleotide), is the unit recognized by Fur, with a minimum of three units, each capable of binding a Fur dimer, being required for the complex to be fully functional. Subsequently, Lavrrar *et al.*

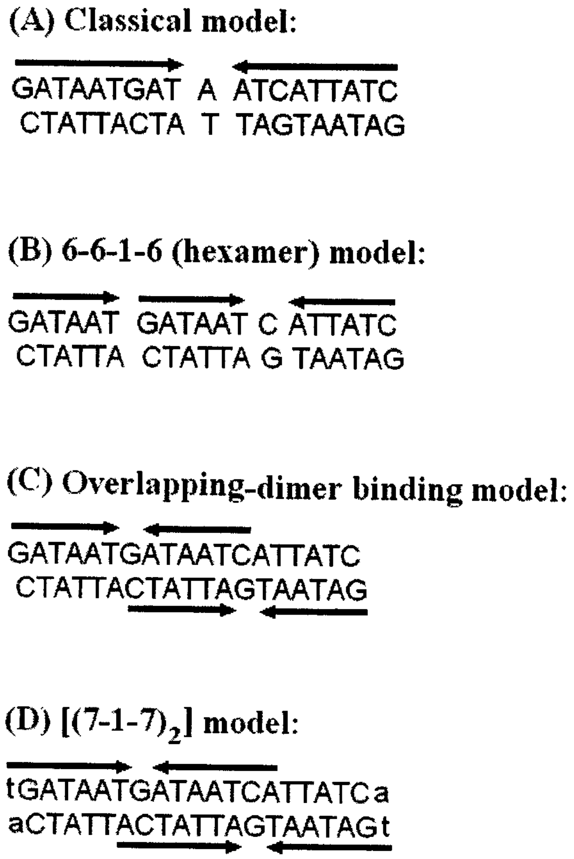


Fig. 1.2. Models for Fur-DNA interactions. In A, C and D, the arrows indicate the sequences recognized by Fur monomers. In B, the arrows indicate the sequences recognized by Fur dimers.

(2002) proposed the overlapping-dimer binding model where overlapping Fur dimers, positioned on opposite faces of the DNA helix, interact with the Fur box (Fig. 1.2C). In this model, the 19-bp Fur box consensus is viewed as overlapping 13-mer sequences, with each 13-mer consisting of two hexamers interrupted by a single nucleotide (Lavrrar *et al.*, 2002). Almost simultaneously, Baichoo and Helmann (2002) presented a very similar model, referred to as the $[(7\text{-}1\text{-}7)_2]$ model (Fig. 1.2D), but here, it was proposed that each Fur monomer binds to a 7-mer. Recent studies have confirmed that the 19-bp consensus sequence does, indeed, bind two overlapping Fur dimers (Lavrrar and McIntosh, 2003). Also, while both of the latter models, the overlapping-dimer binding and $[(7\text{-}1\text{-}7)_2]$ models, can be used to explain the initial binding event, the results of Lavrrar and McIntosh (2003) indicate that the overlapping-dimer binding model is a closer representation of reality.

1.6 Transferrin and haemoglobin receptors as vaccine components

TbpA and TbpB are considered promising vaccine targets because they are very important for the survival of organisms *in vivo*, they are surface-accessible and usually, are not subject to high-frequency phase or antigenic variations (Cornelissen, 2003). Tbps are necessary for *A. pleuropneumoniae* to cause disease in experimental animals (Baltes *et al.*, 2002) and attempts to elicit protective immune responses using Tbps from *A. pleuropneumoniae* (Rossi-Campos *et al.*, 1992), and from *N. meningitidis* (Danve *et al.*, 1993; West *et al.*, 2001), have been successful. Whether TbpA or TbpB is the better candidate for a vaccine formulation is a matter of debate. While Cornelissen (2003) has argued that TbpA, the less antigenically variable and more conserved outer membrane protein, is a better vaccine target than TbpB, others have suggested that TbpB is the better potential target for immunoprophylaxis (Ala'Aldeen *et al.*, 1994; Potter *et al.*, 1999; Yu *et al.*, 1999). In this context, it has been demonstrated that recombinant TbpB (rTbpB)-based vaccines, containing *N. meningitidis* TbpB fused to the maltose-binding protein as an adjuvant, are able to elicit the production of efficient and broadly cross-bactericidal antibodies (Rokbi *et al.*, 2000). Nonetheless, caution must be exercised when assessing the practicality and *in vivo* efficacy of such vaccines since to date, most investigations relating to their immunogenicity have been conducted under laboratory conditions and using purified proteins as antigens rather than *in vivo*-grown organisms (Rokbi *et al.*, 2000). Regarding the use of Hb receptors as vaccine components, little information is available. Notably, however, Bosch *et al.*, (2004) have investigated several *P. multocida* haemin- and Hb-binding proteins for their potential as immunogens in mice. Unfortunately, while these proteins elicited the production of specific antibodies, they did not protect the animals against a subsequent challenge with virulent *P. multocida*.

1.7 Aims of the research project

At the outset of this project, little information was available on the virulence determinants of *A. suis* and except for a single publication (Schryvers and Gonzalez, 1990) that mentioned the binding of porcine Tf, information relating to iron acquisition by this organism appeared to be non-existent. The binding of Tf by *A. suis* (Schryvers and Gonzalez, 1990) suggested that this organism, like other members of the *Pasteurellaceae*, can acquire Tf-bound iron by means of a siderophore-independent, receptor-mediated mechanism. The aims of the present study were therefore to confirm the existence of such a system, and subsequently, the existence of a comparable system for the acquisition of

Hb-bound iron, in *A. suis*, and, if present, to identify and characterize some of the components involved in the iron acquisition processes. It was also envisaged that the discovery of novel receptor components could lead to the production of recombinant receptor proteins that could then be tested for their potential as vaccine components.

Chapter 2. Identification and characterization of transferrin receptor proteins and encoding genes

This chapter was adapted from the following, with permission from Elsevier and the co-authors:

Bahrami, F., Ekins, A. and Niven D. F. 2003. Iron acquisition by *Actinobacillus suis*: identification and characterization of transferrin receptor proteins and encoding genes. Vet. Microbiol. **94:** 79-92.

2.1 Abstract

A. suis is an important pathogen of swine, especially in high-health-status herds. A published report mentioning the binding of porcine Tf by at least one strain of *A. suis* suggested that *A. suis*, like other members of the *Pasteurellaceae*, can acquire Tf-bound iron by means of a siderophore-independent, receptor-mediated mechanism. The objective of the present study was to characterize the components involved in this process, if present. Growth assays, with seven strains, confirmed that *A. suis* can use porcine (but not human or bovine) Tf as an iron source for growth. In solid phase binding assays, total membranes derived from all strains exhibited strong binding of porcine Tf but only if the membranes were from organisms grown under iron-restricted conditions. An affinity-isolation procedure allowed the isolation of putative Tf-binding polypeptides (~100 kDa and ~63 kDa) from comparable membranes from all strains. PCR approaches allowed the amplification, cloning and sequencing of *A. suis tonB*, *tonB2*, *exbB*, *exbB2*, *exbD*, *exbD2*, *tbpB* and *tbpA* homologues. RT-PCR, using RNA from organisms grown

under iron-replete and iron-restricted conditions, revealed that *tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* are transcribed as a single unit with expression being up-regulated in response to iron restriction. The calculated molecular masses of the predicted, mature TbpA (104.3 kDa) and TbpB (63.4 kDa) proteins suggest strongly that the affinity-isolated, ~100-kDa and ~63-kDa Tf-binding polypeptides represent TbpA and TbpB, respectively. It is concluded that the acquisition of Tf-bound iron by *A. suis* involves mechanisms analogous to those found in other members of the *Pasteurellaceae*.

2.2 Introduction

An iron-requiring pathogen must be capable of acquiring iron from its host despite the iron-restricted environment. While many pathogens rely on siderophores for iron acquisition, pathogenic members of the *Pasteurellaceae* (and *Neisseriaceae*) can obtain iron directly from host proteins, most notably from Tfs, by means of siderophore-independent, receptor-mediated mechanisms (see e.g., Griffiths, 1987; Gray-Owen and Schryvers, 1996; Ratledge and Dover, 2000). Receptor-mediated acquisition of Tf-bound iron involves two surface receptor proteins referred to as Tf-binding proteins A and B (TbpA and TbpB, respectively; Gray-Owen and Schryvers, 1996) or a single receptor protein, now referred to as TbpA2 (Ogunnariwo and Schryvers, 2001; Ekins and Niven, 2002; Ekins *et al.*, 2004*a*). In organisms that produce TbpA and TbpB, with the exception of *M. catarrhalis* (Myers *et al.*, 1998), the genes encoding these proteins are arranged in an operon with *tbpB* upstream of *tbpA* (see e.g., Legrain *et al.*, 1993; Gonzalez *et al.*, 1995; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997). Tbps are typically iron-repressible (see e.g., Gray-Owen and Schryvers, 1996) and putative promoter and regulatory sequences believed to be involved in the regulation of gene expression have been located immediately upstream of *tbpBA* (Legrain *et al.*, 1993; Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997) and *tbpA2* (Ekins and Niven, 2002; Ekins *et al.*, 2004*a*). In *A. pleuropneumoniae*, however, it would appear that *tbpBA* is preceded by *tonB-exbBD* and that transcription of all five genes (*tonB-exbBD-tbpBA*) is controlled by an iron-regulated promoter upstream of *tonB* (Tonpitak *et al.*, 2000; Beddek *et al.*, 2004). Iron-regulated production of Tbps is presumed to involve homologues of the *E. coli* ferric uptake regulation (Fur) protein. Notably, putative Fur boxes have been identified in the promoter regions of several *tbp* genes (Legrain *et al.*, 1993; Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997; Ekins and Niven, 2002; Ekins *et al.*, 2004*a*).

A. suis is now recognized as an important pathogen of swine, especially in high-health-status herds (see e.g., MacInnes and Desrosiers, 1999; Rycroft and Garside, 2000). The clinical signs and pathologies associated with *A. suis* disease can take many forms (MacInnes and Desrosiers, 1999) and may, on occasion, resemble those observed in *A. pleuropneumoniae* pleuropneumonia (Yaeger, 1996; Taylor, 1999). While relatively little is known regarding *A. suis* virulence determinants, it would appear that lipopolysaccharides, capsular polysaccharides, and most likely, RTX toxins, have important roles to play in pathogenesis (MacInnes and Desrosiers, 1999; Slavić *et al.*, 2000*a*,*b*). The ability to obtain iron within the host is also crucial for virulence and in this context, a published report (Schryvers and Gonzalez, 1990), mentioning the binding of porcine Tf by at least one strain of *A. suis*, suggested that this organism, like other members of the *Pasteurellaceae*, can acquire Tf-bound iron by means of a siderophore-independent, receptor-mediated mechanism. The objectives of the present study were to confirm the existence of such a system in *A. suis* and, if present, to characterize at least some of the components involved in the iron acquisition process.

2.3 Materials and methods

2.3.1 Organisms

A. suis strain SO4 (serotype O1/K1) was provided by Dr. S. Rosendal (University of Guelph, ON) and strains B49 (serotype O1/K1), C84 (serotype O1/K2), H89-1173 (serotype O2/K3), H91-0380 (serotype O2/K2), VSB 3714 (serotype rough/K?) and ATCC 15557 (serotype O1/K1) were provided by Dr. J.I. MacInnes (University of Guelph). Storage cultures and inocula were stored (-80 °C) as small volumes (~1.0 ml) of late exponential phase cultures supplemented with glycerol (50 ml of a 75% (w/v) solution per 200-ml culture); the growth medium (TYE-H/HCO_3) was HEPES-buffered tryptone-yeast extract (TYE-H; Niven *et al.*, 1989) supplemented, just prior to inoculation, with $NaHCO_3$ (to 10 mM). *E. coli* strain DH5α was handled similarly except that the growth medium was the tryptone broth medium (TY) described by Stojiljkovic *et al.* (1994).

2.3.2 Transferrins and conjugates

Human and bovine apo-Tfs were from Calbiochem and porcine Tf was from First Link (UK) Ltd. The ovine and caprine Tfs were the preparations described previously (Ekins and Niven, 2001). The Tfs were ferrated (to 40-65% saturation for growth assays; 60-90% for biotinylation) and dialyzed as described by Caldwell and Archibald (1987) and once sterilized by filtration, the iron saturation levels were verified by the method of Mazurier and Spik (1980). The Tfs were biotinylated by the method of Schryvers and

Morris (1988), as modified by Ricard *et al.* (1991). Our previous studies (e.g., Niven *et al.*, 1989; Ricard *et al.*, 1991; D'Silva *et al.*, 1995; Ekins and Niven, 2001) have established that in our hands, these methods do yield Tf (bovine, caprine, human, ovine and porcine) preparations that are biologically active.

2.3.3 Growth conditions and preparation of membranes

To investigate the abilities of the *A. suis* strains to acquire iron from Tfs, TYE-H containing sufficient (50 μM) ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDA; Sigma Chemical Corp.) to completely inhibit growth was prepared and autoclaved (25-ml volumes in 125-ml screw-capped Nalgene flasks) and just prior to inoculation, this medium was supplemented with either $NaHCO_3$ (to 10 mM) or $NaHCO_3$ (to 10 mM) plus Tf (to 80 μg/ml). All cultures (0.1% (v/v) inocula) were incubated at 37 °C with agitation on a gyratory shaker (200 rpm); growth was monitored turbidimetrically at 660 nm (Gilford Stasar II Spectrophotometer, Gilford Instrument Laboratories, Inc.) using diluted samples, where appropriate.

For the preparation of membrane fractions, the organisms were grown (0.1% inocula; 37 °C; 200 rpm) under both iron-replete and iron-restricted conditions. TYE-H/HCO_3 was used for growth of organisms under iron-replete conditions; for growth under iron-restricted conditions, the TYE-H/HCO_3 also contained EDDA (14-20 μM; added to the TYE-H prior to autoclaving) such that the final cell yields were approximately 80% of those obtained with iron-replete cultures. These media (200-ml volumes) were contained in 1-l screw-capped Nalgene flasks. The organisms were harvested in early stationary phase, disrupted by sonication and fractionated to yield total membrane and outer membrane preparations essentially as described by Niven *et al.* (1989); protein concentrations were estimated by the method of Gornall *et al.* (1949) using bovine serum albumin (fraction V powder; Boehringer-Mannheim) as the standard.

2.3.4 Transferrin-binding assays

Tf-binding assays (dot blots) were performed essentially as described by Ekins and Niven (2001). Briefly, total membranes were diluted (to 0.5 mg/ml protein) in Tris-buffered saline (TBS; 145 mM NaCl, 100 mM Tris/HCl, pH 7.4) and applied (50-µl volumes) to nitrocellulose sheets (0.45 µm pore size; Bio-Rad) held in a dot blot apparatus (Minifold I; Schleicher and Schuell). Non-specific binding sites were saturated by incubating the nitrocellulose sheets (1 h, 37 °C, gentle agitation) in TBS containing 0.15 % (v/v) Tween 20 (TTBS). An appropriate biotinylated Tf (500 ng in 250 µl TTBS) was added to each well and following incubation (1 h, 37 °C), the Tf solutions were removed and each well rinsed individually with 500 µl TBS. The nitrocellulose sheets were then subjected to three 10-min washes with TBS (37 °C, gentle agitation), incubation (1 h, 37 °C, gentle agitation) with streptavidin-HRP (Gibco BRL; diluted 1:1000 in TTBS) and a final three washes with TBS. Reactions were visualized using 4-chloro-1-naphthol and H_2O_2, as previously described (Niven *et al.*, 1989).

2.3.5 Isolation and identification of transferrin-binding polypeptides

Tf-binding polypeptides were isolated from total membranes using biotinylated porcine Tf plus streptavidin-agarose (Gibco BRL) and the batch affinity procedure and wash system 3 developed by Schryvers and Morris (1988), as modified by Ricard *et al.* (1991). The isolated polypeptides were identified by means of SDS-PAGE, as described by Ekins and Niven (2001) except that silver staining of gels was performed according to Harlow and Lane (1988).

To aid in the identification of the ~75-kDa affinity-isolated polypeptide, membranes from *A. suis* strain SO4 grown under iron-restricted conditions were subjected to the affinity procedure and the polypeptides in the supernatant fractions from 5 isolations were concentrated using Ultrafree-0.5 Biomax-30 (30 kDa MWCO) Centrifugal Filter Devices (Millipore; 12000×*g*, 30 min). The concentrated polypeptides were subjected to SDS-PAGE and transferred to nitrocellulose (Bio-Rad; 50 V, 24 h) using 20 mM Tris, 150 mM glycine, 20% (v/v) methanol, 0.1% (w/v) SDS as transfer buffer. The nitrocellulose sheet was probed with streptavidin-HRP, as above, and developed with 4-chloro-1-naphthol/H_2O_2 as described by Niven *et al.* (1989).

2.3.6 Amplification and sequencing of receptor and affiliated genes

Genomic DNA was isolated from *A. suis* strains C84 and SO4 using a DNeasy Tissue Kit (Qiagen) or Genomic-tip 500/G columns and the recommended buffer system (Qiagen). Amplification of a partial *tbpA* homologue was accomplished using DNA from strain C84 as template and primers (APPF and APPR; Table 2.1) based on sequences within *tbpA* of *A. pleuropneumoniae* (GenBank accession number Z49708); the reaction mixture (50 µl) contained 4 µl of template, 0.5 µM APPF, 0.5 µM APPR, 0.2 mM dNTP, 3 mM $MgCl_2$, 5 µl of 10×*Taq*-buffer and 2.5 U of *Taq* polymerase (Gibco BRL) and was subjected to 3 min at 95 °C, 25 cycles of 30 s at 95 °C, 30 s at 45 °C and 2 min at 72 °C, and a final 3 min at 72 °C using a thermocycler. Following gel electrophoresis (1% agarose), an appropriately sized fragment (~ 2.8 kbp) was excised from the gel and

Table 2.1. Primers used in PCR and RT-PCR

Primer	Description	Sequence (5' → 3')
APPF	Ap[a] *tbpA*; forward	CTGATTAGCCTTGCTCTTCTTAGC
APPR	Ap *tbpA*; reverse	GCATACCTTGCATAGTCTTTTACG
AR1	As[b] *tbpA*; reverse	CGTTGCACCTCTCCCTTGTTCTAC
AR2	As *tbpA*; reverse	CCACTCCTACTCGGGTGATATTCT
BA6[c]	Ap *tonB*; forward	AGTGGGATCCTGAAAGTTACTATTGGAG
BF1	As *tbpB*; forward	TGGTAATGGCTTTATCGGCAGTGC
BR1	As *tbpB*; reverse	CCAGAACAAGCGACAAGAAACAGC
CF1	As *tbpA*; forward	TAGATGCAGGCTCTAGCCAACACG
CR1	As *tbpA*; reverse	GCAGGGCGAACAAAGGTATCTCTG
EBF1	Ap *exbB*; forward	CACTTGCTTCTATTGGTGCGGTTG
EBF2	As *exbB*; forward	GACTATTTGTCGCAATTCCGGCAG
EBR1	As *exbB*; reverse	CAGACCGTTCCAAATAACCCAATGAACG
EBR2	As *exbB* ; reverse	CTGCCGGAATTGCGACAAATAGTC
EDF	Ap *exbD*; forward	AGAGTGGCGATTAATCCTACGGGC
EDR	As *exbD*; reverse	GCCCGTAGGATTAATCGCCACTCT
TF1	As *tonB*; forward	GTCTGATCCATCTAGGAGTAGC
TF2	As DNA[d]; forward	GTCAGGTAGTATTCAAGTTGTGCC
TF3	As *tonB*; forward	GAATCTGATGATGTCGGTAGTGTG
TR1	As *tonB*; reverse	GGTTATCTAGGCGTTTGATGCCTG

Table 2.1. Primers used in PCR and RT-PCR (continued)

Primer	Description	Sequence (5' → 3')
TR2	As *tonB*; reverse	GCTACTCCTAGATGGATCAGAC
UGLF	Ap *glpE*; forward	CCTGATGCGGGCTGATCTCAG
UEB2R	Ap *exbB2*; reverse	GAATAGCGACTAAGATACCGGCTG
UEB2F	Ap *exbB2*; forward	AGGCTTATTACTATTAATGAGTGTTGTGTTGG
UTB2R	Ap *tonB2*; reverse	GCATTATCAAGATTACCGTTACCGGAAC
UTB2F	Ap *tonB2*; forward	GTCCTGAAGCAAAACAAGGTATTGTC
UYBR	Ap *ybaB*; reverse	ACGCTCTTGCATTTGCTGAGCTTG
UEB2F2	As *exbB2*; forward	CACCACTATCTCAACTATCGGTGC
UTB2R2	As *tonB2*; reverse	CGCTTGTGTAGGTTGTTCTTCTGC

[a] *A. pleuropneumoniae*

[b] *A. suis*

[c] Tonpitak *et al.*, 2000.

[d] Upstream of *tonB*.

purified using a QIAEX II Gel Extraction Kit (Qiagen). Purified fragments were ligated into pGEM-T Easy (Promega), as specified by the manufacturer, and the ligation products were used to transform *E. coli* DH5α. A recombinant plasmid containing the appropriate insert was isolated and purified using a QIAprep Spin Miniprep Kit (Qiagen) and sequencing of the insert was initiated using pUC sequencing primers and a BigDye sequencing kit (Applied Biosystems); as sequence information became available, sequencing was continued using custom-made primers. A BlastX search confirmed that the sequenced fragment was a fragment of a *tbpA* homologue and primers based on the acquired sequence were then used for direct genome sequencing (Heiner *et al.*, 1998) of the remainder of the gene and upstream and downstream regions. Primers CF1 and CR1 (Table 2.1), based on direct sequence information, and the Expand High Fidelity PCR System (Roche Diagnostics), were then used in touch-down PCR (3 min at 95 °C, 20 cycles of 1 min at 92 °C, 1 min at 74 °C −1 °C /cycle and 3 min at 72 °C, and a final 3 min at 72 °C) with genomic DNA from strain C84, and subsequently, with DNA from strain SO4. The resulting fragments (~3 kbp) were cloned (as above) to give plasmids pGEMC84A and pGEMSO4A, respectively, and both strands of each insert were then sequenced, as above. Any differences in the sequences of the inserts were checked using genomic DNA from strains C84 and SO4 as templates in PCR followed by double-stranded sequencing of the purified (QIAquick PCR Purification Kit; Qiagen) products.

Amplification of a *tbpB* homologue, and bordering regions, from strain C84 was accomplished using primers based on sequences within exbB (EBF1; Table 2.1) of *A. pleuropneumoniae* (GenBank accession number Y17916) and *tbpA* (AR1; Table 2.1) of strain C84, and the Expand High Fidelity PCR System, in touch-down PCR (as above); a *tbpB* homologue, and bordering regions, from strain SO4 was amplified similarly except that the forward primer (EDF; Table 2.1) was based on sequence within exbD of *A. pleuropneumoniae* (GenBank accession number Y17916). The PCR products were cloned (as above) to give plasmids pGEMC84B and pGEMSO4B, respectively, and both strands of each insert were then sequenced, as above.

To obtain additional sequence, upstream of *tbpB* of strain SO4, three sets of primers (EBF1/EDR, EBF2/BR1, BA6/EBR1), and the Expand High Fidelity PCR System, were used sequentially to amplify fragments of interest and to perform double-stranded sequencing on the purified PCR products. A primer based on the acquired sequence was then used to initiate two rounds of direct genome sequencing farther upstream and as sequence information became available, primers based on this information (TF1 and TR1 and subsequently, TF2 and TR2; Table 2.1) were used to amplify the appropriate fragments and to perform double-stranded sequencing on the products. Additional sequence upstream of *exbD* of strain C84 was obtained similarly except that the initial amplification step involved BA6 and EBR1. For comparative purposes, the *tonB-tbpA* gene clusters (3893 bp) of all seven strains were amplified using touch-down PCR, as above, primers TF1 and AR1 (Table 2.1) and *Taq* polymerase (Invitrogen).

Amplification of *exbB2*, *exbD2* and *tonB2* homologues from *A. suis* strains C84 and SO4 was accomplished using the Expand High Fidelity PCR System and three sets of primers, UGLF/UEB2R, UEB2F/UTB2R and UTB2F/UYBR (Table 2.1), based on *A. pleuropneumoniae exbB2*, *exbD2* and *tonB2*, and the flanking genes, *glpE* and *ybaB*, located upstream of *exbB2* and downstream of *tonB2*, respectively (GenBank accession number AY428647). The amplicons, corresponding to overlapping fragments of *exbB2*, *exbD2* and *tonB2*, that were obtained with genomic DNA from *A. suis* strains C84 and SO4 were used to perform double-stranded sequencing. For comparative purposes, the *exbB2-tonB2* gene clusters (915 bp) of all seven strains were amplified using conventional PCR, primers UEB2F2 and UTB2R2 (Table 2.1) and *Taq* polymerase (Invitrogen). The concentrations of $MgCl_2$ in the reaction mixtures were 1.5 mM (UEB2F/UTB2R; UTB2F/UYBR) and 3 mM (UGLF/UEB2R).

Custom primers and pUC sequencing primers were from Gibco BRL/Invitrogen. Sequence analyses were performed by the Applied Biotechnology Laboratory, McGill University (Macdonald Campus), using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), or by the Genome Québec Innovation Centre (McGill University; *exbB2*, *exbD2* and *tonB2*) using an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems). Sequence data were assembled into contiguous segments using Sequencher 3.0 (Gene Codes) and submitted to GenBank (accession numbers AY101604, AY101605, AY838763 and AY838764; Appendices 1.1, 1.2, 1.3 and 1.4).

2.3.7 Preparation of RNA and RT-PCR

A. suis strains C84 and SO4 were grown (25-ml volumes) under iron-replete and iron-restricted conditions, as described above except that the EDDA concentration was 30 μM. Cultures were grown to the exponential phase of the growth cycle (optical density ~ 0.6 to 0.8) and the organisms from 1-ml volumes of each culture were harvested by centrifugation, washed immediately with phosphate buffered saline (0.17 M NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and resuspended in RNA*later* RNA Stabilization Reagent as described by the manufacturer (Qiagen). RNA was extracted from the organisms using a RNeasy Mini Kit (Qiagen); contaminating DNA was removed by incubating (2 h; room temperature) each RNA sample (50 µl) with 10 µl RNase-free, DNase I (10 units/µl; Roche Diagnostics) followed by re-isolation of the RNA using the RNeasy Mini Protocol for RNA cleanup. RNA concentrations were estimated spectrophotometrically (260 nm; Perkin-Elmer Lambda 3B UV/VIS Spectrophotometer; Perkin-Elmer Corp.). RT-PCR was carried out with the OneStep RT-PCR Kit (Qiagen), as recommended by the manufacturer, with 100 ng of the appropriate RNA sample, or an aliquot of DNA (positive controls), and primers AR2, BF1, BR1, EBF2, TF3 and EBR2 (Table 2.1); in RT-PCR with DNA as template, the TF3-EBR2, EBF2-BR1 and BF1-AR2 primer pairs were expected to amplify a 799-bp *tonB-exbB* fragment, a 639-bp *exbB-tbpB* fragment and a 933-bp *tbpB-tbpA* fragment, respectively. PCR, with the appropriate primers and RNA preparations as template material, served as negative controls. Products were analyzed by means of gel electrophoresis (1% agarose) and staining with ethidium bromide.

2.3.8 Imaging

Images of gels and blots were obtained using an AlphaImager 1200 documentation and analysis system and AlphaEase software (Alpha Innotech) or a ChemiGenius^{Q} bioimaging system and GeneSnap software (Syngene).

2.4 Results

2.4.1 Iron acquisition from transferrins

Liquid cultures were used to investigate the abilities of the *A. suis* strains to acquire iron from animal Tfs. All strains could acquire iron from porcine Tf but not from human or bovine Tf (Figs. 2.1-2.7). This specificity suggests that the acquisition of Tf-bound iron by *A. suis* is siderophore-independent and, most probably, receptor-mediated.

2.4.2 Binding of transferrins

Tf-binding assays were used to investigate the binding of Tfs by total membranes derived from all seven strains of *A. suis* grown under iron-replete and iron-restricted conditions. Membranes derived from all strains exhibited strong binding of only porcine Tf and only if the membranes were from organisms grown under iron-restricted conditions (Fig. 2.8). These results are in keeping with those of Schryvers and Gonzalez (1990) and serve as proof of the existence, in *A. suis*, of iron-repressible receptors specific for porcine Tf.

2.4.3 Isolation and identification of transferrin-binding polypeptides

An affinity-isolation procedure based on biotinylated porcine Tf plus streptavidin-agarose allowed the isolation of three, putative, Tf-binding polypeptides from total membranes derived from all seven strains of *A. suis* but, to a great extent, only if the membranes were from organisms grown under iron-restricted conditions (Figs. 2.9- 2.15). In all cases, the isolated polypeptides had estimated molecular masses of 100, 75 and 63 kDa. The sizes, iron-repressibility and outer membrane location of the large (100 kDa)

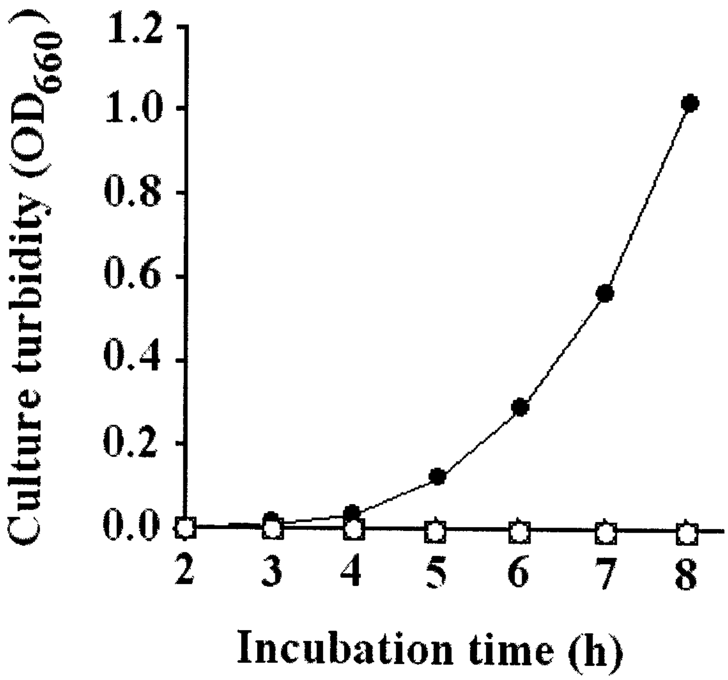


Fig. 2.1. Utilization of Tf-bound iron by *A. suis* strain ATCC 15557. *A. suis* was grown in medium containing EDDA (open circles), or EDDA plus porcine (closed circles), human (closed triangles) or bovine (open squares) Tf. Culture turbidity values are the means of triplicates.

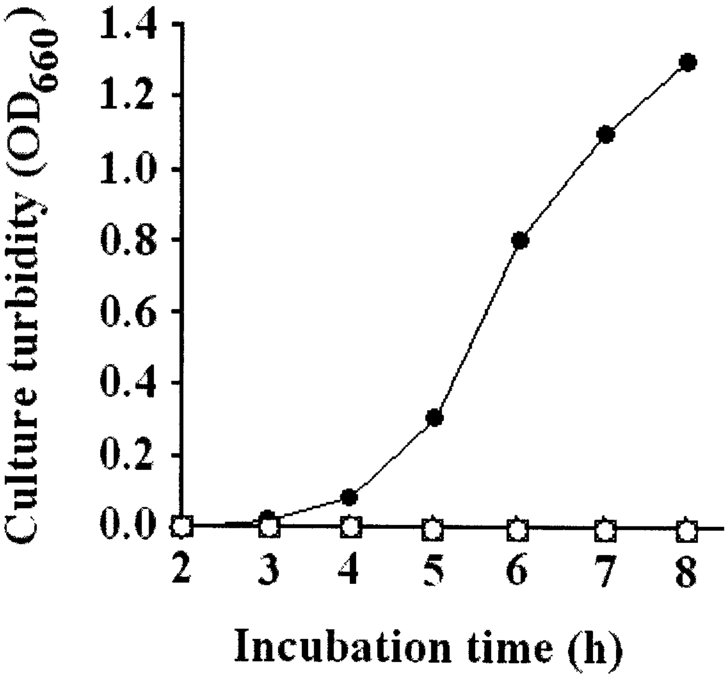


Fig .2.2. Utilization of Tf-bound iron by *A. suis* strain B49. *A. suis* was grown in medium containing EDDA (open circles), or EDDA plus porcine (closed circles), human (closed triangles) or bovine (open squares) Tf. Culture turbidity values are the means of triplicates.

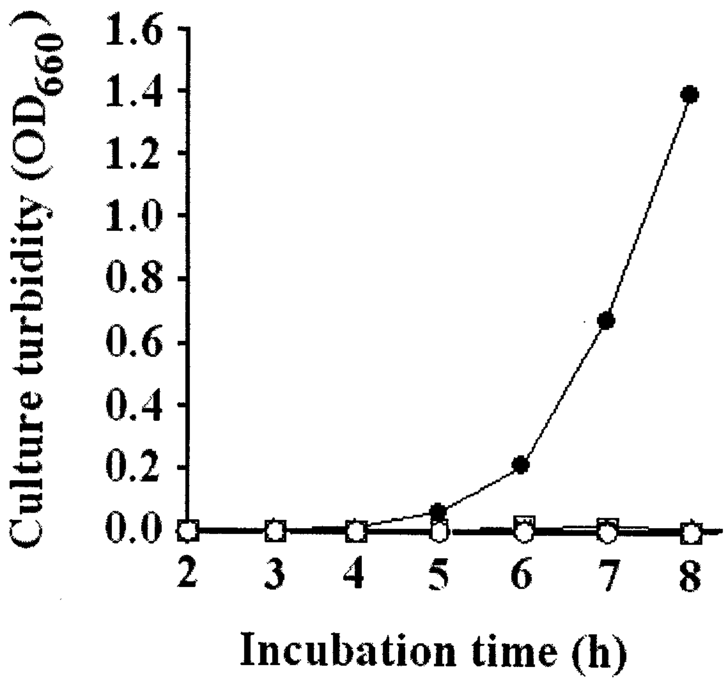


Fig. 2.3. Utilization of Tf-bound iron by *A. suis* strain C84. *A. suis* was grown in medium containing EDDA (open circles), or EDDA plus porcine (closed circles), human (closed triangles) or bovine (open squares) Tf. Culture turbidity values are the means of triplicates.

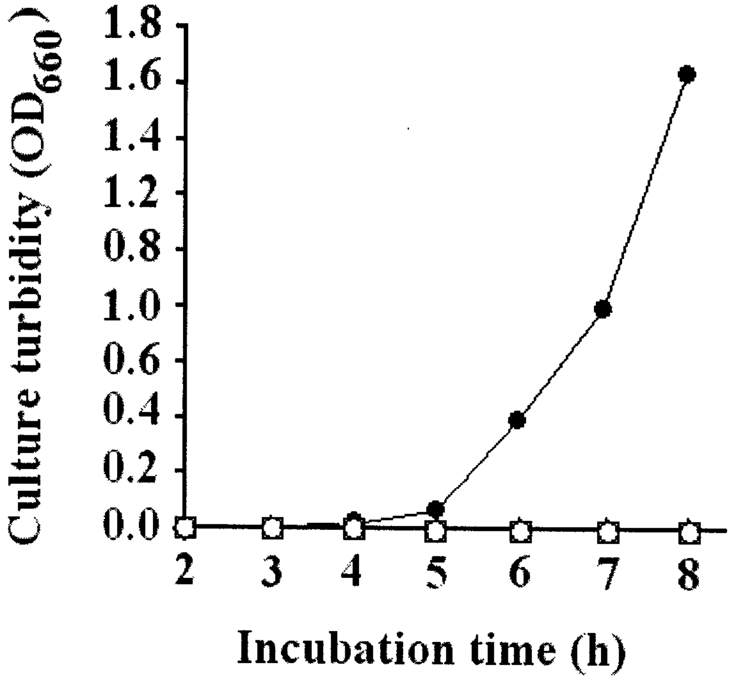


Fig. 2.4. Utilization of Tf-bound iron by *A. suis* strain H89-1173. *A. suis* was grown in medium containing EDDA (open circles), or EDDA plus porcine (closed circles), human (closed triangles) or bovine (open squares) Tf. Culture turbidity values are the means of triplicates.

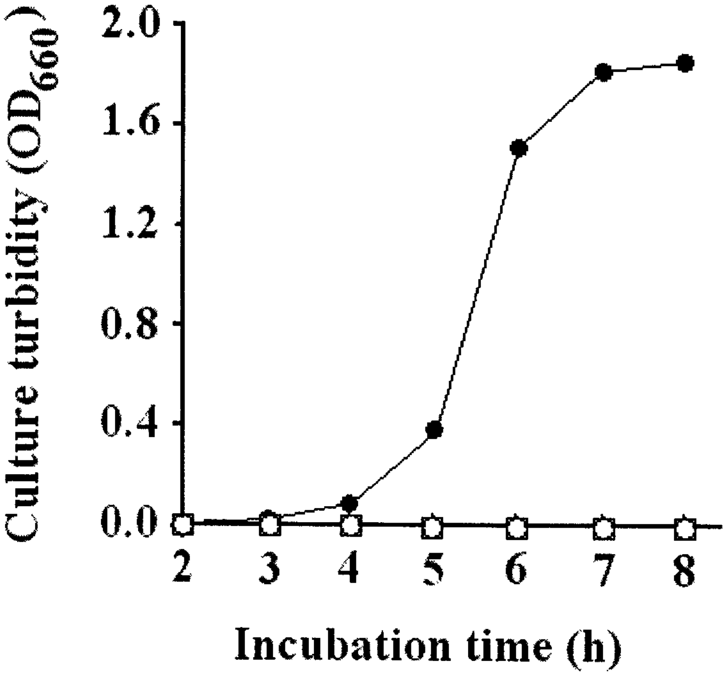


Fig. 2.5. Utilization of Tf-bound iron by *A. suis* strain H91-0380. *A. suis* was grown in medium containing EDDA (open circles), or EDDA plus porcine (closed circles), human (closed triangles) or bovine (open squares) Tf. Culture turbidity values are the means of triplicates.

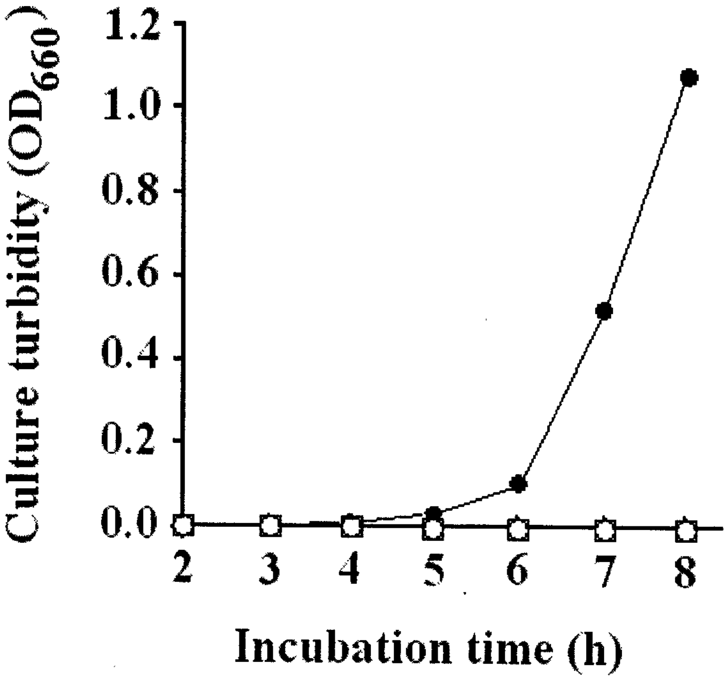


Fig. 2.6. Utilization of Tf-bound iron by *A. suis* strain SO4. *A. suis* was grown in medium containing EDDA (open circles), or EDDA plus porcine (closed circles), human (closed triangles) or bovine (open squares) Tf. Culture turbidity values are the means of triplicates.

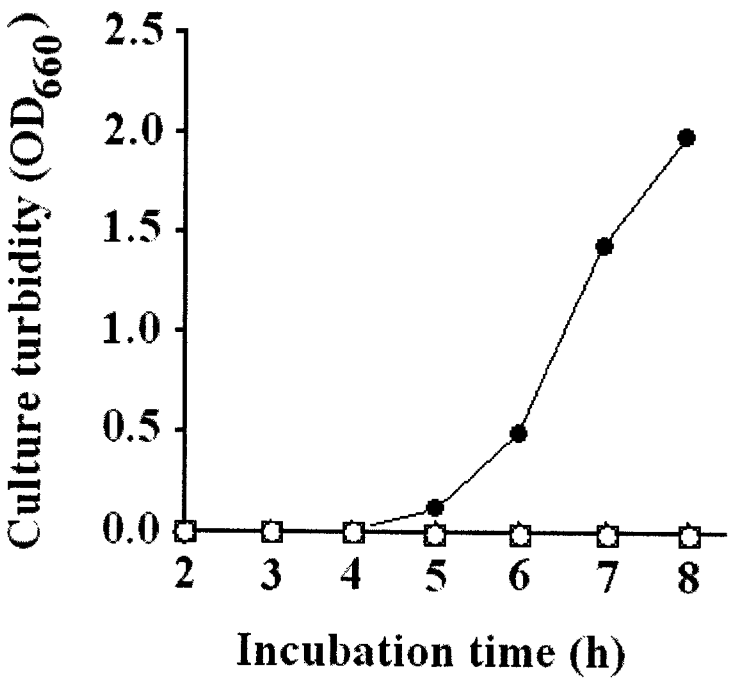


Fig. 2.7. Utilization of Tf-bound iron by *A. suis* strain VSB 3714. *A. suis* was grown in medium containing EDDA (open circles), or EDDA plus porcine (closed circles), human (closed triangles) or bovine (open squares) Tf. Culture turbidity values are the means of triplicates.

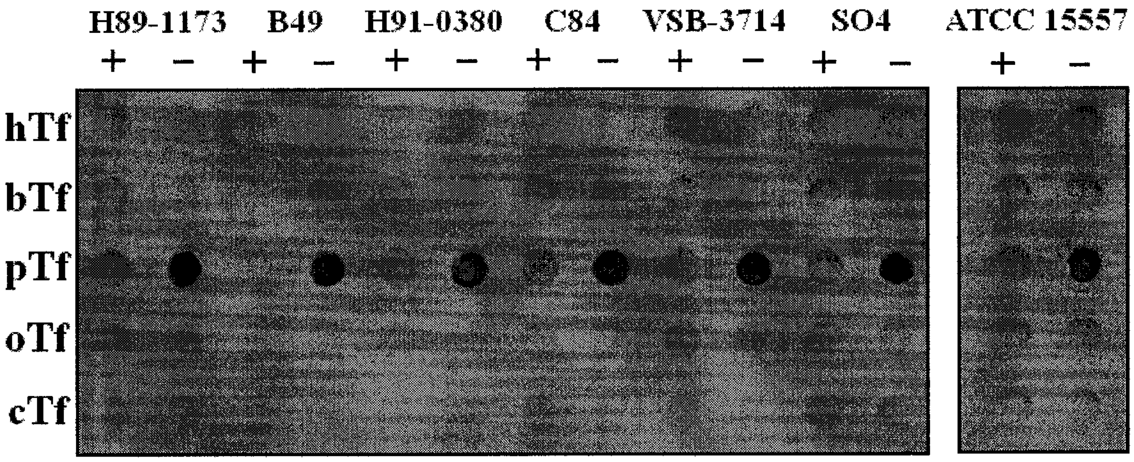


Fig. 2.8. Dot blot depicting binding of the indicated Tfs by total membranes from 7 strains of *A. suis* grown under iron-replete (+) and iron-restricted (–) conditions. The Tfs are abbreviated as follows: h, human; b, bovine; p, porcine; o, ovine; c, caprine.

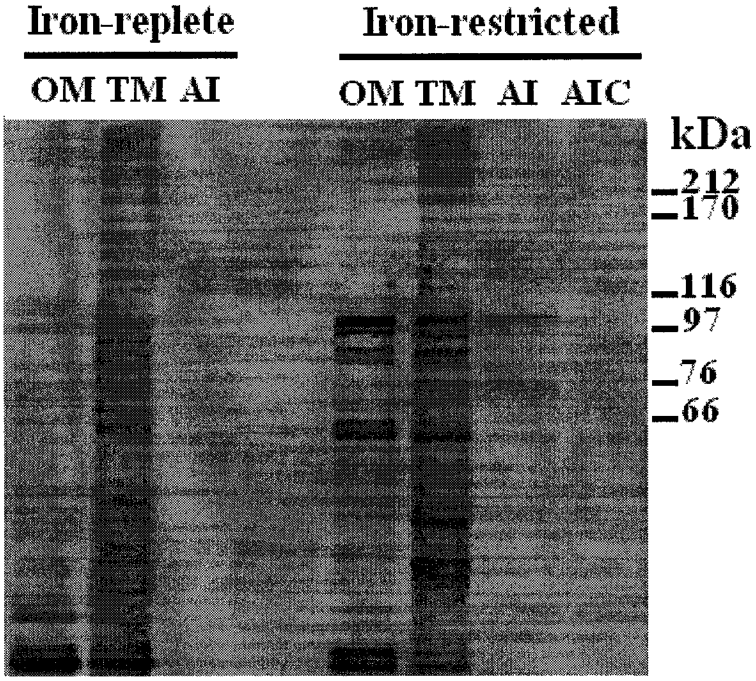


Fig. 2.9. Identification of Tf-binding polypeptides from *A. suis* strain ATCC 15557. SDS-PAGE was performed with total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *A. suis* strain SO4 grown under the indicated conditions, and a control sample (AIC) where the affinity-isolation procedure was performed in the absence of biotinylated Tf.

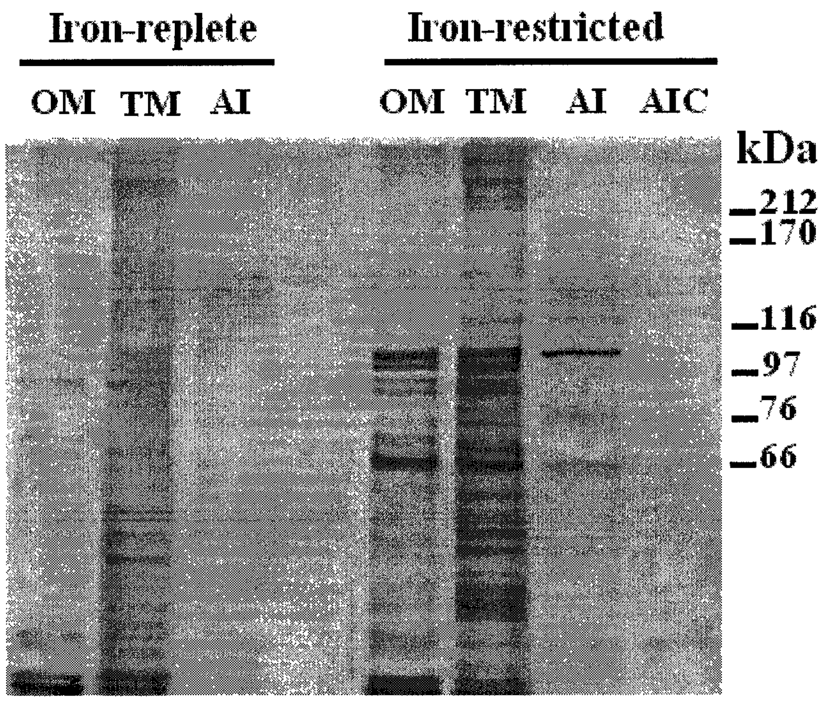


Fig. 2.10. Identification of Tf-binding polypeptides from *A. suis* strain B49. SDS-PAGE was performed with total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *A. suis* strain SO4 grown under the indicated conditions, and a control sample (AIC) where the affinity-isolation procedure was performed in the absence of biotinylated Tf.

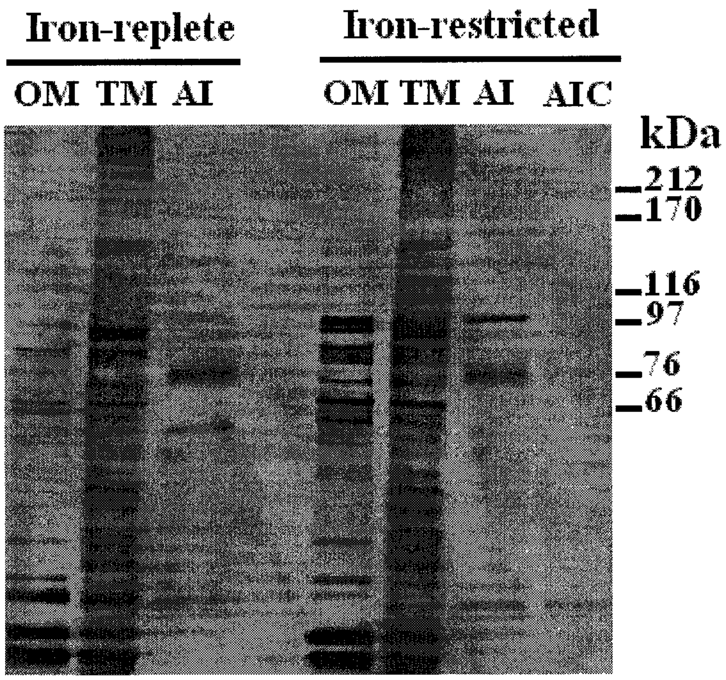


Fig. 2.11. Identification of Tf-binding polypeptides from *A. suis* strain C84. SDS-PAGE was performed with total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *A. suis* strain SO4 grown under the indicated conditions, and a control sample (AIC) where the affinity-isolation procedure was performed in the absence of biotinylated Tf.

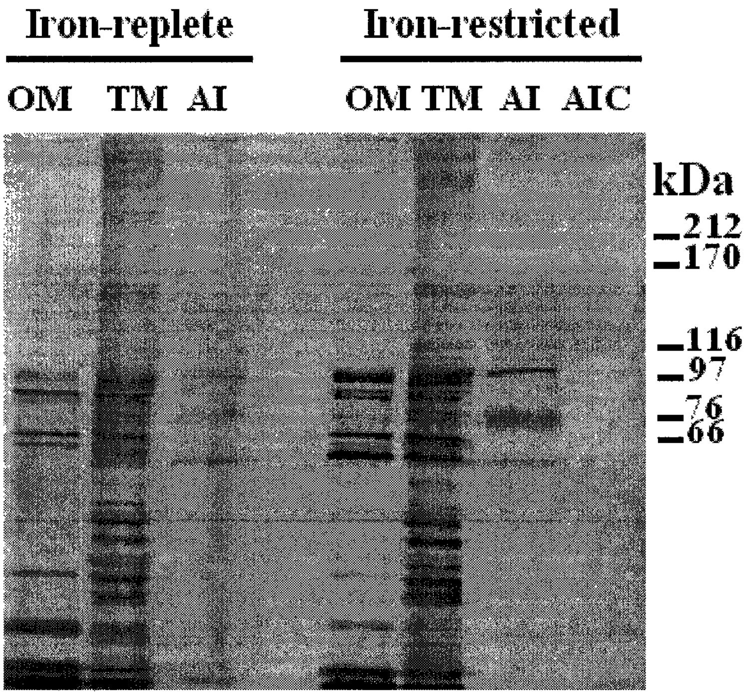


Fig. 2.12. Identification of Tf-binding polypeptides from *A. suis* strain H89-1173. SDS-PAGE was performed with total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *A. suis* strain SO4 grown under the indicated conditions, and a control sample (AIC) where the affinity-isolation procedure was performed in the absence of biotinylated Tf.

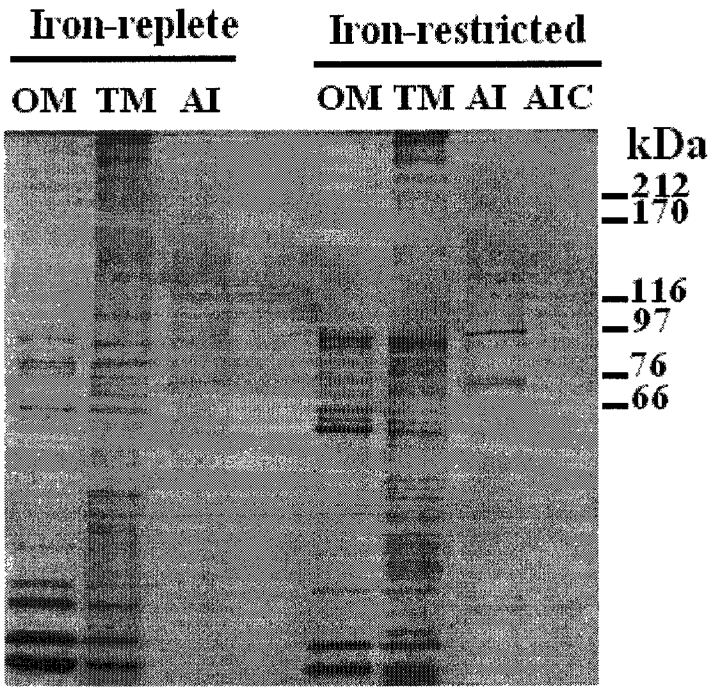


Fig. 2.13. Identification of Tf-binding polypeptides from *A. suis* strain H91-0380. SDS-PAGE was performed with total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *A. suis* strain SO4 grown under the indicated conditions, and a control sample (AIC) where the affinity-isolation procedure was performed in the absence of biotinylated Tf.

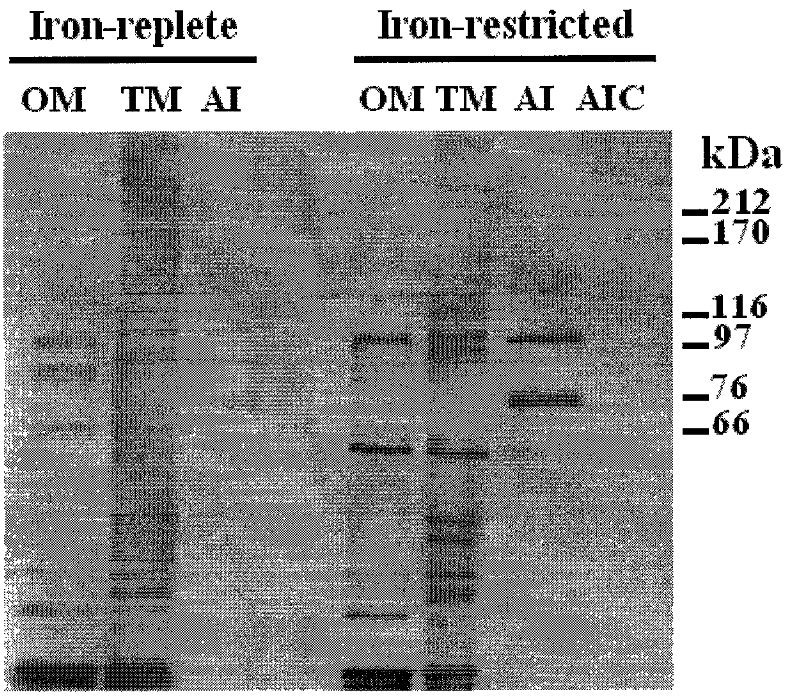


Fig. 2.14. Identification of Tf-binding polypeptides from *A. suis* strain SO4. SDS-PAGE was performed with total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *A. suis* strain SO4 grown under the indicated conditions, and a control sample (AIC) where the affinity-isolation procedure was performed in the absence of biotinylated Tf.

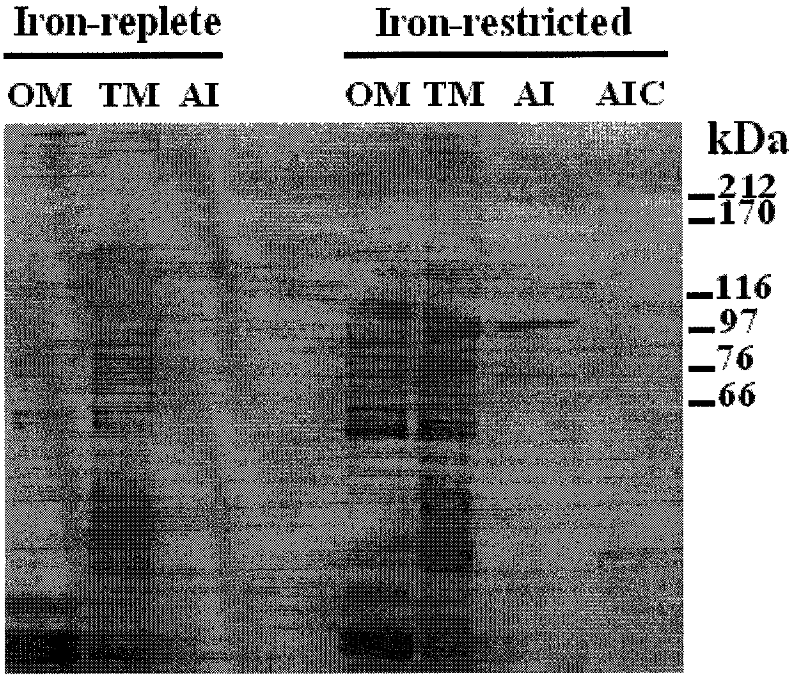


Fig. 2.15. Identification of Tf-binding polypeptides from *A. suis* strain VSB 3714. SDS-PAGE was performed with total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *A. suis* strain SO4 grown under the indicated conditions, and a control sample (AIC) where the affinity-isolation procedure was performed in the absence of biotinylated Tf.

and small (63 kDa) polypeptides (Figs. 2.9-2.15) suggest that these polypeptides represent TbpA and TbpB homologues, respectively. Following transfer to nitrocellulose, the 75-kDa polypeptide reacted strongly with streptavidin-HRP (Fig 2.16) and since it was not present when the affinity-isolation procedure was performed with streptavidin-agarose in the absence of biotinylated Tf (see e.g., Fig. 2.14), it would appear that the 75-kDa polypeptide is almost certainly biotinylated porcine Tf.

2.4.4 Identification and transcription of *tbpA*, *tbpB*, *exbB*, *exbD* and *tonB* homologues

PCR, using genomic DNA from *A. suis* strains C84 and SO4 and primers based initially on the sequences of *exbB* and *tbpA* of *A. pleuropneumoniae*, and later on the sequence data obtained with strain C84, allowed the amplification, and subsequently, the cloning and/or sequencing of *tbpA* (2808 bp), *tbpB* (1791 bp), *exbB* (669 bp), *exbD* (411 bp) and *tonB* (741 bp) homologues from both strains; the acquired sequences spanned 6938 bp and were found to be identical. The organization of these genes is outlined in Fig. 2.17. Notably, while putative Shine-Dalgarno sequences could be identified upstream of all five genes, putative promoter sequences, and a putative Fur box (16 of 19 bases match the *E. coli* consensus, GATAATGATAATCATTATC (see e.g., Escolar *et al.*, 1999)), could be identified upstream of only *tonB* (Fig. 2.17; GenBank accession numbers AY101604 and AY101605; Appendices 1.1 and 1.2). PCR, using primers based on *exbB* and *tbpA* of *A. suis* strains SO4 and C84 and genomic DNA from each of the seven strains of *A. suis*, yielded similarly sized amplicons (Fig. 2.18).

The lack of obvious promoters suggested that *tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* may be transcribed as a single unit, as is believed to be the case in *A. pleuropneumoniae*

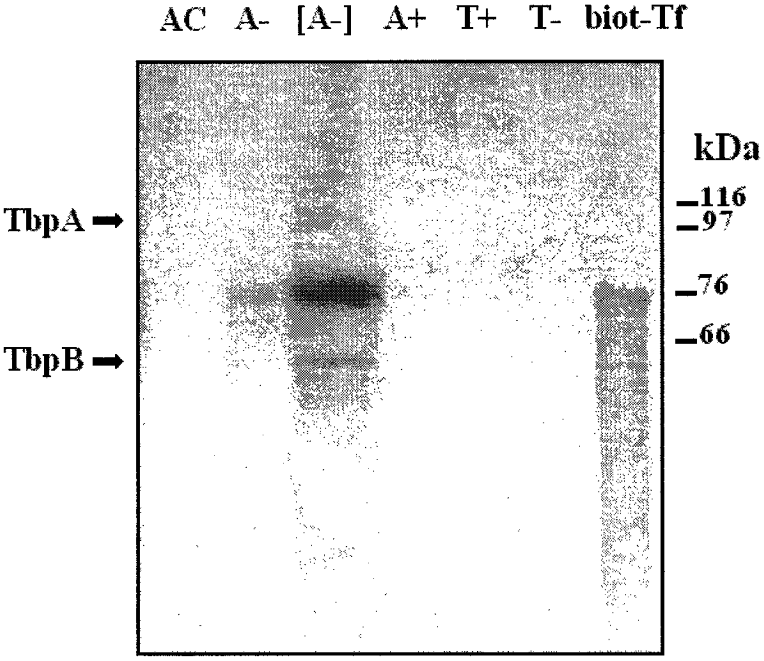


Fig. 2.16. Recognition of the ~75-kDa polypeptide by streptavidin-HRP. Total membranes (T) and affinity-isolated polypeptides (A) from *A. suis* strain SO4 grown under iron replete (+) and iron-restricted (–) conditions, concentrated affinity-isolated polypeptides from *A. suis* strain SO4 grown under iron-restricted conditions ([A-]) and biotinylated porcine Tf (biot-Tf) were subjected to SDS-PAGE. AC represents a control sample where membranes from organisms grown under iron restricted conditions were subjected to the affinity-isolation procedure in the absence of biotinylated Tf. The separated polypeptides were transferred to nitrocellulose, probed with streptavidin-HRP and visualized using 4-chloro-1-naphthol/H_2O_2.

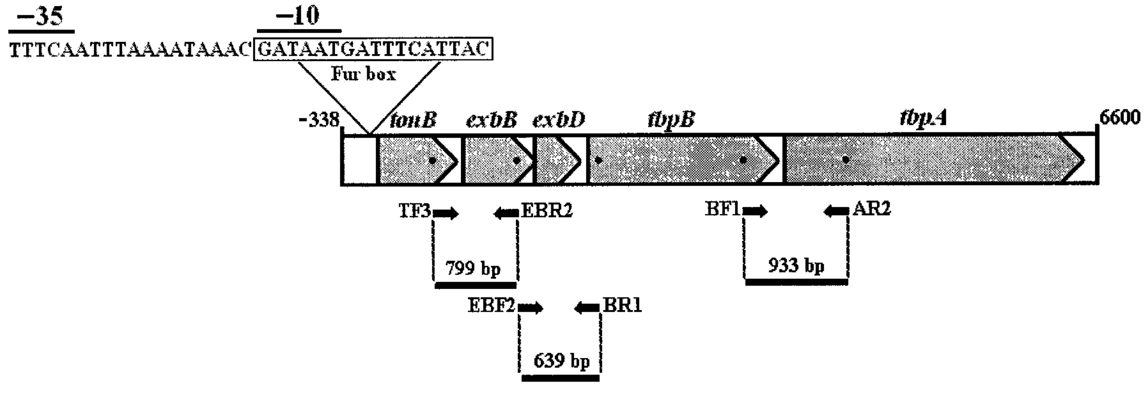


Fig. 2.17. Organization of *tbpA*, *tbpB*, *exbB*, *exbD* and *tonB* in *A. suis* strains SO4 and C84. All five genes are preceded by typical Shine-Dalgarno sequences. The numbers refer to the base pair coordinates relative to the first base of the start codon of *tonB*. The locations and directions of the primers used in RT-PCR are indicated and are represented in the schematic by black dots and arrows, respectively.

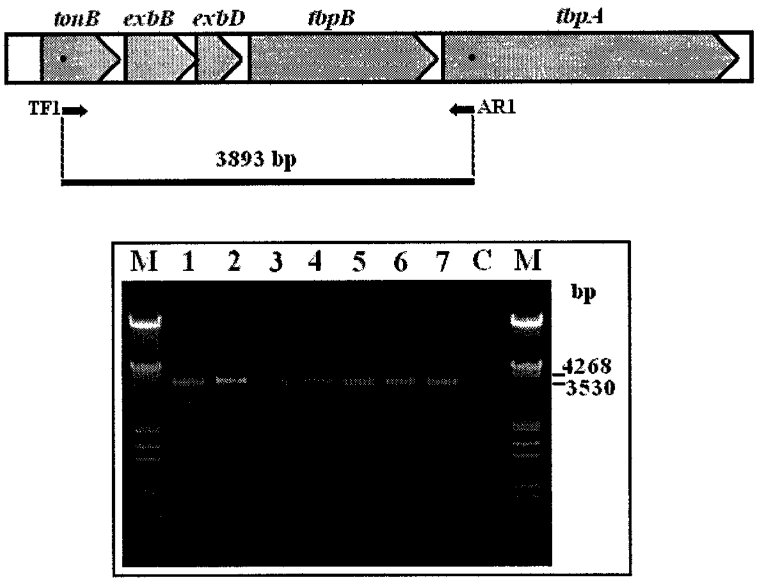


Fig. 2.18. Amplification of *tonB-tbpA* gene clusters. Lanes 1-7 represent the amplicons that were obtained when samples of genomic DNA from *A.* suis strains ATCC 15557, B49, C84, H89-1173, H91-0380, SO4 and VSB 3714, respectively, were used as templates. Lanes M contained DNA Molecular Weight Marker III (0.21-21.2 kbp; Roche Diagnostics) and lane C represents a negative control in which DNA was omitted from the amplification mixture. The locations and directions of the primers used in PCR are indicated in the schematic by black dots and arrows, respectively.

(Tonpitak *et al.*, 2000; Beddek *et al.*, 2004). To investigate this possibility, RT-PCR experiments were performed using RNA from strains C84 and SO4, grown under iron-replete and iron-restricted conditions, and primers based on sequences within *tonB*, *exbB*, *tbpB* and *tbpA*. In all RT-PCR experiments (Figs. 2.19 and 2.20), each primer pair yielded appropriately sized amplification products indicating that *tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* are transcribed as a single unit, and the relative amounts of the products indicated that the expression of these genes is up-regulated in response to iron restriction.

2.4.5 Identification of *exbB2*, *exbD2* and *tonB2* homologues

PCR, using genomic DNA from *A. suis* strains C84 and SO4 and primers based on the sequences of *glpE*, *exbB2*, *exbD2*, *tonB2* and *ybaB* of *A. pleuropneumoniae*, allowed the amplification and sequencing of *exbB2* (453 bp), *exbD2* (390 bp) and *tonB2* (846 bp) homologues from both strains; the acquired sequences spanned 2046 bp and were found to be identical. The organization of these genes is outlined in Fig. 2.21. PCR, using primers based on *exbB2* and *tonB2* of *A. suis* strains SO4 and C84 and genomic DNA from each of the seven strains of *A. suis*, yielded similarly sized amplicons (Fig. 2.21). Comparative analyses revealed that the inferred ExbB2, ExbD2 and TonB2 proteins of *A. suis* share 97%, 96% and 84% identity with the analogous predicted proteins of *A. pleuropneumoniae* (GenBank accession number AY428647), respectively.

2.4.6 Comparative analysis of the predicted TbpA and TbpB proteins

The nucleotide sequences of the comparable *tbp* genes of strains C84 and SO4, and hence, the amino acid sequences of the comparable, predicted Tbp precursor proteins, were found to be identical. BlastP analyses revealed that while the predicted TbpA

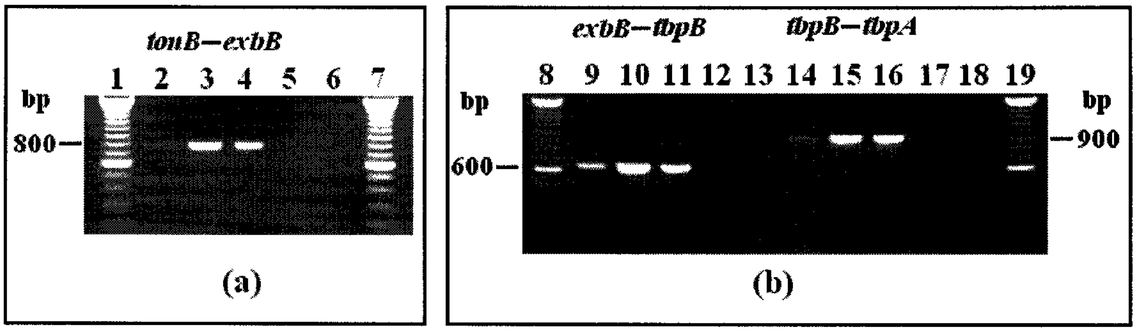


Fig. 2.19. RT-PCR with RNA isolated from *A. suis* strain C84 grown under iron-replete (lanes 2, 9 and 14) or iron-restricted (lanes 3, 10 and 15) conditions, or with total DNA (lanes 4, 11 and 16). Lanes 5, 6, 12, 13, 17 and 18 represent negative controls in which RNA from organisms grown under iron-replete (lanes 5, 12 and 17) or iron-restricted (lanes 6, 13 and 18) conditions served as templates in PCR experiments. The primers were chosen to amplify (a) a 799-bp *tonB-exbB* fragment and (b) a 639-bp *exbB-tbpB* fragment (lanes 9-13) or a 933-bp *tbpB-tbpA* fragment (lanes 14-18). Lanes 1, 7, 8 and 19 contained a 100-bp DNA ladder.

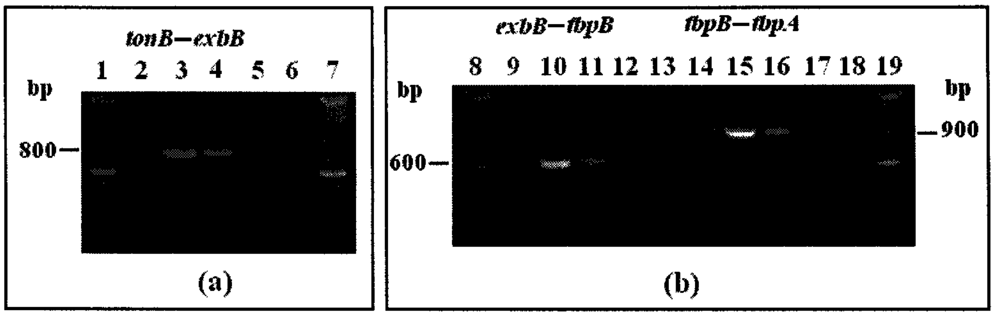


Fig. 2.20. RT-PCR with RNA isolated from *A. suis* strain SO4 grown under iron-replete (lanes 2, 9 and 14) or iron-restricted (lanes 3, 10 and 15) conditions, or with total DNA (lanes 4, 11 and 16). Lanes 5, 6, 12, 13, 17 and 18 represent negative controls in which RNA from organisms grown under iron-replete (lanes 5, 12 and 17) or iron-restricted (lanes 6, 13 and 18) conditions served as templates in PCR experiments. The primers were chosen to amplify (a) a 799-bp *tonB-exbB* fragment and (b) a 639-bp *exbB-tbpB* fragment (lanes 9-13) or a 933-bp *tbpB-tbpA* fragment (lanes 14-18). Lanes 1, 7, 8 and 19 contained a 100-bp DNA ladder.

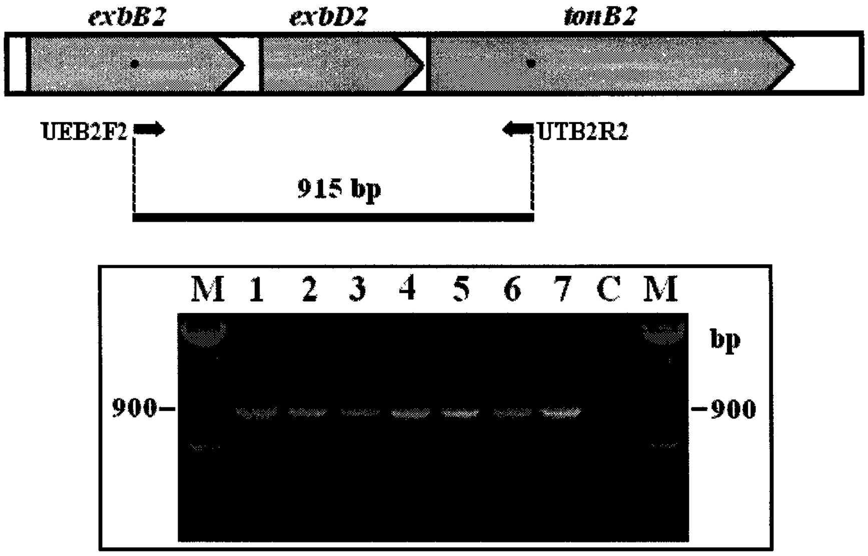


Fig. 2.21. Amplification of *exbB2-tonB2* gene clusters. Lanes 1-7 represent the amplicons that were obtained when samples of genomic DNA from *A.* suis strains ATCC 15557, B49, C84, H89-1173, H91-0380, SO4 and VSB 3714, respectively, were used as templates. Lanes M contained a 100-bp DNA ladder and lane C represents a negative control in which DNA was omitted from the amplification mixture. The locations and directions of the primers used in PCR are indicated in the schematic by black dots and arrows, respectively.

precursor proteins of *A. suis* share 90% and 98% identity with the predicted TbpA precursors of *A. pleuropneumoniae* strains H49 (GenBank accession number U16017) and H171 (GenBank accession number U16019), respectively, the predicted TbpB precursor proteins of *A. suis* and a variety of *A. pleuropneumoniae* strains share only 54-86% identity. Putative cleavage sites, resulting in mature TbpA and TbpB proteins, were identified using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen *et al.*, 2004); the molecular masses of the predicted, mature TbpA and TbpB proteins of *A. suis* were calculated (Compute pI/Mw; http://us.expasy.org/tools/pi_tool.html) to be 104.3 kDa and 63.4 kDa, respectively, suggesting strongly that the affinity-isolated, 100- and 63-kDa Tf-binding polypeptides do represent TbpA and TbpB.

2.5 Discussion

Seven strains of *A. suis* were investigated with respect to iron acquisition from animal Tfs. All strains could use porcine, but not human or bovine, Tf as an iron source for growth and in keeping with the results of Schryvers and Gonzalez (1990), total membranes from all strains bound, specifically, porcine Tf, but only if the membranes were from organisms grown under iron-restricted conditions. Collectively, these results indicated that the acquisition of Tf-bound iron by *A. suis* does not involve siderophores and suggested the involvement of iron-repressible receptors analogous to the Tbps (TbpA and TbpB) of *A. pleuropneumoniae* and other members of the *Pasteurellaceae*. Subsequently, an affinity procedure allowed the isolation of two putative Tf-binding polypeptides, with estimated molecular masses of 100 kDa and 63 kDa, from membranes derived from all strains, and the sizes, iron-repressibility and outer membrane location of these polypeptides allowed them to be identified, tentatively, as TbpA and TbpB, respectively; TbpA proteins, typically, have molecular masses of ~100 kDa (Gray-Owen and Schryvers, 1996) and at least in *A. pleuropneumoniae*, the TbpBs have molecular masses of ~56-65 kDa (Gonzalez *et al.*, 1990; Ricard *et al.*, 1991; Gerlach *et al.*, 1992*b*). The apparent similarities in the Tbps of *A. suis* and *A. pleuropneumoniae* suggested that oligonucleotides based on the sequences of *tbpA* and/or *tbpB* of *A. pleuropneumoniae* might enable the detection and amplification of comparable genes of *A. suis*. Indeed, PCR, using genomic DNA from *A. suis* strains C84 and SO4 and primers based initially on the sequences of *tbpA* (and *exbB*) of *A. pleuropneumoniae*, and later, on the sequence data obtained with strain C84, allowed the amplification, and subsequently, the cloning and/or sequencing of *tbpA* and *tbpB* homologues from both strains. The predicted products were found to be homologues of the TbpA (≥ 90% identity) and TbpB (54-86% identity) precursors of *A. pleuropneumoniae* and the molecular masses of the predicted, mature TbpA and TbpB proteins of *A. suis* were calculated to be 104.3 kDa and 63.4 kDa, respectively, providing additional support for the conclusion that the affinity-isolated, 100- and 63-kDa Tf-binding polypeptides do represent TbpA and TbpB. Interestingly, in *A. pleuropneumoniae*, there would appear to be two distinct groups of TbpB proteins with one group, represented by the 65-kDa TbpB of strain AP37 (serotype 1), being typical of serotypes 1, 6 and 12 and the other, represented by the 60-kDa TbpB of strain AP205 (serotype 7), being typical of serotypes 2, 3, 4, 7, 8, 9, 10 and 11 (Gerlach *et al.*, 1992*b*). Such heterogeneity was not observed with the TbpBs of *A. suis*; the deduced amino acid sequences of the TbpBs of strains SO4 and C84 were identical as were the apparent molecular masses of the TbpBs isolated from all seven strains. It is also notable that the predicted TbpB precursors of strains SO4 and C84 share 86% identity with the predicted

TbpB precursor of *A. pleuropneumoniae* strain AP37 (GenBank protein accession number B44796) but only 55% identity with the predicted TbpB precursor of *A. pleuropneumoniae* strain AP205 (GenBank protein accession number A44796).

Sequencing of *tbpA*, *tbpB* and sequences upstream of both, revealed that in *A. suis* strains SO4 and C84, as in most other organisms investigated (Legrain *et al.*, 1993; Gonzalez *et al.*, 1995; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997), *tbpB* is located upstream of *tbpA* and both genes are preceded by typical Shine-Dalgarno sequences. However, while *tbpA* and *tbpB* are usually arranged as an operon (*tbpBA*) with the promoter and regulatory (Fur box) sequences being located immediately upstream of *tbpB* (Legrain *et al.*, 1993; Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997), and while the expression of the *tbp* genes in *A. suis* is clearly iron-repressible, the sequence upstream of *tbpB* in *A. suis* lacked any obvious promoter sequence or a Fur box. This suggested that *tbpBA* in *A. suis*, as in *A. pleuropneumoniae* (Tonpitak *et al.*, 2000), may be part of a larger operon that includes *tonB* and *exbBD* and sequencing upstream of the *tbpB* genes of strains SO4 and C84 was continued. The acquired sequences confirmed the existence of *tonB*, *exbB* and *exbD* homologues, arranged in such order, in both strains. The comparable sequences were found to be identical and the sequence (1290 bp) from inside *tonB* to the start codon of *tbpB*, and the comparable sequence from *A. pleuropneumoniae* strain AP205 (GenBank accession number Y17916) were found to differ at only three positions, one within *tonB* and the others, adjacent to each other, within *exbD* . In addition, PCR, using primers based on *exbB*, *tbpB* and *tbpA* of *A. suis* strains SO4 and C84 and genomic DNA from each of the seven strains of *A. suis* yielded comparable products indicating that all seven strains of *A. suis*, like *A. pleuropneumoniae* (Tonpitak *et al.*, 2000), possess *exbB*, *exbD*, *tbpB* and *tbpA*, in that order. Similarly, PCR, using primers based on *exbB2* and *tonB2* of *A. suis* strains SO4 and C84, revealed that all seven strains of *A. suis*, like *A. pleuropneumoniae* (Beddek *et al.*, 2004), possess *exbB2*, *exbD2* and *tonB2*. Putative Shine-Dalgarno sequences preceded the *tonB*, *exbB* and *exbD* genes of both strains of *A. suis* and in keeping with the suggestion that *tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* are present on the same operon, there were no obvious promoter elements upstream of *exbB* or *exbD*. RT-PCR experiments also revealed that all five genes can be transcribed as a single unit and since the transcription of these genes was up-regulated in response to iron restriction, it would appear that the expression of these genes must be regulated by the iron-regulated promoter upstream of *tonB*. Notably, it has been suggested that the expression of *tonB-exbBD-tbpBA* by *A. pleuropneumoniae* is regulated similarly (Tonpitak *et al.*, 2000). While this might suggest that the acquisition of iron from Tf, and perhaps the receptor-mediated acquisition of iron from other sources, by *A. pleuropneumoniae* and *A. suis* may be mediated by a dedicated TonB system, it would appear that the situation may, in fact, be more complex than this in that the acquisition of Tf-bound iron by *A. pleuropneumoniae* at least, requires TonB2 (Beddeck *et al.*, 2003).

In conclusion, the results presented here indicate that the acquisition of Tf-bound iron by *A. suis* involves genes, proteins and mechanisms that are analogous to those found in other members of the *Pasteurellaceae* and in particular, in *A. pleuropneumoniae*.

Connecting statement 1

In the previous chapter, it was reported that *A. suis* can acquire iron from Tf but only if the Tf is of host origin. Acquisition of Tf-bound iron was shown to be siderophore-independent and receptor-mediated and to involve two-component (TbpA/TbpB) iron-repressible receptors. The next chapter reports on the ability of *A. suis* to acquire host iron from a second potential source, Hb.

Chapter 3. Identification and characterization of a single-component haemoglobin receptor and encoding gene

This chapter was adapted from the following, with permission from Elsevier and the co-author:

Bahrami, F., and Niven, D.F. 2005. Iron acquisition by *Actinobacillus suis*: identification and characterization of a single-component haemoglobin receptor and encoding gene. Microb. Pathog. **39:** 45-51.

3.1 Abstract

A. suis is an important swine pathogen. As with other pathogens, the ability of *A. suis* to acquire iron within the host is crucial for virulence. Here, the ability of seven strains of *A. suis* to acquire iron from Hbs was investigated. In growth assays, all strains could use porcine, bovine and human Hbs as iron sources for growth. Using solid phase binding assays, membranes derived from all strains, grown under iron-restricted conditions, were shown to bind all three Hbs. Competition binding assays indicated that these Hbs were bound by the same receptor and an affinity procedure allowed the isolation and identification of an iron-repressible, Hb-binding polypeptide (~105 kDa) from all strains. Nucleotide sequence analyses revealed that *A. suis* possesses a gene (*hgbA*) that encodes a homologue of the *A. pleuropneumoniae* Hb-binding protein, HgbA. *hgbA*, encoding a mature protein of 105 kDa, was shown to be preceded by a *hugZ* homologue; putative promoter sequences and a putative Fur box were located upstream of *hugZ* and RT-PCR revealed that *hugZ* and *hgbA* are co-transcribed and iron-repressible. It

is concluded that the acquisition of Hb-bound iron by *A. suis* involves a single-component receptor that is up-regulated in response to iron restriction.

3.2 Introduction

The family *Pasteurellaceae* contains a number of iron-requiring pathogens of animals. Although iron is plentiful in animal hosts, it is not readily available to pathogens due to the iron-withholding properties of Tf and Lf (see e.g., Griffiths, 1987; Gray-Owen and Schryvers, 1996). Despite this, pathogens are still able to obtain the iron that they require and like the pathogenic neisseriae, several members of the *Pasteurellaceae* are known to acquire iron directly from host Tfs, typically, by means of bipartite receptors containing the Tf-binding proteins, TbpA and TbpB (see e.g., Gray-Owen and Schryvers, 1996). While Hb represents another potential source of iron for pathogenic bacteria, studies pertaining to the acquisition of Hb-bound iron by members of the *Pasteurellaceae* have been confined, largely, to *P. multocida* (Bosch *et al.*, 2002; Cox *et al.*, 2003), the haemophili, *H. influenzae* and *H. ducreyi* (see e.g., Elkins *et al.*, 1995; Ren *et al.*, 1998; Jin *et al.*, 1999; Morton *et al.*, 1999; Cope *et al.*, 2000), and most recently, *A. pleuropneumoniae* (Srikumar *et al.*, 2004). Once again, iron acquisition is receptor-mediated but all of the receptors that have been identified to date are single-component; while *H. influenzae* and *P. multocida* are known to produce at least two distinct Hb receptors (Morton *et al.*, 1999; Cope *et al.*, 2000; Cox *et al.*, 2003), *H. ducreyi* and *A. pleuropneumoniae* produce only one (HgbA) (Elkins *et al.*, 1995; Srikumar *et al.*, 2004). Notably, the Hb receptors produced by *P. multocida* (HgbA and HgbB) and *A. pleuropneumoniae* (HgbA) are iron-repressible and putative Fur boxes have been identified immediately upstream of *P. multocida hgbB* (Cox *et al.*, 2003) and *A.*

pleuropneumoniae hgbA (Srikumar *et al.*, 2004); in *P. multocida*, *hgbA* is on an operon that includes *P. shigelloides hugX* and *hugZ* homologues, both upstream of *hgbA*, and the putative Fur box is located upstream of *hugX* (Bosch *et al.*, 2002). In contrast, the Hb receptors of *H. influenzae* and *H. ducreyi* appear to be repressible by haem and/or iron (Frangipane *et al.*, 1994; Elkins, 1995; Hasan *et al.*, 1997). Interestingly, putative Fur boxes have been located immediately upstream of the genes encoding several of these proteins (Elkins *et al.*, 1995; Ren *et al.*, 1998; Jin *et al.*, 1999; Cope *et al.*, 2000) but the role of Fur in their expression remains to be established.

A. suis is a swine pathogen that is gaining prominence as a multifaceted agent of disease, especially in high-health-status herds (see e.g., MacInnes and Desrosiers, 1999; Taylor, 1999; Rycroft and Garside, 2000; Mauch and Bilkei, 2004). While the virulence determinants of *A. suis* are not well defined, lipopolysaccharides, capsular polysaccharides and RTX toxins are believed to play important roles in pathogenesis (MacInnes and Desrosier, 1999; Slavić *et al.*, 2000*a*,*b*). The ability of *A. suis* to obtain iron from the host is also important for pathogenesis and in this context, it has been demonstrated that *A. suis* can obtain iron from porcine Tf by using mechanisms analogous to those described for other members of the *Pasteurellaceae* (Bahrami *et al.*, 2003; Chapter 2). Nothing, however, is known regarding iron acquisition from other host components. With this in mind, the objectives of the present study were to determine if *A. suis* can acquire iron from Hb, and if so, to characterize at least some of the components involved in this process.

3.3 Materials and methods

3.3.1 Organisms and storage conditions

The *A. suis* strains, and the methods used for the preparation and storage of cultures and inocula, were as described above (section 2.3.1).

3.3.2 Haemoglobins and conjugates

Human, bovine and porcine Hbs, and bovine Hb-agarose, were from Sigma-Aldrich. For growth assays, the human, bovine and porcine Hbs were dissolved (to ~10 mg/ml) in 2 mM $NaHCO_3$, 40 mM Tris/HCl, pH 7.4 and 10-ml volumes were dialyzed (Spectra/Por dialysis membrane, Spectrum Medical Industries; 6000-8000 MWCO) against 145 mM NaCl, 5 mM Tris/HCl, pH 7.4 (3 × 1-l volumes, 4 °C). Following dialysis, the Hb preparations were sterilized by filtration (0.22 μm pore size) and the protein concentrations then estimated by *DC* Protein Assay (Bio-Rad). For biotinylation, the human, bovine and porcine Hbs were dissolved (to ~1 mg/ml) in phosphate-buffered saline (PBS; 0.17 M NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and dialyzed against PBS, as above. The dialyzed Hbs were then biotinylated using an ECL Protein Biotinylation Module (protocol A) as directed by the manufacturer (Amersham Biosciences).

3.3.3 Growth conditions and preparation of membranes

To investigate the abilities of the *A. suis* strains to acquire iron from Hbs, the organisms were grown under iron-replete (TYE-H/HCO_3) and iron-restricted (TYE-H/HCO_3 containing 50 μM EDDA) conditions in the presence and absence of each Hb

(80 µg/ml), as described above for analogous experiments with Tfs (section 2.3.3). For the preparation of membrane fractions, the organisms were grown to early stationary phase under iron-replete and iron-restricted conditions, as above (section 2.3.3). The organisms were then harvested, disrupted by sonication and fractioned to yield total membrane and outer membrane preparations essentially as described by Niven *et al.* (1989); protein concentrations were estimated by *DC* Protein Assay.

3.3.4 Solid phase binding assays

Hb-binding assays and competition assays were performed essentially as described by Ekins and Niven (2001). Briefly, total membranes were diluted (to 0.05 mg/ml protein) in TBS and applied (50-µl volumes) to nitrocellulose sheets (0.45 µm pore size; Bio-Rad) held in a dot blot apparatus (Minifold I). Non-specific binding sites were saturated by incubating the nitrocellulose sheets (1 h, 37 °C, gentle agitation) in TTBS. An appropriate biotinylated Hb (5 µg in 250 µl TTBS), or biotinylated Hb (5 µg) plus native Hb (0.1 mg) (250 µl TTBS), was added to each well and following incubation (1 h, 37 °C), the Hb solutions were removed and each well rinsed individually with 500 µl TBS. The nitrocellulose sheets were then subjected to three 10-min washes with TBS (37 °C, gentle agitation), incubation (1 h, 37 °C, gentle agitation) with ImmunoPure streptavidin-HRP (Pierce; diluted 1:1000 in TTBS) and a final three washes with TBS. Reactions were visualized using 4-chloro-1-naphthol and H_2O_2, as previously described (Niven *et al.*, 1989).

3.3.5 Isolation and identification of haemoglobin-binding polypeptides

Hb-binding polypeptides were isolated from total membranes using a modification of a previously described protocol (Elkins, 1995). Briefly, total membranes (~750 µg protein) from cells grown under iron-replete and iron-restricted conditions were solubilized in 1 % Zwittergent 3-14 (Calbiochem), 50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5 (2 h, 37 °C, gentle rocking). These samples (~1-ml volumes) were subjected to centrifugation (14000×*g*, 10 min) and the resulting soluble fractions were mixed with bovine Hb-agarose (100-µl volumes) and incubated (2 h, room temperature, gentle rocking). The Hb-agarose and bound proteins were sedimented by centrifugation (1100×*g*, 3 min), washed once by resuspension and incubation (10 min, room temperature) in, and centrifugation (1100×*g*, 3 min) from, 1 % Zwittergent 3-14, 50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5 and then twice more using a high-salt buffer (1 % Zwittergent 3-14, 50 mM Tris/HCl, 1M NaCl, 5 mM EDTA, pH 7.5) to promote the removal of non-specifically bound proteins. The resulting pellets were resuspended in 2 % SDS, 200 mM Tris/HCl (pH 6.8), immersed in boiling water (5 min), cooled on ice, and centrifuged (1100×*g*, 3 min). Supernatant fractions were subjected to SDS-PAGE and silver staining, as described above (section 2.3.5).

For N-terminal amino acid sequence analyses, Hb-binding polypeptides were affinity-isolated from membranes of *A. suis* strain SO4, grown under iron-restricted conditions, and the polypeptides in the supernatant fractions from 10 or 12 affinity isolations were concentrated using Ultrafree-0.5 Biomax-30 (30 kDa MWCO) Centrifugal Filter Devices (Millipore; 12000×*g*, 30 min). The concentrated polypeptides (~0.2 mg) were subjected to SDS-PAGE and transferred to Sequi-Blot PVDF membranes (Bio-Rad; 50 V, 24 h) as described by the manufacturer of the PVDF membranes except that the transfer buffer was that described in section 2.3.5. An ~105-kDa, PVDF-bound polypeptide was visualized by means of staining with Coomassie Brilliant Blue R-250 (Bio-Rad) and was subjected to N-terminal amino acid sequence analysis (Sheldon Biotechnology Centre, McGill University) by repetitive cycles of Edman degradation followed by detection of the PTH-derivatized amino acids using an on-line C_{18} HPLC column and an Applied Biosystems 492 gas-phase/pulsed-liquid Procise Automated Sequencer.

3.3.6 Amplification and sequencing of receptor and affiliated genes

Primers M+ and M– (Table 3.1), based on the nucleotide sequence of a Hb receptor gene of *A. pleuropneumoniae*, were generously provided by Dr. J. W. Coulton (McGill University). Custom-made primers were from Invitrogen Life Technologies and the Expand High Fidelity PCR System was used for all DNA amplifications. Genomic DNA was isolated from *A. suis* strains C84 and SO4, as described above (section 2.3.6). Using DNA from strain C84 as template, and primers M+ and M–, a 495-bp fragment of *A. suis* DNA was PCR amplified, and cloned and sequenced, using standard procedures. The sequence of this fragment was then used to locate a homologous fragment in the publicly available *A. pleuropneumoniae* serovar 1 strain 4074 genome (http://microgen.ouhsc.edu/a_pleuro/a_pleuro_home.htm). Using primers based on the ORF encompassing this fragment, and genomic DNA from *A. suis* strains SO4 and C84 as templates, a region corresponding to ~80 % of *hgbA* of *A. pleuropneumoniae* (GenBank accession number AF468020) was PCR amplified from both DNA samples; the PCR products were purified (QIAquick PCR Purification Kit) and then sequenced

Table 3.1. Primers used in PCR and RT-PCR

Primer	Description	Sequence (5' → 3')
M+	Ap[a] *hgbA*; forward	CGGAAGAAGTCATTACTTG
M-	Ap *hgbA*; reverse	GTGTAATCCGTTATGCCAT
RTF	As[b] *hugZ*; forward	GGTAATCCGAATGTGAGTTATGC
RTR	As *hgbA*; reverse	CGTGAGTCTTGTACTTGCTG

[a] *A. pleuropneumoniae*

[b] *A. suis*

using standard procedures. Primers based on the acquired sequence information and genomic DNA from *A. suis* strain C84 were then used to initiate direct genome sequencing (Heiner *et al.*, 1998) farther upstream and downstream of the C84 *hgbA* fragment, and subsequently, the SO4 *hgbA* fragment. Primers based on these extended sequences and genomic DNA from strains SO4 and C84 were then used to amplify fragments corresponding to the regions upstream and downstream of the sequenced *hgbA* fragments. The resulting amplicons were purified and used as templates for double-stranded sequencing according to standard protocols. For comparative purposes, the *hugZ-hgbA* gene clusters (673 bp) of all seven strains were amplified using conventional PCR, primers RTF and RTR (Table 3.1) and *Taq* polymerase (Invitrogen Life Technologies). Sequence analyses were performed by the Applied Biotechnology Laboratory, McGill University. Sequence data were assembled into contiguous segments using Sequencher 3.0 and submitted to GenBank (accession numbers AY335901 and AY335902; Appendices 1.5 and 1.6). Putative cleavage sites in predicted, precursor proteins were identified using SignalP 3.0 and the molecular masses of predicted proteins were calculated using Compute pI/Mw.

3.3.7 RT-PCR

A. suis strains C84 and SO4 were grown under iron-replete and iron-restricted conditions, and RNA was extracted and purified, as described above (section 2.3.7). RT-PCR was carried out with the OneStep RT–PCR Kit, as recommended by the manufacturer, with 20 ng of the appropriate RNA sample, or an aliquot of DNA (positive controls), and primers RTF and RTR (Table 3.1); in RT–PCR with DNA as template, the RTF-RTR primer pair was expected to amplify a 673-bp *hugZ–hgbA* fragment. PCR, with the appropriate primers and RNA preparations as template material, served as negative controls. Products were analyzed by means of gel electrophoresis (1 % agarose) and staining with ethidium bromide.

3.3.8 Imaging

Images were obtained as described in section 2.3.8.

3.4 Results

3.4.1 Acquisition of haemoglobin-bound iron and binding of haemoglobins by *A. suis*

Liquid cultures were used to investigate the abilities of the seven strains of *A. suis* to acquire iron from human, bovine and porcine Hbs. All strains acquired iron from all three Hbs and with each strain, growth on all three Hbs exhibited similar kinetics (Figs. 3.1-3.7). In solid phase binding assays, total membranes derived from all seven strains, grown under both iron-replete and iron-restricted conditions, bound all three biotinylated Hbs, human, bovine and porcine, but membranes from organisms grown under iron-restricted conditions exhibited greater binding than those from iron-replete organisms (Figs. 3.8-3.14). Collectively, these results indicate that the acquisition of Hb-bound iron by *A. suis* involves iron-repressible receptors. Excess native human, bovine and porcine Hbs were also able to compete effectively with each of the biotinylated Hbs for the binding sites on the membranes indicating that all three Hbs are recognized and bound by the same receptor(s) (Figs. 3.8-3.14).

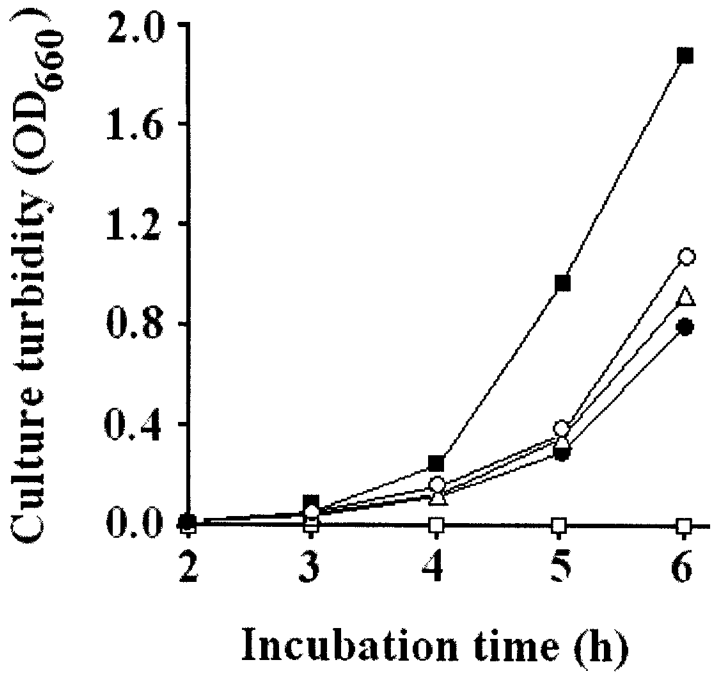


Fig. 3.1. Utilization of Hb-bound iron by *A. suis* strain ATCC 15557. *A. suis* was grown under iron-replete (closed squares) and iron-restricted (open squares) conditions and under iron-restricted conditions in the presence of porcine (closed circles), bovine (open circles) or human (open triangles) Hb. Culture turbidity values are the means of triplicates.

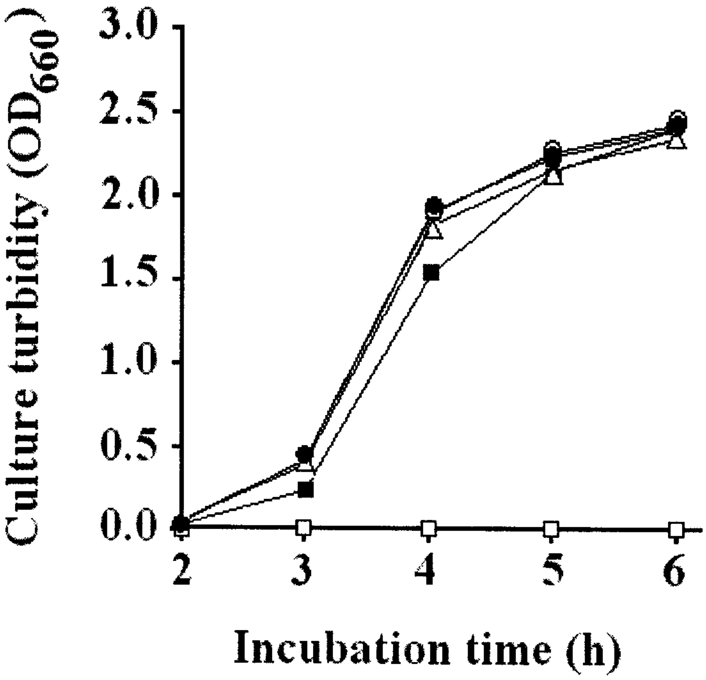


Fig. 3.2. Utilization of Hb-bound iron by *A. suis* strain B49. *A. suis* was grown under iron-replete (closed squares) and iron-restricted (open squares) conditions and under iron-restricted conditions in the presence of porcine (closed circles), bovine (open circles) or human (open triangles) Hb. Culture turbidity values are the means of triplicates.

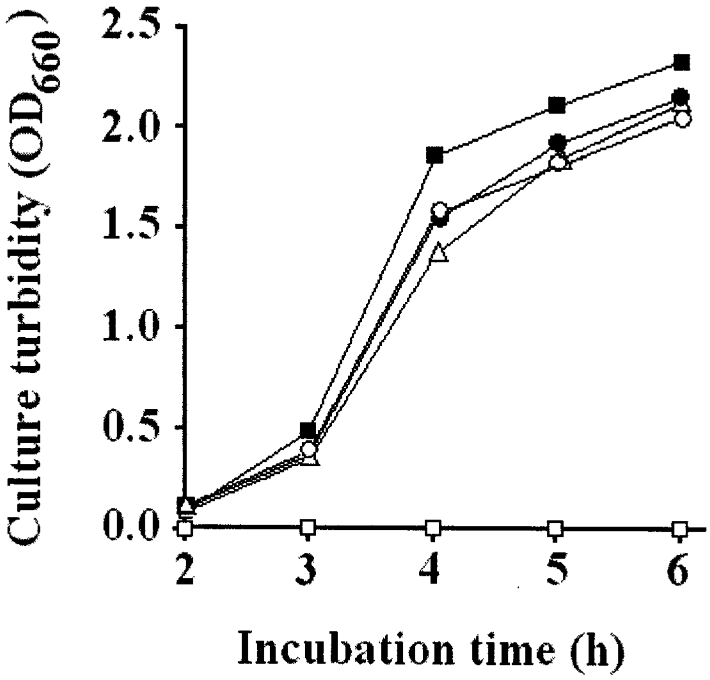


Fig. 3.3. Utilization of Hb-bound iron by *A. suis* strain C84. *A. suis* was grown under iron-replete (closed squares) and iron-restricted (open squares) conditions and under iron-restricted conditions in the presence of porcine (closed circles), bovine (open circles) or human (open triangles) Hb. Culture turbidity values are the means of triplicates.

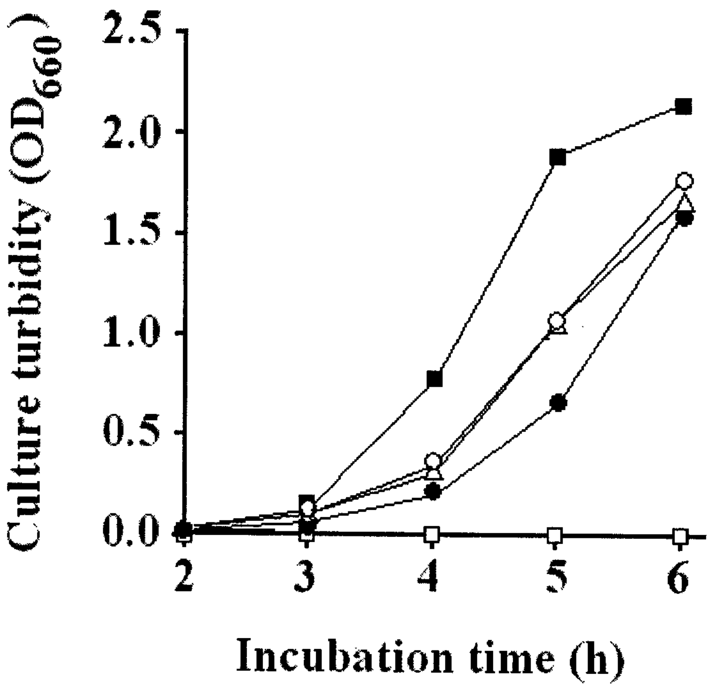


Fig. 3.4. Utilization of Hb-bound iron by *A. suis* strain H89-1173. *A. suis* was grown under iron-replete (closed squares) and iron-restricted (open squares) conditions and under iron-restricted conditions in the presence of porcine (closed circles), bovine (open circles) or human (open triangles) Hb. Culture turbidity values are the means of triplicates.

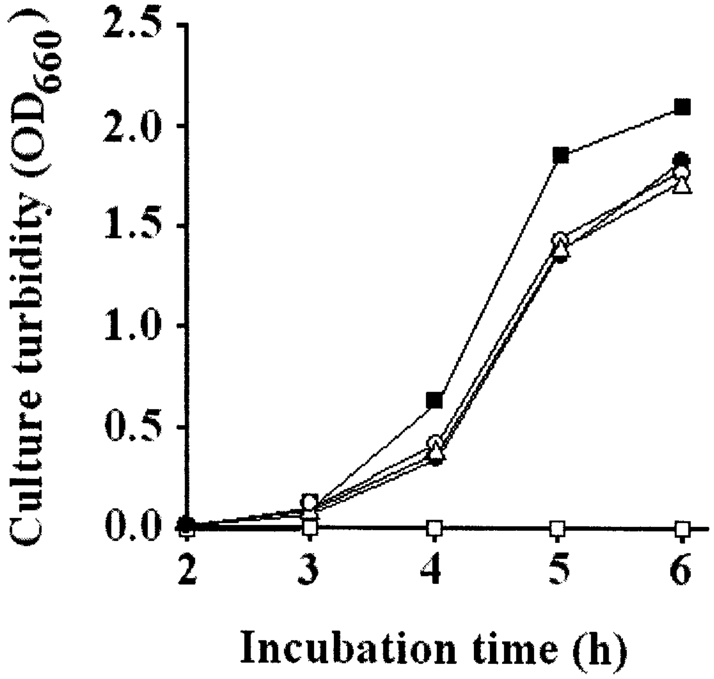


Fig. 3.5. Utilization of Hb-bound iron by *A. suis* strain H91-0380. *A. suis* was grown under iron-replete (closed squares) and iron-restricted (open squares) conditions and under iron-restricted conditions in the presence of porcine (closed circles), bovine (open circles) or human (open triangles) Hb. Culture turbidity values are the means of triplicates.

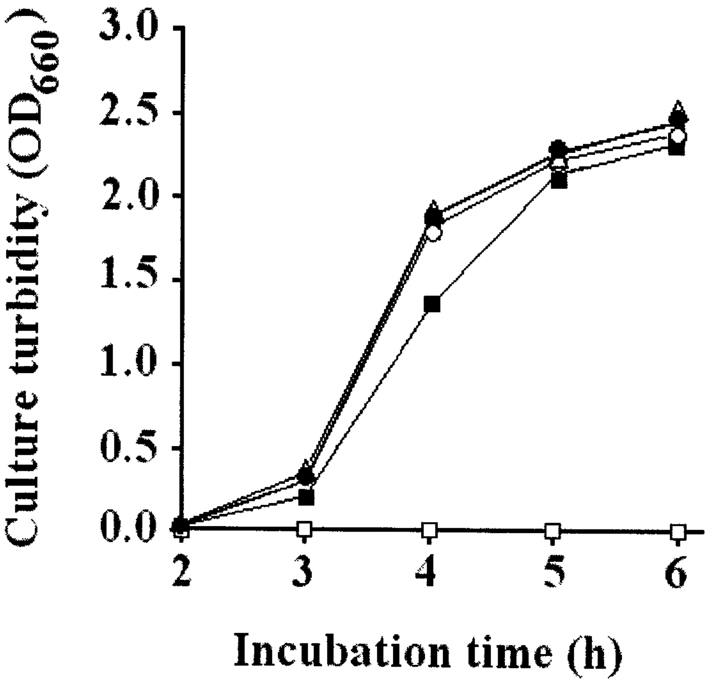


Fig. 3.6. Utilization of Hb-bound iron by *A. suis* strain SO4. *A. suis* was grown under iron-replete (closed squares) and iron-restricted (open squares) conditions and under iron-restricted conditions in the presence of porcine (closed circles), bovine (open circles) or human (open triangles) Hb. Culture turbidity values are the means of triplicates.

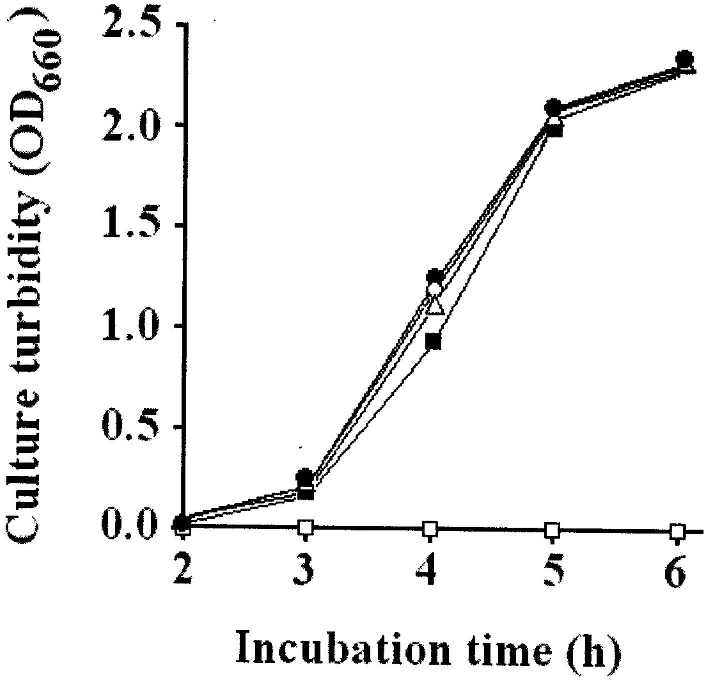


Fig. 3.7. Utilization of Hb-bound iron by *A. suis* strain VSB 3714. *A. suis* was grown under iron-replete (closed squares) and iron-restricted (open squares) conditions and under iron-restricted conditions in the presence of porcine (closed circles), bovine (open circles) or human (open triangles) Hb. Culture turbidity values are the means of triplicates.

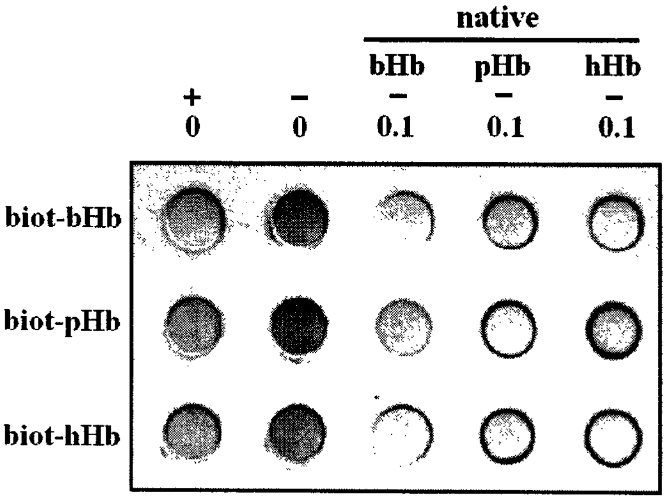


Fig. 3.8. Solid phase binding assay demonstrating binding of biotinylated (biot) Hbs by membranes from *A. suis* strain ATCC 15557 grown under iron-replete (+) and iron-restricted (–) conditions and competition between native Hbs and biotinylated Hbs for binding sites on total membranes. The presence (0.1 mg) and absence (0) of the competing Hbs are indicated and the Hbs are abbreviated as follows: b, bovine; p, porcine; h, human.

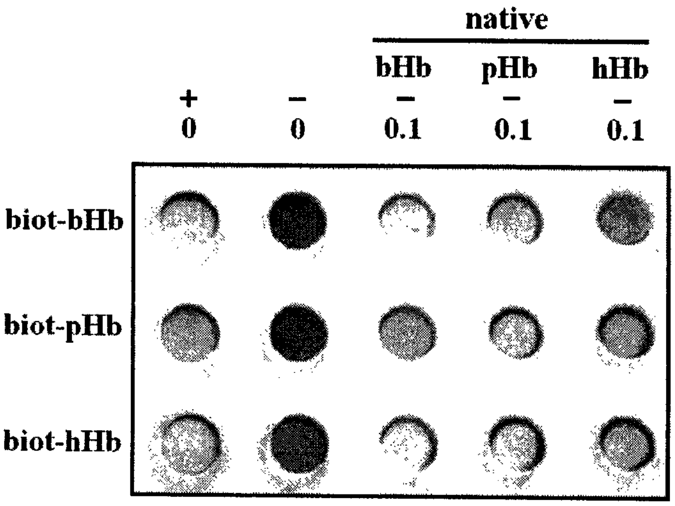


Fig. 3.9. Solid phase binding assay demonstrating binding of biotinylated (biot) Hbs by membranes from *A. suis* strain B49 grown under iron-replete (+) and iron-restricted (–) conditions and competition between native Hbs and biotinylated Hbs for binding sites on total membranes. The presence (0.1 mg) and absence (0) of the competing Hbs are indicated and the Hbs are abbreviated as follows: b, bovine; p, porcine; h, human.

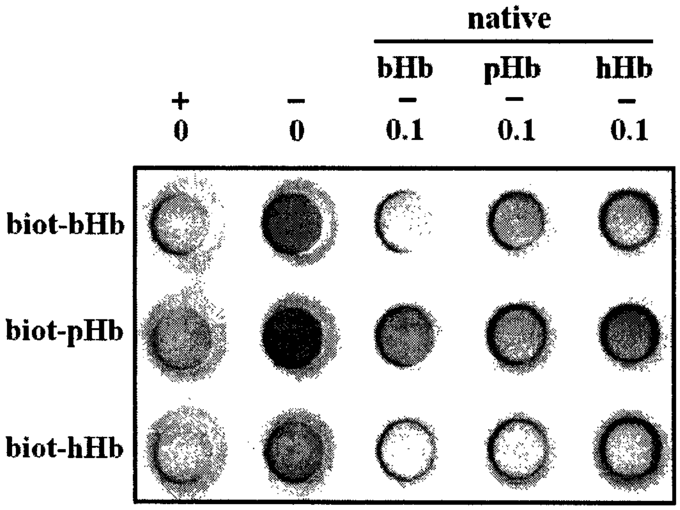


Fig. 3.10. Solid phase binding assay demonstrating binding of biotinylated (biot) Hbs by membranes from *A. suis* strain C84 grown under iron-replete (+) and iron-restricted (–) conditions and competition between native Hbs and biotinylated Hbs for binding sites on total membranes. The presence (0.1 mg) and absence (0) of the competing Hbs are indicated and the Hbs are abbreviated as follows: b, bovine; p, porcine; h, human.

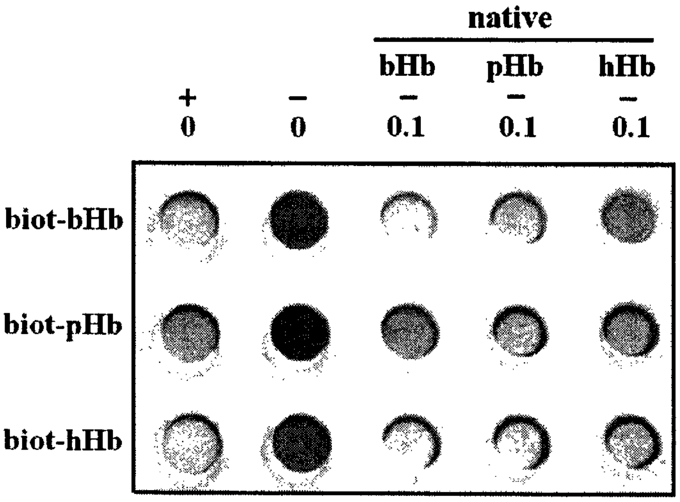


Fig. 3.11. Solid phase binding assay demonstrating binding of biotinylated (biot) Hbs by membranes from *A. suis* strain H89-1173 grown under iron-replete (+) and iron-restricted (–) conditions and competition between native Hbs and biotinylated Hbs for binding sites on total membranes. The presence (0.1 mg) and absence (0) of the competing Hbs are indicated and the Hbs are abbreviated as follows: b, bovine; p, porcine; h, human.

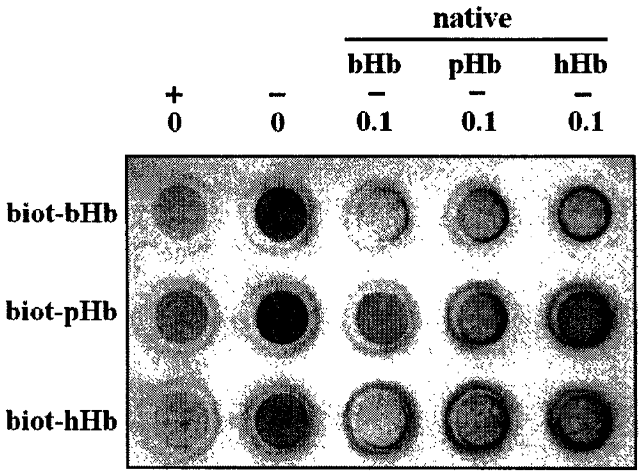


Fig. 3.12. Solid phase binding assay demonstrating binding of biotinylated (biot) Hbs by membranes from *A. suis* strain H91-0380 grown under iron-replete (+) and iron-restricted (–) conditions and competition between native Hbs and biotinylated Hbs for binding sites on total membranes. The presence (0.1 mg) and absence (0) of the competing Hbs are indicated and the Hbs are abbreviated as follows: b, bovine; p, porcine; h, human.

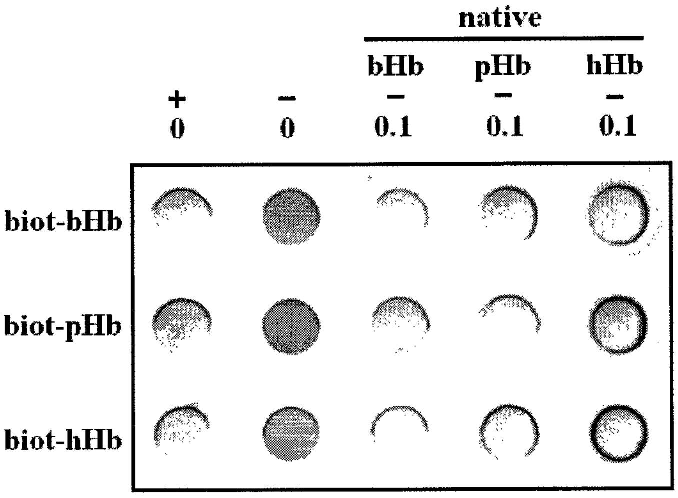


Fig. 3.13. Solid phase binding assay demonstrating binding of biotinylated (biot) Hbs by membranes from *A. suis* strain SO4 grown under iron-replete (+) and iron-restricted (–) conditions and competition between native Hbs and biotinylated Hbs for binding sites on total membranes. The presence (0.1 mg) and absence (0) of the competing Hbs are indicated and the Hbs are abbreviated as follows: b, bovine; p, porcine; h, human.

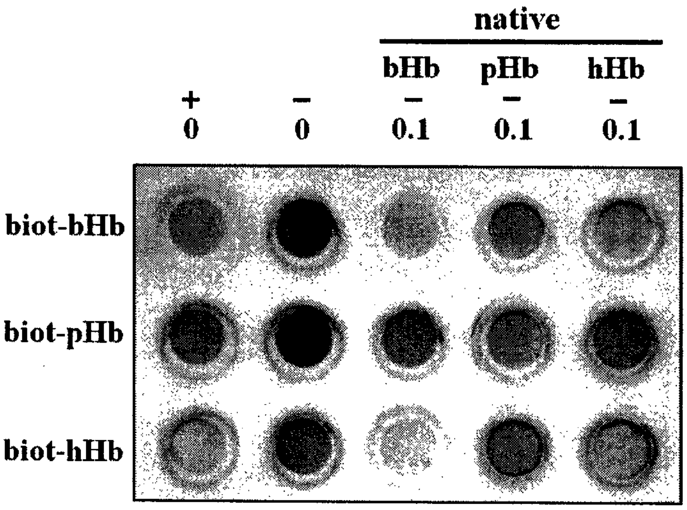


Fig. 3.14. Solid phase binding assay demonstrating binding of biotinylated (biot) Hbs by membranes from *A. suis* strain VSB 3714 grown under iron-replete (+) and iron-restricted (–) conditions and competition between native Hbs and biotinylated Hbs for binding sites on total membranes. The presence (0.1 mg) and absence (0) of the competing Hbs are indicated and the Hbs are abbreviated as follows: b, bovine; p, porcine; h, human.

3.4.2 Isolation and identification of haemoglobin-binding polypeptides

An affinity procedure based on bovine Hb-agarose as binding ligand and Zwittergent 3-14 as solubilizing agent allowed the isolation of a single putative Hb-binding polypeptide from total membranes derived from each of the seven strains of *A. suis* but, to a great extent, only if the membranes were from organisms grown under iron-restricted conditions (Figs. 3.15 and 3.16). In all cases, the isolated polypeptides had estimated molecular masses of ~105 kDa and their sizes, iron-repressibility and outer membrane location (e.g., Fig. 3.15) suggested that these polypeptides do represent Hb-binding proteins. Duplicate affinity-isolations and N-terminal amino acid sequence analyses allowed the identification of 10 of the first 12 amino acids of the ~105-kDa polypeptide from *A. suis* strain SO4; in brief, the N-terminal sequence was determined to be QEQMQLDTVXVX, where X represents unidentified amino acids.

3.4.3 Identification and transcription of *hugZ* and *hgbA* homologues

PCR approaches followed by DNA sequence analyses allowed the amplification, sequencing and identification of *hugZ* and *hgbA* homologues from *A. suis* strains C84 and SO4. In both strains, *hugZ* (522 bp) was located upstream of *hgbA* (2841 bp) and an unidentified, partial ORF, in reverse orientation, was located upstream of both *hugZ* genes (Fig. 3.17; GenBank accession numbers AY335901 and AY335902; Appendices 1.5 and 1.6). Except for 1 bp within *hgbA*, the sequence obtained with strain SO4 (4164 bp) was found to be identical to the comparable region in strain C84 and PCR, using primers based on *hugZ* and *hgbA* of *A. suis* strains SO4 and C84 and genomic DNA from each of the seven strains of *A. suis*, yielded similarly sized amplicons (Fig. 3.18).

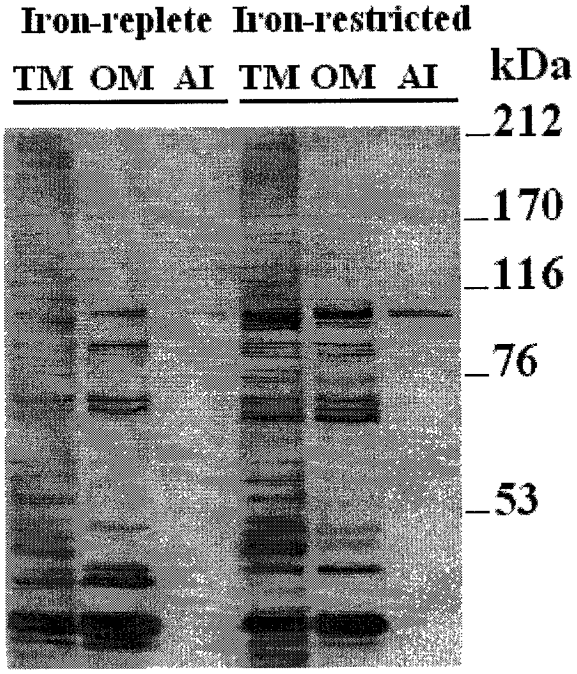


Fig. 3.15. Identification of Hb-binding polypeptides from *A. suis* strain SO4. SDS-PAGE was performed with total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *A. suis* strain SO4 grown under the indicated conditions.

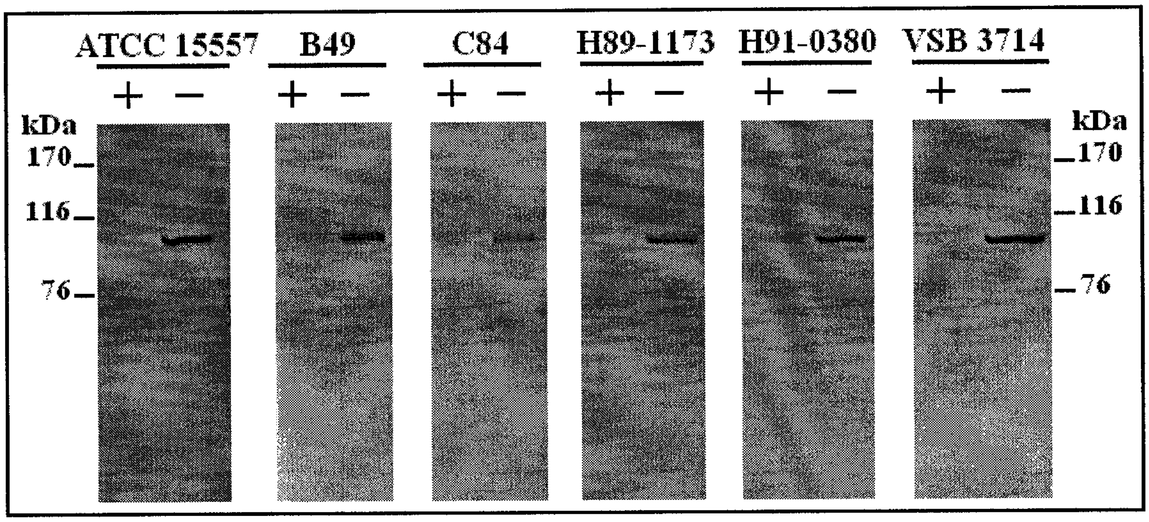


Fig. 3.16. Identification of Hb-binding polypeptides from six strains of *A. suis*. SDS-PAGE was performed with affinity-isolated polypeptides from the indicated strains of *A. suis* grown under iron-replete (+) and iron-restricted (–) conditions.

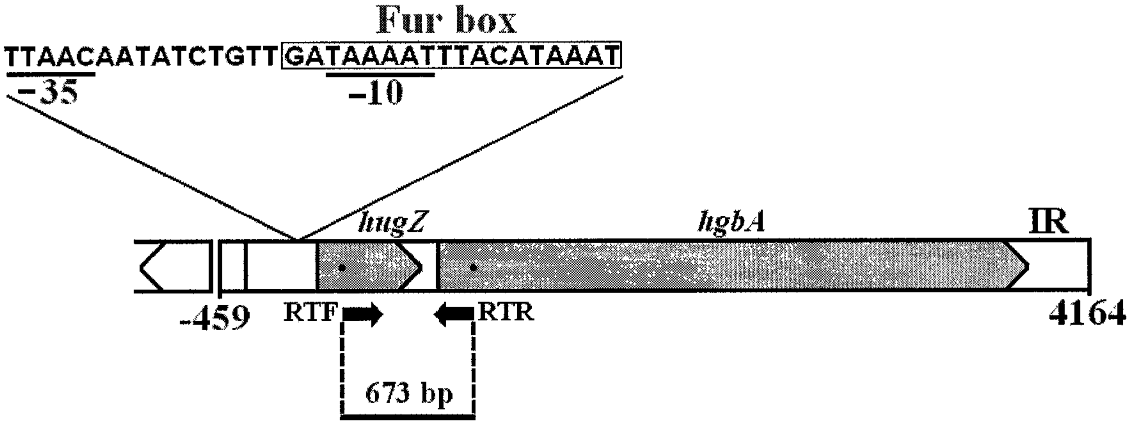
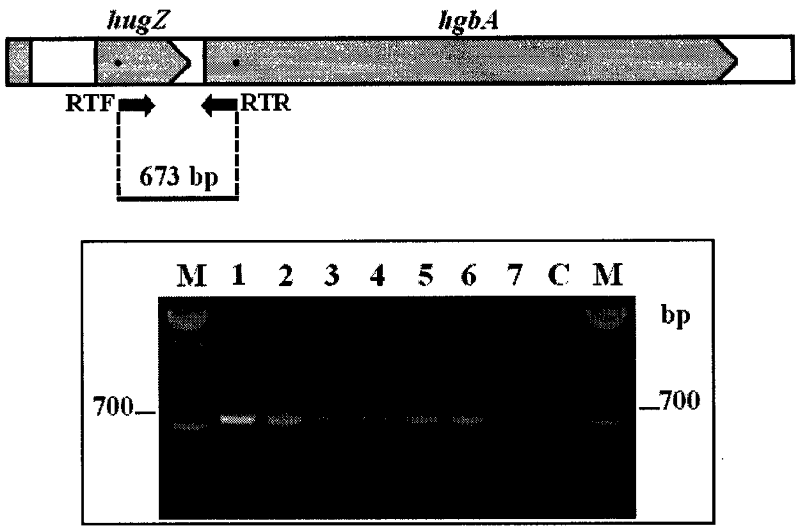


Fig. 3.17. Organization of *hugZ* and *hgbA* in *A. suis* strains SO4 and C84. The numbers, –459 and 4164, are base pair coordinates relative to the first base of the start codon of *hugZ* and IR identifies a region that contains inverted repeats possibly involved in transcriptional termination. The locations and directions of the primers used in RT–PCR are indicated and are represented in the schematic by black dots and arrows, respectively.



3.18. Amplification of *hugZ-hgbA* gene clusters. Lanes 1-7 represent the amplicons that were obtained when genomic DNA from *A.* suis strains ATCC 15557, B49, C84, H89-1173, H91-0380, SO4 and VSB 3714, respectively, were used as templates. Lanes M contained a 100-bp DNA ladder and lane C represents a negative control in which DNA was omitted from the amplification mixture. The locations and directions of the primers used in PCR are indicated and are represented in the schematic by black dots and arrows, respectively.

Putative Shine-Dalgarno sequences were identified upstream of both *hugZ* and *hgbA* and the uncommon start codon, GTG, was predicted to initiate translation of *hgbA*; putative promoter elements, however, and a putative Fur box, could be identified upstream of only *hugZ* (Fig. 3.17; GenBank accession numbers AY335901 and AY335902; Appendices 1.5 and 1.6). A putative cleavage site was identified in the predicted product of the *hgbA* gene and cleavage was predicted to yield a mature HgbA with the N-terminal amino acid sequence, QEQMQLDTVIVK. The molecular masses of the predicted HugZ and mature HgbA proteins were calculated to be 19.7 and 105 kDa, respectively.

The lack of obvious promoter elements upstream of *hgbA* suggested that *hugZ* and *hgbA* might be co-transcribed. To investigate this possibility, RT-PCR experiments were performed using RNA from strains SO4 and C84, grown under iron-replete and iron-restricted conditions, and primers based on sequences within *hugZ* and *hgbA*. RT-PCR with RNA from both strains yielded appropriately sized amplicons indicating that *hugZ* and *hgbA* are co-transcribed, and the relative amounts of the products indicated that the expression of these genes is up-regulated in response to iron restriction (Fig. 3.19).

3.5 Discussion

Studies pertaining to the acquisition of Hb-bound iron by members of the *Pasteurellaceae* have been confined to a relatively small number of species. In the present study, the acquisition of Hb-bound iron by seven strains of *A. suis* was investigated. All strains could use human, bovine and porcine Hbs as iron sources for growth and indicating the existence of receptors, total membranes derived from all strains bound all three Hbs. Since haem is embedded in Hbs, recognition of Hbs by bacterial receptors is

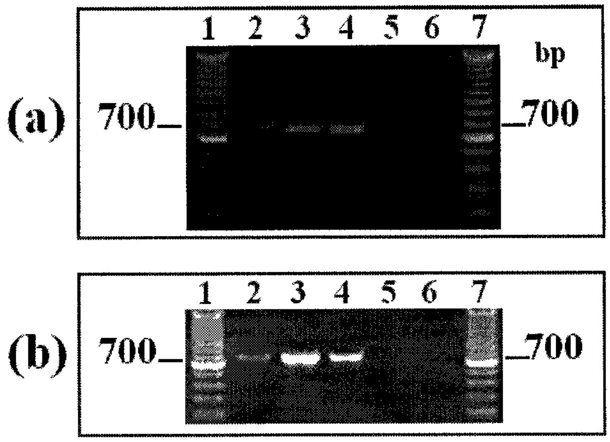


Fig. 3.19. Transcription of *hugZ* and *hgbA* in *A. suis* strains C84 and SO4. RT-PCR was performed with RNA from *A. suis* strains C84 (a) and SO4 (b) grown under iron-replete (lanes 2) or iron-restricted (lanes 3) conditions, or with total DNA (lanes 4). Lanes 5 and 6 represent negative controls in which RNA from organisms grown under iron-replete (lanes 5) or iron-restricted (lanes 6) conditions served as templates in PCR experiments. The primers were chosen to amplify a 673-bp *hugZ–hgbA* fragment. Lanes 1 and 7 contained a 100-bp DNA ladder.

believed to result largely from recognition of the protein itself (Genco and Dixon, 2001) and in particular, recognition of conserved residues in the globin chains surrounding the haem crevice (Lee, 1995). Porcine, bovine and human Hbs can also serve as iron sources for the growth of *A. pleuropneumoniae* (D'Silva *et al.*, 1995; Srikumar *et al.*, 2004). Interestingly, however, while D'Silva *et al.* (1995) reported that these Hbs are equally effective as iron sources, Srikumar *et al.* (2004) indicate that *A. pleuropneumoniae* exhibits a preference for the porcine protein. Preference for a particular Hb is relatively unusual but among the *Pasteurellaceae*, *H. influenzae* would also appear to prefer a particular Hb, in this case, human Hb (Frangipane *et al.*, 1994); *H. ducreyi*, on the other hand, exhibits no such specificity (Elkins, 1995). While the present results do not allow a preference for a particular Hb to be assigned to *A. suis*, they do indicate that porcine, bovine and human Hbs are recognized and bound by the same receptor(s).

A. suis and *A. pleuropneumoniae* are closely-related and with respect to iron acquisition processes, the TbpA and TbpB precursor proteins, and encoding genes, are highly homologous (Bahrami *et al.*, 2003; Chapter 2). *A. pleuropneumoniae* produces a single, iron-repressible, Hb-binding protein, HgbA, and following affinity-isolation and SDS-PAGE, the molecular mass of this protein has been estimated at ~105 kDa (Srikumar *et al.*, 2004); based on the nucleotide sequence of the encoding gene, *hgbA*, the molecular mass is 104.9 kDa (Srikumar *et al.*, 2004). Using Hb-agarose as binding ligand, a single, ~105-kDa, iron-repressible outer membrane protein, a possible HgbA homologue, could be isolated from total membranes derived from each of the seven strains of *A. suis* and with this in mind, it was reasoned that primers based on the sequence of *hgbA* of *A. pleuropneumoniae* might enable the detection and amplification of comparable genes in *A. suis*. Such an approach led to the amplification, sequencing and identification of *hgbA* and subsequently, *hugZ* homologues from *A. suis* strains C84 and SO4. *hugZ* was located upstream of *hgbA* and RT-PCR experiments revealed that these genes are co-transcribed and iron-regulated. In addition, PCR, using primers based on *hugZ* and *hgbA* of *A. suis* strains C84 and SO4 and genomic DNA from each of the seven strains, yielded comparable products suggesting that all seven strains of *A. suis* possess *hugZ* and *hgbA*, in that order. *hgbA* is predicted to encode a 946-amino-acid HgbA precursor that is processed to yield a mature HgbA with a deduced molecular mass of 105 kDa. The N-terminal amino acid sequence of the affinity-isolated ~105-kDa polypeptide from *A. suis* strain SO4 (QEQMQLDTVXVX) was found to compare favourably with that predicted for HgbA (QEQMQLDTVIVK) suggesting strongly that this affinity-isolated ~105-kDa polypeptide is the product of the *hgbA* gene. In effect, it would appear that the ~105-kDa Hb-binding polypeptides that were affinity-isolated from each of the *A. suis* strains do represent HgbA homologues. Notably, the predicted HgbA precursors of *A. suis* (GenBank accession numbers AY335901 and AY335902; Appendices 1.5 and 1.6) and *A. pleuropneumoniae* (GenBank accession number F468020) share 99 % identity and the Ton box and TonB boxC sequences identified in HgbA of *A. pleuropneumoniae* (Srikumar *et al.*, 2004) have identical counterparts in HgbA of *A. suis*. The predicted HgbA of *A. suis* was also found to contain FRAP (residues 655-658) and NPNL (NVDL in *A. suis*; residues 677-680) motifs; these motifs/domains are typical of haem/Hb receptors that transport haem to the bacterial periplasm (Bracken *et al.*, 1999; Wandersman and Stojiljkovic, 2000) and are predicted to be intimately involved in HgbA-mediated Hb binding/haem utilization by *A. pleuropneumoniae* (Pawelek and Coulton, 2004).

hugZ was first identified in *P. shigelloides* where it is preceded by three additional haem utilization genes, *hugA*, which encodes a haem receptor, *hugW* and *hugX*; notably, *hugAW* and *hugZ* appear to be Fur-regulated (Henderson *et al.*, 2001). While functions have yet to be assigned to HugW, HugX and HugZ, it is believed that one of them, at least, is involved in the protection of the organism against haem toxicity (Henderson *et al.*, 2001). Interestingly, *hugX* and *hugZ* homologues have been located immediately upstream of the *hgbA* genes of *P. multocida* (Bosch *et al.*, 2002) and *H. somni* (see e.g., GenBank accession number AY380576) and in both of these organisms, *hugX*, *hugZ* and *hgbA* constitute a transcriptional unit that is up-regulated in response to iron restriction (Bosch *et al.*, 2002; Y.D.N. Tremblay and D.F. Niven, unpublished data). As indicated above, *A. suis* also possesses a *hugZ* homologue upstream of *hgbA* and this is also the case in *A. pleuropneumoniae* (Srikumar *et al.*, 2004); the predicted HugZ proteins of both organisms, *A. suis* (GenBank accession numbers AY335901 and AY335902; Appendices 1.5 and 1.6) and *A. pleuropneumoniae* (http://microgen.ouhsc.edu/a_pleuro/a_pleuro_home.htm), appear to consist of 173 amino acids and to share 97 % identity. Despite the similarities in the *hugZ-hgbA* regions of *A. suis* and *A. pleuropneumoniae,* there appear to be differences in the organization of these genes and in the locations of the regulatory elements. In *A. suis*, *hugZ* and *hgbA* appear to be located on an operon, with expression being controlled by a Fur-regulated promoter upstream of *hugZ*; in contrast, expression of *hgbA* in *A. pleuropneumoniae* is predicted to be controlled by a Fur-regulated promoter immediately upstream of *hgbA* (Srikumar *et al.*, 2004), and apparently, overlapping the 3'-end of *hugZ* (Fig. 3.20). Putative promoter sequences and a putative Fur box have also been located immediately upstream of *hgbA* of *H. ducreyi* (Elkins *et al.*, 2005) but in this organism, *hugZ* and *hgbA* are far removed

As *hugZ*-AATATCGTTCACCTAGATGAAGGTCATAAAACA
N I V H L D E G H K T

Ap AATATCGTTCACCTAGATGAAGGTCATAAAACA

As GAA**TAA**CTATATAATTAAACATTTACTGTGCTAGTTTTGGCTA
E

Fur box
Ap GAATAA......................TTAAACATTTACTGTGCTAGTTTTGGCTA
−35 region −10 region

As GCACAGTTTTTTATCTTGTCTAAAGGAGAATGATT**GTG**-*hgbA*
RBS

Ap GCACAGTTTTTTATCTTGTCTAAAGGAGAATGATT**GTG**-*hgbA*
RBS

Fig. 3.20. Comparison of the 3'-end of *hugZ* and the *hugZ-hgbA* intergenic region in *A. suis* (As) with the corresponding regions in *A. pleuropneumoniae* (Ap). Stop and start codons are indicated in bold. A putative Fur box is indicated and putative promoter elements (−35 and −10 regions) and putative Shine-Dalgarno sequences (RBS) are indicated and underlined. Data were abstracted from Srikumar *et al.* (2004) and GenBank entries AF468020 and AY335901 (Appendix 1.5).

(GenBank accession number NC_002940). In brief, the reason(s) for the differences in the regulation of expression of *hgbA* in *A. suis* and *A. pleuropneumoniae* remains obscure although they may, perhaps, stem from the 8-bp difference in the intergenic regions immediately upstream of the *hgbA* genes (Fig. 3.20); it is also possible, in both organisms, that the expression of *hgbA* is controlled by Fur-regulated promoters upstream of both *hgbA* and *hugZ*. Finally, while *hugX* (ORF PM0298) and *hugZ* (ORF PM0299) would appear to be essential for *P. multocida* viability (Bosch *et al.*, 2002), no trace of *hugX* homologues could be found in the *H. ducreyi* genome (GenBank accession number NC_002940) or in the region immediately upstream of *hugZ* in *A. suis* (GenBank accession numbers AY335901 and AY335902; Appendices 1.5 and 1.6) or *A. pleuropneumoniae* (http://microgen.ouhsc.edu/a_pleuro/a_pleuro_home.htm). If HugX homologues are truly absent from these organisms, it may be that HugZ alone is able to perform an equivalent detoxifying role or alternatively, some other protein is able to compensate for the lack of HugX.

In conclusion, the results presented here indicate that the acquisition of Hb-bound iron by *A. suis* involves iron-repressible TonB-dependent receptors that can recognize and bind Hbs from at least three different animal species, porcine, bovine and human. These receptors and their encoding genes were shown to be homologues of Hb receptors and receptor genes in other members of the *Pasteurellaceae* and in particular, in *A. pleuropneumoniae* and it is envisaged that the mechanisms used by *A. suis* to effect the transport of haem from Hb to the bacterial cytoplasm are correspondingly similar.

Connecting statement 2

In the previous chapter, it was reported that *A. suis* can acquire iron from porcine, human and bovine Hbs. Acquisition of Hb-bound iron was shown to be receptor-mediated and to involve single-component (HgbA) iron-repressible receptors. These receptors, and the Tf receptors described in Chapter 2, represent potential targets for immunoprophylaxis. With subunit vaccines in mind, the next chapter reports on the cloning and expression, in *E. coli*, of polynucleotides encoding TbpB and HgbA.

Chapter 4. Cloning and expression of TbpB and HgbA

4.1 Abstract

Polynucleotides encoding mature TbpB and HgbA of *A. suis* were cloned in the expression vector, pQE80L. The recombinant plasmids were used to transform *E. coli* DH5α and isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce expression of the cloned "genes". While hexahistidine-tagged rTbpB could be purified from lysates of transformed *E. coli*, attempts to isolate a hexahistidine-tagged recombinant HgbA (rHgbA) were unsuccessful. Following SDS-PAGE and transfer to nitrocellulose, the hexahistidine-tagged rTbpB was detectable using mouse monoclonal anti-his$_6$-peroxidase and swine sera raised against *A. pleuropneumoniae* rTbpB.

4.2 Introduction

Iron acquisition mechanisms have been investigated extensively in the pathogenic neisseriae, especially *N. meningitidis* (see e.g. Cornelissen, 2003, Perkins-Balding *et al.*, 2004). Under the iron-restricted conditions in the host, pathogenic neisseriae express a number of surface receptors that bind specific iron-containing ligands such as Tf , Lf, Hb and the exogenous siderophore, enterobactin (Perkins-Balding *et al.*, 2004). The receptor system for the acquisition of Tf-bound iron consists of TbpA and TbpB. While TbpA and TbpB are both outer membrane proteins, TbpB (65 to 85 kDa) has no obvious membrane-spanning feature and compared to TbpA (~98 kDa), an integral membrane protein, exhibits considerable antigenic variability (Evans and Oakhill, 2002). Indeed, between strains, the TbpBs of *N. meningitidis* and *N. gonorrhoeae* may share as little as 57 to 75% identity (Cornelissen, 2003). Despite this heterogeneity, alignment of the amino acid sequences of the neisserial TbpB precursors characterized to date, has allowed the identification of six regions of sequence conservation. The first and second regions are in the N-terminal half of the protein; while the first (~50 amino acids) includes the putative signal peptide at the N-terminus, the second consists of a shorter stretch of amino acids (<50 amino acids) and is located near the middle of the protein. The remaining regions, each spanning a shorter stretch of amino acids, are in the C-terminal half of the protein (Cornelissen, 2003). Interestingly, the C-terminal half of *N. meningitidis* TbpB has been shown to contain conserved surface-exposed epitopes in that immunization of rabbits with the C-terminal half of the receptor induced the production of bactericidal antibodies that were effective against a variety of strains (Rokbi *et al.*, 2000). In brief, it is now beginning to appear that the development of a broadly cross-reactive, rTbpB-based meningococcal vaccine is feasible and imminent (Rokbi *et al.*, 2000). The vaccine potential of Hb or Hb-haptoglobin receptors, on the other hand, has not been well explored owing, to a great extent, to the fact that in the pathogenic neisseriae at least, the expression of such receptors (HmbR and HpuAB) is subject to phase variation (Perkins-Balding *et al.*, 2004).

Considering the limited homology between the TbpBs of *A. suis* and *A. pleuropneumoniae* and the apparent, non-phase-variable expression of *A. suis* HgbA, the objectives of the present study were to clone and express *A. suis* TbpB and HgbA in *E. coli* and to isolate and purify the recombinant proteins for future studies pertaining to their potential use for immunoprophylaxis.

4.3 Materials and methods

4.3.1 Organisms and sera

A. suis strain SO4 and *E. coli* strain DH5α were handled as described in section 2.3.1. *A. pleuropneumoniae* strain ATCC 27088 was handled as for *A. suis* except that the growth medium contained 25 μM EDDA and 10 μM NAD (Roche Diagnostics). Swine anti-rTbpB (91-092) and normal adult swine serum (98-764) were generously provided by Dr. Philip Willson, Vaccine and Infectious Disease Organization, Saskatoon, SK.

4.3.2 Cloning of polynucleotides encoding TbpB and HgbA

Polynucleotides encoding mature *A. suis* TbpB and HgbA were amplified using conventional PCR, genomic DNA from *A. suis* strain SO4 (section 2.3.6), the Expand High Fidelity PCR System and the primer pairs BSacIF/BPstIR and HSacIF/HPstIR (Table 4.1), respectively. The concentrations of $MgCl_2$ in the reaction mixtures were 1.5 mM (BSacIF/BPstIR) and 3 mM (HSacIF/HPstIR). BSacIF and HSacIF were designed to generate *Sac*I restriction sites at the 5' ends of the polynucleotides encoding TbpB and HgbA, respectively. Primers BPstIR and HPstIR were designed to generate *Pst*I restriction sites 55 bp and 197 bp, respectively, downstream of the corresponding stop codons. The purified (QIAquick PCR purifying kit) amplicons were ligated into pGEM-T Easy, as specified by the manufacturer, and the ligation products were used to transform competent *E. coli* DH5α. Colonies harbouring recombinant plasmids containing the appropriate polynucleotide inserts (pGtbpB, encoding TbpB; pGhgbA, encoding HgbA) were transferred to liquid TY medium containing 100 μg/ml ampicillin (TY/amp) and following growth, the plasmids were isolated and purified (QIAprep Spin Miniprep Kit). Following digestion of pGtbpB, pGhgbA and the expression vector, pQE80L (The QIAexpressionist, Qiagen) with

Table 4.1. Primers used in the construction of polynucleotides encoding rTbpB and rHgbA

Primer	Description	Sequence[a] (5' → 3')
BPstIR	*tbpB*; reverse	GAACTGCAGGCTAAGAAGAGCAAGGCTAATCAG
BSacIF	*tbpB*; forward	CCTTGAGCTCTGTTCTGGCGGAAAAGGAGGTTTTG
BXR3	*tbpB*; reverse	GCACTGCCGATAAAGCCATTACCA
HbG2CR	*hgbA*; reverse	CTTGCAGCATATCATCCGGAATCTTC
HPstIR	*pheA*[b]; reverse	GGAACTGCAGTGGAACTAGAGGCGAATACGCATGC
HSacIF	*hgbA*; forward	CCTTGAGCTCCAAGAGCAAATGCAACTCGACACCG
PQEF	pQE; forward	CGGATAACAATTTCACACAG

[a] The underlined sequences denote *Sac*I and *Pst*I restriction sites.

[b] The sequence immediately downstream of *hgbA* appears to encode a PheA (prephenate dehydratase) homologue.

*Sac*I and *Pst*I (Invitrogen; 2 h, 37 °C), the products, as appropriate, were gel-purified (MinElute Gel Extraction Kit, Qiagen) and then ligated using T4 DNA ligase (Invitrogen Life Technologies; overnight, 4 °C). The ligation products (pQE80tbpB, encoding hexahistidine-tagged TbpB; pQE80hgbA, encoding hexahistidine-tagged HgbA) were used to transform competent *E. coli* DH5α and transformants were grown on solid TY/amp. Plasmids containing inserts in the appropriate orientation were identified using colony-touch PCR and primers PQEF (based on sequence upstream of the pQE80L multiple cloning site) and either BXR3 (based on sequence within the polynucleotide encoding TbpB) or HbG2CR (based on sequence within the polynucleotide encoding HgbA) (Table 4.1). To verify the sizes of the inserts in pQE80tbpB and pQE80hgbA, these plasmids were digested with *Sac*I and *Pst*I (2 h, 37 °C) and the restriction digests subjected to gel electrophoresis. The inserts in pQE80tbpB and pQE80hgbA were checked for integrity by means of nucleotide sequencing using primers based on the polynucleotide sequences encoding TbpB and HgbA and sequences flanking the multiple cloning site of pQE80L.

Putative cleavage sites in TbpB and HgbA precursors were identified using SignalP 3.0. Custom-made primers were from Invitrogen Life Technologies. Sequence analyses were performed by the Applied Biotechnology Laboratory, McGill University (pQE80tbpB) or by the Genome Québec Innovation Centre, McGill University (pQE80hgbA). Sequence data were assembled into contiguous segments using Sequencher 3.0. The molecular masses of predicted proteins were calculated using Compute pI/Mw.

4.3.3 Expression and purification of recombinant TbpB and HgbA

Overnight cultures of *E. coli* DH5α containing pQE80tbpB or pQE80hgbA (8-ml volumes) were used to inoculate duplicate 200-ml volumes of TY/amp and the resulting cultures were then incubated at 37°C, with agitation on a gyratory shaker (200 rpm). When the OD_{660} of the cultures reached ~0.6, protein expression by the organisms in one of the duplicate cultures was induced by adding IPTG (Roche Diagnostics) to 1 mM; the other culture (without IPTG) served as a control. After 2 h of induction, the organisms in 35-ml volumes of each of the cultures were harvested by centrifugation (5000×*g*, 4 °C, 5 min) and the pellets were then washed twice by resuspension in, and centrifugation from, 35-ml volumes of cold 50 mM Tris-HCl, pH 8.0. Following storage at -80 °C, each pellet was thawed quickly and resuspended in a buffer solution containing 10 ml of binding buffer (10 mM imidazole, 0.5 M NaCl, 20 mM phosphate buffer, pH 7.4; Amersham Biosciences) and 50 µl of an ethanolic solution of 0.1 M phenylmethylsulfonyl fluoride (Sigma-Aldrich). The organisms in each sample were treated with lysozyme (10 mg/ml in 25 mM Tris-HCl, pH 8.0; 30 min, room temp., with gentle rocking) and then disrupted by sonication (3×10-s bursts of 80W, separated by 30-s cooling periods; Sonifier Cell Disruptor, model 185D, Heat Systems-Ultrasonics Inc.); unbroken cells and large debris were sedimented by centrifugation (12000×*g*, 4 °C, 15 min) and the top 80% of each supernatant fraction, referred to as the cell lysate, was retained. Recombinant proteins were purified from filtered (0.45 µm pore size) cell lysates using a HisTrap kit (1-ml columns; Amersham Biosciences), as directed by the manufacturer. Eluates (12.5-µl volumes) and filtered cell lysates (12.5-µl volumes) were subjected to SDS-PAGE and separated polypeptides were visualized by means of silver staining, as described above (section 2.3.5), or by staining with Coomassie Brilliant Blue R-250 (Bio-Rad).

4.3.4 Immunoblotting of recombinant TbpB

rTbpB (~150 µg protein), concentrated affinity-isolated Tf-binding polypeptides (section 2.3.5) and outer membranes (50 µg protein) from *A. suis* strain SO4 and *A.*

pleuropneumoniae strain ATCC 27088 , both grown under iron-restricted conditions, were subjected to SDS-PAGE, in duplicate, as described above (section 2.3.5). Separated polypeptides were transferred to nitrocellulose, as described in section 2.3.5, and non-specific binding sites were blocked by incubation in TTBS (1 h, 37 °C, gentle agitation). Half of the nitrocellulose sheet, containing one set of the electrotransferred polypeptides, was incubated (2 h, 37 °C, gentle agitation) in swine polyclonal serum raised against *A. pleuropneumoniae* rTbpB (91-092; diluted 1:100 in TTBS); the other half was incubated (2 h, 37 °C, gentle agitation) in normal adult swine serum (98-764; diluted 1:100 in TTBS). The nitrocellulose sheets were rinsed thrice with TTBS (10 min, 37 °C, gentle agitation), incubated in protein A-HRP conjugate (Bio-Rad; diluted 1:500 in TTBS; 2 h, 37 °C, gentle agitation), rinsed thrice with TBS (10 min, 37 °C, gentle agitation) and then incubated in BM blue POD substrate (precipitating) (Roche Diagnostics; 3 min, room temp.) for colour development. rTbpB, alone, was also subjected to SDS-PAGE and immunoblotting, as described above, except that the nitrocellulose was blocked in TTBS for 2 h and the antibody was mouse monoclonal anti-his_6-peroxidase (Roche Diagnostics, 100 mU/ml).

4.3.5 Alignment

Amino acid sequence alignment was performed using CLUSTAL W (1.81) Multiple Sequence Alignments (San Diego Supercomputer Center Biology WorkBench; http://workbench.sdsc.edu/; Thompson *et al.*, 1994).

4.3.6 Imaging

Images were obtained as described in section 2.3.8.

4.4 Results

4.4.1 Cloning and expression of TbpB

A polynucleotide believed to encode an *A. suis* TbpB was cloned in the expression vector, pQE80L. Nucleotide sequencing confirmed that the insert had the correct orientation and revealed that the nucleotide sequence was identical to that determined (Chapter 2; Bahrami *et al.*, 2003) for the corresponding fragment of *A. suis tbpB*. The QIAexpressionist system is designed in such a way that, here, it should have allowed the expression, in *E. coli* DH5α, of an N-terminally hexahistidine-tagged rTbpB. The expected size of hexahistidine-rTbpB was calculated to be 64.2 kDa and the recovery of a ~64-kDa polypeptide, notably, following induction with IPTG, suggested the successful production and purification of the recombinant protein (Fig. 4.1). SDS-PAGE followed by immunoblotting with mouse monoclonal anti-his$_6$-peroxidase supported the thesis that the ~64-kDa polypeptide is hexahistidine-rTbpB (Fig. 4.2).

4.4.2 Recognition of rTbpB (*A. suis*) by anti-rTbpB (*A. pleuropneumoniae*)

In immunoblot assays, swine polyclonal antisera, raised against *A. pleuropneumoniae* rTbpB, reacted strongly with *A. suis* rTbpB and also with affinity-isolated Tf-binding polypeptides and similarly sized outer membrane polypeptides from both *A. suis* and *A. pleuropneumoniae* (Fig. 4.3). These data indicate that the TbpB proteins of *A. suis* and *A. pleuropneumoniae* share common epitopes and in addition, they provide additional support for the conclusion (Chapter 2) that the ~63-kDa Tf-binding polypeptides that were affinity-isolated from all seven strains of *A. suis* are the products of the respective *tbpB* genes.

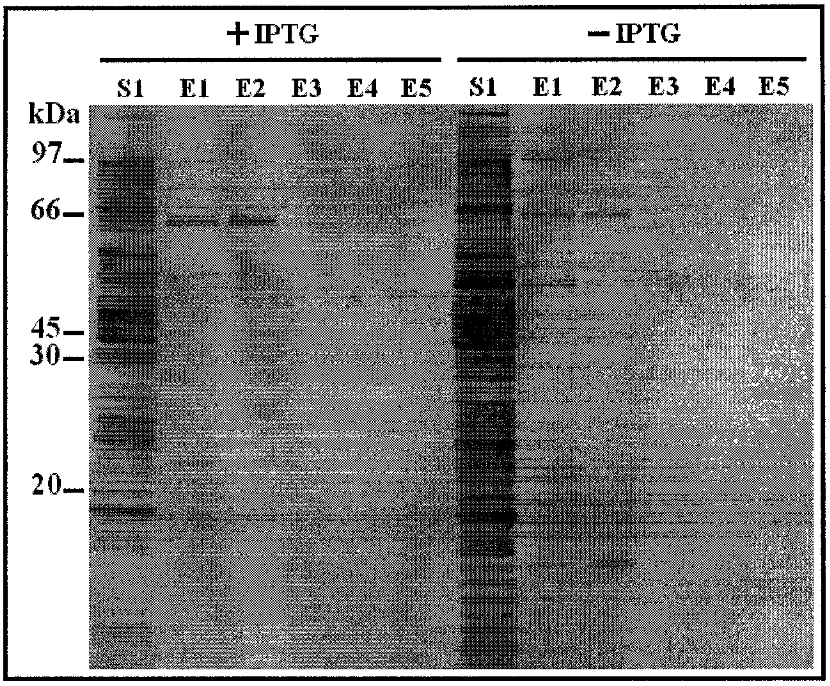


Fig. 4.1. Effect of IPTG on the expression of rTbpB. *E. coli* harbouring the recombinant plasmid, pQE80tbpB, was incubated in the presence (+) or absence (–) of IPTG. Filtered cell lysates (S1) and sequential samples of eluates from HisTrap columns (E1-E5) were subjected to SDS-PAGE and silver stained.

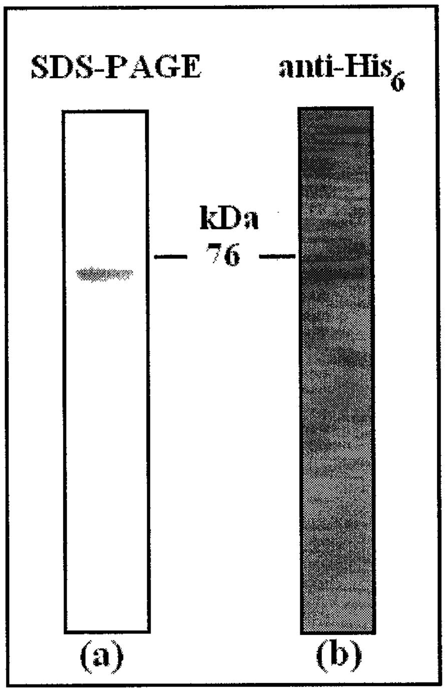


Fig. 4.2. SDS-PAGE and immunoblotting of rTbpB. rTbpB of *A. suis* was subjected to SDS-PAGE and either stained with Coomassie Blue (a) or transferred to nitrocellulose and reacted with mouse monoclonal anti-his$_6$-peroxidase followed by protein A-HRP (b).

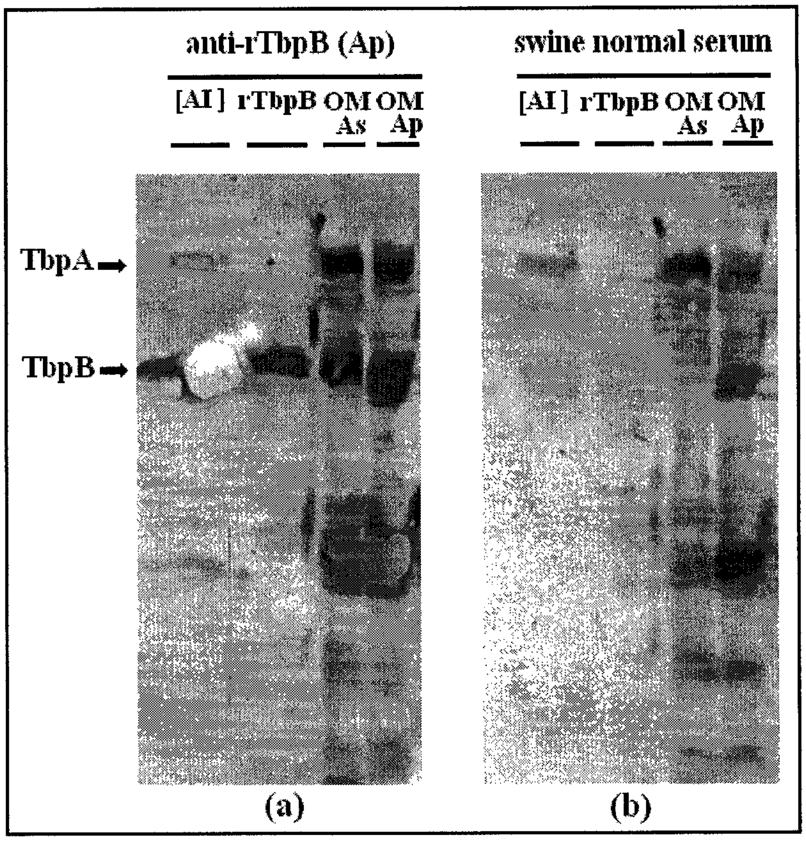


Fig. 4.3. SDS-PAGE and immunoblotting of rTbpB and affinity-isolated polypeptides from *A. suis* strain SO4. rTbpB, concentrated affinity-isolated Tf-binding polypeptides from *A. suis* strain SO4 grown under iron-restricted conditions [AI] and outer membranes (OM) from *A. suis* strain SO4 (As) and *A. pleuropneumoniae* strain ATCC 27088 (Ap) were subjected to SDS-PAGE, transferred to nitrocellulose and reacted with swine polyclonal anti-rTbpB of *A. pleuropneumoniae* (a) or adult swine normal serum (b) followed by protein A-HRP.

4.4.3 Cloning and expression of HgbA

A polynucleotide believed to encode an *A. suis* HgbA was cloned in the expression vector, pQE80L. While nucleotide sequencing confirmed that the insert had the correct orientation, the sequence obtained for the complete polynucleotide and that determined (Chapter 3; Bahrami and Niven, 2005) for the corresponding fragment of *A. suis* strain SO4 *hgbA* differed at 5 positions; as a consequence, the cloned polynucleotide was predicted to encode an HgbA with a histidine at position 215 rather than a tyrosine. The expected size of the hexahistidine-rHgbA was calculated to be 105.8 kDa and while it was envisaged, perhaps naively, that this rHgbA would still be well expressed in *E. coli*, the polypeptide mix that was isolated from the *E. coli* lysates contained only minimal amounts of polypeptide of the appropriate size (Fig. 4.4). Attempts to improve the yield of the ~106-kDa polypeptide by using a protease inhibitor cocktail (P-8340; Sigma-Aldrich), CelLytic B-II bacterial cell lysis reagent (Sigma-Aldrich), incubation with IPTG overnight, and at 25 °C, and His-Select Cartridge (1 ml) purification columns (Sigma-Aldrich) were unsuccessful.

4.5 Discussion

Compared to the highly homologous TbpA precursors of *A. suis* and *A. pleuropneumoniae*, sharing identities of >90%, the TbpB precursors constitute a relatively heterogeneous group sharing identities of only 54-86%. Nevertheless, like the neisserial TbpB precursors (Cornelissen, 2003), alignment of the amino acid sequences of the TbpB precursors of *A. suis* and six strains of *A. pleuropneumoniae* revealed six major regions of homology (Fig. 4.5). Except for region I, located at the N-terminus and extending beyond the predicted cleavage site (between residues 19 and 20), all of the homologous regions (II-VI) are located

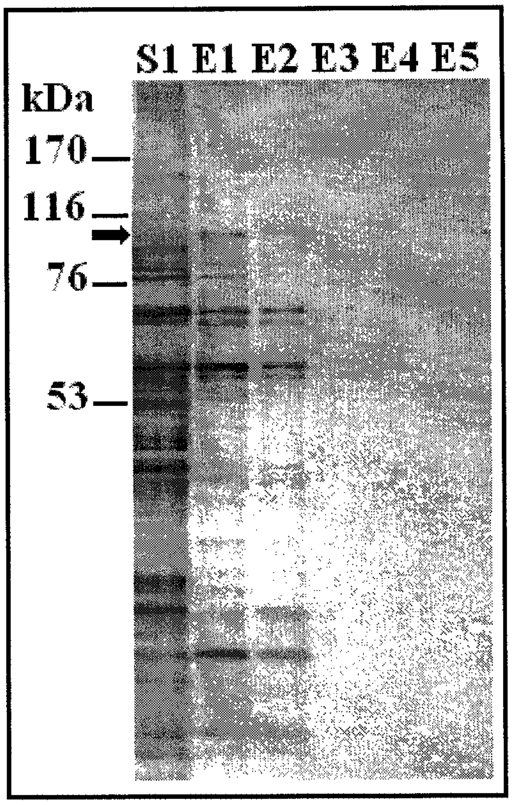


Fig. 4.4. Expression of rHgbA. *E. coli* harbouring the recombinant plasmid, pQE80hgbA, was incubated in the presence of IPTG. Filtered cell lysate (S1) and sequential samples of eluate from a HisTrap column (E1-E5) were subjected to SDS-PAGE and silver stained. The arrow indicates the expected position (~106 kDa) of a hexahistidine-tagged rHgbA.

```
      I
1     MHFKLNPYALAFTSLFLVACSGGKGSFDLEDVRPN  KTTGVSKE--EYKDVETAKKEKEQL
2     MHFKLNPYALAFTSLFLVACSGGKGSFDLEDVRPN  KTTGVSKE--EYKDVETAKKEKEQL
3     MHFKLNPYALAFTSLFLVACSGGKGSFDLEDVRPN  KTTGVSKE--EYKDVETAKKEKEQL
4     MHFKLNPYALAFTSLFLVACSGGKGSFDLEDVRPN  QTAKAEKATTSYQDEETKKKTKEEL
5     MHFKLNPYALAFTSLFLVACSGGKGSFDLEDVRPN  QTAKAEKATTSYQDEETKKKTKEEL
As    MHFKLNPYALAFTSLFLVACSGGKGGFDLEDVRPN  QTAKAEKATTSYQDEETKKKTKEEL
6     MHFKSDLYIVALTSTFLVACSGGKGSFDLDDVQPN  KAVEPEKTKVNYTDEETQKRKKEEL
      **** : * :*:** **********.***:**:**  ::.  .*   .* * ** *: **:*

1     GELMEPALGYVVKVPVSSFENKK------------VDIS--DIEVITNGNLDDVPYKANS
2     GELMEPALGYVVKVPVSSFENKK------------VDIS--DIEVITNGNLDDVPYKANS
3     GELMEPALGYVVKVPVSSFENKK------------VDIS--DIEVITNGNLDDVPYKANS
4     DKLMEPALGYETQILRRNKAPKTETGEKRN--ERVVELSEDKITKLYQESVEIIPHLDEL
5     DKLMEPALGYETQILRRNKAPKTETGEKRN--ERVVELSEDKITKLYQESVEIIPHLDEL
As    DKLMEPTLGVEAKIPRRNRALFDKEGNRKATPDTTDELSEAQIMAIWNENIDEIPHLKEL
6     DKLMEPALGYVTKIP-VNIPSVRKT-----------EIS--EIDTVTDESLSLVPNEDKL
      .:****:**  .::   .                  ::*  .*  : : .:. :*   :

1     -----SKYNYPDIKTK----DSSLQYVRSGYVIDGEHSGSNEK-------------GYVY
2     -----SKYNYPDIKTK----DSSLQYVRSGYVIDGEHSGSNEK-------------GYVY
3     -----SKYNYLDIKTK----DSSLQYVRSGYVIDGEHSGSNEK-------------GYVY
4     NGKTTSNDVYHSHDSKRLDKNRDLKYVRSGYVYDGSFNEIRRNDSGF-HVFKQGIDGYVY
5     NGKTTSNDVYHSHDSKRLDKNRDLKYVRSGYVYDGSFNEIRRNDSGF-HVFKQGIDGYVY
As    NDKTTSGLIYHSHDGKQEDKKRNLQYVRSGYVFDESYSEIVKNKNGVPYIFKNGIDGYIY
6     R--TIANENYGSVVTK--SGSNTMNFVRSGYTIDVDHYGLRDK-------------GYVY
           :   * .   *    .  :::*****. * ..     :             **:*

1     YKGNSPAKELPVNQLLTYTGSWDFTSNANLNNEEGRPNYLNDDYY--------TKFIGKR
2     YKGNSPAKELPVNQLLTYTGSWDFTSNANLNNEEGRPNYLNDDYY--------TKFIGKR
3     YKGNSPAKELPVNQLLTYTGSWDFTSNANLNNEEGRPNYLNDDYY--------TKFIGKR
4     YLGVTPSKELPKGKVISYKGTWDFVSNINLEREIDGFDTSGDGKNVSATSITETVNRDHK
5     YLGVTPSKELPKGKVISYKGTWDFVSNINLEREIDGFDTSGDGKNVSATSITETVNRDHK
As    YLGTSPSKELPKGNKVTYKGTWDFTSDVKTSYELSGFSDAGNGKNVAATSISDNVNRDHK
6     YKGVHPSKELPKGNIIVYQGEWDFTSNADLDAKRPNYNPEFNG-----------YGAGQR
      * *  *:**** .: : * * ***.*: . . :    .   :.              .::

1     VGLVSGDAKPAKHKYTSQFEVDFATKKMTGKLS----------DKEKTIYTVNADIRGNR
2     VGLVSGDAKPAKHKYTSQFEVDFATKKMTGKLS----------DKEKTIYTVNADIRGNR
3     VGLVSGDAKLAKHKYTSQFEVDFATKKMTGKLS----------DKEKTIYTVNADIRGNR
4     VGEKLGDNEVKGVAHSSEFAVDFDNKKLTGSLYRNGYINRNKAQEVTKRYSIEADIAGNR
5     VGEKLGDNEVKGVAHSSEFAVDFDNKKLTGSLYRNGYINRNKAQEVTKRYSIEADIAGNR
As    VGEKLGDNEVKGVAHSSEFAVDFDNKKLTGSLYRNGYINRNKAQEVTKRYSIEADITGNR
6     VGVTSADAKER--TYISKFNIDFSNKKLNGQLLT-----KTKENQEKLRYTVEANISGNR
      **   .* :     : *:* :** .**:.*.*           ::  . *:::*:* ***

                                 II
1     FTGAATASDKNKGKGESYNFFSADSQS  LEGGFYGPKAEEMAGKFVANDKSLFAVFSAK  HN
2     FTGAATASDKNKGKGESYNFFSADSQS  LEGGFYGPKAEEMAGKFVANDKSLFAVFSAK  HN
3     FTGAATASDKNKGKGESYNFFSADSQS  LEGGFYGPKAEEMAGKFVANDKSLFAVFSAK  HN
4     FRGKAKAEKAGDP------IFT-DSNY  LEGGFYGPKAEEMAGKFFTNNKSLFAVFAAK  SE
5     FRGKAKAEKAGDP------IFT-DSNY  LEGGFYGPKAEEMAGKFFTNNKSLFAVFAAK  SE
As    FRGKAKAEKAGDP------IFT-DSNY  LEGGFYGPKAEEMAGKFFTNNKSLFAVFAAK  SE
6     FRGKATATDKTDP------ILGKDSEH  LEGGLYGPKSEELAGKFVAHDKSLFAVFSGK  RG
      * * *.* .  .       ::  **:   ****:****:**:****.:::*******:.*

        III
1     GSNVD  TVRIIDASKIDLTNFSISELNNFGDASVLIIDGKKIKLAGSGFTNKHTIEINGKT
2     GSNVN  TVRIIDASKIDLTNFSISELNNFGDASVLIIDGKKIKLAGSGFTNKHTIEINGKT
3     GSNVD  TVRIIDASKIDLTNFSISELNNFGDASVLIIDGKKIKLAGSGFTNKHTIEINGKT
4     NGETT  TERIIDATKIDLTQFNAKELNNFGDASVLIIDGQKIDLAGVNFKNSKTVEINGKT
5     NGETT  TERIIDATKIDLTQFNAKELNNFGDASVLIIDGQKIDLAGVNFKNSKTVEINGKT
As    NGETT  TERIIDATKIDLTQFNAKELNNFGDASVLIIDGQKIDLAGVNFKNSKTVEINGKT
6     NDVLE  TVKIIDASKIDLTTFESSELNNFGNANVLIIDGQKIDLAGADFKNRKTVDINGKT
      ..     * :****:***** *. .******:*.******:**.*** .*.* :*::*****
```

```
                                              IV
1     MVAVACCSNLEYMKFGQLWQ QAEGGKPEN NSLFLQGERTATDKMPKGGNYKYIGTWDAQV
2     MVAVACCSNLEYMKFGQLWQ QAEGGKPEN NSLFLQGERTATDKMPKGGNYKYIGTWDAQV
3     MVAVACCSNLEYMKFGQLWQ QAEGGKPEN NSLFLQGERTATDKMPKGGNYKYIGTWDAQV
4     MVAVACCSNLEYMKFGQLWQ KEGKQQVKD NSLFLQGERTATDKMPAGGNYKYVGTWDALV
5     MVAVACCSNLEYMKFGQLWQ KEGKQQVKD NSLFLQGERTATDKMPAGGNYKYVGTWDALV
As    MVAVACCSNLEYMKFGQLWQ KEGKQQVKD NSLFLQGERTATDKMPAGGNYKYVGTWDALV
6     MVAIACCSNLEYMKFGQLWQ KEG-EQTKD NSLFLQGERTATDKIPVGGNYKYVGTWDALV
      ***:**************** :    : :: **************:* ******:***** *

                     V
1     SKENNWVATADDDRKA GYRTEFDVDFGNKNLSGKLFDKNGVNPVFTVDPKIDGNGFTGKA
2     SKENNWVATADDDRKA GYRTEFDVDFGNKNLSGKLFDKNGVNPVFTVDAKIDGNGFTGKA
3     LKENNWVATADDDRKA GYRTEFDVDFGNKNLSGKLFDKNGVNPVFTVDAKIDGNGFTGKA
4     SKGTNWIAEADNNRES GYRTEFDVNFSDKKVNGKLFDKGGVNPVFTVDATINGNGFIGSA
5     SKGTNWIAEADNNRES GYRTEFDVNFSDKKVNGKLFDKGGVNPVFTVDATINGNGFIGSA
As    SKGTNWIAEADNNRES GYRTEFDVNFSDKKVNGKLFDKGGVNPVFTVDATINGNGFIGSA
6     SKGTNWVAEADNNRES GYRSEFDVNFGDKKVSGKLFDKGGIVPVFMINADIKGNGFTGTA
       * .**:* **::*:: ***:****:*.:*::.******.*: *** ::. *.**** *.*

                                VI
1     KTSDAGFALDSGSS RYENVKFNDVAVS GGFYGPTAAELGGQFHHKSENGSVGAVFGAKQQ
2     KTSDEGFALDSGSS RYENVKFNDVAVS GGFYGPTAAELGGQFHHKSENGSVGAVFGAKQQ
3     KTSDAGSALDSGSS RYENVKFNDVAVS GGFYGPTAAELGGQFHHKSENGSVGAVFGAKQQ
4     KTSDSGFALDAGSS QHGNAVFSDIKVN GGFYGPTAGELGGQFHHKSDNGSVXAVFGAKRQ
5     KTSDSGFALDAGSS QHGNAVFSDIKVN GGFYGPTAGELGGQFHHKSDNGSVGAVFGAKRQ
As    KTSDSGFALDAGSS QHGNAVFSDIKVN GGFYGPTAGELGGQFHHKSDNGSVGAVFGAKRQ
6     NTTDTGFALDSGSS QHGNAVFSDIKVN GGFYGPTAGELGGQFHHKSDNGSVGAVFGAKRQ
      :*:* * ***:*** :: *. *.*: *. ********.**********:**** ******:*


1     VKK
2     VKK
3     VKK
4     IEK
5     IEK
As    IEK
6     IEK
      ::*
```

Fig. 4.5. Alignment of predicted TbpB precursors of *A. suis* (As) and six strains of *A. pleuropneumoniae* (1-6). The GenBank protein accession numbers for the *A. pleuropneumoniae* proteins are S49815, AAC43484, A44796, S27483, B44796 and S49814, respectively. Asterisks indicate single, fully conserved residues; as defined by CLUSTAL W, colons indicate residues that are fully conserved within a strongly related group and periods indicate residues that are fully conserved within a weakly related group. The boxes identified by roman numerals enclose major regions of homology.

in the C-terminal half (Fig 4.5). As in almost all TbpB precursors listed in the database, there are two tandem cysteine residues in region III but the significance of these conserved residues in the function of the receptor remains to be elucidated (Cornelissen, 2003). Unlike neisserial TbpB precursors (see Cornelissen, 2003), where the longest conserved region is region I, regions III, V and VI of the *A. suis* and *A. pleuropneumoniae* Tbp precursors each contain more amino acids than are present in region I (Fig. 4.5). Since the C-terminal half of neisserial TbpB is perceived to be surface-exposed and immunogenic (Rokbi *et al.*, 2000), it would seem reasonable to suggest that this is also the case for the TbpBs of *A. suis* and *A. pleuropneumoniae* and that the conserved regions may serve as common epitopes and be accessible by antibodies raised against TbpBs of either organism.

Use of the QIAexpressionist and *E. coli* DH5α as host allowed the production, isolation and purification of a hexahistidine-tagged rTbpB. Perhaps not surprisingly, based on the above discussion, this rTbpB was recognized by swine sera raised against *A. pleuropneumoniae* rTbpB. It would appear, therefore, that rTbpBs may prove to be useful components for inclusion in subunit vaccines. During the course of this study, an added bonus was that SDS-PAGE of *A. suis* rTbpB and affinity-isolated Tf-binding polypeptides followed by immunoblotting with anti-rTbpB helped to substantiate the earlier conclusion (Chapter 2; Bahrami *et al.*, 2003) that the ~63-kDa Tf-binding polypeptide that was affinity-isolated from *A. suis* was almost certainly the product of the *tbpB* gene.

In contrast to TbpB, a relatively small protein that is anchored in the outer membrane via a lipid moiety (Gray-Owen and Schryvers, 1996), HgbA is a large, integral transmembrane protein (Pawelek and Coulton, 2004). While these differences offer possible explanations for the lack of success in the isolation of rHgbA, there may well be others. In effect, it would appear that the QIAexpressionist and *E. coli* DH5α may not be suitable for the expression of proteins such as HgbA. Notably, a C-terminally histidine-tagged rHgbA of *A. pleuropneumoniae* has been produced successfully using the expression vector, pET24b, produced by Novagen, and *E. coli* BL21(DE3) as host (Srikumar *et al.*, 2004), and it would be interesting to apply this system to the production of rHgbA of *A. suis*.

Chapter 5. Conclusions and general discussion

All seven strains of *A. suis* used in this study were shown to use porcine, but not human or bovine, Tf as an iron source for growth. Since siderophores cannot discriminate between different animal Tfs (Byers and Arceneaux, 1998; Ratledge and Dover, 2000), the results of the growth experiments alone indicated that *A. suis* does not produce siderophores and that the acquisition of Tf-bound iron is receptor-mediated. While all strains exhibited an absolute specificity for host Tf, no such specificity was observed with Hbs, all strains being able to use bovine and human Hbs, as well as porcine, as sources of iron. These Tf and Hb specificities were also observed in the binding assays, all strains being capable of binding only those proteins that could serve as iron sources for growth. These results indicated that the acquisition of Hb-bound iron is also receptor-mediated and the results obtained in the competition binding assays indicated that all three Hbs, porcine, bovine and human, are bound by the same receptor(s). Notably, membranes from organisms grown under iron-restricted conditions bound Tf and the Hbs to much greater extents than membranes from organisms grown under iron-replete conditions, suggesting the existence of iron-repressible receptors, and subsequently, affinity procedures allowed the isolation of iron-repressible Tf- (~100 and ~63 kDa) and Hb-binding (~105 kDa) polypeptides from all strains.

The strategy employed for the identification and nucleotide sequencing of genes encoding Tf and Hb receptors of *A. suis* benefited from information available (Gonzalez *et al.*, 1995, Tonpitak *et al.*, 2000) on the related swine pathogen, *A. pleuropneumoniae*, the provision of *A. pleuropneumoniae hgbA*-specific primers, prior to publication (Srikumar *et al.*, 2004) of the *hgbA* sequence, and the rapid advances that were made in the *A. pleuropneumoniae* genome project (Microgen Laboratory for Genomics and Bioinformatics). Since the molecular masses of the Tf-binding polypeptides that were affinity-isolated from *A. suis* were similar to those obtained for the Tbps of *A. pleuropneumoniae* (Gonzalez *et al.*, 1990; Ricard *et al.*, 1991; Gerlach *et al.*, 1992*b*; Gray-Owen and Schryvers, 1996), and recognizing the relatedness of *A. suis* and *A. pleuropneumoniae*, it was reasoned that primers based on the nucleotide sequences of *A. pleuropneumoniae tbpA* and *tbpB* might enable the identification and sequencing of homologous genes of *A. suis*. This approach led to the identification and sequencing of *tbpA* and *tbpB* of *A. suis* strains C84 and SO4 and subsequently, genes encoding TonB systems (*exbB*, *exbD* and *tonB*), immediately upstream of the *tbpB* genes. Similarly, primers based on *hgbA* of *A. pleuropneumoniae* allowed the identification and sequencing of *A. suis hgbA* and *hugZ*. Interestingly, the sequenced genes constituted two clusters, *tonB-exbB-exbD-tbpB-tbpA* and *hugZ-hgbA*, and putative promoter elements and Fur boxes could be identified upstream of only *tonB* and *hugZ*; in keeping with the locations of these regulatory elements, the genes in each cluster were shown to be co-transcribable and iron-repressible. Notably, the affinity-isolated Tf- and Hb-binding polypeptides were also found to be iron-repressible and the molecular masses of these polypeptides, determined experimentally, and the N-terminal sequence of the affinity-isolated Hb-binding polypeptide, compared favourably with those predicted from the nucleotide sequences of *tbpA*, *tbpB* and *hgbA*; collectively, these data suggest strongly, that the affinity-isolated polypeptides were, indeed, the products of the *tbpA*, *tbpB* and *hgbA* genes. Genes encoding TonB2 systems (*exbB2*, *exbD2* and *tonB2*) in *A. suis* strains C84 and SO4 were also identified and sequenced but the extents to which the products of these genes, or for that matter, TonB, ExbB and ExbD, are involved in the acquisition of iron from Tf and Hb remain to be established.

Except for 1 bp within *hgbA*, the nucleotide sequences that were obtained for *A. suis* strains C84 and SO4 were identical over 13148 bp. Following deposition of the sequences for *tbpA* and *tbpB* of strains C84 and SO4 in GenBank, nucleotide sequences for *tbpA* and *tbpB* of the type strain of *A. suis* (CCM 5586) were made available by two different research groups, one in Spain (GenBank accession number AF375821) and one in Japan (GenBank accession number AB089839). Based on the sequence information provided by the Spanish group, *tbpA* and *tbpB* of strains C84 (or SO4) and CCM 5586 exhibit 100 % and 99.66 % identity, respectively, at the nucleotide level; the corresponding values based on the sequence information provided by the Japanese group are 99.96 % and 100 %, respectively.

Based on predicted amino acid sequences, the TbpA precursors of *A. suis* exhibit a high degree of homology (>90% identity) to their counterparts in *A. pleuropneumoniae* and *H. parasuis* but a much lower degree of homology (40-42% identity) to the TbpA precursors in other members of the *Pasteurellaceae* (Fig. 5.1). Interestingly, the TbpB precursors of *A. suis* clustered (~86% identity) with representatives of a group of similarly sized (~65 kDa) *A. pleuropneumoniae* TbpB precursors and exhibited only 54-58% identity with representatives of the second group of smaller (~60 kDa) *A. pleuropneumoniae* TbpB precursors (Table 5.1; Fig. 5.2). Nevertheless, alignment of the amino acid sequences of the TbpB precursors of *A. suis* and *A. pleuropneumoniae* reveals the existence of several conserved regions exhibiting high degrees of homology; not surprisingly, rTbpB of *A. suis* was recognized by swine sera raised against rTbpB of *A. pleuropneumoniae*. When TbpB precursors were compared within the *Pasteurellaceae*, a

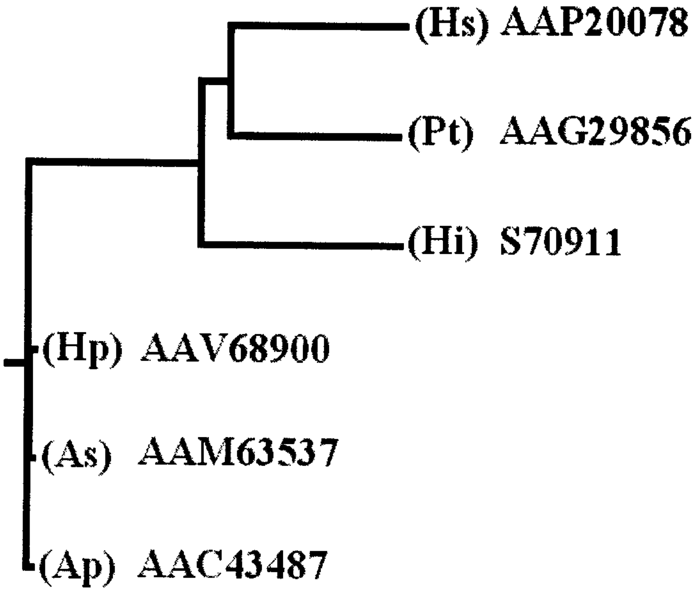


Fig. 5.1. Dendrogram depicting amino acid sequence relatedness between the TbpA precursor of *A. suis* strain SO4 and representative TbpA precursors of other members of the *Pasteurellaceae*. The amino acid sequences that were used are identified by their GenBank protein accession numbers and the names of the organisms are abbreviated as follows: Ap, *A. pleuropneumoniae*; As, *A. suis*; Hi, *H. influenzae*; Hp, *H. parasuis*; Hs, *H. somni*; Pt, *P. trehalosi*. Alignments of amino acid sequences were performed using CLUSTAL W (Biology WorkBench). The dendrogram was constructed at the Biology WorkBench using DRAWGRAM and the CLUSTAL W alignments to generate a rooted phylogenetic tree. Branch lengths separating the sequences reflect the degree of similarity between them.

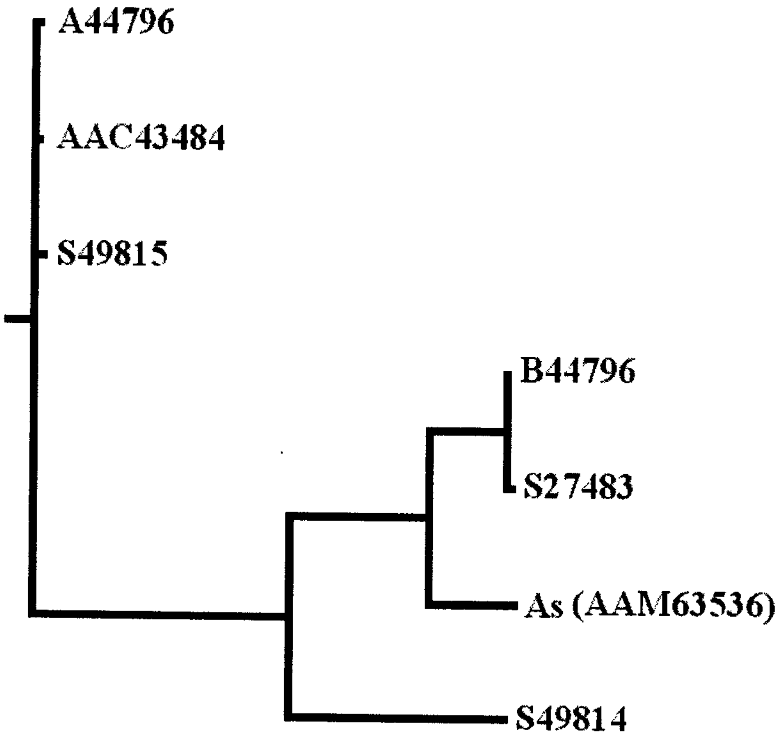
Table 5.1. Comparison of *A. pleuropneumoniae* and *A. suis* TbpB precursors

Species, strain (serotype)	GenBank protein accession no.	% identity vs. *A. suis*	Publication date	Mw (kDa)
Ap[a], nr[b]	S49815	54	1994	59.8
Ap, H49 (7)	AAC43484	54	1994	59.8
Ap, AP205 (7)	A44796	55	1992	59.8
Ap, nr	S49814	58	1995	60.0
Ap, nr	S27483	86	1992	65.5
Ap, AP37 (1)	B44796	86	1993	65.5
As[c], SO4	AAM63536	100	2003	65.6

[a] *A. pleuropneumoniae*

[b] not reported

[c] *A. suis*



5.2. Dendrogram depicting amino acid sequence relatedness between the TbpB precursor of *A. suis* strain SO4 (As) and TbpB precursors of representative strains of *A. pleuropneumoniae*. The amino acid sequences that were used are identified by their GenBank protein accession numbers. The dendrogram was constructed as indicated in the legend to Fig. 5.1. Branch lengths separating the sequences reflect the degree of similarity between them.

total of three phylogenetically related groups could be identified (Fig. 5.3). One contains *A. suis* and *A. pleuropneumoniae*, as described above, with the TbpB precursors exhibiting ~86% identity. The others contain *H. parasuis* and *A. pleuropneumoniae*, with the TbpB precursors sharing ~54% identity with the TbpB precursors of *A. suis*, and *Pasteurella trehalosi* and *H. influenzae*, where the TbpB precursors share 36% identity and 30% identity, respectively, with the TbpB precursors of *A. suis*. Comparisons of the amino acid sequence of the *A. suis* strain SO4 HgbA precursor with the annotated sequences available in the database for the HgbA precursors of other members of the *Pasteurellaceae* revealed that the HgbA precursors of *A. suis* are related most closely to the HgbA precursor of *A. pleuropneumoniae* (99% identity), followed by the HgbA precursor of *P. multocida* (57% identity) and then the HgbA precursors of *H. influenzae*, *H. ducreyi*, *H. somni* and *A. actinomycetemcomitans* (Fig. 5.4).

Recent homology modelling of *N. meningitidis* TbpA (Oakhill *et al.*, 2005) and *A. pleuropneumoniae* HgbA (Pawelek and Coulton, 2004) prompted comparison of the amino acid sequences of these proteins with those of *A. suis* TbpA and HgbA and the construction of similar models (Figs. 5.5 and 5.6). As depicted in the models, *A. suis* TbpA and HgbA are predicted to exist as 22-stranded β-barrel outer membrane receptors, each with a globular N-terminal cork domain and eleven extracellular loops (Tables 5.2 and 5.3). Notably, while the 1-bp difference in the *hgbA* genes of *A. suis* strains C84 and SO4 translates as a difference of 1 amino acid (position 756 in the HgbA precursors), threonine in strain C84 versus methionine in strain SO4, these amino acids are located extracellularly in loops 8 (Table 5.3). Interestingly, it has been proposed that extracellular loops 2, 6 and 9 of *A. pleuropneumoniae* HgbA are the loops that interact with haem

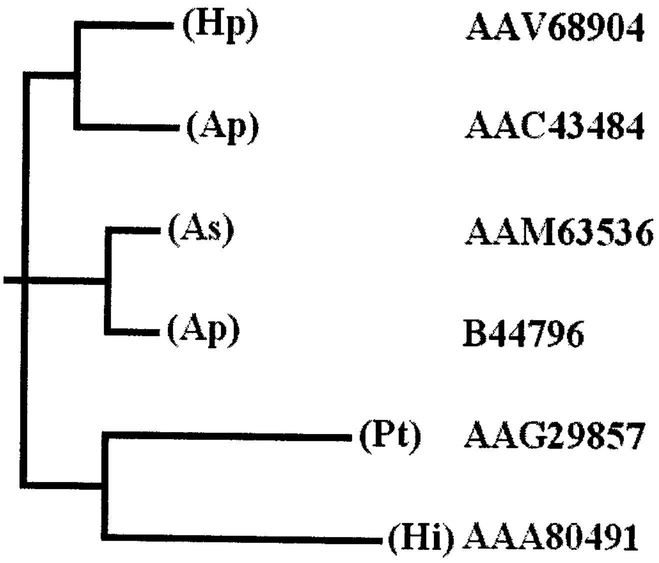


Fig. 5.3. Dendrogram depicting amino acid sequence relatedness between the TbpB precursor of *A. suis* strain SO4 and representative TbpB precursors of other members of the *Pasteurellaceae*. The amino acid sequences that were used are identified by their GenBank protein accession numbers and the names of the organisms are abbreviated as follows: Ap, *A. pleuropneumoniae*; As, *A. suis*; Hi, *H. influenzae*; Hp, *H. parasuis*; Pt, *P. trehalosi*. The dendrogram was constructed as indicated in the legend to Fig. 5.1. Branch lengths separating the sequences reflect the degree of similarity between them.

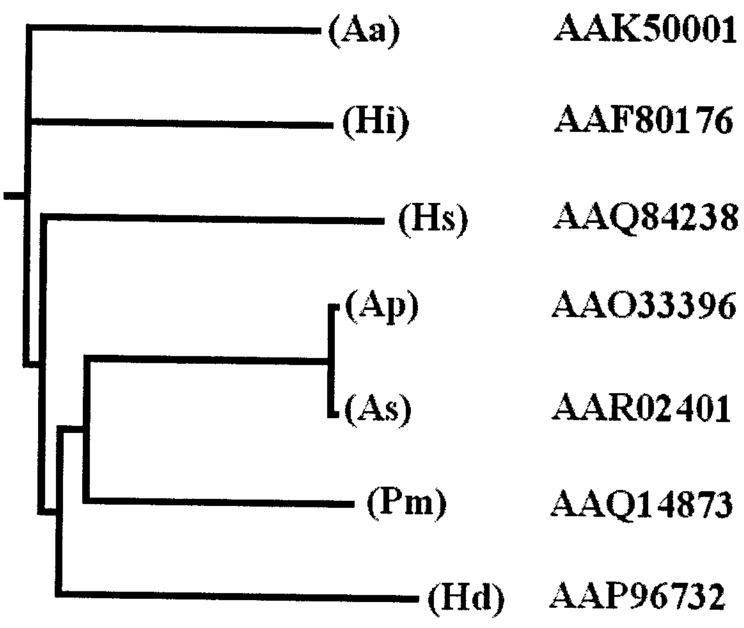


Fig. 5.4. Dendrogram depicting amino acid sequence relatedness between the HgbA precursor of *A. suis* strain SO4 and representative HgbA precursors of other members of the *Pasteurellaceae*. The amino acid sequences that were used are identified by their GenBank protein accession numbers and the names of the organisms are abbreviated as follows: Aa, *A. actinomycetemcomitans*; Ap, *A. pleuropneumoniae*; As, *A. suis*; Hd, *H. ducreyi*; Hi, *H. influenzae*; Hs, *H. somni*; Pm, *P. multocida*. The dendrogram was constructed as indicated in the legend to Fig. 5.1. Branch lengths separating the sequences reflect the degree of similarity between them.

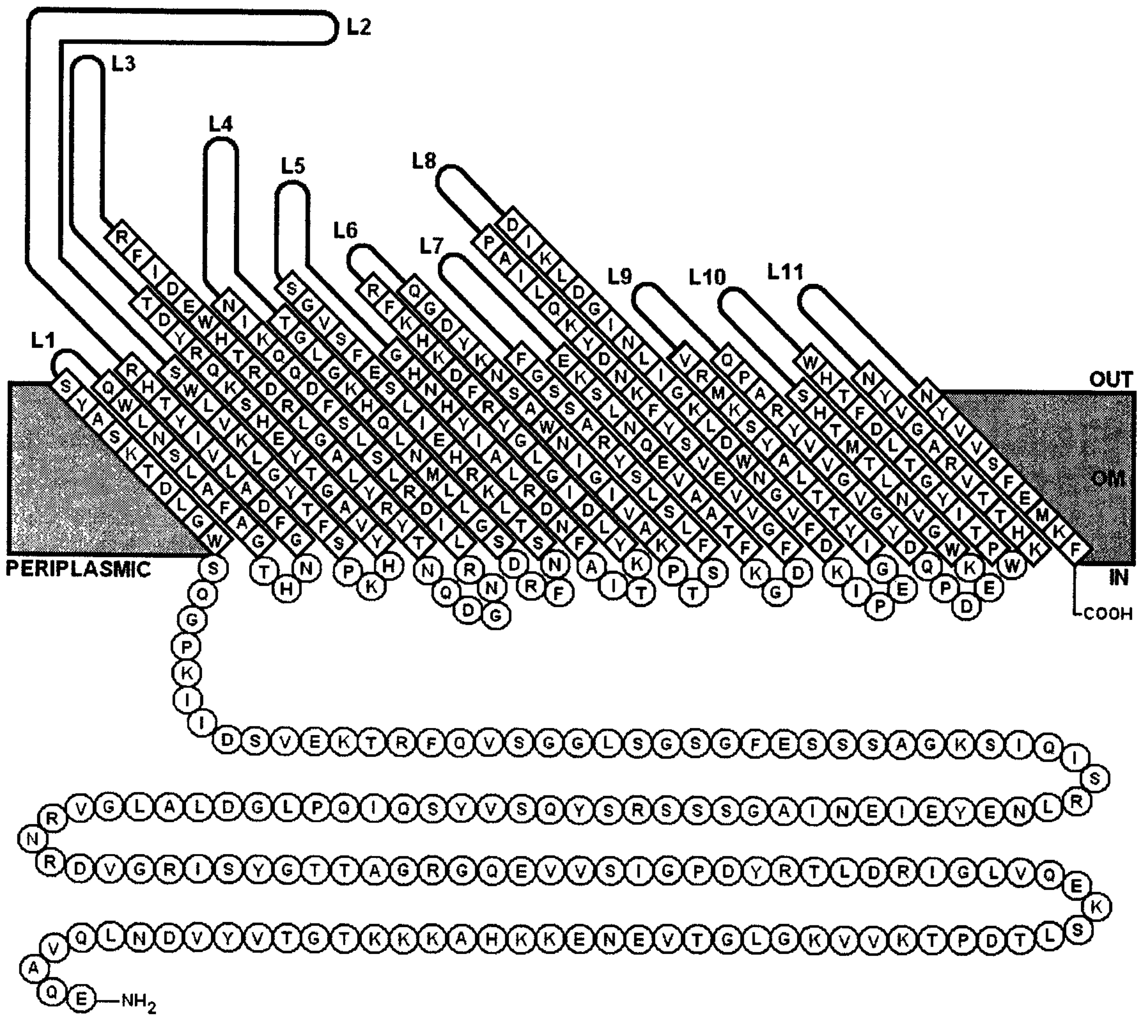


Fig. 5.5. Topological model of *A. suis* TbpA. L1-L11 represent the putative surface-exposed loops (Table 5.2). Residues in diamonds represent the transmembrane β-strands of the β-barrel and residues in circles represent the periplasmic loops and the putative cork-like domain. The model was prepared based on the homology model of *N. meningitidis* TbpA (Oakhill *et al.*, 2005) and alignment (CLUSTAL W) of the primary sequences of the TbpA proteins of *A. suis* strain SO4 and *N. meningitidis* strain K454 (GenBank protein accession numbers AAM63537 and AAF81744, respectively). The figure was constructed using Microsoft Paint.

Table 5.2. Putative extracellular loop sequences of *A. suis* TbpA (based on Oakhill *et al.*, 2005)

TbpA loop	Sequence (N → C)
L1	SKNQ
L2	DGKETKAHKDAESRSQNITRVGVETNELDTSNRYTATTNNQHTYGWFLIKDECPTLDCTPKQMARVTKDTPSFRSYPEYTPEEKQAYENQKHITERLNAQDYTGEYRALPDPLKYKSD
L3	RDMTYTAYWQPSDLLRTGRNWYPMNNAKGLYRDNALDGVAIDYFTEDGVKSSKGLRWAKA
L4	LSTRLRENNCSEYPTIDKNCRATLDKLWSSTKNEQSSYEEKHDTIQLSLDKTVQ
L5	WGLVRDIGYRNGSYNNPYVYELKDQAIYSKNECDYSGTIAGRADCATSKIK
L6	STHRWAN
L7	RVPSFQELFGLRYDGAMKGSSDAYQKTEKLSP
L8	AERMHQTQSMINYFNVQ
L9	KEVKNYQGYMNIRSPLLDTI
L10	SGKNPDELRGNEQVGFANYERTATKKRTRS
L11	LMNYRYTTWESVRQSSLNAIHQHTNVKDYARYAAPGR

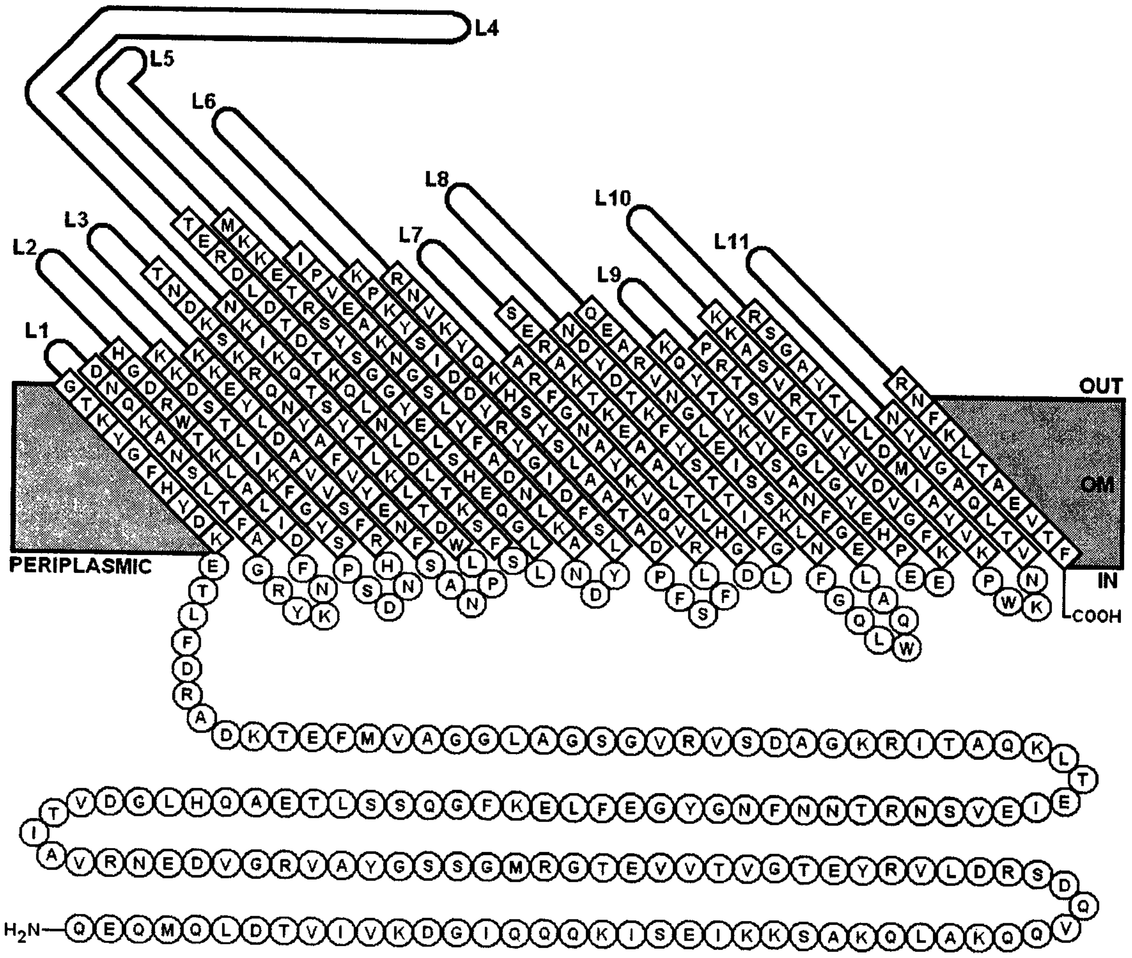


Fig. 5.6. Topological model of *A. suis* HgbA. L1-L11 represent the putative surface-exposed loops (Table 5.3). Residues in diamonds represent the transmembrane β-strands of the β-barrel and residues in circles represent the periplasmic loops and the putative cork-like domain. The model was prepared based on the homology model of *A. pleuropneumoniae* HgbA (Pawelek and Coulton, 2004), information pertaining to the proposed sequences of extracellular loop and β-barrel domains (Pawelek and Coulton, 2004) and alignment (CLUSTAL W) of the primary sequences of the HgbA proteins of *A. suis* strain SO4 and *A. pleuropneumoniae* (GenBank protein accession numbers AAR02401 and AAO33396, respectively). The figure was constructed using Microsoft Paint.

Table 5.3. Putative extracellular loop sequences of *A. suis* strain SO4 HgbA (based on Pawelek and Coulton, 2004)

HgbA loop	Sequence (N → C)
L1	YSSA
L2	ELENYDYKTADGSVQGKEREKADPYSI
L3	NRGHDFSYNLKPTTYINVDEYELRH
L4	RARTDDYCDGSHCKETENLAGLQLKDGKVVDRDGNQPNLGVDELGLTTITDSKGTYTEGVNLVRAYWFDCSVFDCNKSVTAYYKDYSNNITSEEVALTKTYTDEKGRKFATLDPKSKFKSILLPGSKGYTENIY
L5	VNRGGFGATSNTQWWTKRFLGMRNNFFKGTEEVITCKNATGSDQWNGLICPNEDTFSFL
L6	YVAGQTAKIPDDMLQGLFVPLPDRPTKEQIRQNAEENIKYLS
L7	PTSDELYFTFKHPDFTVLPNVDLK
L8	FIDLKYLGPKNLMNAFGGSAMARPYQIYQNVNR
L9	GKMEGNLPLNAIQ
L10	AEDTYNMYHSEEKAKDSYLKW
L11	LQNRKYLTWESVRSIRPFGTSNLINQTTGKGLNRFTAPG

(Pawelek and Coulton, 2004) and based on their similarity, it would seem reasonable to propose that this is also the case for HgbA of *A. suis*.

References

Abdul-Tehrani, H., Hudson, A.J., Chang, Y.-S., Timms, A.R., Hawkins, C., Williams, J.M., Harrison, P.M., Guest, J.R., and Andrews, S.C. 1999. Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and *fur* mutants are iron deficient. J. Bacteriol. **181:** 1415-1428.

Ahmer, B.M.M., Thomas, M.G., Larsen, R.A., and Postle, K. 1995. Characterization of the *exbBD* operon of *Escherichia coli* and the role of ExbB and ExbD in TonB function and stability. J. Bacteriol. **177:** 4742-4747.

Aisen, P. 1998. Transferrin, the transferrin receptor, and the uptake of iron by cells. Met. Ions. Biol. Syst. **35:** 585-631.

Ala'Aldeen, D.A.A, Stevenson, P., Griffiths, E., Gorringe, A.R., Irons, L.I., Robinson, A., Hyde, S., and Borriello, S.P. 1994. Immune responses in humans and animals to meningococcal transferrin-binding proteins: implications for vaccine design. Infect. Immun. **62:** 2984-2990.

Alm, R.A., Ling, L.S., Moir, D.T., King, B.L., Brown, E.D., Doig, P.C., Smith, D.R., Noonan, B., Guild, B.C., deJonge, B.L., Carmel, G., Tummino, P.J., Caruso, A., Uria Nickelsen, M., Mills, D.M., Ives, C., Gibson, R., Merberg, D., Mills, S.D., Jiang, Q., Taylor, D.E., Vovis, G.F., and Trust, T.J. 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori.* Nature **397:** 176-180.

Anderson, J.E., Sparling, P.F., and Cornelissen, C.N., 1994. Gonococcal transferrin-binding protein 2 facilitates but is not essential for transferrin utilization. J. Bacteriol. **176:** 3162-3170.

Andrews, S.C., Robinson A.K., and Rodríguez-Quiñones, F. 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. **27:** 215-237.

Archibald, F.S.1983. *Lactobacillus plantarum*, an organism not requiring iron. FEMS Microbiol. Lett. **19:** 29-32.

Arnoux, P., Haser, R., Izadi, N., Lecroisey, A., Delepierre, M., Wandersman, C., and Czjzek, M. 1999. The crystal structure of HasA, a hemophore secreted by *Serratia marcescens*. Nat. Struct. Biol. **6:** 516-520.

Bahrami, F., and Niven, D.F. 2005. Iron acquisition by *Actinobacillus suis*: identification and characterization of a single-component haemoglobin receptor and encoding gene. Microb. Pathog. **39:** 45-51.

Bahrami, F., Ekins, A., and Niven, D.F. 2003. Iron acquisition by *Actinobacillus suis*: identification and characterization of transferrin receptor proteins and encoding genes. Vet. Microbiol. **94:** 79-92.

Baichoo, N., and Helmann, J.D. 2002. Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. J. Bacteriol. **184:** 5826-5832.

Baker, E.N., Baker, H.M., and Kidd, R.D. 2002 Lactoferrin and transferrin: functional variations on a common structural framework. Biochem. Cell Biol. **80:** 27-34.

Baltes, N., Henning-Pauka, I., and Gerlach, G.-F. 2002. Both transferrin binding proteins are virulence factors in *Actinobacillus pleuropneumoniae* serotype 7 infection. FEMS Microbiol. Lett. **209:** 283-287.

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:** 701-708.

Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S. 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340:** 783-795.

Biswas, G.D., Anderson, J.E., and Sparling, P.F. 1997. Cloning and functional characterization of *Neisseria gonorrhoeae tonB*, *exbB* and *exbD* genes. Mol. Microbiol. **24:** 169-179.

Biville, F., Cwerman, H., Létoffé, S., Rossi, M.S., Drouet, V., Ghigo, J.M., and Wandersman, C. 2004. Haemophore-mediated signalling in *Serratia marcescens*: a new mode of regulation for an extra cytoplasmic function (ECF) sigma factor involved in haem acquisition. Mol. Microbiol. **53:** 1267-1277.

Blackall, P.J., Klaasen, H.L., van den Bosch, H., Kuhnert, P., and Frey, J. 2002. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet. Microbiol. **84:** 47-52.

Bosch, M., Garrido, M.E., Llagostera, M., Pérez de Rozas, A.M., Badiola, I., and Barbé, J. 2002. Characterization of the *Pasteurella multocida hgbA* gene encoding a hemoglobin-binding protein. Infect. Immun. **70:** 5955-5964.

Bosch, M., Garrido, M.E., Pérez de Rozas, A.M., Badiola, I., Barbé, J., and Llagostera, M. 2004. *Pasteurella multocida* contains multiple immunogenic haemin- and haemoglobin-binding proteins. Vet. Microbiol. **99:** 103-112.

Boyer, E., Bergevin, I., Malo, D., Gros, P., and Cellier, M.F. 2002. Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of *Salmonella enterica* serovar *typhimurium*. Infect. Immun. **70:** 6032-6042.

Bracken, C.S., Baer, M.T., Abdur-Rashid, A., Helms, W., and Stojiljkovic, I. 1999. Use of heme-protein complexes by the *Yersinia enterocolitica* HemR receptor: histidine residues are essential for receptor function. J. Bacteriol. **181:** 6063-6072.

Bradbeer, C. 1993. The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli.* J. Bacteriol. **175:** 3146-3150.

Braun, V. 1995. Energy-coupled transport and signal transduction through the Gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. FEMS Microbiol. Rev. **16:** 295-307.

Braun, V., and Braun, M. 2002. Iron transport and signaling in *Escherichia coli.* FEBS Lett. **529:** 78-85.

Braun, V., Gaisser, S., Herrmann, C., Kampfenkel, K., Killmann, H., and Traub, T. 1996. Energy-coupled transport across the outer membrane of *Escherichia coli*: ExbB binds ExbD and TonB *in vitro*, and leucine 132 in the periplasmic region and aspartate 25 in the transmembrane region are important for ExbD activity. J. Bacteriol. **178:** 2836-2845.

Braun, V., Hantke, K., and Wolfgang, K. 1998. Bacterial iron transport: mechanisms, genetics, and regulation. *In* Metal Ions in Biological Systems, Vol. 35. *Edited by* A. Sigel, and H. Sigel. Marcel Dekker, Inc., New York, NY, U.S.A. pp. 67-145.

Bsat, N., and Helmann, J.D. 1999. Interaction of *Bacillus subtilis* Fur (ferric uptake repressor) with the *dhb* operator *in vitro* and *in vivo.* J. Bacteriol. **181:** 4299-4307.

Buchanan, S.K., Smith, B.S., Venkatramani, L., Xia, D., Esser, L., Palnitkar, M., Chakraborty, R., van der Helm, D., and Deisenhofer, J. 1999. Crystal structure of the outer membrane active transporter FepA from *Escherichia coli.* Nat. Struct. Biol. **6:** 56-63.

Burrows, L.L., and Lo, R.Y. 1992. Molecular characterization of an RTX toxin determinant from *Actinobacillus suis.* Infect. Immun. **60:** 2166-2173.

Byers, B.R., and Arceneaux, J.E.L. 1998. Microbial iron transport: iron acquisition by pathogenic microorganisms. *In* Metal Ions in Biological Systems, Vol. 35. *Edited by* A. Sigel, and H. Sigel. Marcel Dekker, Inc., New York, NY, U.S.A. pp. 37-66.

Calderwood, S.B., and Mekalanos, J.J. 1988. Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J. Bacteriol. **170:** 1015-1017.

Caldwell, M., and Archibald, F. 1987. The effect of the hypoferremic response on iron acquisition by and growth of murine lymphoma cells. Biochem. Cell Biol. **65:** 651-657.

Camejo, G., Halberg, C., Manschik-Lundin, A., Hurt-Camejo, E., Rosengren, B., Olsson, H., Hansson, G.I., Forsberg, G.B., and Ylhen, B. 1998. Hemin binding and oxidation of lipoproteins in serum: mechanisms and effect on the interaction of LDL with human macrophages. J. Lipid. Res. **39:** 755-766.

Campagnari, A.A., Shanks, K.L., and Dyer, D.W. 1994. Growth of *Moraxella catarrhalis* with human transferrin and lactoferrin: expression of iron-repressible proteins without siderophore production. Infect. Immun. **62:** 4904-4914.

Carrondo, M.A. 2003. Ferritins, iron uptake and storage from the bacterioferritin viewpoint. EMBO J. **22:** 1959-1968.

Chang, C., Mooser, A., Pluckthun, A., and Wlodawer, A. 2001. Crystal structure of the dimeric C-terminal domain of TonB reveals a novel fold. J. Biol. Chem. **276:** 27535-27540.

Chen, C.-J., Sparling, P.F., Lewis, L.A., Dyer, D.W., and Elkins, C. 1996. Identification and purification of a hemoglobin-binding outer membrane protein from *Neisseria gonorrhoeae*. Infect. Immun. **64:** 5008-5014.

Chen, C.-J., Elkins, C., and Sparling, P.F. 1998. Phase variation of haemoglobin utilization in *Neisseria gonorrhoeae*. Infect. Immun. **66:** 987-993.

Chen, C.Y., and Morse, S.A. 1999. *Neisseria gonorrhoeae* bacterioferritin: structural heterogeneity, involvement in iron storage and protection against oxidative stress. Microbiology **145:** 2967-2975.

Chipperfield, J.R., and Ratledge, C. 2000. Salicylic acid is not a bacterial siderophore: a theoretical study. Biometals **13:** 165-168.

Christensen, H., and Bisgaard, M. 2004. Revised definition of *Actinobacillus sensu stricto* isolated from animals. A review with special emphasis on diagnosis. Vet. Microbiol. **99:** 13-30.

Chu, G.C., Katakura, K., Zhang, X., Yoshida, T., and Ikeda-Saito, M. 1999. Heme degradation as catalyzed by a recombinant bacterial heme oxygenase (Hmu O) from *Corynebacterium diphtheriae*. J. Biol. Chem. **274:** 21319-21325.

Cope, L.D., Thomas, S.E., Latimer, J.L., Slaughter, C.A., Mulle-Eberhard, U., and Hansen, E.J. 1994. The 100 kDa haem:haemopexin-binding protein of *Haemophilus influenzae*: structure and localization. Mol. Microbiol. **13:** 863-873.

Cope, L.D., Yogev, R., Muller-Eberhard, U., and Hansen, E.J. 1995. A gene cluster involved in the utilization of both free heme and heme:hemopexine by *Haemophilus influenzae* type B. J. Bacteriol. **177:** 2644-2653.

Cope, L.D., Thomas, S.E., Hrkal, Z., and Hansen, E.J. 1998. Binding of heme-hemopexin complexes by soluble HxuA protein allows utilization of this complexed heme by *Haemophilus influenzae*. Infect. Immun. **66:** 4511-4516.

Cope, L.D., Hrkal, Z., and Hansen, E.J. 2000. Detection of phase variation in expression of proteins involved in hemoglobin and hemoglobin-haptoglobin binding by nontypeable *Haemophilus influenzae*. Infect. Immun. **68:** 4092-4101.

Cope, L.D., Love, R.P., Guinn, S.E., Gilep, A., Usanov, S., Estabrook, R.W., Hrkal, Z., and Hansen, E.J. 2001. Involvement of HxuC outer membrane protein in utilization of hemoglobin by *Haemophilus influenzae*. Infect. Immun. **69:** 2353-2363.

Cornelissen, C.N. 2003. Transferrin-iron uptake by Gram-negative bacteria. Front. Biosci. **8:** d836-d847.

Cornelissen, C.N., Biswas, G.D., Tsai, J., Paruchuri, D.K., Thompson, S.A., and Sparling, P.F. 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. J. Bacteriol. **174:** 5788-5797.

Cox, A.J., Hunt, M.L., Boyce, J.D., and Adler, B. 2003. Functional characterization of HgbB, a new hemoglobin binding protein of *Pasteurella multocida*. Microb. Pathogen. **34:** 287-296.

Danve, B., Lissolo, L., Mignon, M., Dumas, P., Colombani, S., Schryvers, A.B., and Quentin-Millet, M.-J. 1993. Transferrin-binding proteins isolated from *Neisseria meningitidis* elicit protective and bactericidal antibodies in laboratory animals. Vaccine **11:** 1214-1220.

de Lorenzo, V., Wee, S., Herrero, M., and Neilands, J.B. 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J. Bacteriol. **169:** 2624-2630.

del Río, M.L., Gutiérrez-Martín, C.B., Rodríguez-Barbosa, J.I., Navas, J., and Rodríguez-Ferri. 2005. Identification and characterization of the TonB region and its role in transferrin-mediated iron acquisition in *Haemophilus parasuis*. FEMS Immunol. Med. Microbiol. **45:** 75-86.

Deneer, H.G., Healey, V., and Boychuk, I. 1995. Reduction of exogenous ferric iron by a surface-associated ferric reductase of *Listeria* spp. Microbiology **141:** 1985-1992.

Dockal, M., Carter, D.C., and Ruker, F. 1999. The three recombinant domains of human serum albumin. Structural characterization and ligand binding properties. J. Biol. Chem. **274:** 29303-29310.

D'Silva, C.G., Archibald, F.S., and Niven, D.F. 1995. Comparative study of iron acquisition by biotype 1 and biotype 2 strains of *Actinobacillus pleuropneumoniae*. Vet. Microbiol. **44:** 11-23.

Eick-Helmerich, K., and Braun, V. 1989. Import of biopolymers into *Escherichia coli*: nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of the *tolQ* and *tolR* genes, respectively. J. Bacteriol. **171:** 5117-5126.

Ekins, A., and Niven, D.F. 2001. Production of transferrin receptors by *Histophilus ovis*: three of five strains require two signals. Can. J. Microbiol. **47:** 417-423.

Ekins, A., and Niven, D.F. 2002. Identification of *fur* and *fldA* homologs and a *Pasteurella multocida tbpA* homolog in *Histophilus ovis* and effects of iron availability on their transcription. J. Bacteriol. **184:** 2539-2542.

Ekins, A., and Niven, D.F. 2003. Transferrin-dependent expression of TbpA by *Histophilus ovis* involves a poly G tract within *tbpA*. FEMS Microbiol. Lett. **220:** 95-98.

Ekins, A., Bahrami, F., Sijercic, A., Maret, D., and Niven, D.F. 2004*a*. *Haemophilus somnus* possesses two systems for acquisition of transferrin-bound iron. J. Bacteriol. **186:** 4407-4411.

Ekins, A., Khan, A.G., Shouldice, S.R., and Schryvers, A.B. 2004*b*. Lactoferrin receptors in Gram-negative bacteria: insights into the iron acquisition process. Biometals **17:** 235-243.

Elkins, C. 1995. Identification and purification of a conserved heme-regulated hemoglobin-binding outer membrane protein from *Haemophilus ducreyi*. Infect. Immun. **63:** 1241-1245.

Elkins, C., Chen, C.-J., and Thomas, C. E. 1995. Characterization of the *hgbA* locus encoding a hemoglobin receptor from *Haemophilus ducreyi*. Infect. Immun. **63:** 2194-2200.

Elkins, C., Totten, P.A., Olsen, B., and Thomas, C.E. 1998. Role of the *Haemophilus ducreyi* Ton system in internalization of heme from hemoglobin. Infect. Immun. **66:** 151-160.

Ernst, J.F., Bennett, R.L., and Rothfield, L.I. 1978. Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of *Salmonella typhimurium*. J. Bacteriol. **135:** 928-934.

Escolar, L., Pérez -Martin, J., and de Lorenzo, V. 1998. Binding of the Fur (ferric uptake regulator) repressor of *Escherichia coli* to arrays of the GATAAT sequence. J. Mol. Biol. **283:** 537-547.

Escolar, L., Pérez -Martin, J., and de Lorenzo, V. 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. J. Bacteriol. **181:** 6223-6229.

Evans, R.W., and Oakhill, J.S. 2002. Transferrin-mediated iron acquisition by pathogenic *Neisseria*. Biochem. Soc. Trans. **30:** 705-707.

Fanucci, G.E., Cadieux, N., Piedmont, C.A., Kadner, R.J., and Cafiso, D.S. 2002. Structure and dynamics of the β-barrel of the membrane transporter BtuB by site-directed spin labeling. Biochemistry **39:** 11543-11551.

Fanucci, G.E., Coggshall, K.A., Cadieux, N., Kim, M., Kadner, R.J., and Cafiso, D.S. 2003. Substrate-induced conformational changes of the periplasmic N-terminus of an outer membrane transporter by site-directed spin-labeling. Biochemistry **42:** 1391-1400.

Ferguson, A.D., Hofmann, E., Coulton, J.W., Diederichs, K., and Welte, W. 1998. Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. Science **282:** 2215-2220.

Frangipane, M.E., Morton, D.J., Wooten, J.A., Pozsgay, J.M., and Stull, T.L. 1994. Binding of human hemoglobin by *Haemophilus influenzae*. FEMS Microbiol. Lett. **118:** 243-248.

Fridovich, I. 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem. **64:** 97-112.

Fridovich, I. 1997. Superoxide anion radicals (O_2^-) superoxide dismutases (SODs), and related matters. J. Biol. Chem **272:** 18515-18517.

Furrer, J., Sanders, D., Hook-Barnard, I., and McIntosh, M. 2002. Export of the siderophore enterobactin in *Escherichia coli*: involvement of a 43 kDa membrane exporter. Mol. Microbiol. **44:** 1225-1234.

Genco, C.A., and Dixon, D.W. 2001. Emerging strategies in microbial haem capture. Mol. Microbiol. **39:** 1-11.

Gerlach, G.-F., Anderson, C., Potter, A.A., Klashinsky, S., and Willson, P.J. 1992*a*. Cloning and expression of a transferrin-binding protein from *Actinobacillus pleuropneumoniae*. Infect. Immun. **60:** 892-898.

Gerlach, G.-F., Klashinsky, S., Anderson, C., Potter, A.A., and Willson, P.J. 1992*b*. Characterization of two genes encoding distinct transferrin-binding proteins in different *Actinobacillus pleuropneumoniae* isolates. Infect. Immun. **60:** 3253-3261.

Ghigo, J.M., Létoffé, S., and Wandersman, C. 1997. A new type of hemophore-dependent heme acquisition system of *Serratia marcescens* reconstituted in *Escherichia coli.* J. Bacteriol. **179:** 3572-3579.

Gonzalez, G.C., Caamano, D.L., and Schryvers, A.B. 1990. Identification and characterization of a porcine-specific transferrin receptor in *Actinobacillus pleuropneumoniae.* Mol. Microbiol. **4:** 1173-1179.

Gonzalez, G.C., Yu, R.-H., Rostek, P.R., and Schryvers, A.B. 1995. Sequence, genetic analysis, and expression of *Actinobacillus pleuropneumoniae* transferrin receptor genes. Microbiology **141:** 2405-2416.

Gornall, A.G., Bardawill, C.S., and David, M.M. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177:** 751-763.

Graham, M.R., and Lo, R.Y. 2002. A putative iron-regulated TonB-dependent receptor of *Mannheimia* (*Pasteurella*) *haemolytica* A1: possible mechanism for phase variation. Vet. Microbiol. **84:** 53-67.

Gray-Owen, S.D., and Schryvers, A.B. 1995. Characterization of transferrin binding proteins 1 and 2 in invasive type b and nontypeable strains of *Haemophilus influenzae.* Infect. Immun. **63:** 3809-3815.

Gray-Owen, S.D., and Schryvers, A.B. 1996. Bacterial transferrin and lactoferrin receptors. Trends Microbiol. **4:** 185-192.

Gray-Owen, S.D., Loosmore, S., and Schryvers, A.B. 1995. Identification and characterization of genes encoding the human transferrin-binding proteins from *Haemophilus influenzae.* Infect. Immun. **63:** 1201-1210.

Griffiths, E. 1987. The iron-uptake systems of pathogenic bacteria. *In* Iron and Infection. Molecular, Physiological and Clinical Aspects. *Edited by* J.J. Bullen, and E. Griffiths. John Wiley & Sons Ltd., Chichester, U.K. pp. 69-137.

Haesebrouck, F., Chiers, K., Van Overbeke, I., and Ducatelle, R. 1997. *Actinobacillus pleuropneumoniae* infections in pigs: the role of virulence factors in pathogenesis and protection. Vet. Microbiol. **58:** 239-249.

Haesebrouck, F., Pasmans, F., Chiers, K., Maes, D., Ducatelle, R., and Decostere, A. 2004. Efficacy of vaccines against bacterial diseases in swine: what can we expect? Vet. Microbiol. **100:** 255-268.

Hantke, K. 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol. Gen. Genet. **182:** 288-292.

Hantke, K. 1984. Cloning of the repressor protein gene of iron-regulated systems in *Escherichia coli* K12. Mol. Gen. Genet. **197:** 337-341.

Hantke, K. 2001. Iron and metal regulation in bacteria. Curr. Opin. Microbiol. **4:** 172-177.

Harlow, E., and Lane, D. 1988. Silver staining of gels-ammoniacal silver staining. *In* Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, NY, U.S.A. pp. 651-652.

Harrison, S.C., and Aggarwal, A.G. 1990. DNA recognition by proteins with the helix-turn-helix motif. Annu. Rev. Biochem. **59:** 933-969.

Hasan, A.A., Holland, J., Smith, A., and Williams, P. 1997. Elemental iron does repress transferrin, haemopexin and haemoglobin receptor expression in *Haemophilus influenzae*. FEMS Microbiol. Lett. **150:** 19-26.

Hayashida, H., Poulsen, K., and Kilian, M. 2002. Differences in iron acquisition from human haemoglobin among strains of *Actinobacillus actinomycetemcomitans*. Microbiology **148:** 3993-4001.

Heiner, C.R., Hunkapiller, K.L., Chen, S.-M., Glass, J.I., and Chen, E.Y. 1998. Sequencing multimegabase-template DNA with BigDye terminator chemistry. Genome Res. **8:** 557-561.

Helmann, J.D. 1997. Metal cation regulation in Gram-positive bacteria, *In* Metal Ions in Gene Regulation. *Edited by* S. Silver, and W. Walden. Chapman & Hall, New York, NY, U.S.A. pp. 45-76.

Henderson, D.P., Wyckoff, E.E., Rashidi, C.E., Verlei, H., and Oldham A.L. 2001. Characterization of the *Plesiomonas shigelloides* genes encoding the heme iron utilization system. J. Bacteriol. **183:** 2715-2723.

Hentze, M.W., Muckenthaler, M.U., and Andrews, N.C. 2004. Balancing acts: molecular control of mammalian iron metabolism. Cell **117:** 285-297.

Higgs, P.I., Larsen, R.A., and Postle, K. 2002. Quantification of known components of the *Escherichia coli* TonB energy transduction system: TonB, ExbB, ExbD and FepA. Mol. Microbiol. **44:** 271-281.

Hohmuth, M., Valentin-Weiganz, P., Rohde, M., and Gerlach, G.-F. 1998. Identification and characterization of a novel extracellular ferric reductase from *Mycobacterium paratuberculosis*. Infect. Immun. **66:** 710-716.

Jacobsen, I., Gerstenberger, J., Gruber, A.D., Bossé, J.T., Langford, P.R., Hennig-Pauka, I., Meens, J., and Gerlach, G.-F. 2005. Deletion of the ferric regulator Fur impairs the *in vitro* growth and virulence of *Actinobacillus pleuropneumoniae*. Infect. Immun. **73:** 3740-3744.

Jarosik, G.P., Sanders, J.D., Cope, L.D., Muller-Eberhard, U., and Hansen, E.J. 1994. A functional *tonB* gene is required for both utilization of heme and virulence expression by *Haemophilus influenzae* Type B. Infect. Immun. **62:** 2470-2477.

Jarosik, G.P., MacIver, I., Cope, L.D., and E.J. Hansen. 1995. Utilization of transferrin-bound iron by *Haemophilus influenzae* requires an intact *tonB* gene. Infect. Immun. **63:** 710-713.

Jin, H., Ren, Z., Whitby, P.W., Morton, D.J., and Stull, T.L. 1999. Characterization of *hgpA*, a gene encoding a haemoglobin/haemoglobin-haptoglobin-binding protein of *Haemophilus influenzae*. Microbiology **145:** 905-914.

Kammler, M., Schon, C., and Hantke, K. 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. J. Bacteriol. **175:** 6212-6219.

Kamp, E.M., Vermeulen, T.M., Smits, M.A., and Haagsma, J. 1994. Production of Apx toxins by field strains of *Actinobacillus pleuropneumoniae* and *Actinobacillus suis*. Infect. Immun. **62:** 4063-4065.

Kampfenkel, K., and Braun, V. 1992. Membrane topology of the *Escherichia coli* ExbD protein. J. Bacteriol. **174:** 5485-5487.

Kampfenkel, K., and Braun, V. 1993. Topology of the ExbB protein in the cytoplasmic membrane of *Escherichia coli*. J. Biol. Chem. **268:** 6050-6057.

Kaplan, J. 2002. Mechanisms of cellular iron acquisition: another iron in the fire. Cell **111:** 603-606.

Keyer, K., Gort, A.S., and Imlay, J.A. 1995. Superoxide and the production of oxidative DNA damage. J. Bacteriol. **177:** 6782-6790.

Lambert, L.A., Perri, H., and Meehan, T.J. 2005. Evolution of duplications in the transferrin family of proteins. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **140:** 11-25.

Larsen, R.A., Foster-Hartnett, D., McIntosh, M.A., and Postle, K. 1997. Regions of *Escherichia coli* TonB and FepA proteins essential for *in vivo* physical interactions. J. Bacteriol. **179:** 3213-3221.

Lavrrar, J.L., and McIntosh, M.A. 2003. Architecture of a Fur binding site: a comparative analysis. J. Bacteriol. **185:** 2194-2202.

Lavrrar, J.L., Christoffersen, C.A., and McIntosh, M.A. 2002. Fur-DNA interactions at the bidirectional *fepDGC-entS* promoter region in *Escherichia coli*. J. Mol. Biol. **322:** 983-995.

Lee, B.C. 1995. Quelling the red menace: haem capture by bacteria. Mol. Microbiol. **18:** 383-390.

Legrain, M., Mazarin, V., Irwin, S.W., Bouchon, B., Quentin-Millet, M.-J., Jacobs, E., and Schryvers, A.B. 1993. Cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. Gene **130:** 73-80.

Létoffé, S., Ghigo, J.M., and Wandersman, C. 1994. Secretion of the *Serratia marcescens* HasA protein by an ABC transporter. J. Bacteriol. **176:** 5372-5377.

Létoffé, S., Redeker, V., and Wandersman, C. 1998. Isolation and characterization of an extracellular heme binding protein from *Pseudomonas aeruginosa* that shares functional and sequence similarities with the *Serratia marcescens* HasA hemophore. Mol. Microbiol. **28:** 1223-1234.

Létoffé, S., Nato, F., Goldberg, M.E., and Wandersman, C. 1999. Interactions of HasA, a bacterial haemophore, with haemoglobin and with its outer membrane receptor HasR. Mol. Microbiol. **33:** 546-555.

Lewis, L.A., Gray, E., Wang, Y.-P., Roe, B.A., and Dyer, D.W. 1997. Molecular characterization of *hpuAB*, the haemoglobin-haptoglobin-utilization operon of *Neisseria meningitidis*. Mol. Microbiol. **23:** 737-749.

Lewis, L.A., Sung, M.H., Gipson, M., Hartman, K., and Dyer, D.W. 1998. Transport of intact porphyrin by HpuAB, the hemoglobin-haptoglobin utilization system of *Neisseria meningitidis*. J. Bacteriol. **180:** 6043-6047.

Lewis, L.A., Gipson, M., Hartman, K., Ownbey, T., Vaughn, J., and Dyer, D.W. 1999. Phase variation of HpuAB and HmbR, two distinct haemoglobin receptors of *Neisseria meningitidis* DNM2. Mol. Microbiol. **32:** 977-989.

Locher, K.P., Rees, B., Koebnik, R., Mitschler, A., Moulinier, L., Rosenbusch, J.P., and Moras, D. 1998. Transmembrane signalling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes. Cell **95:** 771-778.

MacInnes, J.I., and Desrosiers, R. 1999. Agents of the "suis-ide diseases" of swine: *Actinobacillus suis*, *Haemophilus parasuis*, and *Streptococcus suis*. Can. J. Vet. Res. **63**: 83-89.

MacInnes, J.I., and Rosendal, S. 1987. Analysis of major antigens of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* and related organisms. Infect. Immun. **55:** 1626-1634.

Mazurier, J., and Spik, G. 1980. Comparative study of the iron-binding properties of human transferrins. I. Complete and sequential iron saturation and desaturation of the lactotransferrin. Biochim. Biophys. Acta. **629:** 399-408.

Mickelsen, P.A., Blackman, E., and Sparling, P.F. 1982. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from lactoferrin. Infect. Immun. **35:** 915-920.

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:** 2911-2915.

Miller, Y.I., and Shaklai, N. 1999. Kinetics of hemin distribution in plasma reveals its role in lipoprotein oxidation. Biochim. Biophys. Acta. **1454:** 153-164.

Miniats, O.P., Spinato, M.T., and Sanford, S.E. 1989. *Actinobacillus suis* septicemia in mature swine: two outbreaks resembling erysipelas. Can. Vet. J. **30:** 943-947.

Moeck, G.S., Coulton, J.W., and Postle, K. 1997. Cell envelope signaling in *Escherichia coli*. Ligand binding to the ferrichrome-iron receptor *fhua* promotes interaction with the energy-transducing protein TonB. J. Biol. Chem. **272:** 28391-28397.

Monteiro, M.A., Slavić, Đ., St. Michael, F., Brisson, J.-R., MacInnes, J.I., and Perry, M.B. 2000. The first description of a (1→6)-β-*D*-glucan in prokaryotes: (1→6)-β-*D*-glucan is a common component of *Actinobacillus suis* and is the basis for a serotype system. Carbohydr. Res. **329:** 77-86.

Morrissey, J.A., Cockayne, A., Brummell, K., and Williams, P. 2004. The staphylococcal ferritins are differentially regulated in response to iron and manganese and via PerR and Fur. Infect. Immun. **72:** 972-979.

Morton, D.J., Musser, J.M., and Stull, T.L. 1993. Expression of the *Haemophilus influenzae* transferrin receptor is repressible by hemin but not elemental iron alone. Infect. Immun. **61:** 4033-4037.

Morton, D.J., Whitby, P.W., Jin, H., Ren, Z., and Stull, T.L. 1999. Effect of multiple mutations in the hemoglobin- and hemoglobin-haptoglobin-binding proteins, HgpA, HgpB, and HgpC, of *Haemophilus influenzae* type b. Infect. Immun. **67:** 2729-2739.

Muller-Eberhard, U. 1988. Hemopexin. Methods Enzymol. **163:** 536-565.

Myers, L.E., Yang, Y.-P., Du, R.-P., Wang, Q., Harkness, R.E., Schryvers, A.B., Klein, M.H., and Loosmore, S.M. 1998. The transferrin binding protein B of *Moraxella catarrhalis* elicits bactericidal antibodies and is a potential vaccine antigen. Infect. Immun. **66:** 4183-4192.

Nevin, K.P., and Lovley, D.R. 2002. Mechanisms for accessing insoluble Fe(III) oxide during dissimilatory Fe(III) reduction by *Geothrix fermentans*. Appl. Environ. Microbiol. **68:** 2294-2295.

Newman, D.K., and Kolter, R. 2000. A role for excreted quinones in extracellular electron transfer. Nature **405:** 94-97.

Nicholson, M.L., and Beall, B. 1999. Disruption of *tonB* in *Bordetella bronchiseptica* and *Bordetella pertussis* prevents utilization of ferric siderophores, haemin and haemoglobin, as iron sources. Microbiology **145:** 2453-2461.

Nielson, H., Engelbrecht, J., Brunak, S., and von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:** 1-6.

Niven, D.F., Donga, J., and Archibald, F.S. 1989. Responses of *Haemophilus pleuropneumoniae* to iron restriction: changes in the outer membrane protein profile and the removal of iron from porcine transferrin. Mol. Microbiol. **3:** 1083-1089.

Niven, D.F., Ekins, A., and Al-Samaurai, A.A.-W. 1999. Effects of iron and manganese availability on growth and production of superoxide dismutase by *Streptococcus suis*. Can. J. Microbiol. **45:** 1027-1032.

Nørskov-Lauritsen, N., Christensen, H., Okkels, H., Kilian, M., and Bruun, B. 2004. Delineation of the genus *Actinobacillus* by comparison of partial *infB* sequences. Int. J. Syst. Evol. Mivrobiol. **54:** 635-644.

Oakhill, J.S., Sutton, B.J., Gorringe, A.R., and Evans, R.W. 2005. Homology modelling of transferrin-binding protein A from *Neisseria meningitidis*. Protein Eng. Des. Sel. **18:** 221-228.

O'Brien-Simpson, N.M., Veith, P.D., Dashper, S.G., and Reynolds, E.C. 2003. *Porphyromonas gingivalis* gingipains: the molecular teeth of a microbial vampire. Curr. Protein Pept. Sci. **6:** 409-426.

Occhino, D.A., Wyckoff, E.E., Henderson, D.P., Wrona, T.J., and Payne, S.M. 1998. *Vibrio cholerae* iron transport: haem transport genes are linked to one of two sets of *tonB*, *exbB*, and *exbD* genes. Mol. Microbiol. **29:** 1493-1507.

Octave, J.-N., Schneider, Y.-J., Trouet, A., and Crichton, R.R. 1983. Iron uptake and utilization by mammalian cells. I: cellular uptake of transferrin and iron. Trends Biochem. Sci. **8:** 217-220.

Ogunnariwo, J.A., and Schryvers, A.B. 2001. Characterization of a novel transferrin receptor in bovine strains of *Pasteurella multocida*. J. Bacteriol. **183:** 890-896.

Ogunnariwo, J.A., Woo, T.K.W., Lo, R.C.Y., Gonzalez, G.C., and Schryvers, A.B. 1997. Characterization of the *Pasteurella haemolytica* transferrin receptor genes and the recombinant receptor proteins. Microb. Pathog. **23:** 273-284.

Paoli, M., Anderson, B.F., Baker, H.M., Morgan,W.T., Smith, A., and Baker, E.N. 1999. Crystal structure of hemopexin reveals a novel high-affinity heme site formed between two beta-propeller domains. Nat. Struct. Biol. **6:** 926-931.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M.A., Rutherford, K.M., van Vliet, A.H., Whitehead, S., and Barrell, B.G. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:** 665-668.

Pawelek, P.D., and Coulton, J.W. 2004. Hemoglobin-binding protein HgbA in the outer membrane of *Actinobacillus pleuropneumoniae*: homology modelling reveals regions of potential interactions with hemoglobin and heme. J. Mol. Graph. Model. **23:** 211-221.

Peacock, R.S., Weljie, A.M., Howard, S.P., Price, F.D., and Vogel, H.J. 2005. The solution structure of the C-terminal domain of TonB and interaction studies with TonB box peptides. J. Mol. Biol. **345:** 1185-1197.

Perkins-Balding, D., Baer, M.T., and Stojiljkovic, I. 2003. Identification of functionally important regions of a haemoglobin receptor from *Neisseria meningitidis.* Microbiology **149:** 3423-3435.

Perkins-Balding, D., Ratliff-Griffin, M., and Stojiljkovic, I. 2004. Iron transport systems in *Neisseria meningitidis.* Microbiol. Mol. Biol. Rev. **68:** 154-171.

Perutz, M.F., Rossman, M.G., Cullis, A.F., Muirhead, H., and North, A.C.T. 1960. Structure of haemoglobin: a three-dimensional Fourier synthesis at 5.5-Å. resolution, obtained by X-ray analysis. Nature **185:** 416-422.

Phillips, J.E. 1984. Genus III. *Actinobacillus* Brumpt 1910, 849^{AL}. *In* Bergey's manual of systematic bacteriology. Vol. I. *Edited by* N.R. Krieg, and J.G. Holt. Williams & Wilkins Co., Baltimore, MD, U.S.A. pp. 570-576.

Pohl, E., Haller, J.C., Mijovilovich, A., Meyer-Klaucke, W., Garman, E., and Vasil, M.L. 2003. Architecture of a protein central to iron homeostasis: crystal structure and spectroscopic analysis of the ferric uptake regulator. Mol. Microbiol. **47:** 903-915.

Posey, J.E., and Gherardini, F.C. 2000. Lack of a role for iron in the Lyme disease pathogen. Science **288:** 1651-1653.

Posey, J.E., Hardham, J.M., Norris, S.J., and Gherardini, F.C. 1999. Characterization of a manganese-dependent regulatory protein, TroR, from *Treponema pallidum.* Proc. Natl. Acad. Sci. USA **96:** 10887-10892.

Postle, K. 1993. TonB protein and energy transduction between membranes. J. Bioenerg. Biomembr. **25:** 591-601.

Postle, K., and Good, R.F. 1983. DNA sequence of the *Escherichia coli tonB* gene. Proc. Natl. Acad. Sci. USA **80:** 5235-5239.

Potter, A.A., Schryvers, A.B., Ogunnariwo, J.A., Hutchins, W.A., Lo, R.Y.C., and Watts, T. 1999. Protective capacity of the *Pasteurella haemolytica* transferrin-binding proteins TbpA and TbpB in cattle. Microb. Pathog. **27:** 197-206.

Pradel, E., Guiso, N., Menozzi, F.D., and Locht, C. 2000. *Bordetella pertussis* TonB, a Bvg-independent virulence determinant. Infect. Immun. **68:**1919-1927.

Pressler, U., Staudenmaier, H., Zimmermann, L., and Braun, V. 1988. Genetics of the iron dicitrate transport system of *Escherichia coli*. J. Bacteriol. **170:** 2716-2724.

Ratledge, C., and Dover, L.G. 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. **54:** 881-941.

Ratliff, M., Zhu, W., Deshmukh, R., Wilks, A., and Stojiljkovic, I. 2001. Homologues of neisserial heme oxygenase in Gram-negative bacteria: degradation of heme by the product of the *pigA* gene of *Pseudomonas aeroginosa*. J. Bacteriol. **183:** 6394-6403.

Reeves, S.A., Torres, A.G., and Payne, S.M. 2000. TonB is required for intracellular growth and virulence of *Shigella dysenteriae*. Infect. Immun. **68:** 6329-6336.

Ren, Z., Jin, H., Morton, D.J., and Stull, T.L. 1998. *hgpB*, a gene encoding a second *Haemophilus influenzae* hemoglobin- and hemoglobin-haptoglobin-binding protein. Infect. Immun. **66:** 4733-4741.

Ren, Z., Jin, H., Whitby, P.W., Morton, D.J., and Stull, T.L. 1999. Role of CCAA nucleotide repeats in regulation of hemoglobin and hemoglobin-haptoglobin binding protein genes of *Haemophilus influenzae*. J. Bacteriol. **181:** 5865-5870.

Repine, J.E., Fox, R.B., and Berger, E.M. 1981. Hydrogen peroxide kills *Staphylococcus aureus* by reacting with staphylococcal iron to form hydroxyl radicals. J. Biol. Chem. **256:** 7094-7096.

Ricard, M.A., Archibald, F.S., and Niven, D.F. 1991. Isolation and identification of a putative porcine transferrin receptor from *Actinobacillus pleuropneumoniae* biotype 1. J. Gen. Microbiol. **137:** 2733-2740.

Richardson, A.R., and Stojiljkovic, I. 1999. HmbR, a hemoglobin-binding outer membrane protein of *Neisseria meningitidis*, undergoes phase variation. J. Bacteriol. **181:** 2067-2074.

Rohde, K.H., Gillaspy, A.F., Hatfield, M.D., Lewis, L.A., and Dyer, D.W. 2002. Interactions of haemoglobin with *Neisseria meningitidis* receptor HpuAB: the role of TonB and an intact proton motive force. Mol. Microbiol. **43:** 335-354.

Rokbi, B., Renauld-Mongenie, G., Mignon, M., Danve, B., Poncet, D., Chabanel, C., Caugant, D.A., and Quentin-Millet, M.J. 2000. Allelic diversity of the two transferrin binding protein B gene isotypes among a collection of *Neisseria meningitidis* strains representative of serogroup B disease: Implication for the composition of a recombinant TbpB-based vaccine. Infect. Immun. **68:** 4938-4947.

Ross, R.F., Hall, J.E., Orning, A.P., and Dale, S.E. 1972. Characterization of an *Actinobacillus* isolated from the sow vagina. Int. J. Syst. Bacteriol. **22:** 39-46.

Rossi, M.S., Paquelin, A., Ghigo, J.M., and Wandersman, C. 2003. Haemophore-mediated signal transduction across the bacterial cell envelope in *Serratia marcescens*: the inducer and the transported substrate are different molecules. Mol. Microbiol. **48:** 1467-1480.

Rossi-Campos, A., Anderson, C., Gerlach, G.-F., Klashinsky, S., Potter, A.A., and. Willson, P.J. 1992. Immunization of pigs against *Actinobacillus pleuropneumoniae* with two recombinant protein preparations. Vaccine **10:** 512-518.

Rouault, T.A. 2004. Pathogenic bacteria prefer heme. Science **305:** 1577-1578.

Rycroft, A.N., and Garside, L.H., 2000. *Actinobacillus* species and their role in animal disease. Vet. J. **159:** 18-36.

Saier, M.H. 2003. Tracing pathways of transport protein evolution. Mol. Microbiol. **48:** 1145-1156.

Sanford, S.E. 1987. *Actinobacillus suis* septicemia in piglets. Can. Vet. J. **28:** 654.

Sanford, S.E., Josephson, K.A., Rehmtulla, A.J., and Tilker, A.M.E. 1990. *Actinobacillus suis* septicemia in mature swine: two outbreaks resembling erysipelas. Can. Vet. J. **31:** 443-447.

Seliger, S.S., Mey, A.R., Valle, A.M., and Payne, S.M. 2001. The two TonB systems of *Vibrio cholerae*: redundant and specific functions. Mol. Microbiol. **39:** 801-812.

Schaffer, S., Hantke, K., and Braun, V. 1985. Nucleotide sequence of the iron regulatory gene *fur*. Mol. Gen. Genet. **200:** 110-113.

Schaible, U.E., and Kaufmann, S.H.E. 2004. Iron and microbial infection. Nat. Rev. microbiol. **2:** 946-953.

Schroder, I., Johnson, E., and de Vries, S. 2003. Microbial ferric iron reductases. FEMS Microbiol. Rev. **27:** 427-447.

Schryvers, A.B., and Gonzalez, G.C. 1990. Receptors for transferrin in pathogenic bacteria are specific for the host's protein. Can. J. Microbiol. **36:** 145-147.

Schryvers, A.B., and Morris, L.J. 1988. Identification and characterization of the human lactoferrin-binding protein from *Neisseria meningitidis*. Infect. Immun. **56:** 1144-1149.

Schryvers, A.B., and Stojiljkovic, I. 1999. Iron acquisition systems in the pathogenic *Neisseria*. Mol. Microbiol. **32:** 1117-1123.

Shouldice, S.R., Skene, R.J., Dougan, D.R., Snell, G., McRee, D.E., Schryvers, A.B., and Tari, L.W. 2004. Structural basis for iron binding and release by a novel class of periplasmic iron-binding proteins found in Gram-negative pathogens. J. Bacteriol. **186:** 3903-3910.

Skare, J.T., Ahmer, B.M.M., Seachord, C.L., Darveau, R.P., and Postle, K. 1993. Energy transduction between membranes. TonB, a cytoplasmic membrane protein, can be chemically cross-linked *in vivo* to the outer membrane receptor FepA. J. Biol. Chem. **268:** 16302–16308.

Slavić, Đ., DeLay, J., Hayes, M.A., and MacInnes, J.I. 2000*a*. Comparative pathogenicity of different *Actinobacillus suis* O/K serotypes. Can. J. Vet. Res. **64:** 81-87.

Slavić, Đ., Toffner, T.L., Monteiro, M.A., Perry, M.B., and MacInnes, J.I. 2000*b*. Prevalence of O1/K1- and O2/K3-reactive *Actinobacillus suis* in healthy and diseased swine. J. Clin. Microbiol. **38:** 3759-3762.

Srikumar, R., Mikael, L.G., Pawelek, P.D., Khamessan, A., Gibbs, B.F., Jacques, M., and Coulton, J.W. 2004. Molecular cloning of haemoglobin-binding protein HgbA in the outer membrane of *Actinobacillus pleuropneumoniae*. Microbiology **150:** 1723-1734.

Stellwagen, E. 1978. Haem exposure as the determinate of oxidation-reduction potential of haem proteins. Nature **275:** 73-74.

Stojiljkovic, I., and Hantke, K. 1995. Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). Mol. Gen. Genet. **247:** 199-205.

Stojiljkovic, I., and Perkins-Balding, D. 2002. Processing of heme and heme-containing proteins by bacteria. DNA Cell Biol. **21:** 281-295.

Stojiljkovic, I., and Srinivasan, N. 1997. *Neisseria meningitidis tonB*, *exbB*, and *exbD* genes: Ton-dependent utilization of protein-bound iron in Neisseriae. J. Bacteriol. **179:** 805-812.

Stojiljkovic, I., Bäumler, A.J., and Hantke, K., 1994. Fur regulon in Gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. J. Mol. Biol. **236:** 531-545.

Stojiljkovic, I., Hwa, V., de Saint Martin, L., O'Gaora, P., Nassif, X., Heffron, F., and So., M. 1995. The *Neeisseria* spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J. Bacteriol. **178:** 4670-4678.

Stojiljkovic, I., Larson, J., Hwa, V., Anic, S., and So, M. 1996. HmbR outer membrane receptors of pathogenic *Neisseria* spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J. Bacteriol. **178:** 4670-4678.

Stork, M., Di Lorenzo, M., Mouriño, S., Osorio, C.R., Lemos, M.L., and Crosa, H. 2004. Two *tonB* systems function in iron transport in *Vibrio anguillarum*, but only one is essential for virulence. Infect. Immun. **72:** 7326-7329.

Taylor, D.J. 1999. Miscellaneous bacterial infections. *Actinobacillus suis*. *In* Diseases of Swine, 8th edn. *Edited by* B.E. Straw, S. D'Allaire, W.L. Mengeling, and D.J. Taylor. Iowa State University Press, Ames, IA, U.S.A. pp. 624-627.

Theil, E.C. 1987. Ferritin: structure, gene regulation and cellular function in animals, plants and microorganisms. Annu. Rev. Biochem. **56:** 289-316.

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:** 4673-4680.

Tonpitak, W., Thiede, S., Oswald, W., Baltes, N., and Gerlach, G.-F. 2000. *Actinobacillus pleuropneumoniae* iron transport: a set of *exbBD* genes is transcriptionally linked to the *tbpB* gene and required for utilization of transferrin-bound iron. Infect. Immun. **68:** 1164-1170.

Torres, A.G., and Payne, S.M. 1997. Haem iron-transport system in enterohaemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. **23:** 825-833.

Touati, D. 2000. Iron and oxidative stress in bacteria. Arch. Biochem. Biophys. **373:** 1-6.

Touati, D., Jacques, M., Tardat, B., Bouchard, L., and Despied, S. 1995. Lethal oxidative damage and mutagenesis are generated by iron in Δ*fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. J. Bacteriol. **177:** 2305-2314.

van Berkel, P.H.C., Geerts, M.E.J., van Veen, H.A., Mericskay, M., de Boer, H.A., and Nuijens, J.H. 1997. N-terminal stretch Arg^2, Arg^3, Arg^4 and Arg^5 of human lactoferrin is essential for binding to heparin, bacterial lipopolysaccharide, human lysozyme and DNA. Biochem. J. **328:** 145-151.

Van Ostaaijen, J., Frey, J., Rosendal, S., and MacInnes, J.I. 1997. *Actinobacillus suis* strains isolated from healthy and diseased swine are clonal and carry *apxICABD*$_{\text{var.suis}}$ and *apxIICA*$_{\text{var.suis}}$ toxin genes. J. Clin. Microbiol. **35:** 1131-1137.

Van Overbeke, I., Chiers, K., Ducatelle, R., and Haesebrouck, F. 2001. Effect of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with a vaccine containing Apx toxins and transferrin-binding proteins. J. Vet. Med. B. **48:** 15-20.

Velayudhan, J., Hughes, N.J., McColm, A.A., Bagshaw, J., Clayton, C.L., Andrews, S.C., and Kelly, D.J. 2000. Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. Mol. Microbiol. **37:** 274-286.

Wai, S.N., Nakayama, K., Umene, K., Moriya, T., and Amako, K. 1996. Construction of a ferritin-deficient mutant of *Campylobacter jejuni*: contribution of ferritin to iron storage and protection against oxidative stress. Mol. Microbiol. **20:** 1127-1134.

Waidner, B., Greiner, S., Odenbreit, S., Kavermann, H., Velayudhan, J., Stahler, F., Guhl, J., Bisse, E., van Vliet, A.H., Andrews, S.C., Kusters, J.G., Kelly, D.J., Haas, R., Kist, M., and Bereswill, S. 2002. Essential role of ferritin Pfr in *Helicobacter pylori* iron metabolism and gastric colonization. Infect. Immun. **70:** 3923-3929.

Wandersman, C., and Delepelaire, P. 2004. Bacterial iron sources: from siderophores to hemophores. Annu. Rev. Microbiol. **58:** 611-647.

Wandersman, C., and Stojiljkovic, I. 2000. Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. Curr. Opin. Microbiol. **3:** 215-220.

Ward, P.P., and Conneely, O.M. 2004. Lactoferrin: role in iron homeostasis and host defense against microbial infection. Biometals **17:** 203-208.

Weinberg, E.D. 1997. The *Lactobacillus* anomaly: total iron abstinence. Perspect. Biol. Med. **40:** 578-583.

West, D., Reddin, K., Matheson, M., Heath, R., Funnell, S., Hudson, M., Robinson, A., and Gorringe, A. 2001. Recombinant *Neisseria meningitidis* transferrin binding protein A protects against experimental meningococcal infection. Infect. Immun. **69:** 1561-1567.

Wilks, A., and Schmitt, M.P. 1998. Expression and characterization of a heme oxygenase (Hmu O) from *Corynebacterium diphtheriae*. J. Biol. Chem. **273:** 837-841.

Wilson, R.J., and McOrist, S. 2000. *Actinobacillus suis* infection in pigs in Australia. Aust. Vet. J. **78:** 317-319.

Woodmansee, A.N., and Imlay, J.A. 2002. Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. J. Biol. Chem. **277**: 34055-34066.

Yaeger, M.J. 1995. *Actinobacillus suis* septicemia: an emerging disease in high-health status herds. Swine Health Prod. **3:** 209-210.

Yaeger, M.J. 1996. An outbreak of *Actinobacillus suis* septicemia in grow/finish pigs. J. Vet. Diagn. Invest. **8:** 381-383.

Yu, R.-H., and Schryvers, A.B. 1994. Transferrin receptors on ruminant pathogens vary in their interaction with the C-lobe and N-lobe of ruminant transferrins. Can. J. Microbiol. **40:** 532-540.

Yu, R.-H., Gray-Owen, S.D., Ogunnariwo, J., and Schryvers, A.B. 1992. Interaction of ruminant transferrins with transferrin receptors in bovine isolates of *Pasteurella haemolytica* and *Haemophilus somnus*. Infect. Immun. **60:** 2992-2994.

Yu, R.-H., Bonnah, R.A., Ainsworth, S., and Schryvers, A.B. 1999. Analysis of the immunological responses to transferrin and lactoferrin proteins from *Moraxella catarrhalis*. Infect. Immun. **67:** 3793-3799.

Zhao, Q., and Poole, K. 2000. A second *tonB* gene in *Pseudomonas aeruginosa* is linked to the *exbB* and *exbD* genes. FEMS Microbiol. Lett. **184:** 127-132.

Zhu, W., Wilks, A., and Stojiljkovic, I. 2000. Degradation of heme in Gram-negative bacteria: the product of the *hemO* gene of Neisseriae is a heme oxygenase. J. Bacteriol. **182:** 6783-6790.

Appendix 1. GenBank entries

Appendix 1.1

A. suis strain SO4 TonB (*tonB*, 339-1079), ExbB (*exbB*, 1101-1769), ExbD (*exbD*, 1769-2179), TbpB (*tbpB*, 2207-3997) and TbpA (*tbpA*, 4011-6818) genes, complete cds. Start and stop codons are in bold type and underlined, respectively. GenBank accession number AY101604 (6938 bp).

```
   1 tttggcttta tcatcaactt agtcgtgcca attttattag catttttat tggattaaca
  61 taacaaatac gctcaagcga tctgtttttg tggaaatttt acaaaaacag actgcttatt
 121 gaatcttacg ctaacgtaat tagatcataa aaccctaact aacctaactg atactcttcg
 181 ctatagccat taatcacaca gctcacaata cctcaaatct agcccgaaca aacattattt
 241 acataaaaag caaaaaacat ttgcaaatct tacaattttc aatttaaaat aaacgataat
 301 gattttcatt aacttaagat gcaagtaaaa atagagatat gaaaagaagt ttttatcatt
 361 tttttatcat cttggctgta agtctgatcc atctaggagt agcatattca atctcacgat
 421 tttcctggga caaaaatctt gctctctttg gaggactaaa aaatttacac atgatagaag
 481 ttaatttagg tgttgaagag tcccttacgg taaatacacc cttaaaacct aaaatagcag
 541 aaccacctga agaattttta aatactacag taaaagatgt aactcagtca aacttagccc
 601 ccaaattagc agttcctaaa aaaaagcctc cctcaaagga agtaaaaaaa gcgcctgaaa
 661 agatcatgaa aaaacatgag acaaagaaag atgtttctcc aacaaacaaa agcagtcgta
 721 ctcagcagcc gatccaacaa ggaaatccaa atggtataaa tggaagcaaa tcaacctatg
 781 gaaatgccaa agtcgatgcc tcccttggtg ctggttatgg caatgcaatg cgagggcgtt
 841 gctctgatat ttcagatgaa tctgatgatg tcggtagtgt gaaattgaaa gttactattg
 901 gagcaaacgg caaagcaatc aatgttgaaa ttttatcttc ttcaggcatc aaacgcctag
 961 ataaccaagc tagcagaatg gcaacaggac atacgtatca accggcaaaa attaatggga
1021 atactattgt aggcagtgta actttcaata ttcattttaa atgtggtgca gcagcctaaa
1081 ttatttgagg aaattaattt atgaatttaa cagaacttat caaacaacaa gatattgtac
1141 tctcggtatc tttcttcata ttagttttta tgagtatttt atcttggagt attattttaa
1201 taagattaat taaaataatc ggagtaaaga aaaacaatca ggtaacattt aatcttatta
1261 aatctgatga aaatatgaat caagcaaaga aatacactca aaataagcgg tctgatattt
1321 ctgaaatttt gcaaaccact tttaaacaat gcgaacttta tcaattaaat gggaaatata
1381 atttatctca tcaacttcct tttaaagatt ttttaacctt acatttacga aatgcaataa
1441 cacagtccct tcgtaaatat gatggtggtt tagttgcact tgcttctatt ggtgcggttg
1501 caccgttcat tgggttattt ggaacggtct ggggaatttt taatgcactt atggacatca
1561 gtcagcaagg acaagtcagt atcgcaactg tatctgcacc tataggagaa gcattagttg
1621 caacagcaat gggactattt gtcgcaattc cggcagtact tgcttataac ttaattgctc
1681 gccttaaccg taatctttct caagatttag atcattacgc aaacgagtta tttatctatt
1741 tattacatca ctatggagaa aataaataat ggcatttgga agttttgata aaaatgaaaa
1801 ttcggtaatg tcagaaatta atgttacgcc attagttgat gtaatgttgg tgttattaat
1861 tgtttttatg atcacaatgc caatattaac ccaatctatt ccacttgaat taccaacctc
1921 cagtattgag aaaaaagatg agccaaaaga tatcttcaga gtggcgatta atcctacggg
1981 catttattta ggcgagaagc tagtgaatga agaagaatta aaacaatctt ttctgacaaa
2041 atttcaggaa aataaaaata ccgttattgc tatttctgcg gatatttccg tggaatatca
2101 acatatcgtg aaagtccttg aattagctca aaacgtcggg ctaacgaaaa taggctttgt
2161 gactcaacct agtaaataaa agcagaaatt ttatattgga ggcaatatgc attttaaact
2221 taatccctat gcgttagcgt ttacttcgct gtttcttgtc gcttgttctg gcggaaaagg
2281 aggttttgat ttagaagatg ttcggccaaa tcaaactgca aaagcagaaa aagcaacaac
2341 ctcttatcaa gatgaggaaa cgaagaaaaa gacaaaggaa gaattagata agttgatgga
2401 gcctactttg ggagttgagg caaagatacc tcgccggaat agagctttat ttgataaaga
2461 aggaaatcga aaagcaacac ctgatacaac tgatgaatta tctgaagcac aaattatggc
2521 gatttggaat gaaaatatag atgaaattcc tcacttgaaa gagttaaacg ataaaacaac
2581 gagtggttta atttatcact ctcacgatgg taaacaggag gacaaaaaac gaaatttaca
```

2641 atatgtgcgt tcaggctatg ttttcgatga atcttatagt gaaatagtaa aaaataagaa
2701 tggtgtaccc tatattttta aaaatgggat cgatggttac atttactatt tagggacaag
2761 cccgtcaaaa gaactcccta aaggaaataa agttacctat aaaggtactt gggatttcac
2821 cagcgatgtt aaaacaagct acgagttaag tggatttagt gatgctggta atggtaaaaa
2881 tgttgcagct acatctattt cggataatgt caatcgagat cataaagttg gtgaaaaact
2941 aggtgataat gaagttaaag ggtagctca ttctagtgaa tttgcagtag attttgataa
3001 caaaaaattg acaggtagtt tatatcgtaa tggttatatc aacagaaata aagcgcaaga
3061 agtaacgaaa cgctatagca ttgaagctga tataacaggc aaccgtttta gaggaaaagc
3121 caaagcagaa aaagcaggtg atccgatctt tactgattca aattatcttg aaggggggatt
3181 ctatggtcct aaagctgaag aaatggcagg gaaatttttc acaaataata aatctctctt
3241 tgcagtattt gcagctaaaa gtgaaaacgg cgagacgacc acagaacgaa tcattgatgc
3301 aactaaaatt gatttaaccc aatttaatgc taaagaactc aacaattttg gtgatgcctc
3361 tgttttaatt attgatggac aaaaaataga tctagcaggt gtcaatttta aaaatagtaa
3421 aacggttgaa atcaacggca aaacaatggt agccgtagct tgctgtagta atctggaata
3481 tatgaaattt ggtcaattgt ggcaaaaaga gggcaaacaa caagttaaag ataatagttt
3541 attcctacaa ggtgaacgta ctgcaacgga taaaatgccc gcaggaggta actataagta
3601 tgttggaact tgggatgcac tcgtatctaa agggacgaac tggatagcgg aagcagataa
3661 taatcgagaa tcgggctatc gcactgaatt tgatgttaat tttagtgata aaaaagtaaa
3721 cggtaagtta tttgataaag gcggtgtaaa tcctgtattt accgtagatg cgacaattaa
3781 tggtaatggc tttatcggca gtgcgaaaac ctctgatagt ggctttgctt tagatgcagg
3841 ctctagccaa cacggaaatg cggtatttag tgatataaaa gtcaatggtg gcttctatgg
3901 tccaaccgct ggagaacttg gcggacaatt ccatcataaa tcagacaatg gcagtgttgg
3961 cgctgtcttt ggtgcaaaac gacaaataga aaaataataa ggaatttgct **atg**aaaaata
4021 aattaaatct gattagcctt gctcttctta gcctatttgc cgtacaaagc tatgcagaac
4081 aagcggtaca attaaatgat gtttatgtca caggtaccaa aaagaaagca cataaaaaag
4141 agaacgaagt cacaggctta gggaaagtag ttaaaacacc agatactctt agtaaggagc
4201 aagtgttagg aatacgagat ctgactcgtt acgaccccgg tatttctgtc gtagaacaag
4261 ggagaggtgc aacgacaggc tactcaattc gcggggtaga tcgtaatcgt gtgggcttgg
4321 cattagacgg tttgccacag attcaatcct atgtaagcca atattcacgt tcctcaagcg
4381 gtgccattaa tgaaatagaa tacgaaaatc tgcgttcgat ccaaattagt aaaggggcta
4441 gttcttctga gtttggtagt ggctcactag gcggttcggt gcaattccgt accaaagagg
4501 taagcgacat tattaagcca gggcaatctt ggggattaga taccaaaagt gcctacagta
4561 gcaaaaatca acaatggtta aactcacttg cttttgcggg tactcacaat ggctttgatg
4621 ctcttgtgat ttacactcac cgtgatggta aggaaacgaa agctcataaa gatgcagaga
4681 gtcgttctca gaatatcacc cgagtaggag tggaaaccaa cgagcttgat acctcaaata
4741 gatatactgc gacgacgaat aatcaacata cttatggctg gtttttgatt aaagatgaat
4801 gtccaacgtt agattgtacg ccgaaacaga tggctagggt gacaaaagat acgccatctt
4861 tccgttctta ccctgaatat actcctgagg aaaaacaggc ttatgagaac caaaaacata
4921 ttacagagcg tctaaatgct caggattaca ctggtgaata tagagcttta cctgatccgc
4981 ttaaatataa atctgattct tggctggtta aattgggata cacattctct ccgaaacatt
5041 atgtcgctgg tacttatgaa catagcaaac agcgttacga cacccgagat atgacctata
5101 ccgcttattg gcaaccatcg gatttactta gaactggtag aaattggtat ccaatgaata
5161 atgctaaagg attatatcgt gataatgctt tagatggtgt tgctattgac tactttacgg
5221 aagatggtgt gaaatcctca aaaggtttac gttgggcaaa agctcgtttt attgacgagt
5281 ggcacactcg tgatcgctta ggtgctttat atcgttatac taatcaagat ggaaatcgtc
5341 tgattgatag actatccttg agtttcgatc agcaaaaaat taatttatct acccgcttga
5401 gagaaaacaa ctgttcggaa tatccaacca tagataagaa ttgccgtgca actcttgata
5461 aactttggtc ttctactaaa aatgagcaaa gttcttatga agaaaaacac gacactattc
5521 agctctcgtt agataaaacc gtacaaacgg gattgggtaa acatcaatta aatatgttat
5581 taggttcaga ccgtttcaat tccaccttaa aacgccacga aattttgagt gaattttctg
5641 tagggagttg gggcttgtt agagacattg gctatcgaaa tggatcttac aataatcctt
5701 atgtgtatga gctaaaagat caggcaattt atagtaaaaa tgaatgtgat tatagtggca
5761 ctattgcagg tagggctgat tgtgctacaa gtaaaatcaa agggcataat cactacatcg
5821 ctctgagaga taattttgcc ataaccaagt atttggatat tggtttgggt taccgtttcg
5881 ataagcataa attccgtagc actcatcgct gggcaaatca aggcgattat aaaaacagtg
5941 cgtggaatat tggcatagtc gcaaaaccaa cgtcattcct atcgctctct tatcgagcat
6001 catctggctt tagagtgcca agtttccaag agctatttgg cttacgttat gatggtgcaa
6061 tgaaaggctc cagcgatgct taccaaaaaa cagagaagtt atctcctgaa aaatccttaa
6121 accaagaggt tgctgcgact ttcaaaggtg attttggtgt cgttgaagtc agttatttca
6181 aaaatgacta taagcagtta attgctccag cagaaagaat gcatcaaact caatcaatga
6241 ttaactattt taatgtgcaa gatattaaat tggacggcat taatcttatt ggtaagctag
6301 attggaatgg ggtatttgat aaaattcctg agggcattta cacaacattg gcttatagca
6361 aaatgcgagt aaaagaggtg aaaaactatc aagggtatat gaatattcgt tctccattgt

```
6421 tagataccat tcagcctgct cgctatgttg taggagtggg gtacgatcag ccagatgaaa
6481 aatggggcgt gaatctaaca atgacacact ccagtggaaa aaatccagat gagttaagag
6541 gtaatgaaca agtcggtttt gccaattatg agcgaactgc cacgaagaaa agaacacgtt
6601 cttggcatac ctttgactta acgggatata tcaccccttg gaaacataca acggtacgag
6661 ctggtgtata taatctgatg aattaccgtt acaccacttg ggaatccgta cgtcaatctt
6721 cgcttaacgc tattcaccag cacactaatg tgaaagatta cgcaagatat gcggctccgg
6781 gtagaaatta tgttgtttca ttcgaaatga aattctaatt tagagtacct tagtttctaa
6841 aaggcatagt agacaaaagt gttgtaaaaa gtgagctaga gccttatggc acaaggcttt
6901 aagcccgctt tttgaaatat gcacaaaaaa ctgtcctt
```

Appendix 1.2

A. suis strain C84 TonB (*tonB*, 339-1079), ExbB (*exbB*, 1101-1769), ExbD (*exbD*, 1769-2179), TbpB (*tbpB*, 2207-3997) and TbpA (*tbpA*, 4011-6818) genes, complete cds. Start and stop codons are in bold type and underlined, respectively. GenBank accession number AY101605 (6938 bp).

1 tttggcttta tcatcaactt agtcgtgcca attttattag catttttttat tggattaaca
61 taacaaatac gctcaagcga tctgtttttg tggaaatttt acaaaaacag actgcttatt
121 gaatcttacg ctaacgtaat tagatcataa aaccctaact aacctaactg atactcttcg
181 ctatagccat taatcacaca gctcacaata cctcaaatct agcccgaaca aacattattt
241 acataaaaag caaaaaacat ttgcaaatct tacaattttc aatttaaaat aaacgataat
301 gattttcatt aacttaagat gcaagtaaaa atagagat**at** **g**aaaagaagt ttttatcatt
361 tttttatcat cttggctgta agtctgatcc atctaggagt agcatattca atctcacgat
421 tttcctggga caaaaatctt gctctctttg gaggactaaa aaatttacac atgatagaag
481 ttaatttagg tgttgaagag tcccttacgg taaatacacc cttaaaacct aaaatagcag
541 aaccacctga agaattttta aatactacag taaaagatgt aactcagtca aacttagccc
601 ccaaattagc agttcctaaa aaaaagcctc cctcaaagga agtaaaaaaa gcgcctgaaa
661 agatcatgaa aaaacatgag acaaagaaag atgtttctcc aacaaacaaa agcagtcgta
721 ctcagcagcc gatccaacaa ggaaatccaa atggtataaa tggaagcaaa tcaacctatg
781 gaaatgccaa agtcgatgcc tcccttggtg ctggttatgg caatgcaatg cgagggcgtt
841 gctctgatat ttcagatgaa tctgatgatg tcggtagtgt gaaattgaaa gttactattg
901 gagcaaacgg caaagcaatc aatgttgaaa ttttatcttc ttcaggcatc aaacgcctag
961 ataaccaagc tagcagaatg gcaacaggac atacgtatca accggcaaaa attaatggga
1021 atactattgt aggcagtgta actttcaata ttcattttaa atgtggtgca gcagcctaaa
1081 ttatttgagg aaattaattt **atg**aatttaa cagaacttat caaacaacaa gatattgtac
1141 tctcggtatc tttcttcata ttagttttta tgagtatttt atcttggagt attattttaa
1201 taagattaat taaaataatc ggagtaaaga aaaacaatca ggtaacattt aatcttatta
1261 aatctgatga aaatatgaat caagcaaaga aatacactca aaataagcgg tctgatattt
1321 ctgaaatttt gcaaaccact tttaaacaat gcgaacttta tcaattaaat gggaaatata
1381 atttatctca tcaacttcct tttaaagatt ttttaacctt acatttacga aatgcaataa
1441 cacagtccct tcgtaaatat gatggtggtt tagttgcact tgcttctatt ggtgcggttg
1501 caccgttcat tgggttattt ggaacggtct ggggaatttt taatgcactt atggacatca
1561 gtcagcaagg acaagtcagt atcgcaactg tatctgcacc tataggagaa gcattagttg
1621 caacagcaat gggactattt gtcgcaattc cggcagtact tgcttataac ttaattgctc
1681 gccttaaccg taatctttct caagatttag atcattacgc aaacgagtta tttatctatt
1741 tattacatca ctatggagaa aataaata**at** **g**gcatttgga agttttgata aaaatgaaaa
1801 ttcggtaatg tcagaaatta atgttacgcc attagttgat gtaatgttgg tgttattaat
1861 tgtttttatg atcacaatgc caatattaac ccaatctatt ccacttgaat taccaacctc
1921 cagtattgag aaaaaagatg agccaaaaga tatcttcaga gtggcgatta atcctacggg
1981 catttattta ggcgagaagc tagtgaatga agaagaatta aaacaatctt ttctgacaaa
2041 atttcaggaa aataaaaata ccgttattgc tatttctgcg gatatttccg tggaatatca
2101 acatatcgtg aaagtccttg aattagctca aaacgtcggg ctaacgaaaa taggctttgt
2161 gactcaacct agtaaataaa agcagaaatt ttatattgga ggcaat**atg**c attttaaact
2221 taatccctat gcgttagcgt ttacttcgct gtttcttgtc gcttgttctg gcggaaaagg
2281 aggttttgat ttagaagatg ttcggccaaa tcaaactgca aaagcagaaa aagcaacaac
2341 ctcttatcaa gatgaggaaa cgaagaaaaa gacaaaggaa gaattagata agttgatgga
2401 gcctactttg ggagttgagg caaagatacc tcgccggaat agagctttat ttgataaaga
2461 aggaaatcga aaagcaacac ctgatacaac tgatgaatta tctgaagcac aaattatggc
2521 gatttggaat gaaaatatag atgaaattcc tcacttgaaa gagttaaacg ataaaacaac
2581 gagtggttta atttatcact ctcacgatgg taaacaggag gacaaaaaac gaaatttaca
2641 atatgtgcgt tcaggctatg ttttcgatga atcttatagt gaaatagtaa aaaataagaa
2701 tggtgtaccc tatattttta aaaatgggat cgatggttac atttactatt tagggacaag
2761 cccgtcaaaa gaactcccta aaggaaataa agttacctat aaaggtactt gggatttcac

2821 cagcgatgtt aaaacaagct acgagttaag tggatttagt gatgctggta atggtaaaaa
2881 tgttgcagct acatctattt cggataatgt caatcgagat cataaagttg gtgaaaaact
2941 aggtgataat gaagttaaag gggtagctca ttctagtgaa tttgcagtag attttgataa
3001 caaaaaattg acaggtagtt tatatcgtaa tggttatatc aacagaaata aagcgcaaga
3061 agtaacgaaa cgctatagca ttgaagctga tataacaggc aaccgtttta gaggaaaagc
3121 caaagcagaa aaagcaggtg atccgatctt tactgattca aattatcttg aagggggatt
3181 ctatggtcct aaagctgaag aaatggcagg gaaatttttc acaaataata aatctctctt
3241 tgcagtattt gcagctaaaa gtgaaaacgg cgagacgacc acagaacgaa tcattgatgc
3301 aactaaaatt gatttaaccc aatttaatgc taaagaactc aacaatttg gtgatgcctc
3361 tgttttaatt attgatggac aaaaaataga tctagcaggt gtcaatttta aaaatagtaa
3421 aacggttgaa atcaacggca aaacaatggt agccgtagct tgctgtagta atctggaata
3481 tatgaaattt ggtcaattgt ggcaaaaaga gggcaaacaa caagttaaag ataatagttt
3541 attcctacaa ggtgaacgta ctgcaacgga taaaatgccc gcaggaggta actataagta
3601 tgttggaact tgggatgcac tcgtatctaa agggacgaac tggatagcgg aagcagataa
3661 taatcgagaa tcgggctatc gcactgaatt tgatgttaat tttagtgata aaaaagtaaa
3721 cggtaagtta tttgataaag gcggtgtaaa tcctgtattt accgtagatg cgacaattaa
3781 tggtaatggc tttatcggca gtgcgaaaac ctctgatagt ggctttgctt tagatgcagg
3841 ctctagccaa cacggaaatg cggtatttag tgatataaaa gtcaatggtg gcttctatgg
3901 tccaaccgct ggagaacttg gcggacaatt ccatcataaa tcagacaatg gcagtgttgg
3961 cgctgtcttt ggtgcaaaac gacaaataga aaaataataa ggaatttgct **atg**aaaaata
4021 aattaaatct gattagcctt gctcttctta gcctatttgc cgtacaaagc tatgcagaac
4081 aagcggtaca attaaatgat gtttatgtca caggtaccaa aaagaaagca cataaaaaag
4141 agaacgaagt cacaggctta gggaaagtag ttaaaacacc agatactctt agtaaggagc
4201 aagtgttagg aatacgagat ctgactcgtt acgaccccgg tatttctgtc gtagaacaag
4261 ggagaggtgc aacgacaggc tactcaattc gcggggtaga tcgtaatcgt gtgggcttgg
4321 cattagacgg tttgccacag attcaatcct atgtaagcca atattcacgt tcctcaagcg
4381 gtgccattaa tgaaatagaa tacgaaaatc tgcgttcgat ccaaattagt aaaggggcta
4441 gttcttctga gtttggtagt ggctcactag gcggttcggt gcaattccgt accaaagagg
4501 taagcgacat tattaagcca gggcaatctt ggggattaga taccaaaagt gcctacagta
4561 gcaaaaatca acaatggtta aactcacttg cttttgcggg tactcacaat ggctttgatg
4621 ctcttgtgat ttacactcac cgtgatggta aggaaacgaa agctcataaa gatgcagaga
4681 gtcgttctca gaatatcacc cgagtaggag tggaaaccaa cgagcttgat acctcaaata
4741 gatatactgc gacgacgaat aatcaacata cttatggctg gtttttgatt aaagatgaat
4801 gtccaacgtt agattgtacg ccgaaacaga tggctagggt gacaaaagat acgccatctt
4861 tccgttctta ccctgaatat actcctgagg aaaaacaggc ttatgagaac caaaaacata
4921 ttacagagcg tctaaatgct caggattaca ctggtgaata tagagcttta cctgatccgc
4981 ttaaatataa atctgattct tggctggtta aattgggata cacattctct ccgaaacatt
5041 atgtcgctgg tacttatgaa catagcaaac agcgttacga cacccgagat atgacctata
5101 ccgcttattg gcaaccatcg gatttactta gaactggtag aaattggtat ccaatgaata
5161 atgctaaagg attatatcgt gataatgctt tagatggtgt tgctattgac tactttacgg
5221 aagatggtgt gaaatcctca aaaggtttac gttgggcaaa agctcgtttt attgacgagt
5281 ggcacactcg tgatcgctta ggtgctttat atcgttatac taatcaagat ggaaatcgtc
5341 tgattgatag actatccttg agtttcgatc agcaaaaaat taatttatct acccgcttga
5401 gagaaaacaa ctgttcggaa tatccaacca tagataagaa ttgccgtgca actcttgata
5461 aactttggtc ttctactaaa aatgagcaaa gttcttatga agaaaaacac gacactattc
5521 agctctcgtt agataaaacc gtacaaacgg gattgggtaa acatcaatta aatatgttat
5581 taggttcaga ccgtttcaat tccaccttaa aacgccacga aattttgagt gaattttctg
5641 tagggagttg gggcttgtt agagacattg gctatcgaaa tggatcttac aataatcctt
5701 atgtgtatga gctaaaagat caggcaattt atagtaaaaa tgaatgtgat tatagtggca
5761 ctattgcagg tagggctgat tgtgctacaa gtaaaatcaa agggcataat cactacatcg
5821 ctctgagaga taattttgcc ataaccaagt atttggatat tggtttgggt taccgtttcg
5881 ataagcataa attccgtagc actcatcgct gggcaaatca aggcgattat aaaaacagtg
5941 cgtggaatat tggcatagtc gcaaaaccaa cgtcattcct atcgctctct tatcgagcat
6001 catctggctt tagagtgcca agtttccaag agctatttgg cttacgttat gatggtgcaa
6061 tgaaaggctc cagcgatgct taccaaaaaa cagagaagtt atctcctgaa aaatccttaa
6121 accaagaggt tgctgcgact ttcaaaggtg attttggtgt cgttgaagtc agttatttca
6181 aaaatgacta taagcagtta attgctccag cagaaagaat gcatcaaact caatcaatga
6241 ttaactattt taatgtgcaa gatattaaat tggacggcat taatcttatt ggtaagctag
6301 attggaatgg ggtatttgat aaaattcctg agggcattta cacaacattg gcttatagca
6361 aaatgcgagt aaaagaggtg aaaaactatc aagggtatat gaatattcgt tctccattgt
6421 tagataccat tcagcctgct cgctatgttg taggagtggg gtacgatcag ccagatgaaa
6481 aatggggcgt gaatctaaca atgacacact ccagtggaaa aaatccagat gagttaagag
6541 gtaatgaaca agtcggtttt gccaattatg agcgaactgc cacgaagaaa agaacacgtt

```
6601 cttggcatac ctttgactta acgggatata tcaccccttg gaaacataca acggtacgag
6661 ctggtgtata taatctgatg aattaccgtt acaccacttg ggaatccgta cgtcaatctt
6721 cgcttaacgc tattcaccag cacactaatg tgaaagatta cgcaagatat gcggctccgg
6781 gtagaaatta tgttgtttca ttcgaaatga aattctaatt tagagtacct tagtttctaa
6841 aaggcatagt agacaaaagt gttgtaaaaa gtgagctaga gccttatggc acaaggcttt
6901 aagcccgctt tttgaaatat gcacaaaaaa ctgtcctt
```

Appendix 1.3

A. suis strain SO4 ExbB2 (*exbB2*, 134-586), ExbD2 (*exbD2*, 631-1020) and TonB2 (*tonB2*, 1030-1875) genes, complete cds. Start and stop codons are in bold type and underlined, respectively. GenBank accession number AY838763 (2046 bp).

1 tatttataaa aaataaacga gaatacctat tattacgagt tgaattttct gcctcactta
61 gtataattac gaaccatttt cattatacag tatcacctgt cttctatatt tttatatcgt
121 ttccgaggat ttt**atg**gaac aaatgcttga acttttacaa gatcatgtag attacattat
181 cttaggttta ttattattaa tgagtgttgt gttagtatgg aaaattattg aacgtgttct
241 attttataaa cagcttgatg tcacaaaata tgaaaccatc caagatttag agatcgatac
301 aactcgtaac ctcaccacta tctcaactat cggtgctaac gcgccttata tcggtttatt
361 aggaaccgta ttaggtatct tacttacctt ctaccattta ggtcattccg gcggtgaaat
421 tgatgccgca tctattatgg ttcacctctc acttgcatta aaagcgacag cagcaggtat
481 cttagttgca attccggcaa tgatgttcta cagcggtttt aaccgtaaag tagatgaaag
541 caaacttaaa tggcaggcca ttcaagcccg taaagccaat caataagcgg tcaaatttca
601 cttatttttt gcaactcaat caggtttctt **atg**aaaaaat ttgacgaaat taatattatt
661 ccgttcatcg acattatgtt ggtgctatta gcgatcgtgc ttgttaccgc ctcatttatt
721 tcacaaggca aaattcaagt gaatgtgccg aaagcaacca cgactcaagc gatgaaagcg
781 gatgaattag ctaaacttct tacgatcaca gaaaacaacg agttctattt caacgatcaa
841 ccaattacta aagaagcact tactactgag attgccactt gggacaaaac ccaaaaagta
901 accttaaaag tagatggtgc ggtggcattt gaaaaatttg ttgaactcac cgatgtactc
961 tctgcgaatg aaattaaaaa cgtcgctatc gtaacgaaga aagatacagc caagaaatag
1021 gatacgagt**a** **tg**aagaaaaa acattctcgt atcgggttaa tcagttctat tgttattcac
1081 acctttgttt ttgccggttt catttcgata gtaaaaagtt ctcattcgga cggtcagcct
1141 gaagataatg taatgtcgat ggaattagtt gccgctttat tagaacagcc tcaagttgcg
1201 gtggcagaag aacaacctac acaagcggaa ccggaaaaac agcaagcaga accggagccg
1261 gaacctgaag cagagccgat tcctgaaccg aagccacagc ctaaacctaa agaaaagccg
1321 aaggaaaagc caaaaccaaa agagcaaccg aagcctaaag aaaaagaaaa gcctaaaaaa
1381 gagaaagcta aagataaacc gattaaggca ttagaaaaag ggccggaagc caaacaaggt
1441 attgtcgcaa aagccatacc gaatgcagtg caaggtactc aagcacgagc gggcatcccg
1501 aatggtaaac caaacggtaa tcctaacggt acatcagcga acggctcaca aaatggtgcg
1561 gcaggtggca gtggcagcgg aagtaatggt agtgaaatcg gagcttataa agctgcatta
1621 cagcgagcct tacaacgtcg agctaacaat gcctatcccg ctcgagaaaa aatgatgcgt
1681 aaaacagggg ttgttactat tggttttacc gtatcgtctt ctggcgattt aaccaatgta
1741 aaagtgttaa acagttccgg taacagtaat cttgataacg cagcagttaa agcagcgcaa
1801 agcaccaaag tgtcaccgcc accggctggt ttcccaagta gtgttactgt tccggtaaaa
1861 ttctcgattg aataaatctt accaataagc tcccctgcgg gagcttattt tttattcgtt
1921 ataagcggtc atttttttact aaaatttgc aaacatcagc aaaagataag cgcttgccta
1981 aagcggggtg cttaagtaaa ataaactgtt tctattttta ttatacaaat tgaggaaaat
2041 gtaatg

Appendix 1.4

A. suis strain C84 ExbB2 (*exbB2*, 134-586), ExbD2 (*exbD2*, 631-1020) and TonB2 (*tonB2*, 1030-1875) genes, complete cds. Start and stop codons are in bold type and underlined, respectively. GenBank accession number AY838764 (2046 bp).

```
   1 tatttataaa aaataaacga gaatacctat tattacgagt tgaattttct gcctcactta
  61 gtataattac gaaccatttt cattatacag tatcacctgt cttctatatt tttatatcgt
 121 ttccgaggat tttatggaac aaatgcttga acttttacaa gatcatgtag attacattat
 181 cttaggttta ttattattaa tgagtgttgt gttagtatgg aaaattattg aacgtgttct
 241 attttataaa cagcttgatg tcacaaaata tgaaaccatc caagatttag agatcgatac
 301 aactcgtaac ctcaccacta tctcaactat cggtgctaac gcgccttata tcggtttatt
 361 aggaaccgta ttaggtatct tacttacctt ctaccattta ggtcattccg gcggtgaaat
 421 tgatgccgca tctattatgg ttcacctctc acttgcatta aaagcgacag cagcaggtat
 481 cttagttgca attccggcaa tgatgttcta cagcggtttt aaccgtaaag tagatgaaag
 541 caaacttaaa tggcaggcca ttcaagcccg taaagccaat caataagcgg tcaaatttca
 601 cttatttttt gcaactcaat caggtttctt atgaaaaaat ttgacgaaat taatattatt
 661 ccgttcatcg acattatgtt ggtgctatta gcgatcgtgc ttgttaccgc ctcatttatt
 721 tcacaaggca aaattcaagt gaatgtgccg aaagcaacca cgactcaagc gatgaaagcg
 781 gatgaattag ctaaacttct tacgatcaca gaaaacaacg agttctattt caacgatcaa
 841 ccaattacta aagaagcact tactactgag attgccactt gggacaaaac ccaaaaagta
 901 accttaaaag tagatggtgc ggtggcattt gaaaaatttg ttgaactcac cgatgtactc
 961 tctgcgaatg aaattaaaaa cgtcgctatc gtaacgaaga aagatacagc caagaaatag
1021 gatacgagta tgaagaaaaa acattctcgt atcgggttaa tcagttctat tgttattcac
1081 acctttgttt ttgccggttt catttcgata gtaaaaagtt ctcattcgga cggtcagcct
1141 gaagataatg taatgtcgat ggaattagtt gccgctttat tagaacagcc tcaagttgcg
1201 gtggcagaag aacaacctac acaagcggaa ccggaaaaac agcaagcaga accggagccg
1261 gaacctgaag cagagccgat tcctgaaccg aagccacagc ctaaacctaa agaaaagccg
1321 aaggaaaagc caaaaccaaa agagcaaccg aagcctaaag aaaaagaaaa gcctaaaaaa
1381 gagaaagcta aagataaacc gattaaggca ttagaaaaag ggccggaagc caaacaaggt
1441 attgtcgcaa aagccatacc gaatgcagtg caaggtactc aagcacgagc gggcatcccg
1501 aatggtaaac caaacggtaa tcctaacggt acatcagcga acggctcaca aaatggtgcg
1561 gcaggtggca gtggcagcgg aagtaatggt agtgaaatcg gagcttataa agctgcatta
1621 cagcgagcct tacaacgtcg agctaacaat gcctatcccg ctcgagaaaa aatgatgcgt
1681 aaaacagggg ttgttactat tggttttacc gtatcgtctt ctggcgattt aaccaatgta
1741 aaagtgttaa acagttccgg taacagtaat cttgataacg cagcagttaa agcagcgcaa
1801 agcaccaaag tgtcaccgcc accggctggt ttcccaagta gtgttactgt tccggtaaaa
1861 ttctcgattg aataaatctt accaataagc tcccctgcgg gagcttattt tttattcgtt
1921 ataagcggtc atttttact aaaattttgc aaacatcagc aaaagataag cgcttgccta
1981 aagcggggtg cttaagtaaa ataaactgtt tctattttta ttatacaaat tgaggaaaat
2041 gtaatg
```

Appendix 1.5

A. suis strain SO4 HugZ (*hugZ*, 460-981) and HgbA (*hgbA*, 1055-3895) genes, complete cds. Start and stop codons are in bold type and underlined, respectively. GenBank accession number AY335901 (4164 bp).

```
   1 gataattagc ttcatcaaat ttataaaaac aagcaaagga ccaaggttgg ttattttctg
  61 tcacagacaa agtacagaga tattttttat taaggaaatt agctatgcgt ttctgcatat
 121 ggacccctat ttattaataa ttgtaagata tgtcaatttt taatgttttg acatactgtt
 181 taataagcaa aatccttttt ggcgaagtaa atattcaaga atgaacagat tcattttgac
 241 acaaaattgt aagtttagta aagtgaaaag ccctttttat ggctattaat aggccttaat
 301 ttcaaagaga tacaaccact cgctgattat ttgttcgtca aaagtaaaaa tttacatttt
 361 taacattgcg aaaaaaaaca gtataaatat aattaaaaat aatttgtatt aacaatatct
 421 gttgataaaa tttacataaa ttttagttga ggagaactta tgactgtgac taatcgtcaa
 481 gaagtcttac aaaatcgttt aggacctgaa attcaagaat taaaagaaaa tattcagaca
 541 attgtgcttt caacagtaga taaagacggt aatccgaatg tgagttatgc gccttttgtg
 601 attaataatg gcgaatacca agtactgatt tcaacgattg ctcgtcatgc tcgtaattta
 661 attgatgtac cgaaagtatc attaatgttg attgatgatg aaacgaaaag ccgtcaaatt
 721 tttgctcgcc gccgtttaac gtttgatgcg acttcacgtt taattgaacg tgaaggtgaa
 781 gagtgggaaa gtggtttaaa agcattaaaa gcacgccatg gtgatcttat tgatgagctt
 841 tcaaaaatga aagatttcaa cttattcagt tttaaaccga cacaaggcct tttcgtaaaa
 901 ggctttggta aagctttcga agtaagttcg gatgatctag tgaatatcgt tcacctagat
 961 gaaggtcata aaacagaata actatataat taaacattta ctgtgctagt tttggctagc
1021 acagtttttt tatcttgtct aaaggagaat gattgtgatt aagcttcgtt ttaatctatc
1081 tattgcagca ctttgtgttt tgcaatacaa cagtgcattg gcacaagagc aaatgcaact
1141 cgacaccgtg attgtaaagg atggtattca gcaacaaaaa atttctgaaa tcaaaaaatc
1201 tgcaaaacaa ctggcaaaac agcaagtaca agactcacga gatctcgtac gttatgaaac
1261 cggtgtgacg gtagttgaga ccggacgtat ggggagtagc ggttatgcgg tgcgaggggt
1321 agatgagaac cgtgttgcaa ttacagtaga tggtttacat caagcggaaa cactttcatc
1381 tcagggtttt aaagaattat ttgaagggta tggcaatttt aataataccc gtaactcggt
1441 agaaattgaa acacttaaac aagcgacgat tcgtaaaggt gcggattccg ttcgtgtcgg
1501 tagcggtgct ttaggcggcg cagtaatgtt tgaaaccaaa gatgctcgtg attttttaac
1561 ggaaaaagat taccactttg gttataaaac cggttattca agtgcagata atcaaaaagc
1621 caactcactg acctttgccg gtcgttataa aaattttgat attttagcgt taaaaacttg
1681 gcgtgacggt catgaattgg aaaactatga ttacaagacg gctgacggta gtgtacaagg
1741 gaaagaacga gaaaaagccg acccttattc aattaagaaa gacagtactt taattaaatt
1801 cggttattca ccttcggata atcaccgttt tagtgttgtt gcggatttgt atgagaagaa
1861 aaatagaggt catgactttt cttacaactt aaaaccgaca acttatatta acgttgacga
1921 atatgagtta cgtcatacga atgataaaag taaacgccag aattatgctt ttgtgtatga
1981 gaattttagt gcgaatccat tatgggatac attaaagctt acctattcaa cacaaaaaat
2041 taaaaatcgt gctaggacag atgattattg tgatggttcg cattgtaagg aaaccgagaa
2101 tttagccggt ttacagctaa aagatggtaa agtggttgat agagacggta atcaaccgaa
2161 cttaggcgtg gatgaattag gtttaaccac aattaccgat tcaaaaggca cttatactga
2221 gggagtaaat ctcgttagag cttattggtt tgattgtagt gtttttgatt gtaataaatc
2281 tgtcacagca tattataaag attattcaaa taatataacc tcggaagagg ttgcacttac
2341 aaaaacgtat acggacgaaa aaggacgtaa gtttgcaact ttagatccaa agagtaaatt
2401 taaaagcatc ctattaccag gaagtaaagg ctatacggaa aatatttata ctgaacgaga
2461 tttagatacg gatacaaagc aactgaatct tgatttaact aaatccttct ctttactcgg
2521 tcaagaacat tcattggaat atggtggtag ctatagccgt acagagaaaa aaatggttaa
2581 ccgtggcggt ttcggtgcaa cgagtaatac tcaatggtgg acaaagcgtt tccttggtat
2641 gcggaataac ttctttaaag gaacggaaga agtcattact tgtaaaaatg cgaccggtag
2701 cgatcaatgg aatggcttaa tttgcccgaa tgaagatacg ttttcgttct taattccggt
2761 tgaagcgaaa aatggttcat tatattttgc ggataatttg aaagcgaatg actatttaag
2821 ttttgatatc ggctaccgct atgatgatat tagctataag cctaaatatg ttgcgggaca
2881 aacggcgaag attccggatg atatgctgca agggttattt gtaccgttac cagatcgacc
2941 aacaaaagag caaattcgcc aaaatgcgga agaaaatatt aaatatttat cacgtaatgt
```

```
3001 taaatatcaa aaacattctt attcattagc ggcaactgcg gatccgttta gcttcttgcg
3061 cgtacaagtt aaatacgcaa atggatttag agcaccgact tctgatgagc tttattttac
3121 ctttaagcac ccggatttta ccgtattgcc taatgtggat cttaaatctg agcgtgcaaa
3181 gacgaaagaa gccgcattaa ctttacatgg tgatttaggc tttattacaa ccagttactt
3241 taaaacggat tatgataatt ttattgattt gaagtatctt ggtcctaaaa atctaatgaa
3301 tgctttcggc ggcagtgcaa tggcaagacc ttatcaaatt tatcaaaatg taaaccgtca
3361 agaggctaga gttaacggct tagaaattag ttctaaattg aatttcgggc aattatggca
3421 agctttagaa gggttcaacg caagttataa atacacttat caaaaaggta agatggaggg
3481 gaatttaccg cttaatgcga ttcagccgag aacttctgtg tttggtttag gctatgaaca
3541 tccggaagag aaatttggcg tagatgtgta tgttacccgt gtaagcgcga aaaaagcgga
3601 agatacttac aatatgtatc atagtgaaga aaaggcaaaa gacagctact taaaatggcg
3661 cagcggtgct tatactttat tagatatgat tgcttatgtt aaaccgtgga aaaacgtcac
3721 cttacaagca ggtgtttata atttacagaa tcgcaaatat ctgacttggg aatcggttcg
3781 ttcaattcgt ccatttggta cgagtaactt gatcaaccaa acgacaggta aaggattaaa
3841 tcgtttcact gctcctggaa gaaactttaa gttaaccgca gaggtaactt tctaagtaaa
3901 atgcccgata agtcattgct tatcgggcat tttttgcaaa aaaatcttaa aatttaaccg
3961 cttgttagag ttttaccggc tcgataattt cactagggta gcaacctaat actttgatat
4021 agcttgtaac atcttctaaa gcttttagtg cttgttgggt attttcagca tgcgtattcg
4081 cctctagttc cacataaaac atttcttccc aaggtttacc gtaaatcggg cgagattcca
4141 atttggtcat acgaatctga tact
```

Appendix 1.6

A. suis strain C84 HugZ (*hugZ*, 460-981) and HgbA (*hgbA*, 1055-3895) genes, complete cds. Start and stop codons are in bold type and underlined, respectively. GenBank accession number AY335902 (4197 bp).

```
   1 gataattagc ttcatcaaat ttataaaaac aagcaaagga ccaaggttgg ttattttctg
  61 tcacagacaa agtacagaga tatttttat taaggaaatt agctatgcgt ttctgcatat
 121 ggacccctat ttattaataa ttgtaagata tgtcaatttt taatgttttg acatactgtt
 181 taataagcaa aatccttttt ggcgaagtaa atattcaaga atgaacagat tcatttgac
 241 acaaaattgt aagtttagta aagtgaaaag cccttttat ggctattaat aggccttaat
 301 ttcaaagaga tacaaccact cgctgattat ttgttcgtca aaagtaaaaa tttacatttt
 361 taacattgcg aaaaaaaaca gtataaatat aattaaaaat aatttgtatt aacaatatct
 421 gttgataaaa tttacataaa ttttagttga ggagaactta tgactgtgac taatcgtcaa
 481 gaagtcttac aaaatcgttt aggacctgaa attcaagaat taaaagaaaa tattcagaca
 541 attgtgcttt caacagtaga taaagacggt aatccgaatg tgagttatgc gcctttgtg
 601 attaataatg gcgaatacca agtactgatt tcaacgattg ctcgtcatgc tcgtaattta
 661 attgatgtac cgaaagtatc attaatgttg attgatgatg aaacgaaaag ccgtcaaatt
 721 tttgctcgcc gccgtttaac gtttgatgcg acttcacgtt taattgaacg tgaaggtgaa
 781 gagtgggaaa gtggtttaaa agcattaaaa gcacgccatg gtgatcttat tgatgagctt
 841 tcaaaaatga aagatttcaa cttattcagt tttaaaccga cacaaggcct tttcgtaaaa
 901 ggctttggta aagctttcga agtaagttcg gatgatctag tgaatatcgt tcacctagat
 961 gaaggtcata aaacagaata actatataat taaacattta ctgtgctagt tttggctagc
1021 acagtttttt tatcttgtct aaaggagaat gattgtgatt aagcttcgtt ttaatctatc
1081 tattgcagca ctttgtgttt tgcaatacaa cagtgcattg gcacaagagc aaatgcaact
1141 cgacaccgtg attgtaaagg atggtattca gcaacaaaaa atttctgaaa tcaaaaaatc
1201 tgcaaaacaa ctggcaaaac agcaagtaca agactcacga gatctcgtac gttatgaaac
1261 cggtgtgacg gtagttgaga ccggacgtat ggggagtagc ggttatgcgg tgcgaggggt
1321 agatgagaac cgtgttgcaa ttacagtaga tggtttacat caagcggaaa cactttcatc
1381 tcagggtttt aaagaattat ttgaagggta tggcaatttt aataataccc gtaactcggt
1441 agaaattgaa acacttaaac aagcgacgat tcgtaaaggt gcggattccg ttcgtgtcgg
1501 tagcggtgct ttaggcggcg cagtaatgtt tgaaaccaaa gatgctcgtg atttttaac
1561 ggaaaaagat taccactttg gttataaaac cggttattca agtgcagata atcaaaaagc
1621 caactcactg acctttgccg gtcgttataa aaattttgat attttagcgt taaaaacttg
1681 gcgtgacggt catgaattgg aaaactatga ttacaagacg gctgacggta gtgtacaagg
1741 gaaagaacga gaaaaagccg acccttattc aattaagaaa gacagtactt taattaaatt
1801 cggttattca ccttcggata atcaccgttt tagtgttgtt gcggatttgt atgagaagaa
1861 aaatagaggt catgactttt cttacaactt aaaaccgaca acttatatta acgttgacga
1921 atatgagtta cgtcatacga atgataaaag taaacgccag aattatgctt ttgtgtatga
1981 gaattttagt gcgaatccat tatgggatac attaaagctt acctattcaa cacaaaaaat
2041 taaaaatcgt gctaggacag atgattattg tgatggttcg cattgtaagg aaaccgagaa
2101 tttagccggt ttacagctaa aagatggtaa agtggttgat agagacggta atcaaccgaa
2161 cttaggcgtg gatgaattag gtttaaccac aattaccgat tcaaaaggca cttatactga
2221 gggagtaaat ctcgttagag cttattggtt tgattgtagt gtttttgatt gtaataaatc
2281 tgtcacagca tattataaag attattcaaa taatataacc tcggaagagg ttgcacttac
2341 aaaaacgtat acggacgaaa aaggacgtaa gtttgcaact ttagatccaa agagtaaatt
2401 taaaagcatc ctattaccag gaagtaaagg ctatacggaa aatatttata ctgaacgaga
2461 tttagatacg gatacaaagc aactgaatct tgatttaact aaatccttct ctttactcgg
2521 tcaagaacat tcattggaat atggtggtag ctatagccgt acagagaaaa aaatggttaa
2581 ccgtggcggt ttcggtgcaa cgagtaatac tcaatggtgg acaaagcgtt tccttggtat
2641 gcggaataac ttctttaaag gaacggaaga agtcattact tgtaaaaatg cgaccggtag
2701 cgatcaatgg aatggcttaa tttgcccgaa tgaagatacg ttttcgttct taattccggt
2761 tgaagcgaaa aatggttcat tatattttgc ggataatttg aaagcgaatg actatttaag
2821 ttttgatatc ggctaccgct atgatgatat tagctataag cctaaatatg ttgcgggaca
2881 aacggcgaag attccggatg atatgctgca agggttattt gtaccgttac cagatcgacc
2941 aacaaaagag caaattcgcc aaaatgcgga agaaaatatt aaatatttat cacgtaatgt
```

```
3001 taaatatcaa aaacattctt attcattagc ggcaactgcg gatccgttta gcttcttgcg
3061 cgtacaagtt aaatacgcaa atggatttag agcaccgact tctgatgagc tttattttac
3121 ctttaagcac ccggatttta ccgtattgcc taatgtggat cttaaatctg agcgtgcaaa
3181 gacgaaagaa gccgcattaa ctttacatgg tgatttaggc tttattacaa ccagttactt
3241 taaaacggat tatgataatt ttattgattt gaagtatctt ggtcctaaaa atctaatgaa
3301 tgctttcggc ggcagtgcaa cggcaagacc ttatcaaatt tatcaaaatg taaaccgtca
3361 agaggctaga gttaacggct tagaaattag ttctaaattg aatttcgggc aattatggca
3421 agctttagaa gggttcaacg caagttataa atacacttat caaaaaggta agatggaggg
3481 gaatttaccg cttaatgcga ttcagccgag aacttctgtg tttggtttag gctatgaaca
3541 tccggaagag aaatttggcg tagatgtgta tgttacccgt gtaagcgcga aaaaagcgga
3601 agatacttac aatatgtatc atagtgaaga aaaggcaaaa gacagctact taaaatggcg
3661 cagcggtgct tatactttat tagatatgat tgcttatgtt aaaccgtgga aaaacgtcac
3721 cttacaagca ggtgtttata atttacagaa tcgcaaatat ctgacttggg aatcggttcg
3781 ttcaattcgt ccatttggta cgagtaactt gatcaaccaa acgacaggta aaggattaaa
3841 tcgtttcact gctcctggaa gaaactttaa gttaaccgca gaggtaactt tctaagtaaa
3901 atgcccgata agtcattgct tatcgggcat tttttgcaaa aaaatcttaa aatttaaccg
3961 cttgttagag ttttaccggc tcgataattt cactagggta gcaacctaat actttgatat
4021 agcttgtaac atcttctaaa gcttttagtg cttgttgggt attttcagca tgcgtattcg
4081 cctctagttc cacataaaac atttcttccc aaggtttacc gtaaatcggg cgagattcca
4141 atttggtcat acgaatctga tactgtttga ataccatcaa cgcatccact aatgcac
```

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: D. NIVEN
TELEPHONE: 398-7886
ADDRESS: MACDONALD CAMPUS
FAX NUMBER: 398-7990
E-MAIL: niven@nrs.mcgill.ca
DEPARTMENT: NATURAL RESOURCE SCIENCES
PROJECT TITLE: RESEARCH NETWORK ON BACTERIAL PATHOGENS OF SWINE

2. FUNDING SOURCE: MRC ☐ NSERC ☒ NIH ☐ FCAR ☐ FRSQ ☐
INTERNAL ☐ OTHER ☐ (specify)
Grant No. Beginning date APRIL 1, 2000 End date MARCH 31, 2005

3. Indicate if this is

☒ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date MARCH 31, 2001.

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 [✓ 2] 3 4

Principal Investigator or course director: date: 19 03 01 (day month year)

Chairperson, Biohazards Committee: SIGNATURE date: 30 03 01 (day month year)

Approved period: beginning 01 04 01 (day month year) ending 31 03 05 (day month year)

* as defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

4. RESEARCH PERSONNEL: **(attach additional sheets if preferred)**

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
D.F. NIVEN	NAT. RES. SCI	✓				
F. BAHRAMI					✓	
F. YOUNES					✓	

5. EMERGENCY: Person(s) designated to handle emergencies

Name: D.F. NIVEN Phone No: work: 398-7886 home: 457-3112

Name: ____ Phone No: work: ____ home: ____

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

Actinobacillus suis Gp 2
Actinobacillus pleuropneumoniae Gp 2
Streptococcus suis Gp 2
Escherichia coli DH5α and H1717 Gp 1.

ii) the procedures involving biohazards

Organisms are grown in liquid culture, harvested and washed by centrifugation, and either used per se or disrupted by sonication and the resulting extracts used in experiments (in vitro only). Centrifuge tubes are closed tubes/bottles.

iii) the protocol for decontaminating spills

Any spill is decontaminated with sodium hypochlorite or 70% ethanol.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent (s)?

NO

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

YES

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

Any manipulation that has the potential to generate an aerosol is conducted in the biological safety cabinet.

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
MACDONALD – STEWART	MS3-050	CANADIAN CABINETS	BM6 2A	7089	NOV17, 1999 AWAITING TECHNICIAN

December 28, 2004

Our ref: BahramiMcGillML1-05

Mr. Fariborz Bahrami
McGill University
Macdonald Campus, Dept. NRS
21111 Lakeshore Rd.
H9X 3V9 Ate. Anne de Bellevue, QC
CANADA
Fax: (514) 398-7990

Publication: VETERINARY MICROBIOLOGY, 94(1):79-92, Bahrami, Ekins, and Niven: "Iron acquisition by...," copyright 2003 Elsevier B.V.

As per your letter dated December 14, 2004, we hereby grant you permission to reprint the aforementioned material at no charge **in your thesis** subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

   "Reprinted from Publication title, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

3. Reproduction of this material is confined to the purpose for which permission is hereby given.

4. This permission is granted for non-exclusive world **<u>English</u>** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.

5. This includes permission for University Microfilms to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely,

for Michael J. Lacovara
for Elsevier

For your convenience, apply online at www.us.elsevierhealth.com
170 S Independence Mall W 300 E– Philadelphia, PA 19106-3399
Phone (215) 238-7869 Fax (215) 238-2239 Email: healthpermissions@elsevier.com

Elsevier Ltd., The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK VAT: 494627212

We, the co-authors of the following publication, grant Mr. Fariborz Bahrami permission to reprint and use the following publication in his Ph.D. Thesis (McGill University) entitled "Iron Acquisition by *Actinobacillus suis*":

Bahrami, F., Ekins, A. and Niven D. F. 2003. Iron acquisition by *Actinobacillus suis*: identification and characterization of transferrin receptor proteins and encoding genes. Vet. Microbiol. **94:** 79-92.

Dr. Donald F. Niven
Dept. of Natural Resource Sciences
McGill University
21111 Lakeshore Road
Ste. Anne de Bellevue, QC H9X 3V9

Dr. Andrew Ekins
Dept. of Food Science and
Agricultural Chemistry
McGill University
21111 Lakeshore Road
Ste. Anne de Bellevue, QC H9X 3V9

ELSEVIER

425·125
YEARS OF PUBLISHING
TRADITION | EXCELLENCE
We commemorate the founding of the House of Elzevir in 1580 and celebrate the establishment of the Elsevier company in 1880.

08 August 2005

Our ref: HG/ct/aug 05.J024

Mr Fariborz Bahrami
McGill University, Macdonald Campus
Dept. NRS, MS3-039, 21111 Lakeshore Rd.
Ste. Anne de Bellevue
Canada H9X 3V9

Dear Mr Bahrami

MICROBIAL PATHOGENESIS, Vol 39, Issue 1-2, 2005, pp 45-51, Bahrami et al, "Iron acquisition by ..."

As per your letter dated 20 July 2005, we hereby grant you permission to reprint the aforementioned material at no charge **in your thesis** subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

   "Reprinted from Publication title, Vol number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier".

3. Reproduction of this material is confined to the purpose for which permission is hereby given.

4. This permission is granted for non-exclusive world **English** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.

5. This includes permission for the National Library of Canada to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely

Helen Gainford
Rights Manager

Your future requests will be handled more quickly if you complete the online form at www.elsevier.com/locate/permissions

Elsevier, Global Rights Department, PO Box 800, Oxford OX5 1DX, UK
Tel +44 (0)1865 843830 | Fax +44 (0)1865 853333 | www.elsevier.com
vat no. 494 6272 12

I, the co-author of the following publication, grant Mr. Fariborz Bahrami permission to reprint and use the following publication in his Ph.D. Thesis (McGill University) entitled "Iron Acquisition by *Actinobacillus suis*":

Bahrami, F., and Niven, D.F. 2005. Iron acquisition by *Actinobacillus suis*: identification and characterization of a single-component haemoglobin receptor and encoding gene. Microb. Pathog. **39:** 45-51.

Dr. Donald F. Niven
Dept. of Natural Resource Sciences
McGill University
21111 Lakeshore Road
Ste. Anne de Bellevue, QC H9X 3V9