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Iron acquisition by *Actinobacillus pleuropneumoniae*

by

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A thesis submitted to

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Doctor of Philosophy

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For my parents

Abstract

Ph. D. **Colin Gerard D'Silva** **Microbiology**

Iron acquisition by *Actinobacillus pleuropneumoniae*

Four strains of the swine pathogen *Actinobacillus pleuropneumoniae*, namely, the type strain (ATCC 27088), the "reference" strain of biotype 2 (Bertschinger 2008/76) and two additional biotype 1 strains, strain BC181, which is less virulent than the type strain, and strain K17 (reference strain of serotype 5A), which was isolated from a lamb, were investigated with respect to iron acquisition. All four strains produced iron-repressible outer membrane proteins. However, only strains ATCC 27088 and Bertschinger 2008/76 could acquire iron from porcine transferrin. No organism could utilize human, bovine or ovine transferrin, or ovine or porcine lactoferrin. Haemoglobin supported good growth of all strains except K17 (which also failed to acquire iron from haemin). In all cases, iron acquisition from transferrin or haemoglobin required direct contact between the organisms and the proteins. Total membranes derived from iron-restricted organisms were subjected to an affinity isolation technique based on biotinylated porcine transferrin and streptavidin-agarose, and the following polypeptides were isolated: 99 kDa and 64 kDa from strain ATCC 27088; 93 kDa from strain Bertschinger 2008/76; 95 kDa (trace amounts) and 60 kDa from strain BC181; none from strain K17. These polypeptides appear to be transferrin receptor components. The 99 kDa polypeptide (TBP1) from the type strain was purified by SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membrane. The N-terminal amino acid sequence of the polypeptide was determined commercially. A commercially-synthesized oligonucleotide probe was used to clone the gene encoding the TBP1 of the type strain in competent *Escherichia coli* DH5α cells.

Résumé

Ph.D. **Colin Gerard D'Silva** **Microbiologie**

Acquisition du fer par *Actinobacillus pleuropneumoniae*

Quatre souches du pathogène du porc *Actinobacillus pleuropneumoniae*, nommément la souche type (ATCC 27088), la souche de référence du biotype 2 (Bertschinger 2008/76) et deux souches additionnelles du biotype 1, la souche BC181, qui est moins virulente que la souche type, et la souche K17 (souche de référence de sérotype 5A), qui a été isolée d'un agneau, ont été examinées pour leur capacité à acquérir le fer. Les quatre souches produisent des protéines de la membrane externe qui sont inhibées par le fer. Cependant, seules les souches ATCC 27088 et Bertschinger 2008/76 peuvent acquérir le fer de la transferrine porcine. Aucun organisme n'a pu utiliser les transferrines humaine, bovine ou ovine, ni les lactoferrines ovine et porcine. L'hémoglobine a supporté une bonne croissance de toutes les souches sauf la souche K17 (qui s'est également révélée incapable d'acquérir le fer de l'hémine. Dans tous les cas, l'acquisition du fer de la transferrine ou de l'hémoglobine a requis un contact direct entre les organismes et les protéines. Les membranes totales extraites d'organismes privés de fer ont été soumises à une technique d'isolement fondée sur l'affinité, reposant sur la transferrine porcine biotinylée et sur la streptavidine-agarose. Les polypeptides suivants furent isolés: 99 kDa et 64 kDa de la souche ATCC 27088; 93 kDa de la souche Bertschinger 2008/76; 95 kDa (traces) et 60 kDa de la souche BC181; aucun de la souche K17. Ces polypeptides sont apparemment des composantes des récepteurs de la transferrine. Le polypeptide de 99 kDa (TBP1) de la souche type a été purifié par SDS-PAGE et transféré par électrophorèse sur une membrane de polyvinylidene difluoride. Les acides aminés à l'extrémité N-terminale de la protéine ont été séquencés. De cette séquence un oligonucléotide fut synthétisé et utilisé comme sonde pour cloner le gène codant pour le polypeptide TBP1

de la souche type, qui fut inséré dans des cellules compétentes d'*Escherichia coli* DH5α.

Contributions to knowledge

(I) *A. pleuropneumoniae* strains Bertschinger 2008/76, BC181 and K17 produce iron-repressible outer membrane proteins when grown under iron-restricted conditions *in vitro*.

(II) *A. pleuropneumoniae* strains cannot acquire iron from porcine lactoferrin.

(III) The "reference" strain of *A. pleuropneumoniae* biotype 2 can acquire iron from porcine transferrin and haemoglobin.

(IV) *A. pleuropneumoniae* strain BC181 can acquire iron from haemoglobin but not from transferrin.

(V) *A. pleuropneumoniae* strain K17 can acquire iron from neither transferrin, haemoglobin nor haemin.

(VI) Acquisition of iron from transferrin and haemoglobin by *A. pleuropneumoniae* requires direct contact between the organisms and the host proteins.

(VII) The ability of *A. pleuropneumoniae* to acquire iron from transferrin may be a virulence determinant but is not essential for a strain to induce disease in swine.

(VIII) The ability of *A. pleuropneumoniae* to acquire iron from a specific transferrin may help to determine host specificity but it is not the only factor involved.

(IX) Transferrin-binding polypeptides of the following molecular masses can be isolated from total membranes of organisms grown under iron-restricted conditions: 93 kDa from strain Bertschinger 2008/76; 95kDa (trace amounts) and 60 kDa from strain BC181; none from strain K17.

(X) Iron acquisition from porcine transferrin involves one or more polypeptides that function as receptor components.

(XI) The inability of a strain of *A. pleuropneumoniae* to use porcine transferrin and the haemoglobins may reflect defects in the corresponding receptor components, or alternatively, defects in, or the absence of, other components involved in the overall iron-acquisition process.

(XII) The N-terminal amino acid sequence of the 99 kDa polypeptide (TBP1) from the type strain (ATCC 27088) was determined commercially and shown to be Glu-Gln-Ala-Val-Gln-Leu-Asn-Asp-Asp-Tyr-Gly-Thr-Thr-.

(XIII) A commercially-synthesized oligonucleotide (corresponding to the N-terminus of TBP1) probe was used to clone the gene encoding the TBP1 of the type strain in competent *E. coli* DH5α cells.

List of Abbreviations

EDDA	: Ethylenediamine di-o-hydroxyphenylacetic acid
IRMP	: Iron-repressible outer membrane protein
NAD	: Nicotinamide adenine dinucleotide
OMP	: Outer membrane protein
PVDF	: Polyvinylidene difluoride
TBP	: Transferrin-binding protein
TMP	: Total membrane protein

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Last, but not least, I thank my parents and family members (despite being thousands of miles away!) for their constant encouragement, support and prayers. Unfortunately, my Dad is not around to see the completion of this degree. May his soul rest in peace.

Table of contents

List of figures

Chapter 1. Literature review

Introduction

Actinobacillus pleuropneumoniae is the causative agent of severe and often fatal swine pleuropneumonia which is responsible for great economic losses to the swine industry worldwide. Like other pathogens, *A. pleuropneumoniae* has an essential requirement for iron for growth and must be capable of obtaining iron from the host despite the iron-restricted environment.

The purpose of the following section is to provide a review of *A. pleuropneumoniae*, of both the organism and its virulence factors, as well as the importance of iron and the mechanisms of iron-acquisition by pathogenic bacteria. This section also reviews the importance of transferrin receptors as vaccine components with a view to the eradication of swine pleuropneumonia.

1.1 *Actinobacillus pleuropneumoniae*

1.1.1 Introduction

Actinobacillus pleuropneumoniae is the causative agent of swine pleuropneumonia. Organisms of the genus *Actinobacillus* belong to the family *Pasteurellaceae* which also includes the genera *Haemophilus* and *Pasteurella* (Zinnemann, 1981). Organisms of the family *Pasteurellaceae* are Gram-negative, facultatively anaerobic, non-spore-forming pleomorphic rods and are obligate parasites in their animal hosts (Shope, 1964). The family does not include any free-living species. The organisms reside on the mucous membranes of the upper respiratory tract and lower genital tract. There are over 40 named species in the family and of these species, *A. pleuropneumoniae* is one of four that is capable of causing disease upon mere introduction, by a natural route (in reasonable numbers), into a normal host animal (Biberstein, 1990).

Organisms of the family *Pasteurellaceae* are spread by several means including aerosols, sexual contact and animal bites. The mechanisms used by these organisms for the colonization of host tissues are not completely understood. Some genera of the family are piliated and piliation could aid in colonization (Tomcik *et al.*, 1988).

In members of the *Pasteurellaceae*, several cytotoxic and leukotoxic proteins have been identified which have been found to be responsible for the invasiveness of these

organisms (Bendixen *et al.*, 1981). In addition, iron-repressible outer membrane proteins (IRMPs) are considered as possible virulence factors (Ikeda and Hirsh, 1988; Deneer and Potter, 1989*a*,*b*). In all members of the *Pasteurellaceae*, the lipopolysaccharide (LPS) and lipooligosaccharide endotoxins have been found to be major factors involved in pathogenesis (Emau *et al.*, 1986; 1987; Fenwick *et al.*, 1986; Rose *et al.*, 1988). Major manifestations of infection by *Haemophilus*, *Actinobacillus* and *Pasteurella* species such as fever (Lin *et al.*, 1982), lung damage (Snapper *et al.*, 1983), vascular derangements (Emau *et al.*, 1986; Fenwick *et al.*, 1986) and meningitis (Syrogiannopoulos *et al.*, 1988), have been shown to be producible by endotoxins or their effector substances such as prostaglandins. However, other factors also contribute to the pathogenesis of the infections (Fenwick *et al.*, 1986; Syrogiannopoulos *et al.*, 1988). Local tissue damage caused during infection is due to lysosomal enzymes released from leukocytes and hence the leukotoxic properties of members of the *Pasteurellaceae* are directly and indirectly responsible for the inflammatory lesions (Movat and Wasi, 1985; Slocombe *et al.*, 1985; Breider *et al.*, 1988).

1.1.2 Genus designation

Prior to 1983, the organism causing porcine pleuropneumonia was referred to as *Haemophilus pleuropneumoniae*. However, as early as 1978, a *Pasteurella haemolytica*-like organism causing porcine necrotizing pleuropneumonia had also been described (Bertschinger and Seifert, 1978) (as cited by Pohl *et al.*, 1983) and designated the BS organism. This organism differed from *H. pleuropneumoniae* in that it was V factor-independent. The disease caused by the BS organism could not be distinguished by either clinical or histological means from that caused by *H. pleuropneumoniae* and the BS strain resembled *H. pleuropneumoniae* in several biochemical properties. Both organisms were not significantly related to *Haemophilus influenzae*, the type species of the genus *Haemophilus*. On the basis of conventional phenotypic characteristics, the BS organism was placed close to *Actinobacillus lignieresii*.

In a study by Pohl *et al.* (1983), *H. pleuropneumoniae* (Matthews and Pattison 1961) Shope 1964 (Kilian *et al.*, 1978) and the BS organism were compared with *A. lignieresii* and *H. influenzae*. On the basis of phenotypic and DNA relatedness between *H. pleuropneumoniae* and *A. lignieresii*, Pohl *et al.* (1983) proposed the transfer of *H. pleuropneumoniae* to the genus *Actinobacillus*. The DNA of the BS organism and those of *H. pleuropneumoniae* and *A. lignieresii* were found to be very similar in size (based on the fact that reassociation rates in reactions with homologous DNA were nearly identical), and the guanine-plus-cytosine contents of their DNA were almost the same.

However, the level of reassociation of the DNA of the type strain of *H. influenzae* with that of either the type strain of *H. pleuropneumoniae*, the type strain of *A. lignieresii* or the BS organism, was extremely low. Moreover, the results of these and additional heterologous reassociation studies indicated that *H. pleuropneumoniae* and the BS organism were very closely related to *A. lignieresii* and hence, that *H. pleuropneumoniae* and the BS organism belonged to the genus *Actinobacillus*. Consequently, Pohl *et al.* (1983) proposed the transfer of *H. pleuropneumoniae* and the BS organism to the genus *Actinobacillus* as *Actinobacillus pleuropneumoniae* (Matthews and Pattison) comb. nov.

1.1.3 Biotypes and serotypes

The family *Pasteurellaceae* includes V factor-dependent and V factor-independent species. *A. pleuropneumoniae* is somewhat unusual in that the species contains V factor-dependent and V factor-independent strains (Pohl *et al.*, 1983). V factor-dependent strains were designated biotype 1, while V factor-independent strains were designated biotype 2. It is possible that the two biotypes differ from each other only with respect to a single enzyme (nicotinamide phosphoribosyltransferase) involved in pyridine nucleotide metabolism (Niven and O'Reilly, 1990). On the basis of agglutination tests (Gunnarsson *et al.*, 1977; Greenway, 1981; Rosendal *et al.*, 1981*b*; Sebunya *et al.*, 1982) and coagglutination tests (Mittal *et al.*, 1983), twelve serotypes of *A. pleuropneumoniae* have been identified. Antisera prepared in rabbits against formalinized whole cell suspensions

of reference strains of serotypes 1 to 12 of biotype 1 have been employed for serotyping (Mittal *et al.*, 1992). Serotype 1 is the most widespread in the world ranging from 55% to 87% (depending on the year) of all serotypes isolated, while serotype 5 is second in prevalence with an average incidence of 23%. Other serotypes account for a small percentage of the serotypes isolated and serotypes 4, 9 and 11 have not been isolated during the past 10 years (Mittal *et al.*, 1992). Serotype 2 is known to occur only in Canada, the Far East and Europe (Nicolet, 1971) (cited by Sebunya and Saunders, 1983).

Studies of various serotypes of *A. pleuropneumoniae* have shown that the serotypes vary in terms of their virulence in swine (Mittal *et al.*, 1984; Rosendal *et al.*, 1985; Brandreth and Smith, 1987; Komal and Mittal, 1990). In general, strains of serotypes 1, 5, 9, 10 and 11 were found to be highly virulent and those of serotypes 2, 3, 4, 6, 7, 8 and 12 were found to be less virulent (Komal and Mittal, 1990). However, some strains of serotypes 3 and 7 were found to be highly virulent while a few strains of serotype 5 caused low mortality in mice (Komal and Mittal, 1990). Highly virulent strains of *A. pleuropneumoniae* are very invasive and appear in the blood within 3 to 6 hours of intranasal inoculation in mice. DNA-DNA hybridization and restriction endonuclease fingerprinting (REF) studies have revealed that representative strains of all 12 serotypes form a homogeneous group, sharing 74% to 90% homology with *A. pleuropneumoniae* serotype 1 (Borr *et al.*, 1991). All the serotypes tested showed a high degree of genetic relatedness (66% to 79%) to the type strain of *A. lignieresii*.

In Quebec, over 2500 strains isolated from the lungs of pigs that died due to acute

pleuropneumonia have been serotyped by Mittal and co-workers (Mittal *et al.* (1989)) (cited by Komal and Mittal, 1990). Approximately 70% of the strains belonged to serotype 1 and another 22% belonged to serotype 5 (Mittal *et al.* (1989)) (cited by Komal and Mittal, 1990). Mittal *et al.* (1989) (cited by Komal and Mittal, 1990) suggest that strains of serotypes 1 and 5 are more virulent than strains of other serotypes. Strong cross-reactions exist between serotypes (Nicolet *et al.*, 1980). Several serotype-specific and species-specific antigens have been detected on the basis of agglutination, immunodiffusion, adsorption or complement fixation tests (Gunnarsson *et al.*, 1977; Gunnarsson *et al.*, 1978; Gunnarsson, 1979; Mittal *et al.*, 1982).

A 1.5-kb DNA fragment from a genomic library of *A. pleuropneumoniae* serotype 1 strain Shope 4074 (ATCC 27088) was found to hybridize to DNA from all 12 serotypes of the organism (Sirois *et al.*, 1991). No cross-hybridization was observed with DNA from other haemolytic organisms of the family *Pasteurellaceae*. Based on the nucleotide sequence of the putative genomic probe, three primers were synthesized for use in the polymerase chain reaction (PCR). PCR amplification products were obtained using the DNA from the 12 known serotypes, but the DNA from any other Gram-negative or Gram-positive organism studied (including those found in the normal flora of the swine respiratory tract) was not amplified. The results of the work of Sirois *et al.* (1991) suggest that amplification of specific *A. pleuropneumoniae* sequences from tissue samples could be used to identify asymptomatic carriers and hence, PCR could be used in efforts to eradicate swine pleuropneumonia.

1.1.4 Virulence of *A. pleuropneumoniae*

A. pleuropneumoniae is the causative agent of acute and often fatal, fibrinous and necrotizing pleuropneumonia in swine in several countries around the world (Sebunya and Saunders, 1983; Nicolet, 1986; MacInnes and Rosendal, 1988). The disease, referred to often as swine pleuropneumonia, has been a major disease of swine in European countries since the late 1960s (Christensen, 1981) (cited by Sebunya and Saunders, 1983) and has been recognized frequently in the United States (Pijoan, 1982) and Canada (Sanford and Josephson, 1981) since the early 1980s. Acute pleuropneumonia is often characterised by extensive haemorrhagic and necrotic lesions and fibrinous exudate in the lungs (Nicolet, 1986; Bertram, 1988). Chronic lesions involve fibrosis and purulent inflammation (Rosendal *et al.*, 1985). Asymptomatic infections involve fibrosis with pleural adhesions (Inzana *et al.*, 1993*a*). Animals that become infected usually die if untreated, and death can occur within 24 to 48 hours of the first signs of illness (although in some cases, death can occur even if no signs of illness are observed) (Shope, 1964). Outbreaks of swine pleuropneumonia caused by biotype 1 strains have resulted in a higher death rate and the severity of clinical infections has been greater than in outbreaks caused by biotype 2 strains (Pohl *et al.*, 1983). Great economic losses to the swine industry result from the inability to control disease spread in epizootics (Shope, 1964). Autogenous vaccines, prepared from locally important serotypes, are used for immunoprophylaxis in various countries (Pijoan *et al.*, 1975; Rosendal *et al.*, 1981*a*).

Several components are believed to contribute to the virulence of *A. pleuropneumoniae*. These include the capsular polysaccharide (Jensen and Bertram, 1986), the LPS (Fenwick *et al.*, 1986; Udeze *et al.*, 1987), as well as haemolytic toxin(s) (Nakai *et al.*, 1984; Kume *et al.*, 1986; Rosendal *et al.*, 1988).

The first step in the colonization of host mucosal surfaces is bacterial adherence which is achieved by the microorganism via adhesins (Beachey, 1981). Respiratory mucosal surfaces are covered with a mucus layer and *A. pleuropneumoniae* has been shown to have an affinity for the same (Belanger *et al.*, 1992). Studies with isolated LPS and crude porcine respiratory tract mucus would indicate that LPS is a component involved in the binding (Belanger *et al.*, 1990). Two low molecular mass polypeptides of 10 and 11 kilodaltons (kDa) have been identified in porcine respiratory tract secretions and these polypeptides appear to be involved in the binding of the LPS of *A. pleuropneumoniae* (Belanger *et al.*, 1994). A protein, designated outer membrane lipoprotein A (OmlA) with a calculated molecular mass of 40 kDa, has been identified as an adhesin of a serotype 1 strain of *A. pleuropneumoniae*. OmlA is encoded by the *omlA* gene, is lipid modified, and is present in the outer membrane and membrane blebs of the organism (Gerlach *et al.*, 1993). The DNA encoding OmlA detected hybridizing sequences in the reference strains of all serotypes of *A. pleuropneumoniae*, and a OmlA-specific serum detected a homologous protein in the reference strains of serotypes 2, 8, 9, 11 and 12.

Several different haemolysins and cytotoxins have been described in the 12

serotypes of *A. pleuropneumoniae* (Kilian, 1976*b*; Kume *et al.*, 1986; Martin *et al.*, 1985*b*; Rosendal *et al.*, 1988; Frey and Nicolet, 1988*a*,*b*; 1990; Devenish *et al.*, 1989; Frey *et al.*, 1989; Kamp *et al.*, 1989; Gygi *et al.*, 1990). Like several other Gram-negative bacteria, *A. pleuropneumoniae* secretes toxins of the RTX (repeat toxin) family (Welch, 1991). Three antigenically distinct RTX toxins have been shown to be present, either singly or in combination, in different isolates of *A. pleuropneumoniae* (Kamp *et al.*, 1991; Frey *et al.*, 1993*a*).

Recently, Frey *et al.* (1993*b*) introduced a unifying system of nomenclature for the pleurotoxin, haemolysins, and cytolysins (and their genes) produced by *A. pleuropneumoniae*. The RTX toxin produced by the reference strains of serotypes 1, 5, 9, 10 and 11 and referred to previously as haemolysin I (HlyI) or cytolysin I (ClyI) was designated ApxI with *apxI* being the structural gene encoding this toxin. The RTX toxin produced by the reference strain of each serotype (except serotype 10) and previously named HlyII, App, Cyt or ClyII is now called ApxII; *apxII* is the structural gene encoding this toxin. The structural gene encoding the ApxIII toxin, the 120 kDa toxin produced by the reference strains of serotypes 2, 3, 4, 6 and 8, is *apxIII*; ApxIII is the toxin known previously as CylIII, pleurotoxin or macrophage toxin (Mat). ApxI is the strongest haemolysin and cytotoxin of the three. ApxII exists in two forms, a toxin which remains intracellular and has both haemolytic and cytotoxic activities, and a secreted toxin which is cytotoxic, but essentially non-haemolytic. ApxIII is cytotoxic but not haemolytic and would appear to belong to the leukotoxin class of the RTX family (Chang *et al.*, 1993;

Jansen *et al.*, 1993). The genes encoding these toxins (*apxI, apxII* and *apxIII*) have been cloned and sequenced (Chang *et al.*, 1989; Frey *et al.*, 1991*b*;Chang *et al.*,1993).

Production of ApxI by serotypes 1 through 12 of *A. pleuropneumoniae* has been studied (Frey and Nicolet, 1990). Strains of serotypes 1, 5, 9, 10 and 11 exhibit strong haemolytic activity (haemolysin production is induced by calcium) whereas strains of serotypes 2, 3, 4, 6, 7, 8 and 12 exhibit only weak haemolytic activity (haemolysin production is not induced by calcium). These same sets of strains were found to be highly virulent and weakly virulent, respectively, when inoculated intranasally/intraperitoneally in mice suggesting that the relative virulence of the different serotypes may be related to their haemolytic activity (Komal and Mittal, 1990). The ApxI toxin from serotype 1 strains is a 105 kDa protein; it has been purified and has haemolytic as well as cytotoxic properties (Frey and Nicolet, 1988*a*,*b*; Rosendal *et al.*, 1988; Chang *et al.*, 1989; Devenish and Rosendal, 1989; Devenish *et al.*, 1989). The structural gene encoding the ApxI of the serotype 1 strain ATCC 27088 has also been isolated, and the nucleotide sequence of the gene has been determined (Frey *et al.*, 1991*b*). A recombinant plasmid, A44, constructed using DNA from a serotype 1 strain (CM5), conferred haemolytic activity on *Escherichia coli* (MacInnes *et al.*, 1990). The nucleotide sequence of the region of the plasmid that conferred haemolytic activity revealed the presence of a gene (*hlyX*) very similar to *fnr*, the gene that encodes the regulatory protein FNR of *E. coli* (Shaw and Guest, 1982). The HlyX protein has been purified and has been found to have a molecular mass of 29 kDa (Green *et al.*, 1992). Gene fusion studies *(fnr:hlyX)* have

shown that like FNR, HlyX can function as an activator or repressor of genes responsible for anaerobic respiratory activities. It has been shown that the ability of some pathogens, such as *Salmonella typhimurium,* to enter mammalian cells is regulated by the concentration of oxygen in the growth medium such that high oxygen represses and low oxygen induces invasiveness (Jones and Falkow, 1994). This tends to suggest that HlyX might play a role in the regulation of genes encoding virulence factors which in turn, may have roles to play in the persistence of *A. pleuropneumoniae* in chronic or acute infections.

ApxII is produced by all serotypes of *A. pleuropneumoniae* except serotype 10 (Frey *et al.*, 1992) and is antigenically distinct from ApxI as confirmed from Western blot experiments. ApxII, a polypeptide with a molecular mass of 103 kDa, was identified in culture supernatants and was found to be responsible for the cytotoxic properties of the organism. The haemolysin (ApxII) from the reference strain of serotype 2 of *A. pleuropneumoniae* was isolated and characterized (Frey *et al.*, 1991*a*). Unlike serotype 1, serotype 2 strains produce only one haemolysin. ApxII from serotype 2, like ApxI of serotype 1, has a molecular mass of 105 kDa, and these two toxins are immunologically cross-reactive. However, DNA-DNA hybridization experiments have shown that the structural genes for these two haemolysins are different and show only 70% homology, thus confirming that the two proteins are distinct. Both ApxI and ApxII toxins lyse erythrocytes and kill porcine lung macrophages (Kamp *et al.*, 1991).

ApxIII is the least well-characterized cytolysin of *A. pleuropneumoniae* and is

secreted by serotypes 2, 3, 4, 6 and 8 (Kamp *et al.*, 1991). ApxIII differs from ApxI and ApxII in several aspects (Jansen *et al.*, 1993). Immunologically, ApxIII seems to be unrelated to ApxI and ApxII. Monoclonal antibodies raised against ApxIII do not cross-react with ApxI or ApxII, and monoclonal antibodies raised against ApxI or ApxII do not cross-react with ApxIII (Kamp *et al.*, 1991). ApxIII kills porcine lung macrophages but does not lyse porcine erythrocytes (Van Leengoed *et al.*, 1992). The gene encoding ApxIII in a serotype 8 strain has been cloned and characterized (Jansen *et al.*, 1993).

The endotoxin of *A. pleuropneumoniae* has been implicated as a virulence factor (Udeze *et al.*, 1987). However, the acute stages of the disease are only partially reproduced using endotoxin alone (Liggett *et al.*, 1986). This suggests that the development of pulmonary lesions during the acute phase of porcine pleuropneumonia may be due to a synergistic action of endotoxin and haemolysin (Udeze *et al.*, 1987).

1.1.5 Control of swine pleuropneumonia

Swine infected with *A. pleuropneumoniae* may be asymptomatic or may suffer from chronic to acute disease. Introduction of asymptomatic carriers into a nonimmune herd is an important cause of pleuropneumonia outbreaks (Sebunya and Saunders, 1983). Pleuropneumonia is costly to the swine industry. Great losses result from decreased productivity due to high mortality (acute disease in a nonimmune herd), reduced rates of gain resulting in variable growth rate and increased marketings of undersized or cull pigs

(chronic disease) (Leman *et al.*, 1982).

Several steps have been taken to prevent the occurrence and spread of pleuropneumonia in pigs. Antibiotic therapy has been useful in treatment of chronic infections. However, plasmids conferring resistance to several antibiotics including streptomycin, tetracycline, ampicillin, chloramphenicol, kanamycin and sulfonamide have now been demonstrated in strains of *A. pleuropneumoniae* (Ishii *et al.*, 1991, 1993). Dihydrofolate reductase inhibitors have been tested *in vitro* against strains of serotypes 2 and 9 (Mengelers *et al.*, 1990). Aditoprim, trimethoprim, sulfadimethoxine and sulfamethoxazole, and combinations of these drugs, have been found to be effective against *A. pleuropneumoniae*. Doxycycline hydrochloride and thiamphenicol at doses of 100 to 200 ppm were found to have a therapeutic effect on coughing and rhinorrhea in infected pigs (Sakai *et al.*, 1992); weight gain and food consumption were also improved and no pulmonary lesions were observed at necropsy on day 17 of drug administration. The administration to piglets of dihydroheptraprenol in combination with a heat-inactivated *A. pleuropneumoniae* preparation, resulted in enhanced resistance to infection (as opposed to a heat-inactivated vaccine preparation alone) following intraperitoneal injection with a homologous strain (Kimura *et al.*, 1993) .

Prevention of pleuropneumonia through vaccination is problematic. Optimal protection of pigs against homologous and heterologous serotypes seems to occur following natural infection with *A. pleuropneumoniae* (Nielsen, 1979) (cited by Sebunya and Saunders, 1983). Current killed *A. pleuropneumoniae* vaccines are known to be

inadequate, particularly in the prevention of the development of lesions and chronic infections (Higgins *et al.*, 1985; Inzana, 1991). Factors responsible for the virulence of *A. pleuropneumoniae* include the LPS or endotoxin, capsular polysaccharide, and the haemolytic and cytotoxic RTX (Apx) toxins (Inzana, 1991). Passive immunization of swine using antibodies to the LPS or capsule has been found to be inadequate, although the capsule is an important virulence factor and prevents opsonization and clearance of bacteria from the lung (Inzana *et al.*, 1988). Heat-labile Apx toxins are responsible for the development of severe lesions and for the development of protective immunity in animals. Nonhaemolytic mutants are avirulent and cannot induce protective immunity (Inzana *et al.*, 1991). Commercial vaccines consisting of killed bacteria that lack the Apx toxins do not provide adequate protection (Nielsen, 1976; Nielsen, 1984 (cited by Sebunya and Saunders, 1983); Higgins *et al.*, 1985). In brief, a truly efficacious vaccine is not available and the search for such a vaccine continues. For example, Gerlach *et al.* (1993) have shown that pigs immunized with a recombinant preparation of the outer membrane lipoprotein A (OmlA) were protected from death in an aerosol challenge with an *A. pleuropneumoniae* serotype 1 isolate. Subcutaneous immunization using either washed, formalinized whole cells, capsular polysaccharide, LPS or purified haemolysin I (105 kDa protein; ApxI), partially protected pigs against intranasal challenge with a lethal dose of *A. pleuropneumoniae* cells of different serotypes. Full protection was obtained if the formalinized whole cells were supplemented with purified haemolysin (Bhatia *et al.*, 1991). A conjugate vaccine was prepared by coupling the capsular polysaccharide of *A.*

pleuropneumoniae serotype 1 ATCC 27088 to the haemolysin, and the LPS to the haemolysin (Byrd and Kadis, 1992). Recipient pigs exhibited a significant increase in IgG antibodies to the capsular polysaccharide, LPS and haemolysin, following a booster vaccination. Such an antibody response to each component of the conjugate could be instrumental in protecting swine against pleuropneumonia. Two antigens of *A. pleuropneumoniae*, in the form of a fusion protein containing the C-terminal 70% of the 105 kDa cytolysin (ApxI) and a full length 60 kDa protein that binds transferrin, were tested for their protective efficacy in an experimental pig model (Rossi-Campos *et al.*, 1992). Pigs developed a very strong humoral response and when challenged with a serotype 7 strain, were less affected by the disease and showed significantly lower mortality rates than unimmunized pigs. An outer membrane preparation (degraded with proteinase K) from an *A. pleuropneumoniae* serotype 5 strain has been found to be more efficacious than untreated outer membranes (Chiang *et al.*, 1991). After vaccination with the treated outer membrane preparations, only a few pigs developed lung lesions upon intranasal challenge with a homologous strain. The lesions were also much less severe than those produced in pigs that were vaccinated with untreated outer membranes. Noncapsulated mutants of *A. pleuropneumoniae* serotypes 1 and 5 which were identical to the parent strain in terms of their LPS, protein composition and haemolytic activity have been tested as live vaccines (Inzana *et al.*, 1993*a*). Pigs vaccinated subcutaneously showed a strong immune response to whole cells as well as to Apx toxins I and II. Immunization of pigs with one or both of the mutant serotypes protected them against

clinical disease following intratracheal challenge with the virulent homologous or heterologous serotype. Nonimmunized pigs or those immunized with a commercial bacterin preparation died within 24 h of challenge (Inzana *et al.*, 1993*a*) indicating that live noncapsulated mutants of *A. pleuropneumoniae* may provide a safe and inexpensive method of protection against swine pleuropneumonia. Temperature-sensitive mutants may also be valid candidates for live pleuropneumonia vaccines. Two temperature-sensitive mutants of *A. pleuropneumoniae* serotype 5 have been shown to be avirulent for swine following intratracheal challenge at doses greater than ten times the LD_{50} of the parent (Inzana *et al.*, 1993*b*). While the results of these most recent studies are truly encouraging, the search for a safe and efficacious vaccine against swine pleuropneumonia remains ongoing.

1.2 Iron and infection

1.2.1 The importance of iron

Several complex interactions occur between a host and a pathogen during an infection. Of these, one of the most important is the ability of the invading microorganism to multiply within the host tissues and thus, succeed in establishing the infection. There are several essential nutrients which the pathogen must acquire in order to successfully establish itself within the host. Iron is such a nutrient and is essential for the growth of

most bacteria (Bullen *et al.*, 1978; Gadd, 1992); it is required in a number of enzymatic reactions, either as a cofactor or as a prosthetic group (Martinez *et al.*, 1990). Iron is present in several bacterial proteins and enzymes including catalases, cytochromes, dehydrogenases, oxidases, and reductases. Iron can exist in two valence states in the cell, with each valence state having two arrangements of d-shell electrons. Hence, a weak and a strong ligand field state exist for both the ferrous (+2) and ferric (+3) oxidation states. Therefore, iron can form a variety of complexes with both organic molecules and inorganic ions and thus, will have a redox potential that is largely dependent on both its oxidation state and molecular environment (Neilands, 1972, 1974). As a result, iron is capable of forming a wide variety of complexes with redox potentials ranging from -500 mV in some iron-sulfur proteins to +350 mV in cytochrome oxidase ($a\ a_3$). The availability of iron can influence gene expression and the production of several bacterial proteins such as toxins (Betley *et al.*, 1986), haemolysins (Cavalieri *et al.*, 1984) and colicin V (Chehade and Braun, 1988).

A certain degree of iron deprivation is bacteriostatic, and extreme iron deficiency over an extended period is often lethal for bacteria (Bullen *et al.*, 1978; Weinberg, 1984). In fact, it is believed that except for a few species of lactobacilli, all living cells have an absolute requirement for iron for growth (Neilands, 1972). Experimental infections have shown that when iron is injected with a bacterial inoculum, the test organisms exhibit enhanced virulence. This has been shown to be the case with organisms from several genera including *Listeria*, *Pasteurella*, *Yersinia*, *Escherichia*, *Klebsiella*, *Neisseria*,

Shigella and *Salmonella* (Barry and Reeve, 1977; Kochan *et al.*, 1978; Miles *et al.*, 1979; Bullen, 1981). Therapeutic administration of iron can contribute to an increased susceptibility to infection (Bullen, 1981). For example, people treated with iron are more susceptible to malaria (Murray *et al.*, 1980) and children receiving iron-dextran injections to prevent iron deficiency have an increased susceptibility to *E. coli* bacteremia and meningitis (Barry and Reeve, 1977; Weinberg, 1984). Liberation of haem compounds from damaged cells can enhance infections. A combination of *E. coli* and lysed erythrocytes, when introduced into the mouse peritoneal cavity, produces a lethal effect (Bornside *et al.*, 1968) and the virulence of *Yersinia pestis* is enhanced by the absorption of haemin by the pathogen (Jackson and Burrows, 1956; Perry and Brubaker, 1979).

1.2.2 Sources of iron in the animal host

1.2.2.1 Introduction

There is an abundance of iron present in extracellular and intracellular "pools" in the mammalian host. However, none of this iron is freely available since at neutral pH, iron forms extremely insoluble $Fe(OH)_3$ with a very low solubility product of 4×10^{-36} (Bullen *et al.*, 1978). As a result, the amount of free ionic iron is of the order of only 10^{-18} M (Williams and Griffiths, 1992). This level of iron is too low to support the growth of most microorganisms (Weinberg, 1978, 1984; Bullen, 1981; Griffiths, 1987*a*, 1991;

Williams, 1988).

The extracellular tissue fluids of man and animals contain iron-binding proteins (Morgan, 1974; Critchton and Charloteaux-Wauters, 1987) such as transferrin in the blood and lymph, and lactoferrin in external secretions such as milk and bronchial mucus (Aisen and Leibman, 1972). These iron-binding proteins have very high association constants for Fe^{3+} (Chaberek *et al.*, 1959) and function as soluble iron-protein complexes (Bullen *et al.*, 1978).

The intracellular "pool" of iron consists of iron sequestered in proteins such as haemoglobin, myoglobin, ferritin, and homosiderin (Bell *et al.*, 1953). Since haemoglobin is located within erythrocytes, iron bound to haemoglobin is accessible to microorganisms only if the haemoglobin is released from the cell (Martinez *et al.*, 1990). However, free haemoglobin is bound by haptoglobin, and since some organisms cannot obtain iron from haemoglobin-haptoglobin complexes (Pidcock *et al.*, 1988), haptoglobin can act as a bacteriostatic agent (Eaton *et al.*, 1982).

1.2.2.2 Transferrin

The discovery of transferrin stemmed from the observation that the inhibition of bacterial growth by raw egg white could be reversed following the addition of iron (Schade and Caroline, 1944). The active ingredient was later discovered to be conalbumin (Alderton et al., 1946). A protein which also inhibited bacterial growth in an iron-

reversible manner was subsequently isolated from fractionated human plasma (Schade and Caroline, 1944). This protein was further characterized in 1947 by Laurell and Ingelman who named it transferrin and since then, transferrins have been found in several of the biological fluids of vertebrates including bile, blood, amniotic fluid, cerebrospinal fluid, lymph, colostrum and milk (Aisen and Listowsky, 1980).

Transferrins are glycoproteins and are made up of a single polypeptide chain and two N-linked complex-type glycan chains (MacGillivray *et al.*, 1983). They range in molecular mass from 76 kDa to 81 kDa. Each molecule consists of two similar (homologous) domains, the N-terminal and C-terminal domains, each containing a single iron-binding site (Williams and Griffiths, 1992). The two iron-binding sites are not identical (Harris and Aisen, 1989). They differ in their affinity for iron and their acid lability. The C-terminal site is more acid stable and releases its iron only at around pH 5.0, whereas iron is released from the N-terminal site at a pH of 6.0 (Princiotto and Zapolski, 1975; Lestas, 1976; Bali and Aisen, 1992). The two sites are functionally equivalent despite any physical or chemical differences (Bezkorovainy, 1987; Harris and Aisen, 1989). All transferrins sequester and solubilize iron, thus exerting their bacteriostatic effect by controlling the amount of available iron. Serum transferrin is the main iron-transporting protein of vertebrates. The protein is involved in the transport of iron from the sites of absorption and haem degradation to those of iron storage and utilization (Bailey *et al.*, 1988). Once transferrin binds to specific cell receptors, the receptor-transferrin complexes are concentrated in clathrin-coated pits on the plasma membrane.

The pits are endocytosed to form vesicles within the cytoplasm. Loss of the clathrin coat results in the formation of endosomes and the acidification of the endosome contents causes the release of the iron from the transferrin. The vescicle-bound apotransferrin-receptor complex is then returned to the cell surface and fuses with the plasma membrane. At the extracellular pH of 7.4, the apotransferrin dissociates from the receptor and is recycled to the blood stream (Williams and Griffiths, 1992).

Each molecule of transferrin binds 2 ions of Fe^{+3} and 2 ions of HCO_3^- (since iron binding requires the presence of an anion) and the resulting transferrin-iron complex possesses a stability constant of approximately 10^{29} (Bezkorovainy and Zschocke, 1974; Bates and Schlabach, 1975). Iron-loaded transferrin has a characteristic salmon-pink colour with an absorption maximum at 465 nm (Williams and Griffiths, 1992). On binding iron, a significant conformational change occurs in the transferrin molecule resulting in a more compact protein with a higher isoelectric point and being more resistant to proteolytic degradation (Bezkorovainy, 1987). Urea polyacrylamide gel electrophoresis has revealed that transferrin can exist in four different forms (Evans and Williams, 1980). These include, apotransferrin, diferric transferrin and monoferric transferrin in which the iron is bound to either the N- or the C-domain binding site. The liver parenchyma is the site of synthesis of transferrin. Normal serum contains about 2.5 mg ml^{-1} of transferrin with an iron-binding capacity of 3 μg Fe^{+3} ml^{-1}; transferrin is usually approximately 35% saturated with iron (Wintrobe, 1974). As a result of this large iron-binding capacity, transferrin can scavenge serum for extracellular iron keeping free iron

concentrations in serum at $<10^{-15}$ M, a level far too low to support bacterial growth (Bullen *et al.*, 1978; Weinberg, 1978).

Transferrins from different animal sources differ in the number, nature and positions of their N-linked oligosaccharide substituents (which are specific for each transferrin type) (Spik *et al.*, 1985; Legrand *et al.*, 1988; De Jong and Van Eijk, 1989). Enzymatic deglycosylation of transferrins does not affect the iron-binding properties of the proteins and the physiological significance of these glycan chains has yet to be established (De Jong and Van Eijk, 1989; Harris and Aisen, 1989). The complete amino acid sequence of several transferrins is known (Bezkorovainy, 1987; Harris and Aisen, 1989). Human transferrin (molecular mass=80 kDa) consists of a single polypeptide chain of 678 amino acids with the N-terminal domain comprising residues 1-336 and the C-terminal domain comprising residues 337-678. Approximately 42% of the amino acid sequences of the N-terminal and C-terminal domains are identical (MacGillivray *et al.*, 1983).

1.2.2.3 Lactoferrin

Lactoferrin, like transferrin, is an iron-binding glycoprotein (Groves, 1960; Montreuil *et al.*, 1960; Aisen and Listowsky, 1980; Brock, 1985). Lactoferrin is present in milk and in mucous secretions (Masson *et al.*, 1966) as well as in the secondary granules of neutrophilic leukocytes (Masson *et al.*, 1969; Baggiolini *et al.*, 1970).

Lactoferrin, by binding iron, prevents growth of iron-requiring bacteria in milk and could account for the bacteriostatic properties of milk. Lactoferrin also binds iron in the intestine (Baker and Rumball, 1977). It may also protect cells from free radical damage by binding potentially-catalytic iron (Baldwin *et al.*, 1984).

The molecular masses of the lactoferrins from different animals range from 75 kDa to as high as 85 kDa (Oram and Reiter, 1968). Lactoferrin is a single-chain glycoprotein, and has two iron-binding sites, each binding one atom of ferric iron (Johansson, 1969; Querinjean *et al.*, 1971; Baker and Rumball, 1977; Bezkorovainy, 1977). The nature of the iron-binding sites are of special interest in that, while lactoferrin binds cupric and other metal ions, its affinity for iron is notably high (the binding constant of lactoferrin for iron is about 10^{20}) (Baker and Rumball, 1977; Cox *et al.*, 1979). Spectroscopic analysis of the unfolding of transition metal-ion complexes of human lactoferrin and transferrin have revealed that the incorporation of Fe^{3+} into the specific metal binding sites offers the greatest resistance to thermal unfolding when compared to other transition metal ions studied such as Cu^{2+}, Mn^{3+} and Co^{3+} (Harrington, 1992). One ion of bicarbonate is incorporated for each Fe^{3+} taken up (Masson and Heremans, 1968) (cited by Querinjean *et al.*, 1971) and three protons are released (Masson, 1970) (cited by Querinjean *et al.*, 1971). Iron-loaded lactoferrin has a characteristic red colour.

Electron paramagnetic resonance spectra as well as optical spectra of lactoferrin and transferrin are almost identical (Windle *et al.*, 1963). The lactoferrin-iron complex is more stable than the transferrin-iron complex at low pH (Groves, 1960; Montreuil *et*

al., 1960). Lactoferrin differs from transferrin in carbohydrate and amino acid composition (Blanc *et al.*, 1963; Montreuil *et al.*, 1965) and antigenic properties (Johansson, 1960) (cited by Querinjean *et al.*, 1971). Data concerning lactoferrin are less abundant and less accurate than for transferrin.

1.2.2.4 Haem-containing/binding proteins

Haem is the prosthetic group of haemoproteins such as haemoglobins, myoglobins, catalases, some peroxidases and cytochromes *b*. It is responsible for the colour furnishing portion of haemoglobin (Merck Index).

Haemoglobin is the major component of red blood cells. It is involved in the transport of oxygen from the lungs to the body tissues and in the transport of carbon dioxide from the tissues to the lungs. Mammalian haemoglobins have molecular masses of about 64.5 kDa. They are composed of four peptide chains referred to as globins; each globin binds a haem prosthetic group. Iron is coordinated to four pyrrole nitrogens of haem, and to an imidazole nitrogen of a histidine residue.

In extracellular tissue fluids, free haem, like iron, is not readily available to an invading pathogen. Tetrameric haemoglobin, liberated by haemolysis or tissue damage, dissociates into dimers in plasma, and is then bound by haptoglobin, a plasma protein. The haemoglobin-haptoglobin complexes are then carried to the liver and the haemoglobin and haptoglobin are catabolized (Kino *et al.*, 1980). Haptoglobin depletion in the plasma

can result in the presence of haemoglobin in the circulation; this haemoglobin quickly becomes oxidised to methaemoglobin, from which the haem dissociates. Free haem gets bound by albumin and haemopexin, the major haem-binding proteins in plasma (Smith, 1990). Haemopexin binds haem more strongly than albumin, with a K_d of <1 pM, and at physiological ratios of albumin to haemopexin, haem becomes bound preferentially to haemopexin (Smith, 1990).

1.3 Iron acquisition by bacterial pathogens

All pathogens must be capable of obtaining iron from their respective hosts despite the iron-restricted environment. This can be accomplished by means of siderophore-dependent or siderophore-independent processes.

1.3.1 Siderophore-dependent iron acquisition processes

Siderophores (Greek, iron carriers) are low-molecular weight, ferric-specific iron-binding compounds excreted under conditions of low iron stress by aerobic and facultative anaerobic bacteria, for the purpose of obtaining iron from the environment (Neilands, 1989). Microorganisms which secrete siderophores produce IRMPs which serve as receptors for the siderophore-iron complexes; both components are produced during growth *in vivo* (e.g. Brown and Williams, 1985; Griffiths, 1987*b*). Several

organisms have been shown to efficiently use the ferrated form of the siderophore they produce. These include *E. coli*, *S. typhimurium* and *Agrobacterium tumefaciens*, as well as species of *Pseudomonas*, *Arthrobacter*, *Mycobacteria*, *Azotobacter*, and *Vibrio* (Neilands, 1989). Siderophores can acquire iron from host proteins such as transferrin and lactoferrin.

Some of the earliest known siderophores were ferrichrome and coprogen which were discovered in the fungi *Ustilago sphaerogena* and *Pilobolus* spp., respectively (Neilands, 1952; Hesseltine *et al.*, 1953). Both of these siderophores were shown to be cyclic trimers of the hydroxylamine derivative, hydroxamic acid. Siderophores which are derivatives of 2,3-dihydroxybenzoate (DHB) have been identified in various species of *Bacillus*, *Enterobacter*, *Escherichia*, *Salmonella*, *Azotobacter*, *Alcaligenes*, *Klebsiella*, *Micrococcus*, *Corynebacterium*, *Nocardia*, *Streptosporangium*, and *Thermoactinomyces* (Lankford, 1973).

The two main classes of siderophores are the phenolates and the hydroxamates. Enterobactin (=enterochelin) is a phenolate-type siderophore and is synthesized by various members of the *Enterobacteriaceae* (including the genera *Escherichia*, *Salmonella*, and *Klebsiella*) in response to iron starvation (Rogers *et al.*, 1977; Perry and San Clemente, 1979; Payne, 1988). Enterobactin is a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine and can remove iron from transferrin (Rosenberg and Young, 1974). A synthetic iron chelator, ethylenediamine di-o-hydroxyphenylacetic acid (EDDA), as well as transferrin, have been shown to stimulate the production of enterobactin by *E. coli* (Rogers, 1973).

Enterobactin production may contribute to bacterial virulence (Yancey *et al.*, 1979; Griffiths and Humphreys, 1980) and the production of this siderophore has been shown to occur *in vivo* during fatal infections with *E. coli* (Griffiths and Humphreys, 1980). Enterobactin-deficient mutants of *E. coli* are avirulent and similar mutants derived from *Salmonella* spp. have been used as live vaccines in mice (Hoiseith and Stocker, 1981) and calves (Robertsson *et al.*, 1983). In experimental infections, injection of enterobactin enhances bacterial virulence by about 600-fold (Kochan *et al.*, 1978). Siderophore production has been implicated in the virulence of organisms such as *Vibrio anguillarum* (Crosa, 1979; Crosa, 1980; Actis *et al.*, 1985), *Y. pestis* (Heeseman, 1987) and *Erwinia chrysanthemi* (Expert and Toussaint, 1985). Recently, siderophore production and the expression of iron-repressible cell-wall-associated proteins have been reported for pathogenic *Staphylococcus aureus* (Lindsay and Riley, 1994). *Aeromonas salmonicida* produces a 2,3-diphenol-catechol siderophore (Hirst *et al.*, 1991). In addition, many other *Aeromonas* spp. produce amonabactin, a phenolate siderophore containing DHB. An amonabactin biosynthetic gene (*amoA*) has been cloned in *Aeromonas hydrophila* (Barghouthi *et al.*, 1991). Phenolate siderophores have been shown to be produced by *Vibrio cholerae* (vibriobactin) (Payne and Finkelstein, 1978), *Mycobacterium* spp. (mycobactin) (Kochan *et al.*, 1971; Hall *et al.*, 1987) and *Pseudomonas aeruginosa* (pseudobactin) (Cox and Graham, 1979). Recently, two polyamine-containing phenolate siderophores (fluvibactins), were identified in *Vibrio fluvialis* (Yamamoto *et al.*, 1993). The freshwater cyanobacterium *Anabaena* sp. 7120 (Hutchins *et al.*, 1991), a cowpea

Rhizobium sp. (Jadhav *et al.*, 1992), and *Azospirillum lipoferum* M (Shah *et al.*, 1992), have been shown to produce phenolate siderophores. Mucoid strains of *P. aeruginosa* isolated from cystic fibrosis patients have been found to synthesize the siderophores known as pyochelin and pyoverdine (Haas *et al.*, 1991).

Aerobacter aerogenes (as well as *E. coli*) produces aerobactin, a hydroxamate-type siderophore (Gibson and Magrath, 1969), the synthesis of which is encoded by ColV plasmids (Williams, 1979; Stuart *et al.*, 1980; Braun, 1981). Aerobactin has been shown to enhance experimental infections with *E. coli* (Williams, 1979; Williams and Warner, 1980), *Shigella flexneri* (Griffiths *et al.*, 1985) and *Klebsiella pneumoniae* (Nassif and Sansonetti, 1986). *Neisseria meningitidis* (Yancey and Finkelstein, 1981; West and Sparling, 1987), *E. coli* strains (Valvano *et al.*, 1986) and *S. typhimurium* strains (Luckey *et al.*, 1972) which cannot produce the siderophore aerobactin can however, grow using the siderophore as a sole source of iron. *Rhizobium leguminosarum* has been shown to produce a hydroxamate siderophore (Carson *et al.*, 1992).

Siderophores are colourless when de-ferrated but become deeply coloured on binding ferric iron with absorption maxima in the range of 400-500 nm (Lankford, 1973). Siderophores may be assayed by colorimetric means due to the presence of their hydroxamic acid or catechol functional groups (Arnow, 1937; Csaky, 1948), or by performing bioassays on culture extracts (Burnham and Neilands, 1961; Peters and Warren, 1968; Luckey *et al.*, 1972).

The iron bound to the siderophore must be transported into the bacterial cell.

Amino acids, monosaccharides, and other small molecules are transported across the outer membrane of most Gram-negative bacteria via water-filled pores lined with proteins known as porins. However, these pores are too "small" to accomodate the siderophores which have molecular masses ranging from approximately 500 to 1000 Da. This problem is overcome by the synthesis of specific IRMPs which first recognize the Fe(III) form of the siderophore molecule and then, with the help of other transport components, guide the coordinated iron to the cytoplasmic membrane or to the cytoplasm.

In the case of microorganisms that produce siderophores, expression of siderophore production is regulated by the product of the *fur* (ferric uptake regulation) gene (Hantke, 1984). The Fur protein has a molecular mass of 17 kDa and is unusually rich in histidine (Wee *et al.*, 1988). Fur functions as an iron-binding repressor, inhibiting siderophore synthesis at high intracellular iron concentrations. Ferrisiderophores are recognized by specific outer membrane receptors (eg. ferrichrome is recognized by FhuA) (Bagg and Neilands, 1987). Once the ferrisiderophore is bound to the receptor, iron uptake may occur by one of three different mechanisms (Critchton and Charloteaux-Wauters, 1987). In the first, the iron is removed from the membrane-bound siderophore and taken into the cell, while the siderophore remains outside. In the second, the ferrisiderophore is taken up by the cell and the chelate is hydrolysed. In the third, the ferrisiderophore is transported into the cell, the iron is released in a reduction step, and the siderophore is secreted again (Critchton and Charloteaux-Wauters, 1987). Two sets of proteins are involved in the translocation of a siderophore that is internalised. The first

set of proteins (FhuC, B and D) is responsible for siderophore secretion and the second (FepB, C, D and G) is responsible for ferric-siderophore uptake (Briat, 1992).

1.3.2 Siderophore-independent iron acquisition processes

Several pathogens do not produce siderophores. Despite this, many of these organisms have been shown to acquire iron from transferrin and/or lactoferrin, and where investigated, these organisms have also been shown to produce IRMPs (Otto *et al.*, 1991; Keumhwa *et al.*, 1991). Such organisms include *N. meningitidis* (Archibald and DeVoe, 1980), *Neisseria gonorrhoeae* (Norrod and Williams, 1978; West and Sparling, 1985), *H. influenzae* (Pidcock *et al.*, 1988; Williams *et al.*, 1990; Lee, 1992*a*), *Haemophilus parainfluenzae* and *Haemophilus paraphrophilus* (Williams *et al.*, 1990), *Haemophilus ducreyi* (Lee, 1991), *Haemophilus somnus* (Ogunnariwo *et al.*, 1990), *Haemophilus parasuis* (Charland *et al.*, 1995), *A. pleuropneumoniae* (Deneer and Potter, 1989*b*; Morton and Williams, 1989; Niven *et al.*, 1989; Morton and Williams, 1990; Schryvers and Gonzalez, 1990; Gonzalez *et al.*, 1990; D'Silva *et al.*, 1995), *Pasteurella multocida* (Ogunnariwo *et al.*, 1991), *Haemophilus paragallinarum* (Ogunnariwo and Schryvers, 1992), and most recently, *Helicobacter pylori* (Husson *et al.*, 1993), and *Moraxella catarrhalis* (Campagnari *et al.*, 1994).

Notably, these organisms exhibit specificities for particular animal (or avian) transferrins (Archibald and DeVoe, 1979; Mickelsen and Sparling, 1981; Herrington and

Sparling, 1985; Redhead *et al.*, 1987; Niven *et al.*, 1989; Schryvers and Gonzalez, 1990; Morton and Williams, 1990; Williams *et al.*, 1990; Ogunnariwo *et al.*, 1991; Ogunnariwo and Schryvers, 1992; Yu *et al.*, 1992; Campagnari *et al.*, 1994; Charland *et al.*, 1995), or particular lactoferrins (Mickelsen *et al.*, 1982; Schryvers, 1988; Husson *et al.*, 1993; Campagnari *et al.*, 1994). This would suggest that the IRMPs may be able to serve as receptors for transferrin and lactoferrin.

Some of these pathogens that do not produce siderophores can also acquire iron from haemoglobin (Mickelsen and Sparling, 1981; Pidcock *et al.*, 1988; Williams *et al.*, 1990) and/or other iron-containing compounds such as haemin (Williams *et al.*, 1990; Lee, 1992*a*,*b*), haem-haemopexin complexes (Wong *et al.*, 1994) and haemoglobin-haptoglobin complexes (Dyer *et al.*, 1987; Pidcock *et al.*, 1988; Williams *et al.*, 1990) suggesting, again, that IRMPs may function as receptors in these organisms. In all of the above cases, siderophore synthesis was not evident suggesting that a mechanism involving a direct interaction between the organism and the host protein was essential for the acquisition of iron by the bacterial cell.

1.3.3 Receptor-mediated acquisition of iron from transferrin and lactoferrin

In addition to acquisition via siderophores, three other mechanisms have been proposed for the acquisition of transferrin-bound iron by bacteria (Griffiths, 1987*b*). These include: (i) proteolytic cleavage of the transferrin resulting in the breakdown of the iron-binding sites; (ii) reduction of bound Fe^{+3} to Fe^{+2} followed by the subsequent release of Fe^{+2} and (iii) a direct surface interaction between the transferrin and a microbial transferrin receptor. Evidence for (iii) was obtained some time ago and it would appear that once bound to the bacterial receptor, the transferrin is not internalised (Archibald and DeVoe, 1979; Archibald and DeVoe, 1980; Simonsen *et al.*, 1982; McKenna *et al.*, 1988). However, the mechanism(s) for the release of iron from the bacterial receptor-bound transferrin is not known.

Acquisition of iron from transferrin and/or lactoferrin by direct contact involves IRMPs which function as receptors. These receptors exhibit specific recognition properties (Lee and Schryvers, 1988; Schryvers, 1988; Schryvers and Morris, 1988*a*; Tsai *et al.*, 1988; Blanton *et al.*, 1990; Morton and Williams, 1990; Ogunnariwo and Schryvers, 1990). The specificities of the receptors for transferrin or lactoferrin from particular animal species may explain the host specificities of the organisms and the capacities of human transferrin and lactoferrin to enhance meningococcal infections in mice (Schryvers and Gonzalez, 1989).

Specific polypeptides of some pathogens have been identified as components of

transferrin or lactoferrin receptors and several of these polypeptides have been affinity-isolated specifically with host transferrin or lactoferrin (Schryvers and Morris, 1988*a,b*; Lee and Bryan, 1989; Schryvers, 1989; Schryvers and Lee, 1989; Ogunnariwo and Schryvers, 1990). Organisms producing such receptors include *A. pleuropneumoniae* (Gonzalez *et al.*, 1990; Ricard *et al.*, 1991), *H. parasuis* (Charland *et al.*, 1995), *H. somnus* (Ogunnariwo *et al.*, 1990), *N. meningitidis* (Schryvers and Morris, 1988*a,b*; Schryvers and Lee, 1989, Ala'Aldeen *et al.*, 1990; Griffiths *et al.*, 1990), *N. gonorrhoeae* (Lee and Bryan, 1989; Schryvers and Lee, 1989), *H. influenzae* (Schryvers, 1989; Morton and Williams, 1990; Holland *et al.*, 1992) and *P. haemolytica* (Ogunnariwo and Schryvers, 1990). The polypeptides isolated with transferrin have included one of high molecular mass (94-106 kDa) and one or more of lower molecular mass (58-86 kDa) and it has been suggested that these polypeptides exist *in situ* in the form of iron acquisition complexes (Lee and Bryan, 1989; Schryvers, 1989; Schryvers and Lee, 1989; Ogunnariwo and Schryvers, 1990). These proteins have been designated transferrin-binding proteins 1 (TBP1) and 2 (TBP2), respectively. TBP2 does not bind transferrin as strongly as TBP1 (Schryvers, 1989). It is still not known whether TBP1 and TBP2 form part of the same receptor.

Porcine TBPs of molecular masses 105 kDa and 56 kDa have been isolated from serotypes 1, 2 and 7 of *A. pleuropneumoniae* (all biotype 1 strains) and both of these polypeptides have been shown to bind transferrin (Gonzalez *et al.*, 1990). TBPs with molecular masses of 99 kDa and 64 kDa have also been affinity-isolated from the type

strain (ATCC 27088; serotype 1) of *A. pleuropneumoniae* (Ricard *et al.*, 1991). It has been shown that the two TBPs of *A. pleuropneumoniae* can bind transferrin independently (Gonzalez *et al.*, 1990; Ricard *et al.*, 1991). An *E. coli* transformant (prepared with DNA from a serotype 7 strain of *A. pleuropneumoniae*) expressing a 60 kDa protein indistinguishable by SDS-PAGE from an IRMP of *A. pleuropneumoniae* serotype 7, has also been identified (Gerlach *et al.*, 1992*a*,*b*). This protein was shown to bind the amino-terminal half of the porcine transferrin molecule, to have a higher affinity for iron-saturated porcine ferritransferrin than for porcine apo-transferrin, to be unable to bind transferrins from other animals, and to bind haem (Gerlach *et al.*, 1992*a*). Since haem could block the transferrin-binding ability of the polypeptide, it was suggested that *A. pleuropneumoniae* may possess a common mechanism for the acquisition of iron from both of these sources (since the organism can use either transferrin or haem as a sole source of iron) (Gerlach *et al.*, 1992*a*). The gene encoding the 60 kDa polypeptide (TBP2) from serotype 7 has been designated *tfbA* and molecular studies have shown that a highly homologous gene and gene product are present in serotypes 2, 3, 4, 8, 9, 10 and 11 (Gerlach *et al.*, 1992*b*). The gene encoding the *A. pleuropneumoniae* serotype 1 TBP2 (*tfbA*) has been cloned (Gerlach *et al.*, 1992*a*). The *tfbA* genes of serotypes 5A and 5B were recognized, under medium stringency conditions of hybridization, by the serotype 1-derived *tfbA* probe. Serotypes 1, 6 and 12 possess a gene with only 65% identity to that of serotype 7 and an immunologically distinct gene product with a molecular mass of approximately 65 kDa rather than 60 kDa (Gerlach *et al.*, 1992*a*). The first 100 base pairs

of the two genes are identical and the first 19 amino acids encode a sequence characteristic of a lipoprotein signal peptide (Gerlach *et al.*, 1992*a*); it would appear, therefore, that *tfbA* encodes an outer membrane lipoprotein. The N-terminus of this protein has a high degree of homology with the TBP2 of *N. meningitidis* and common antigenic domains have been identified in the TBPs2 of *H. influenzae, N. meningitidis* and *N. gonorrhoeae* (Stevenson *et al.*, 1992). The cloned 60 kDa TBP2-like protein of *A. pleuropneumoniae* is capable of binding transferrin without the co-operation of the 105 kDa TBP1 protein (Gerlach *et al.*, 1992*a*).

TBP1 and TBP2 have been identified in *N. gonorrhoeae* (Cornelissen *et al.*, 1992). The gene encoding gonococcal TBP1 (*tbpA*) predicts a protein sequence that is homologous to the TonB-dependent outer membrane receptors of *E. coli* and *P. putida*. Hence, the predicted protein sequence and the function of the TBP1 are consistent with this protein being involved in iron acquisition. The gonococcal TBP1 was cloned in *E. coli* giving rise to an organism that could bind human transferrin (Cornelissen *et al.*, 1993). Lack of TBP1 in *N. gonorrhoeae* resulted in partial reduction of transferrin-binding by whole cells (Cornelissen *et al.*, 1992). While this result indicates that the significant binding was probably due to TBP2, it does not agree with the findings of Tsai *et al.* (1988). Also, in another study involving the TBP1 and TBP2 of *N. gonorrhoeae*, it was shown that both TBP1 and TBP2 were involved in the binding of transferrin (Anderson *et al.*, 1994). However, TBP2 was not essential for the acquisition of transferrin-bound iron since mutants lacking TBP2 showed normal growth on transferrin-

containing medium (Anderson *et al.*, 1994).

The transferrin receptor of *H. influenzae* appears to consist of two IRMPs, TBP1 of approximately 100 kDa, and TBP2 which ranges from 58 to 90 kDa (Schryvers, 1989; Holland *et al.*, 1992; Stevenson *et al.*, 1992). In *N. meningitidis*, TBP1 has a molecular mass of around 98 kDa while TBP2 is between 78 and 85 kDa, although a few strains have a 68 kDa or a 65 kDa TBP2 (Schryvers and Lee, 1989; Ala'Aldeen *et al.*, 1990; Griffiths *et al.*, 1990; Ferreiros *et al.*, 1991). Pintor *et al.* (1993) have indicated that *N. meningitidis* has 700 to 4700 transferrin receptors per cell with affinity constants for transferrin ranging from 0.7×10^{-7} to 4×10^{-7} l mol^{-1}.

Bordetella pertussis does not require direct contact for iron acquisition from human transferrin. However, the acquisition of iron is more efficient when direct contact and siderophore production are both employed by the organism (Gorringe *et al.*, 1990; Redhead and Hill, 1991; Agiato and Dyer, 1992). Affinity chromatography with iron-saturated human transferrin allowed the isolation of an IRMP of 27 kDa from *B. pertussis* (Menozzi *et al.*, 1991).

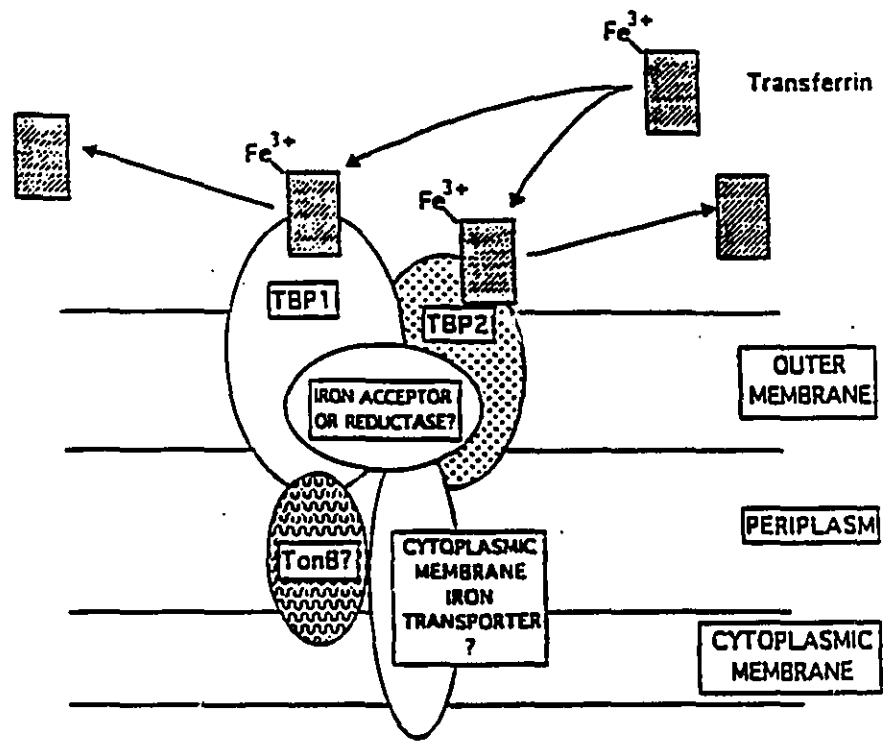
Pasteurella haemolytica can use ovine, caprine or bovine transferrin as a sole source of iron and IRMPs of 71, 77 and 100 kDa have been implicated as possible components of the transferrin receptor of the organism (Ogunnariwo and Schryvers, 1990). Using affinity methods, iron-binding proteins of 94 kDa (TBP1) and 53 kDa (TBP2) were isolated from *H. paragallinarum* specifically with chicken or turkey transferrin (Ogunnariwo and Schryvers, 1991). Biotinylated bovine transferrin allowed

the affinity isolation of an 82 kDa protein from the outer membrane of *Pasteurella multocida* grown under iron-restricted conditions (Ogunnariwo *et al.*, 1991). IRMPs with molecular masses of 105 kDa, 85 kDa and, depending on the strain of the organism, 73 kDa have been isolated from *H. somnus* using bovine transferrin as the binding ligand (Ogunnariwo *et al.*, 1990) and *Moraxella (Branhamella) catarrhalis* has been shown to bind human transferrin (Schryvers and Lee, 1989). The diagrammatic representation of a hypothetical Gram-negative bacterial transferrin receptor and other membrane components that may be involved in the transport of iron from transferrin into the bacterial cell are shown in Figure 1.1.

Unlike Gram-negative organisms, iron acquisition by Gram-positive organisms has received little attention. *Staphylococcus epidermidis* expresses an iron-regulated, human-transferrin-binding protein of 45 kDa (Modun and Williams, 1992) (cited by Williams and Griffiths, 1992). *S. aureus* expresses a specific receptor for human transferrin. A 42 kDa cell wall transferrin-binding protein has been identified in the organism (Modun *et al.*, 1994). *Listeria monocytogenes* can utilize ferritransferrin from a range of animal species as a sole source of iron for growth; SDS-PAGE and Western blotting have revealed the presence of a 126 kDa transferrin-binding protein (Hartford *et al.*, 1993).

Little is known about the molecular biology of the TBPs. The genes *tbp1* and *tbp2*, coding for TBP1 and TBP2, respectively, have been isolated from *N. meningitidis* (Legrain *et al.*, 1993). The *tbp2* and *tbp1* open reading frames are tandemly arranged in the genome with an 87-bp intergenic region. Nucleotide sequence analysis suggests the

FIG. 1.1. Diagrammatic representation of a hypothetical Gram-negative bacterial transferrin receptor.



Williams and Griffiths (1992)

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existence of a TBP1 precursor carrying an N-terminal signal peptide with a peptidase I cleavage site, and a TBP2 precursor with N-terminal homology to lipoproteins and a peptidase II cleavage site. Organisms with mutations in the *tbp1* and *tbp2* genes, and hence deficient in TBP1 and TBP2 respectively, were constructed from *N. meningitidis* strain B16B6 (Irwin *et al.*, 1993). Mutants deficient in either TBP1 or TBP2 showed reduced transferrin binding activity, and neither mutant was capable of using transferrin for growth. While TBP1 could be affinity-isolated from the mutant lacking TBP2, exogenous TBP1 was required for the isolation of TBP2 from the mutant lacking TBP1. The complete gene encoding the TBP1 from *N. meningitidis* has been generated using the polymerase chain reaction, the product has been cloned, and the protein expressed in *E. coli* (Wilton *et al.*, 1993). The protein was surface expressed by *E. coli*, reacted with antiserum to an N-terminal peptide of TBP1, and bound human transferrin. A mutant of *N. meningitidis*, which produced only low amounts of a 65 kDa protein, exhibited normal transferrin binding activity (Tsai *et al.*, 1988). However, both TBP1 and TBP2 are needed for iron acquisition from transferrin in *N. meningitidis* (Irwin *et al.*, 1993). This is in contrast to the findings of Anderson *et al.* (1994), whose experiments demonstrated that TBP2 was not essential in *N. gonorrhoeae* for the acquisition of transferrin-bound iron.

The mechanism by which iron levels control the expression of bacterial genes encoding transferrin-binding proteins, has not been elucidated. In the promoter region of the *N. gonorrhoeae* ferric-binding protein (*fbp*) gene (Morse *et al.*, 1991), a Fur-binding site similar to that in *E. coli* (Griffiths, 1991) has been identified. No such sequence was

observed in the promoter region of the *tbp1* gene of the gonococcus (Cornelissen *et al.*, 1992). It has been suggested that these iron-transport genes may be arranged in tandem as one transcriptional unit and can be coordinately regulated by iron from a single Fur site in the promoter of the first gene. The *tbp1* gene may be in tandem with the *fbp* gene, or perhaps the *tbp2* gene, and may be controlled from a common regulatory element as is the case in *E. coli.*

There is some evidence that the transferrin receptors of pathogenic bacteria are expressed in the host. For example, antibodies to the IRMPs of *A. pleuropneumoniae*, including the TBP1 and TBP2, have been identified in convalescent sera of pigs infected with the pathogen (Niven *et al.*, 1989; Gerlach *et al.*, 1992*a*).

Proteins of 71 kDa and 100 kDa have been isolated from the cell envelope of *P. haemolytica*. The organisms were recovered from the pleural cavity of an infected lamb (Donachie and Gilmour, 1988) and from chambers implanted within the peritoneal cavities of sheep (Sutherland *et al.*, 1990). These two proteins are presumably the same as those that were affinity-isolated from iron-restricted outer membranes of *P. haemolytica* by Ogunnariwo and Schryvers (1990) using bovine transferrin. Convalescent sera from *P. haemolytica*-infected calves contain antibodies to the 71 kDa, 77 kDa and 100 kDa proteins (Deneer and Potter, 1989*a*) that can be affinity-isolated using bovine transferrin.

Convalescent sera from infected rats and patients recovering from *H. influenzae* type b meningitis, showed the presence of antibodies that recognized the affinity-purified

TBP1 (76 kDa) and TBP2 (90 kDa) of strain Eagan (Holland *et al.*, 1992). Outer membranes prepared from *H. influenzae* type b recovered from the peritoneal cavities of infected rats contained TBP1 (76 kDa) and TBP2 (90 kDa) (Holland *et al.*, 1992). Convalescent sera from 75% of patients recovering from *H. influenzae* type b meningitis were shown to contain antibodies to the 90 kDa and 100 kDa transferrin-binding-proteins of the corresponding infecting strain (Holland *et al.*, 1992). In addition, fresh clinical isolates of *H. influenzae* type b recovered from blood and cerebrospinal fluid, showed constitutive expression of TBPs, which became iron-repressible only after prolonged *in vitro* subculture on iron-sufficient medium. In contrast, in the laboratory-adapted strain Eagan, the TBPs remained iron-repressible even after animal passage. Antibodies to the TBPs2 of both *N. meningitidis* and *H. influenzae* have been identified in convalescent sera from humans recovering from meningococcal meningitis (Griffiths *et al.* 1992*a*) (cited by Williams and Griffiths, 1992). Two IRMPs (80 kDa and 81-82 kDa) of *M. catarrhalis* have been shown to be expressed *in vivo* (Helminen *et al.*, 1993*a*; Helminen *et al.*, 1993*b*; Sethi *et al.* 1994) (cited by Campagnari *et al.*, 1994).

Iron-saturated transferrin or lactoferrin of human origin, when injected into mice, enhance *N. meningitidis* infection, confirming that these proteins are effective in supplying iron to the pathogen *in vivo* and thus providing circumstantial evidence that the organism uses the receptor-mediated mechanism of iron transport (Holbein, 1981; Schryvers and Gonzalez, 1989).

Some organisms can utilize lactoferrin as a sole source of iron. Of all *Neisseria*

strains examined, 100% of *N. meningitidis* strains, 53% of *N. gonorrhoeae* strains and around 24% of commensal neisseriae (Mickelsen *et al.*, 1982) can use human lactoferrin as a sole source of iron (Lee and Bryan, 1989; Schryvers and Lee, 1989). In the case of lactoferrin and *Neisseria* species, affinity procedures have allowed the isolation of one (Schryvers and Morris, 1988*b*; Schryvers and Lee, 1989; Lee and Bryan, 1989), or two polypeptides (Schryvers, 1989; Schryvers and Lee, 1989) of high molecular mass (101-106 kDa). Human-lactoferrin-binding proteins with molecular masses of 60 kDa, 40 kDa and 30 kDa have been identified in cell envelope preparations of *Aeromonas hydrophila* (Kishore *et al.*, 1991), and those of 39 kDa, 22 kDa and 16 kDa have been identified in *Shigella flexneri* (Tigyi *et al.*, 1992). IRMPs of 27 kDa and 32 kDa have been affinity-isolated from *B. pertussis* and *B. bronchiseptica* using human lactoferrin (Menozzi *et al.*, 1991). *Treponema pallidum* (Alderete *et al.*, 1988) and *Mycoplasma pneumoniae* (Tyron and Baseman, 1987) have been shown to bind human lactoferrin. *M. catarrhalis* (Schryvers and Lee, 1988*a*) has been shown to bind human lactoferrin.

Little is known about iron acquisition from lactoferrin by Gram-positive bacteria. Although *S. aureus* is known to secrete siderophores, human-lactoferrin-binding proteins of 62 kDa and 67 kDa have been isolated from the organism (Naidu *et al.*, 1992). In addition, bovine-lactoferrin-binding proteins of 92 kDa and 67 kDa have been isolated from strains of *S. aureus* causing bovine mastitis (Naidu *et al.*, 1991). Iron acquisition may possibly involve both mechanisms (i.e. siderophore production and direct contact).

1.3.4 Receptor-mediated acquisition of iron from other proteins

Several pathogens are capable of utilizing free haem, haemoglobin, haemin, haemoglobin-haptoglobin or haem-haemopexin as a sole source of iron for growth ("section 1.3.2").

Lee and Hill (1992*b*) have reported the presence of a haemoglobin-binding protein in the outer membrane of *N. meningitidis* grown under iron-restricted conditions. Two haemin-binding proteins (HmBPs) of molecular masses 97 kDa (HmBP1) and 50 kDa (HmBP2) have recently been isolated from *N. meningitidis* and characterized (Lee, 1994) (cited by Martel and Lee, 1994). It has been proposed that the initial step in the uptake of iron from haem/haemoglobin by the meningococcus involves a specific interaction between haem and/or haemoglobin and the HmBPs. Although the HmBP1 and the TBP1 from *N. meningitidis* are similar in size, the two proteins have been shown to be functionally heterologous (Martel and Lee, 1994). Haemin-binding proteins of 97 kDa and 44 kDa, both of which bound haemin specifically, were affinity-isolated from iron-restricted total membranes of four strains of *N. gonorrhoeae* suggesting interstrain structural and functional homogeneity amongst these polypeptides (Lee, 1992*b*). Mutants of *N. gonorrhoeae* that are defective in the uptake of iron from haemoglobin (or transferrin) are avirulent in mouse subcutaneous chambers (Genco *et al.*, 1991).

H. influenzae is unique among facultatively anaerobic bacteria in that it has an absolute requirement for haem for aerobic growth. Several cell envelope components

appear to be involved in the acquisition of haem by this organism and a 39.5 kDa haemin-binding protein has been isolated from *H. influenzae* type b grown under iron-restricted conditions (Lee, 1992*a*). The haemin-protein interaction was found to be specific and the polypeptide appeared to be structurally and functionally conserved among strains of *H. influenzae*. A 51 kDa periplasmic haem-binding protein (Hanson and Hansen, 1991; Hanson *et al.*, 1992*b*) and a surface-exposed 100 kDa protein required for the acquisition of haem from haem-haemopexin have also been identified (Hanson *et al.*, 1992*a*). These two proteins were shown to be immunologically distinct. Affinity-methods using haem-haemopexin Sepharose 4B followed by SDS-PAGE and Western blotting, have allowed the isolation and identification of 57 kDa and 29 kDa polypeptides from detergent-solubilised cell envelopes of *H. influenzae* (Wong *et al.*, 1994). The *tonB* gene of *H. influenzae*, which codes for a 28 kDa protein, has been found to be essential for haem utilization by the pathogen *in vitro*, as well as for the virulence of the organism in an animal model (Jarosik *et al.*, 1994).

Mutants of *Campylobacter jejuni*, lacking a 71 kDa IRMP, cannot use haemin, haemoglobin, haem-haemopexin, or haemoglobin-haptoglobin, as iron sources for growth in contrast to the wild type which possesses the IRMP (Pickett *et al.*, 1992). A 26 kDa haemin-repressible outer membrane protein has been identified in *Porphyromonas gingivalis* (Bramanti and Holt, 1992*a*). This protein binds haemin and may be involved in haemin transport. The haemin receptor (HemR) of *Yersinia enterocolitica* is a 78 kDa IRMP (Stojiljkovic and Hantke, 1992).

1.3.5 Receptors as vaccine components

One of the problems encountered with the development of an efficacious vaccine stems from the fact that most investigations of bacterial pathogens, including *A. pleuropneumoniae*, are carried out with organisms grown under *in vitro* conditions that do not necessarily reflect those *in vivo*. The low availability of iron in mammalian tissue fluids requires the pathogen to express IRMPs which are involved in the assimilation of protein-bound iron (Griffiths, 1987*a*,*b*). Bacterial IRMPs (TBP1 and TBP2) associated with the acquisition of transferrin-bound iron have been found to be antigenic and to be expressed *in vivo* by several pathogens including *A. pleuropneumoniae* (Donachie and Gilmour, 1988; Deneer and Potter, 1989*b*; Niven *et al.*, 1989; Ogunnariwo and Schryvers, 1990; Sutherland *et al.*, 1990; Stevenson *et al.*, 1992; Holland *et al.*, 1992; Gerlach *et al.*, 1992*a*). These transferrin-binding-proteins may be important virulence determinants and hence are potential targets for immunoprophylaxis (Bhanerjee-Bhatnagar and Frasch, 1990; Griffiths *et al.*, 1990; Stevenson *et al.*, 1992; Ala'Aldeen *et al.*, 1994). The prospect of using TBPs as vaccine components has received considerable attention in recent years. It has been shown that intraperitoneal injection of mice with the TBP1-TBP2 complex of *N. meningitidis* elicits a broadly cross-reactive antibody response to TBP1 and a less cross-reactive antibody response to TBP2 (Ala'Aldeen *et al.*, 1994).

Research has revealed that the deduced N-terminal amino acid sequence of TBP1 of *N. gonorrhoeae* is identical to those of the TBPs1 of two strains of *N. meningitidis*

(Griffiths *et al.* 1992*b*) (cited by Williams and Griffiths, 1992). Antibodies raised against a synthetic peptide based on the first 21 amino acids of the meningococcal TBP1 reacted with all strains of *N. meningitidis* tested, as well as with those of *N. gonorrhoeae* (Griffiths *et al.* 1992*b*) (cited by Williams and Griffiths, 1992). In another study, a comparison of the first 13 amino acids at the N-terminus of meningococcal TBP2 (Griffiths *et al.* 1992*b*) (cited by Williams and Griffiths, 1992) with an analogous TBP of *A. pleuropneumoniae* (Gerlach *et al.*, 1992*a*) revealed strong sequence similarity. These studies are of special interest in that the use of TBPs as vaccine components could conceivably result in protection against more than one infection. However, detailed knowledge of the antigenic structures of the TBP molecules is still required.

1.4 Summary and aims of the present research project

A. pleuropneumoniae is an important respiratory tract pathogen of swine (Sebunya and Saunders, 1983). Two biotype exist, and in general, biotype 1 strains are more virulent than biotype 2 (Pohl *et al.*, 1983). Like other pathogens, *A. pleuropneumoniae*, growing extracellularly *in vivo*, must be capable of obtaining iron from the host. Several biotype 1 strains of *A. pleuropneumoniae* are known to acquire iron from, specifically, porcine transferrin (Morton and Williams, 1989; Niven *et al.*, 1989; Morton and Williams, 1990; Schryvers and Gonzalez, 1990; Gonzalez *et al.*, 1990). This specificity reflects the host specificity of the organism but to what extent transferrin specificity accounts for host specificity remains unknown. None of these organisms produces a conventional siderophore, and where investigated (Gonzalez *et al.*, 1990; Ricard *et al.*, 1991), it would appear that iron acquisition involves two IRMPs, one of high molecular mass (99-105 kDa) and one of lower molecular mass (56-64 kDa), referred to as TBP1 (transferrin binding protein 1) and TBP2, respectively (Williams and Griffiths, 1992). There is evidence for *in vivo* expression of TBPs suggesting that they could be used as vaccines or vaccine components (Niven *et al.*, 1989; Ogunnariwo and Schryvers, 1990).

While there is a limited amount of information on the acquisition of transferrin-bound iron by a few biotype 1 strains of *A. pleuropneumoniae*, transferrin is presumably not the only iron source that can be used for growth. For example, it would appear that biotype 1 strains can also obtain iron from haem compounds, including haemoglobin

(Deneer and Potter, 1989*b*; Niven *et al.*, 1989). Nothing, however, is known regarding iron acquisition from porcine lactoferrin, and nothing is known about iron acquisition in general, by biotype 2. Despite this, and despite the use of haem compounds as iron sources, it is still tempting to speculate that the specificity of *A. pleuropneumoniae* for porcine transferrin (and, perhaps, lactoferrin) might account, at least in part, for the host specificity of the organism and moreover, that the ability of *A. pleuropneumoniae* to use porcine transferrin (and, perhaps, lactoferrin) as an iron source could be an important virulence determinant.

With these points in mind, the objectives of my research project were:

1. To examine iron acquisition, from various transferrins, lactoferrins and haemoglobins, by strains of *A. pleuropneumoniae* that differ in virulence and/or animal source. The strains selected were the type strain (biotype 1; ATCC 27088), the "reference" strain of biotype 2 (strain Bertschinger 2008/76; Pohl *et al.*, 1983), and two additional biotype 1 strains, strain BC181 and strain K17. Strain BC181 was selected because it is known to be associated with chronic disease and to be less virulent than the type strain (Rosendal *et al.*, 1985) and we wanted to determine if these two organisms were equally capable of obtaining iron from host proteins. Strain K17 was originally isolated from a case of arthritis in a lamb (Biberstein *et al.*, 1977) and we anticipated that this strain would allow us to probe the relationship between transferrin (and, perhaps, lactoferrin) specificity and host range.

2. To investigate the production of transferrin and/or lactoferrin receptors by these

organisms.

3. To isolate and identify the TBPs (and/or the porcine lactoferrin-binding proteins) produced by these organisms.

4. To obtain pure receptors (or receptor components) for N-terminal sequence analyses, and subsequently, the construction of gene probes, and to initiate molecular biological studies pertaining to the cloning and sequencing of receptor genes.

Chapter 2. Production of iron-repressible outer membrane proteins by biotype 1 and biotype 2 strains of *Actinobacillus pleuropneumoniae*

Rationale

Iron is essential for the growth of *A. pleuropneumoniae*. The purpose of this study was to compare the reference strain of biotype 2, the type strain (biotype 1) and two additional biotype 1 strains, strains BC181 and K17, of *A. pleuropneumoniae* with respect to the production of IRMPs when grown *in vitro* under iron-restricted conditions.

A prerequisite for this study was to confirm the specificity of ethylenediamine di-o-hydroxyphenylacetic acid (EDDA) as an iron chelating agent and to determine if the nature of the chelating agent (EDDA versus α,α'-dipyridyl) could also influence the OMP profile.

These latter experiments were performed by A. Aurora under my supervision. I extend my sincere thanks to Alex for his contributions.

2.1 Summary

Three biotype 1 strains of *Actinobacillus pleuropneumoniae*, namely, the type strain (ATCC 27088; serotype 1), strain BC181 (serotype 3) and strain K17 (reference strain of serotype 5A) and one biotype 2 strain (Bertschinger 2008/76, reference strain of biotype 2), were shown to produce iron-repressible outer membrane proteins (IRMPs) when grown *in vitro* under iron- restricted conditions. Iron restriction, using ethylenediamine di-o-hydroxyphenylacetic acid (EDDA) as the chelating agent caused these organisms to produce IRMPs of the following molecular sizes: 101 kDa and 99 kDa by strain ATCC 27088; 98 kDa and 93 kDa by strain Bertschinger 2008/76; 98 kDa and 95 kDa by strain BC181; 96 kDa and 92 kDa by strain K17. In all four strains, there were apparent increases in the amounts of polypeptides of 76 kDa and 67 kDa, in response to iron restriction, and except for strain K17, decreases in the amounts of polypeptides of 43 kDa, 38 kDa and 23 kDa. The outer membrane protein (OMP) profiles of *A. pleuropneumoniae* ATCC 27088 grown in the presence of EDDA or α,α'-dipyridyl were found to be essentially identical.

2.2 Introduction

Several biotype 1 strains of *A. pleuropneumoniae* have been shown to increase the production of specific OMPs in response to growth in the presence of an iron chelating agent (Deneer and Potter, 1989*b*; Morton and Williams, 1989; Niven *et al.*, 1989; Morton and Williams, 1990; Schryvers and Gonzalez, 1990; Gonzalez *et al.*, 1990). There has been some question regarding the designation of these OMPs as IRMPs since it is possible that these OMPs may be repressible by some metal other than iron, including, for example, manganese, copper or magnesium. Although EDDA is reported to be very specific for ferric iron (Miles and Khimji, 1975), the aims of this study were to determine if other metal ions could reverse the effects of EDDA and, provided this was not the case, to investigate the production of IRMPs by two biotype 1 strains and one biotype 2 strain of *A. pleuropneumoniae,* and to compare these IRMPs with those produced by the type strain, strain ATCC 27088.

Besides investigating the specificity of EDDA as a chelator of iron, an additional aim of this study was to compare the effects of EDDA and α,α'-dipyridyl on the production of OMPs by the type strain in order to determine whether the OMP profiles of organisms grown under iron-restricted conditions *in vitro* can also be influenced by the iron chelating agent used.

2.3 Materials and Methods

2.3.1 Bacterial strains

A. pleuropneumoniae strains ATCC 27088, BC181, K17 and Bertschinger 2008/76, were stored, and used to prepare frozen inocula, as described by O'Reilly *et al.*, 1984.

For long-term storage, organisms were grown either in liquid (to late logarithmic phase), or on solid (24-36 h) growth medium (tryptone-yeast extract; TYE) containing 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 100 mM NaCl, 10 mM KCl, 10 mM Na_2HPO_4, 10 mM glucose and KOH to pH 7.4. NAD was filter-sterilized and added to a final concentration of 5 μM. The harvested organisms were suspended in 12% (w/v) skim milk powder, 0.5% (w/v) sucrose and freeze-dried.

For the preparation of inocula from freeze-dried cultures, these cultures were reconstituted with 0.5 ml H_20; 50-μl aliquots were transferred to solid TYE, incubated for 24-36 h and colonies were transferred to sterile saline. The saline suspensions were used to inoculate liquid TYE; liquid cultures were grown to late logarithmic phase. 50 ml of 75% (w/v) glycerol was added to each 200-ml culture. The resulting inocula were dispensed into glass vials as volumes of 1-2 ml and then frozen at -80°C.

For experimental cultures, frozen inocula were thawed rapidly at room temperature and an inoculum of 1% (v/v) was used to inoculate liquid media.

2.3.2 Chemicals

Tryptone, yeast extract and agar were from Difco. $FeCl_3$ (in dilute HCl; atomic absorption standard) was from Fisher Scientific Co. NAD (grade II), HEPES and bovine serum albumin (fraction V powder) were from Boehringer-Mannheim. α,α'-dipyridyl, sodium N-lauroyl sarcosine (sarkosyl) and EDDA were from Sigma. EDDA was further purified by the method of Rogers (1973). Tris was from ICN and all other chemicals and stains for SDS-PAGE were from Bio-Rad. Low molecular weight protein standards (Electrophoresis Calibration Kit) were purchased from Pharmacia. Glass-distilled water was used throughout.

2.3.3 Growth conditions and preparation of membrane fractions

The procedures were essentially as described by Niven *et al.* (1989). To provide iron-replete growth conditions for *A. pleuropneumoniae*, the growth medium was HEPES-buffered tryptone-yeast extract (TYE-H) which is similar to TYE except that the concentration of NaCl is only 63 mM, the medium contains 50 mM HEPES, and NaOH is added to bring the pH to 7.8. For growth under iron-restricted conditions, TYE-H was supplemented with 50-70 μM desferrated EDDA (depending on the iron content of the medium) and for EDDA control cultures, TYE-H was supplemented with EDDA and an equimolar amount of $FeCl_3$. These media (200-ml volumes) were contained in 1-l, acid-

washed DeLong flasks fitted with Morton closures (Bellco Glass, Inc., Vineland, NJ). Filter-sterilized NAD and $NaHCO_3$ were added to the media at final concentrations of 5 μM and 10 mM, respectively. Media were inoculated with thawed inoculum and cultures were incubated at 37°C, with agitation on a gyratory shaker (200 rpm), in an atmosphere not enriched with CO_2. Growth was monitored turbidimetrically at 660 nm (model 240 spectrophotometer, Gilford Instrument Laboratories, Inc., Oberlin, OH) and parallel cultures were harvested one hour after stationary phase was reached. The organisms from each 200-ml (iron-replete or EDDA control culture) or 400-ml (iron-restricted culture) volume of culture were pelleted by centrifugation (20,000 x g, 20 min, 4°C), washed with culture volumes of 145 mM NaCl (centrifugation as above) and resuspended with 10 ml of 10 mM HEPES, pH 7.4 with KOH.

Each suspension (10 ml) was placed in an ice bath and the bacterial cells disrupted by sonication (20 x 10s bursts of 80 W, separated by 50s cooling periods, using a Sonifier Cell Disruptor, model W185D; Heat-Systems-Ultrasonics Inc., Plainview, NY). Unbroken cells and large debris were removed by centrifugation (16,000 x g, 10 min, 4°C). The top 90% of the supernatant fraction was carefully recovered and subjected to high-speed centrifugation (180,000 x g, 1 h, 4°C). The pellet obtained was resuspended with 10 ml of 10 mM HEPES, pH 7.4, in a glass homogenizer, to give the total membrane fraction.

To prepare outer membranes, total membranes (10 ml) were mixed with 10 ml of 2% (w/v) sarkosyl in 10 mM HEPES, pH 7.4, and incubated at room temperature for 30

min. After centrifugation (180,000 x g, 1 h, 4°C), the sarkosyl-insoluble material (pellet; outer membrane-enriched fraction) was washed by resuspension in 10 ml H_2O, followed by centrifugation (180,000 x g, 1 h, 4°C). The resulting outer membranes were resuspended with a small volume of H_2O (~0.25-0.5 ml), dispensed as 25-µl aliquots in Eppendorf tubes and frozen (-20°C). Protein was estimated by the method of Peterson (1977) using bovine serum albumin as a standard.

For experiments pertaining to the specificity of EDDA as an iron chelator, TYE-H was supplemented with EDDA and equimolar amounts of either $FeCl_3$, $CaCl_2$, $CuCl_2$, $MnCl_2$, $MgCl_2$, $NiCl_2$ or $ZnSO_4$. To compare the effects of EDDA and α,α'-dipyridyl on the production of OMPs, TYE-H was supplemented with EDDA or filter-sterilised α,α'-dipyridyl (in 95% ethanol) to final concentrations of 50-70 µM or 40-60 µM, respectively (depending on the iron content of the medium); control cultures also received $FeCl_3$, to 50-70 µM or 40-60 µM, respectively. All other procedures were as described above.

2.3.4 SDS-PAGE

SDS-PAGE was performed as described by Niven *et al.* (1989). Briefly, samples to be analyzed were diluted in water to a protein concentration of 4 µg μl^{-1} and mixed with an equal volume of sample buffer containing 10% (w/v) SDS (5 ml), 0.5 M Tris-HCl, pH 6.8 (2.5 ml), H_2O (5 ml), glycerol (2.5 ml), 2-mercaptoethanol (0.25 ml) and 5% (w/v) bromophenol blue (0.2 ml). Samples were immersed in boiling water for 5 min and

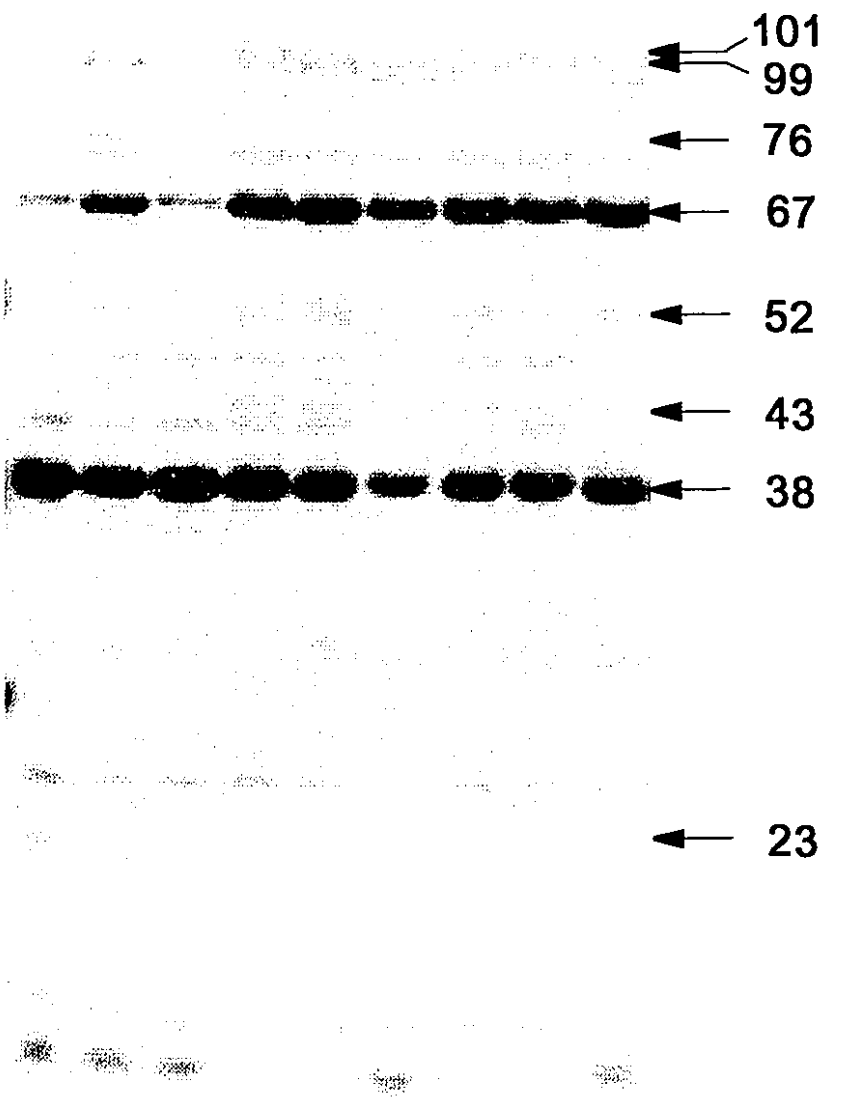
were loaded (40 µg protein per lane) on gels (1.5 mm thick) that were prepared essentially as described by Lugtenberg *et al.* (1975) with 5.2% stacking gels (4 cm long) overlaying 12% separating gels (16 cm long). A Protean II electrophoresis unit (Bio-Rad) was used and the electrode buffer was 25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS. Gels were run at a constant current of 15 mA per gel until the tracking dye just eluted from the gel (~14-17 h). Separated polypeptides were visualized by staining with Coomassie Brilliant Blue R250 and their molecular masses were calculated by comparing their relative electrophoretic mobilities with those of protein standards of known molecular mass.

2.4 Results and Discussion

EDDA, reportedly, has a high specificity for iron; the association constant of EDDA for ferric iron is $10^{33.9}$ and the association constant for the next most avidly held trace metal, copper, is $10^{23.9}$ (Miles and Khimji, 1975).

Figure 2.1 shows the OMP profiles of *A. pleuropneumoniae* ATCC 27088 grown in an EDDA-containing medium and supplemented with either Fe, Mg, Mn, Cu, Ca, Zn or Ni (some of the important metal ions required by most bacteria). Except for Fe, none of the metals chosen for this study could reverse, even to a small extent, the effect of EDDA and the OMP profiles (lanes D-I) are very similar to those obtained with outer membranes from organisms grown in TYE-H plus EDDA alone (lane B). Except when Fe was the metal ion supplement, the growth yield was significantly lower for each of

FIG. 2.1. SDS-PAGs of OMPs derived from *A. pleuropneumoniae* ATCC 27088 grown under iron-replete (lane A; TYE-H alone) and iron-restricted (lane B; TYE-H plus EDDA) conditions and in TYE-H plus EDDA and either $FeCl_3$ (lane C), $MnCl_2$ (lane D), $MgCl_2$ (lane E), $CaCl_2$ (lane F), $CuCl_2$ (lane G), $NiCl_2$ (lane H) or $ZnSO_4$ (lane I). The proteins were stained with Coomassie Blue. The numbers refer to the relative molecular masses (kDa) of some of the separated polypeptides.

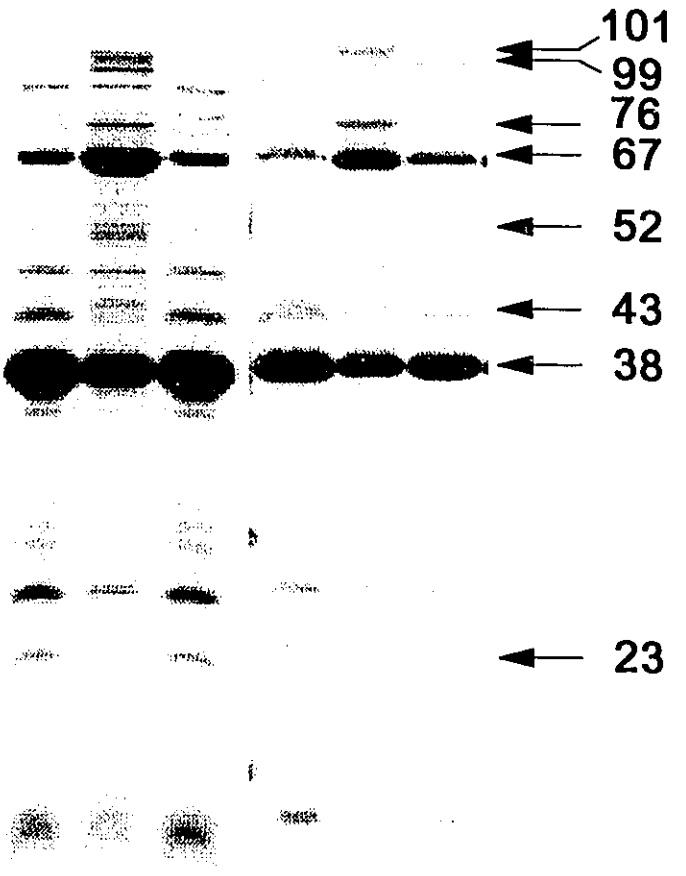

A B C D E F G H I
101
99
76
67
52
43
38
23

the test cultures (TYE-H supplemented with EDDA and either Fe, Mg, Mn, Cu, Ca, Zn or Ni) as compared to the growth yield under iron-replete conditions. When iron was the added metal ion, the growth yield was essentially the same as when the culture was grown in TYE-H alone. These results indicate that EDDA is specific for iron.

EDDA and α,α'-dipyridyl are used commonly as iron chelators. To compare the effects of EDDA and α,α'-dipyridyl on the production of OMPs by *A. pleuropneumoniae*, the type strain (ATCC 27088) was grown under iron-replete and iron-restricted conditions (using either EDDA or α,α'-dipyridyl) and outer membranes were then isolated and analyzed by SDS-PAGE. Fig. 2.2 shows that these chelators have the same or very similar effects on the production of OMPs by the organisms. Except for minor differences in the molecular sizes of the polypeptides, both chelating agents caused the appearance of new polypeptides with molecular masses of 101 kDa and 99 kDa, as well as increased synthesis of the 76 kDa, 67 kDa and 52 kDa polypeptides and decreased synthesis of the 43 kDa, 38 kDa and 23 kDa polypeptides. Hence, EDDA or α,α'-dipyridyl can be used as iron chelators to study the effects of iron-restriction on the OMP profile of *A. pleuropneumoniae.*

Using EDDA as an iron chelator, strains ATCC 27088, Bertschinger 2008/76, BC181 and K17 of *A. pleuropneumoniae* were grown under iron-restricted conditions. For these experiments, EDDA concentrations were chosen so as to obtain a growth yield of 60%-80% of iron-replete growth. EDDA affected neither the growth rate nor the final yield of any of the four organisms grown under iron-replete conditions (EDDA controls)

FIG. 2.2. OMP profiles of *A. pleuropneumoniae* ATCC 27088 grown under iron-replete conditions in the absence (lanes A and D) and presence (lanes C and F) of either EDDA plus $FeCl_3$ (lane C) or α,α'-dipyridyl plus $FeCl_3$ (lane F), and under iron-restricted conditions using either EDDA (lane B) or α,α'-dipyridyl (lane E). The numbers refer to the relative molecular masses (kDa) of some of the separated polypeptides.


A B C D E F
101
99
76
67
52
43
38
23

(Figs. 2.3, 2.4, 2.5 and 2.6).

Figures 2.7, 2.8, 2.9 and 2.10 demonstrate that with respect to TYE-H plus EDDA (lanes B and E) there is no change in the total membrane protein (TMP) or OMP profiles of any of the organisms when grown in TYE-H plus EDDA and $FeCl_3$ (EDDA control; lanes C and F). Figures 2.7, 2.8, 2.9 and 2.10 demonstrate that all four strains of *A. pleuropneumoniae* under study alter their TMP (lanes A-C) and OMP (lanes D-F; Fig 2.11) compositions in response to iron restriction. The TMP profiles (lanes A-C) of all four strains grown under iron-restricted conditions show increases in the amounts of the high molecular mass polypeptides in the 92-101 kDa range, as well as in the amounts of the 76 kDa and 67 kDa polypeptides. No other obvious changes in the profiles could be detected in response to iron-restriction. When the OMP profiles of the organism grown under iron-replete and iron-restricted conditions are compared (lanes D-F), it becomes obvious that the changes in the TMP profiles are due to changes in the outer membrane.

Growth in TYE-H plus EDDA and $FeCl_3$ had no effect on the OMP profile of any of the four strains, as compared to the OMP profiles of the organisms grown in iron-replete medium (TYE-H alone) (Figures 2.7, 2.8, 2.9 and 2.10; lanes D and F). In contrast, growth of all four strains under iron-restricted conditions resulted in changes in the OMP profiles (lanes D and E). The profiles obtained with strain ATCC 27088 revealed noticeable increases in the amounts of the polypeptides of 101 kDa and 99 kDa, as well as those of 76 kDa, 67 kDa and 52 kDa, and decreases in the amounts of others (43 kDa, 38 kDa and 23 kDa) (Fig. 2.7, lanes D and E). Iron restriction was

FIG. 2.3. Growth of *A. pleuropneumoniae* ATCC 27088 in TYE-H plus NAD and $NaHCO_3$ (●), in TYE-H plus NAD and $NaHCO_3$ supplemented with 55 μM EDDA (■), and in TYE-H plus NAD and $NaHCO_3$ supplemented with 55 μM EDDA and 55 μM $FeCl_3$ (▲). Culture turbidity values are the means of triplicates. The standard error bars were plotted for all data points and may be encompassed by the symbols.

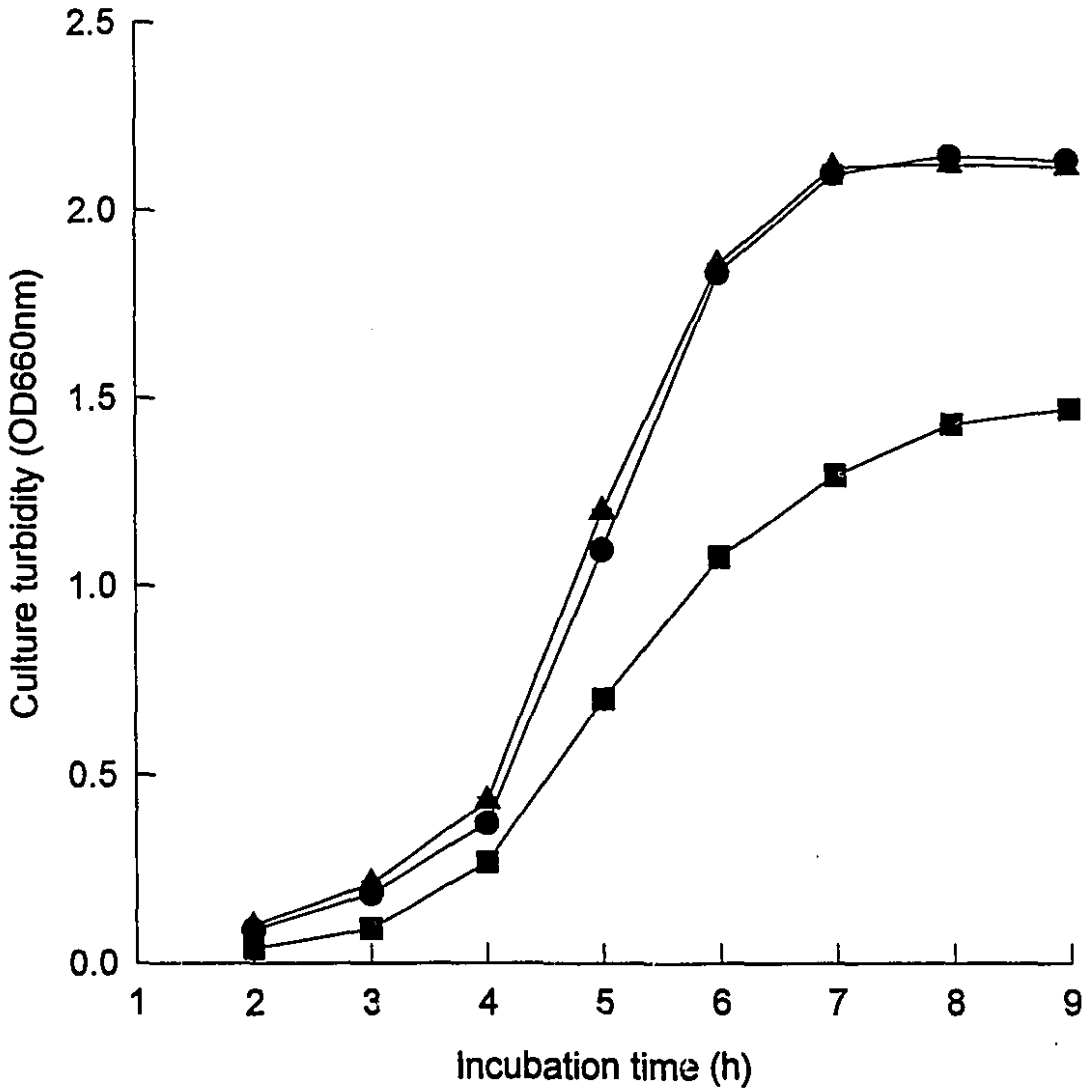

2.5
2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
1
2
3
4
5
6
7
8
9
Incubation time (h)

FIG. 2.4. Growth of *A. pleuropneumoniae* Bertschinger 2008/76 in TYE-H plus NAD and $NaHCO_3$ (●), in TYE-H plus NAD and $NaHCO_3$ supplemented with 60 μM EDDA (■), and in TYE-H plus NAD and $NaHCO_3$ supplemented with 60 μM EDDA and 60 μM $FeCl_3$ (▲). Culture turbidity values are the means of triplicates. The standard error bars were plotted for all data points and may be encompassed by the symbols.

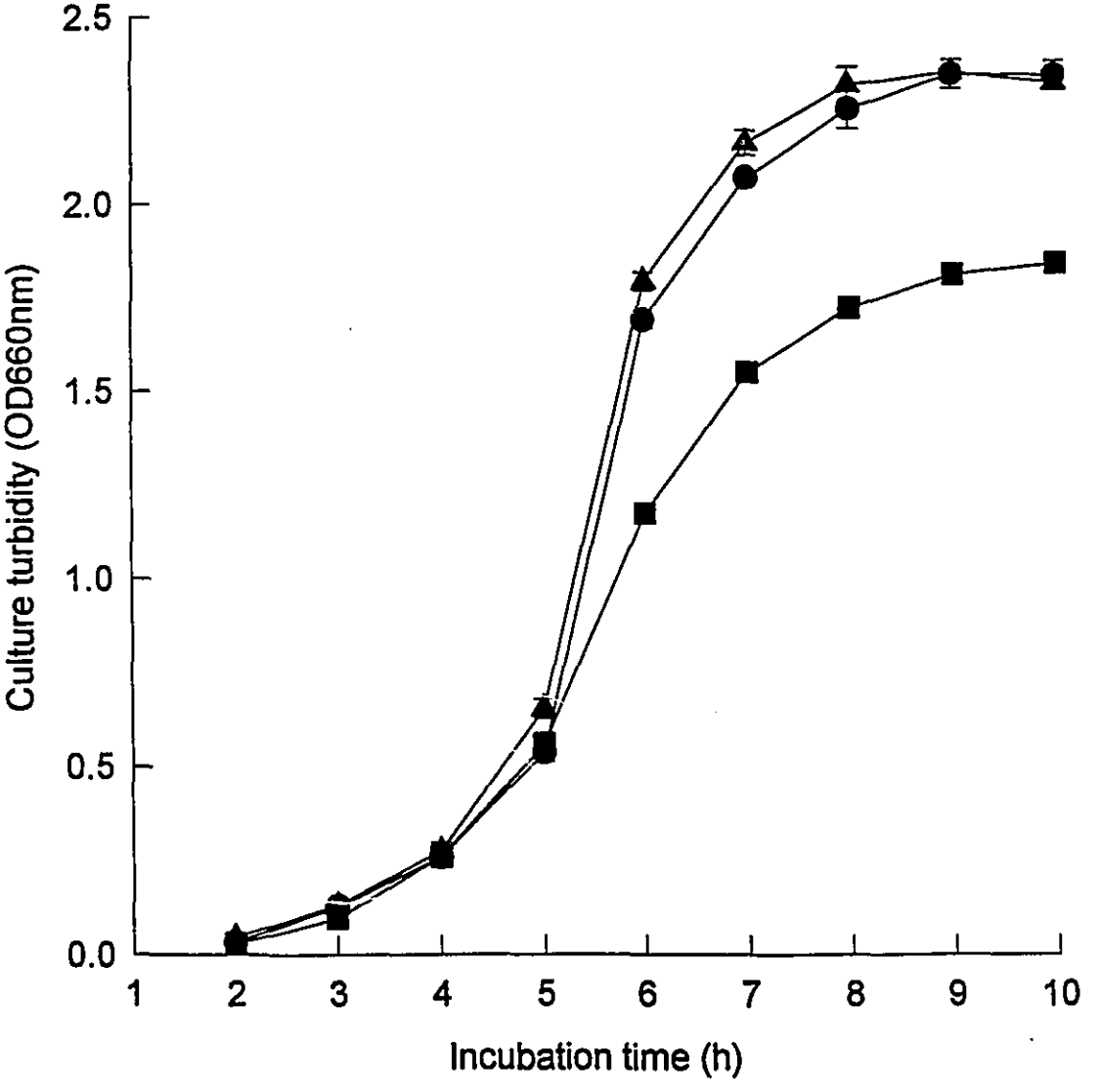

2.5
2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
1
2
3
4
5
6
7
8
9
10
Incubation time (h)

FIG. 2.5. Growth of *A. pleuropneumoniae* BC181 in TYE-H plus NAD and $NaHCO_3$ (●), in TYE-H plus NAD and $NaHCO_3$ supplemented with 60 μM EDDA (■), and in TYE-H plus NAD and $NaHCO_3$ supplemented with 60 μM EDDA and 60 μM $FeCl_3$ (▲). Culture turbidity values are the means of triplicates. The standard error bars were plotted for all data points and may be encompassed by the symbols.

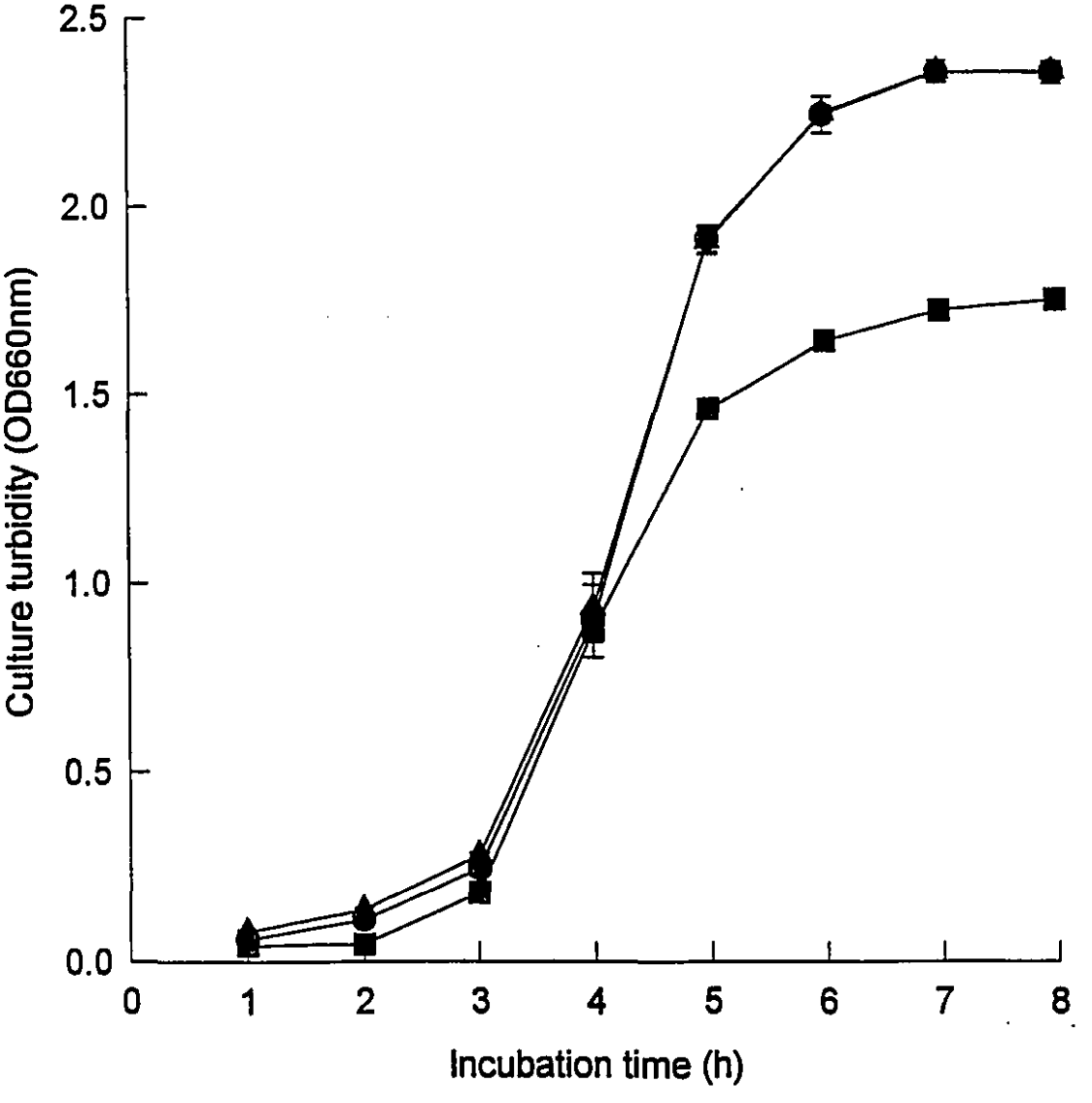

2.5
2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
0
1
2
3
4
5
6
7
8
Incubation time (h)

FIG. 2.6. Growth of *A. pleuropneumoniae* K17 in TYE-H plus NAD and $NaHCO_3$ (●), in TYE-H plus NAD and $NaHCO_3$ supplemented with 60 μM EDDA (■), and in TYE-H plus NAD and $NaHCO_3$ supplemented with 60 μM EDDA and 60 μM $FeCl_3$ (▲). Culture turbidity values are the means of triplicates. The standard error bars were plotted for all data points and may be encompassed by the symbols.

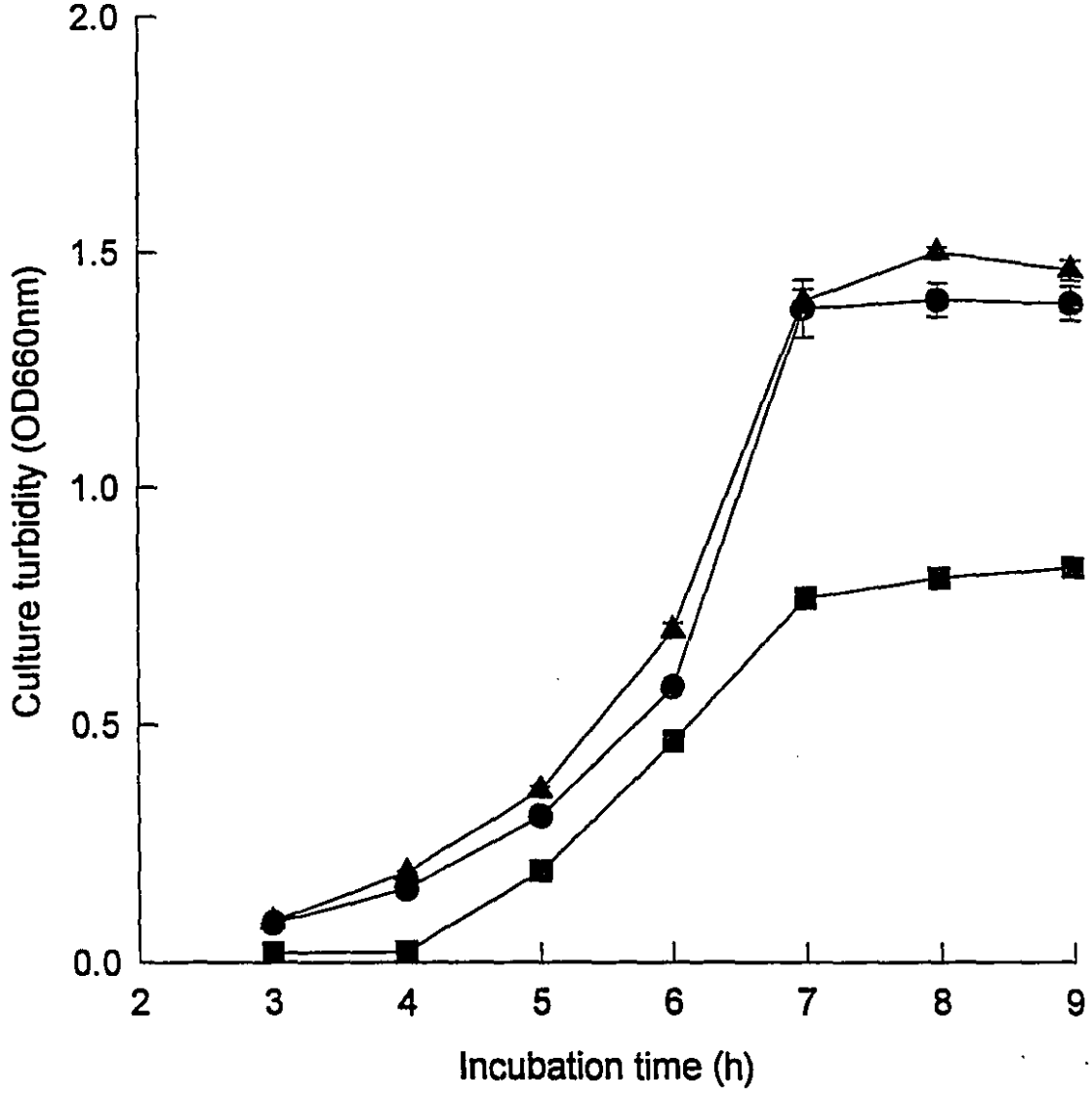

2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
2
3
4
5
6
7
8
9
Incubation time (h)

FIG. 2.7. Protein profiles of total membrane (lanes A-C) and outer membrane (lanes D-F) fractions from *A. pleuropneumoniae* ATCC 27088 grown under iron-replete conditions in the absence (lanes A and D) and presence (lanes C and F) of EDDA plus $FeCl_3$, and under iron-restricted conditions using EDDA (lanes B and E). The proteins were separated by SDS-PAGE and stained with Coomassie Blue. The numbers refer to the relative molecular masses (kDa) of some of the separated polypeptides.

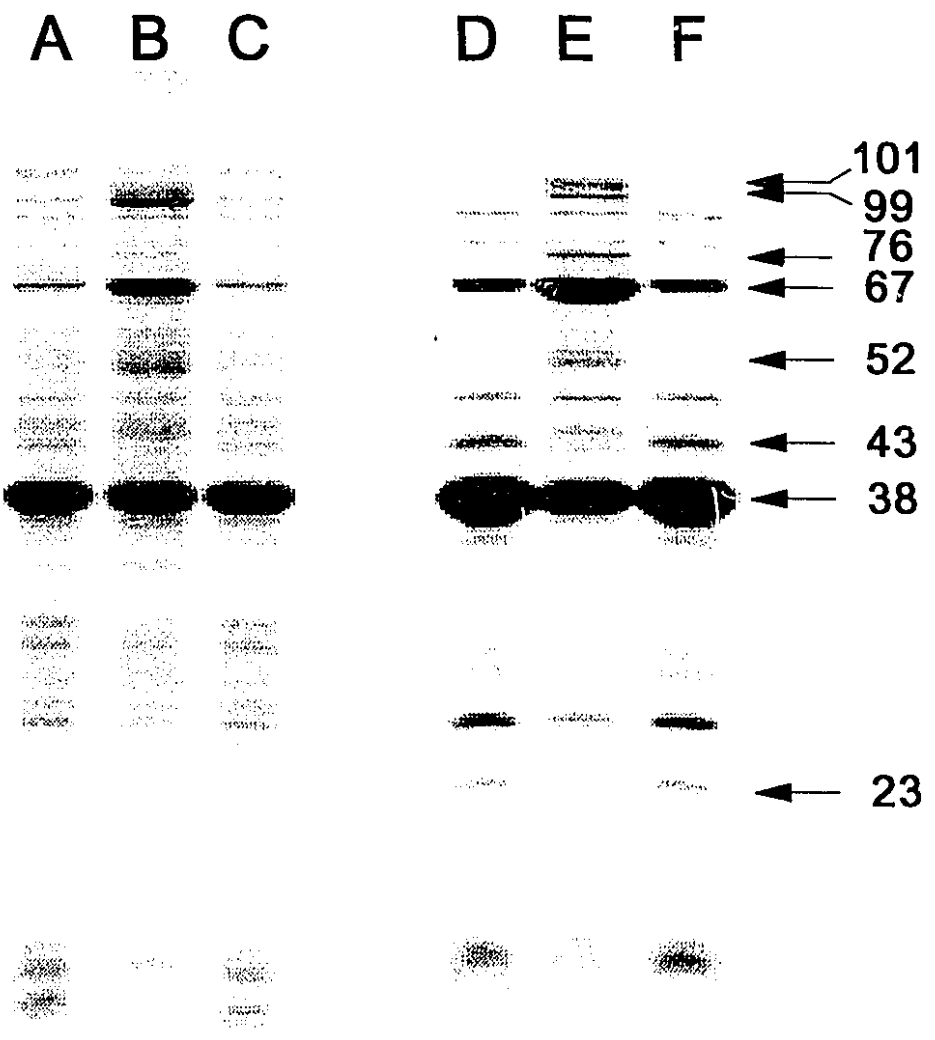

A B C
D E F
101
99
76
67
52
43
38
23

FIG. 2.8. Protein profiles of total membrane (lanes A-C) and outer membrane (lanes D-F) fractions from *A. pleuropneumoniae* Bertschinger 2008/76 grown under iron-replete conditions in the absence (lanes A and D) and presence (lanes C and F) of EDDA plus $FeCl_3$, and under iron-restricted conditions using EDDA (lanes B and E). The proteins were separated by SDS-PAGE and stained with Coomassie Blue. The numbers refer to the relative molecular masses (kDa) of some of the separated polypeptides.

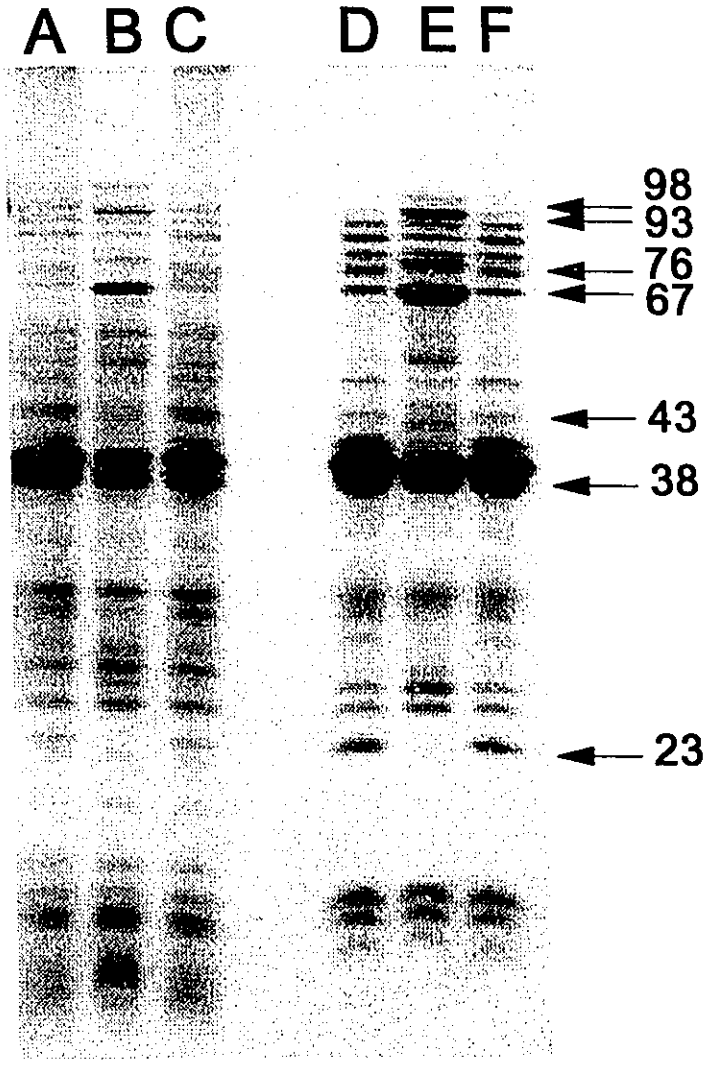

A B C
D E F
98
93
76
67
43
38
23

FIG. 2.9. Protein profiles of total membrane (lanes A-C) and outer membrane (lanes D-F) fractions from *A. pleuropneumoniae* BC181 grown under iron-replete conditions in the absence (lanes A and D) and presence (lanes C and F) of EDDA plus $FeCl_3$, and under iron-restricted conditions using EDDA (lanes B and E). The proteins were separated by SDS-PAGE and stained with Coomassie Blue. The numbers refer to the relative molecular masses (kDa) of some of the separated polypeptides.

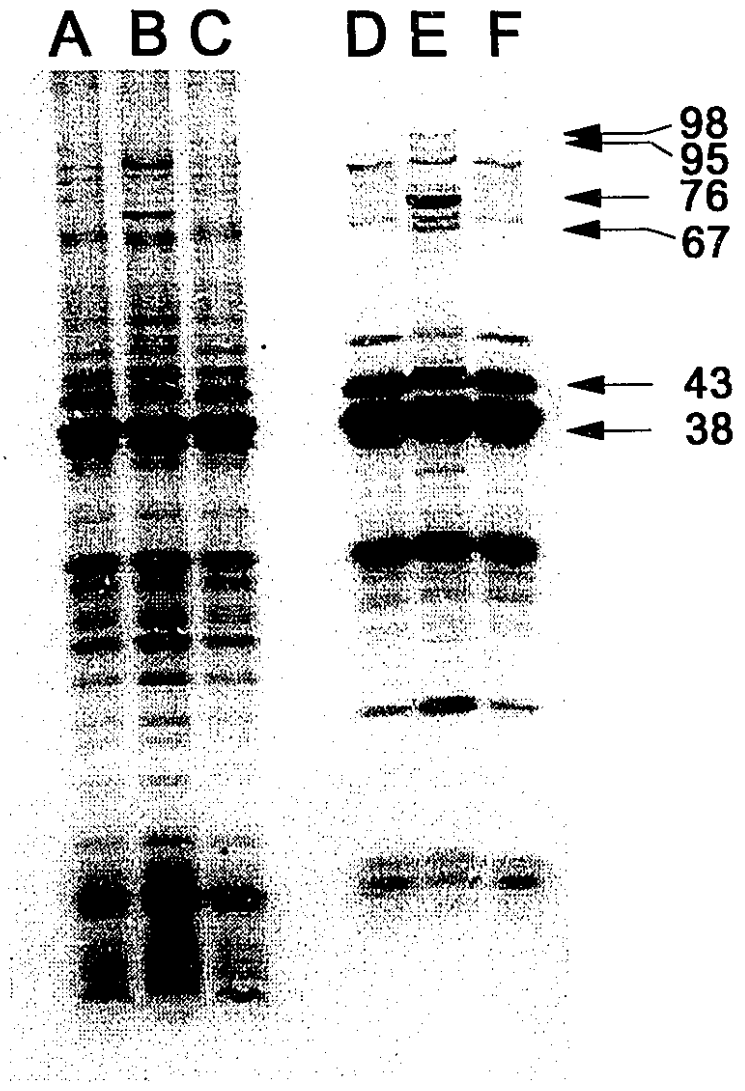

A B C
D E F
98
95
76
67
43
38

FIG. 2.10. Protein profiles of total membrane (lanes A-C) and outer membrane (lanes D-F) fractions from *A. pleuropneumoniae* K17 grown under iron-replete conditions in the absence (lanes A and D) and presence (lanes C and F) of EDDA plus $FeCl_3$, and under iron-restricted conditions using EDDA (lanes B and E). The proteins were separated by SDS-PAGE and stained with Coomassie Blue. The numbers refer to the relative molecular masses (kDa) of some of the separated polypeptides.

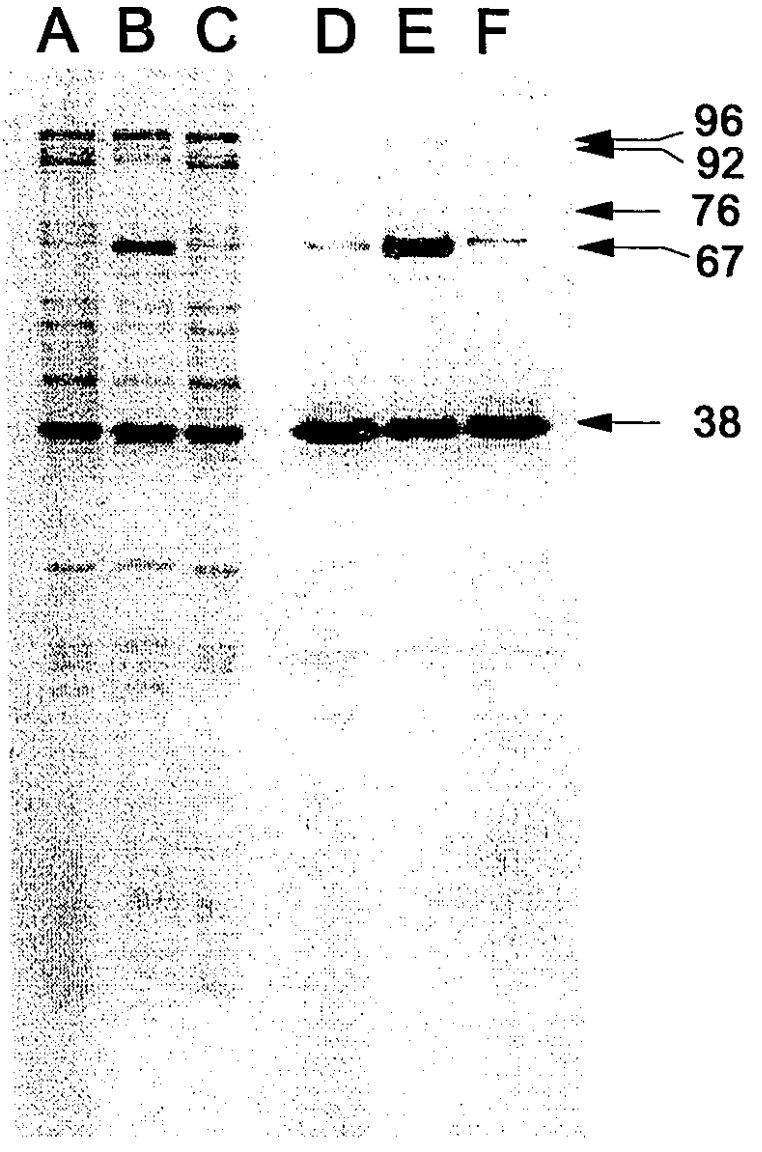
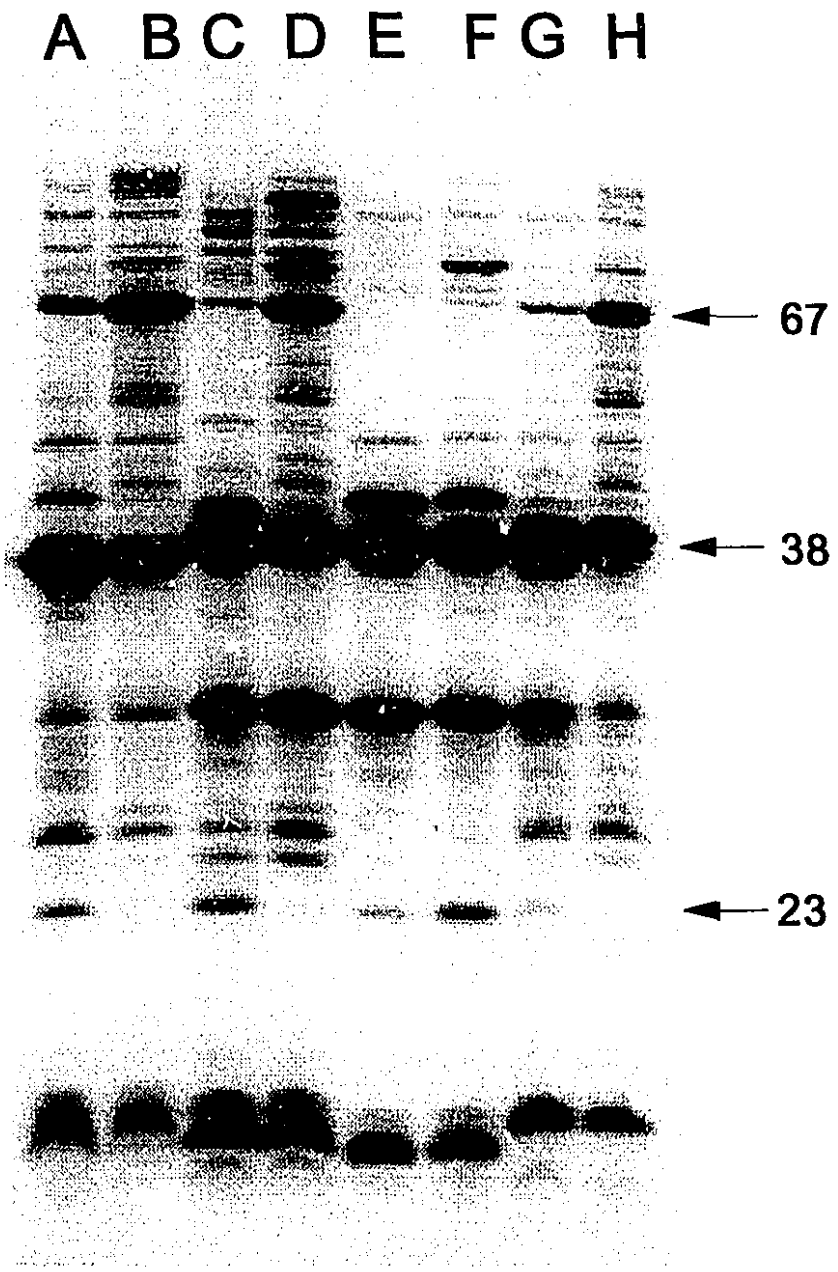

A B C D E F
96
92
76
67
38

FIG. 2.11. A comparison of the OMP profiles of *A. pleuropneumoniae* strains ATCC 27088 (lanes A and B), Bertschinger 2008/76 (lanes C and D), BC 181 (lanes E and F) and K17 (lanes G and H) grown under iron-replete (lanes A, C, E and G) and iron-restricted (lanes B, D, F and H) conditions using EDDA. The numbers refer to the relative molecular masses (kDa) of some of the separated polypeptides.


A B C D E F G H
67
38
23

associated with the production of a novel polypeptide of 98 kDa in strain Bertschinger2008/76, as well as increases in the amounts of the 93 kDa, 76 kDa and 67 kDa polypeptides, and decreases in the amounts of the 38 kDa and 23 kDa polypeptides (Fig. 2.8, lanes D and E). A novel polypeptide of 98 kDa was also synthesized by strain BC181 in response to iron restriction (Fig. 2.9, lanes D and E). Furthermore, there were increases in the amounts of the 95 kDa, 76 kDa and 67 kDa polypeptides and decreases in the amounts of the 43 kDa and 38 kDa polypeptides. Iron restriction was associated with the production of two novel polypeptides of 96 kDa and 92 kDa in strain K17 (Fig. 2.10, lanes D and E). There were increased amounts of polypeptides of 76 kDa and 67 kDa and a decreased amount of the 38 kDa polypeptide.

The changes observed in the OMP profiles in the present study are very similar to those observed with other strains of *A. pleuropneumoniae* (Deneer and Potter, 1989*b*) although there are small differences in the relative molecular masses of the separated polypeptides. Iron restriction, however, may not be responsible for all of these changes since similar changes in the levels of some of these polypeptides (67 kDa, 43 kDa, 38 kDa and 23 kDa) have also been observed to occur in response to a reduction in the glucose or pyridine nucleotide supply to the organisms (O'Reilly *et al.*, 1991). It is possible that several such changes may be related, somehow, to the levels of biomass, rather than the supply of a particular nutrient (O'Reilly *et al.*, 1991). While changes in the levels of polypeptides of 67 kDa, 43 kDa, 38 kDa and 23 kDa may not necessarily be due to iron-restriction, in the case of the strains under investigation, polypeptides in the 92-101 kDa

range and the 76 kDa polypeptide, appear to be true IRMPs. Previous studies with the type strain of *A. pleuropneumoniae* (ATCC 27088) have suggested that these IRMPs are synthesized *in vivo* (Niven *et al.*, 1989).

Chapter 3. Acquisition of iron from host iron-binding proteins by four strains of *Actinobacillus pleuropneumoniae*

Rationale

In the previous section, *A. pleuropneumoniae* strains ATCC 27088, Bertschinger 2008/76, BC181 and K17 were shown to produce IRMPs. The aim of this study was to determine if like the type strain (ATCC 27088), the other three strains could obtain iron from porcine transferrin, and if so, whether the acquisition of iron from transferrin was siderophore-dependent or siderophore-independent, involving specific IRMPs. Other aims were to determine the transferrin specificities of these strains and to examine iron acquisition from haemoglobin and lactoferrin.

This chapter is a component of the research publication:

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. In press.

3.1 Summary

Four strains of the swine pathogen, *Actinobacillus pleuropneumoniae*, namely, the type strain (ATCC 27088), the reference strain of biotype 2 (Bertschinger 2008/76) and two additional biotype 1 strains, strain BC181, which is less virulent than the type strain, and strain K17 (reference strain of serotype 5A), which was isolated from a lamb, were investigated with respect to iron acquisition from host iron-binding proteins. Only strains ATCC 27088 and Bertschinger 2008/76 could acquire iron from porcine transferrin. No organism could utilize human, bovine or ovine transferrin, or ovine or porcine lactoferrin. Haemoglobin supported good growth of all strains except K17, which also failed to acquire iron from haemin. In all cases, iron acquisition from transferrin or haemoglobin required direct contact between the organisms and the proteins.

3.2 Introduction

A. pleuropneumoniae is the causative agent of swine pleuropneumonia (Sebunya and Saunders, 1983). In general, biotype 1 strains are more virulent than biotype 2 (Pohl *et al.*, 1983). *A. pleuropneumoniae*, growing extracellularly *in vivo*, must be capable of obtaining iron from the host. Note that several biotype 1 strains are known to acquire iron from, specifically, porcine transferrin (Morton and Williams, 1989; Niven *et al.*, 1989; Morton and Williams, 1990; Schryvers and Gonzalez, 1990; Gonzalez *et al.*, 1990). This specificity for porcine transferrin indicates that none of these organisms produces a conventional siderophore suggesting that IRMPs are involved in the acquisition of iron from transferrin (Morton and Williams, 1989; Niven *et al.*, 1989; Morton and Williams, 1990; Schryvers and Gonzalez, 1990). These organisms are not unique since a variety of other host-specific pathogens, including, most recently, *Moraxella catarrhalis* (Campagnari *et al.*, 1994), have been shown to acquire iron from specific transferrins and/or lactoferrins by siderophore-independent mechanisms that involve IRMPs as receptors (section 1.3.2; Williams and Griffiths, 1992).

While studies have centered on porcine transferrin as an iron source, it would appear that biotype 1 strains of *A. pleuropneumoniae* can also obtain iron from haem compounds, including haemoglobin (Deneer and Potter, 1989*b*; Niven *et al.*, 1989). However, nothing is known regarding iron acquisition from porcine lactoferrin, and nothing is known about iron acquisition in general, by biotype 2. Based on this

information, and despite the use of haem compounds as iron sources, it is still tempting to speculate that the ability of *A. pleuropneumoniae* to use porcine transferrin (and perhaps, lactoferrin) as an iron source might account, at least in part, for the host specificity of the organism, and moreover, that a strain lacking the ability to acquire iron from porcine transferrin (and/or lactoferrin) might be less virulent than a strain possessing such ability. In this respect, it is notable that unlike the parent, a mutant of *N. gonorrhoeae*, defective in the uptake of iron from human transferrin and haemoglobin, is avirulent in mouse subcutaneous chambers (Genco *et al.*, 1991).

With these points in mind, the purpose of the present study was to examine iron acquisition, from various transferrins, lactoferrins and haemoglobins, by a few selected strains of *A. pleuropneumoniae* that differ with respect to virulence and/or animal source. These strains included the type strain (ATCC 27088; biotype 1), the "reference" strain of biotype 2 (strain Bertschinger 2008/76; Pohl *et al.*, 1983), and two additional biotype 1 strains, strain BC181 and strain K17. Strain BC181 was chosen because it is known to be associated with chronic disease and to be less virulent than the type strain (Rosendal *et al.*, 1985), and we wished to determine if these two organisms were equally capable of obtaining iron from host proteins. Strain K17 was isolated originally from a case of arthritis in a lamb (Biberstein *et al.*, 1977) and we anticipated that this strain would allow us to probe the relationship between transferrin (and, perhaps, lactoferrin) specificity and host range.

3.3 Materials and Methods

3.3.1 Organisms

A. pleuropneumoniae strains ATCC 27088 (biotype 1, type strain), BC181 (biotype 1), K17 (biotype 1) and Bertschinger 2008/76 (biotype 2, reference strain), and also *E. coli* K12, were stored, and used to prepare frozen inocula, as described previously (section 2.3.1).

3.3.2 Chemicals

8-hydroxyquinoline, haemin (bovine) and bovine, human and porcine haemoglobins were purchased from Sigma. Porcine serum was from Gibco BRL. The sources of other chemicals were as described previously (section 2.3.2).

3.3.3 Isolation and purification of porcine and ovine transferrins

The purification of porcine transferrin was essentially as described by Niven *et al.* (1989). Briefly, porcine serum (200 ml) was mixed with an equal volume of saturated ammonium sulphate, pH 7.0, stirred slowly for 30 min, and allowed to stand at room temperature for 2 h. The mixture was subjected to centrifugation (20,000 x g, 10 min,

5°C) and the supernatant fraction was filtered through Whatman No. 4 filter paper. The filtrate was dialysed for 2 h against H_2O (4 l, with two changes; 4°C) and then overnight against 10 mM NaCl, 10 mM Tris-HCl, pH 8.0 (4 l; 4°C). A column of DEAE-Sepharose CL-6B (Pharmacia; 2.4 cm x 30 cm; 4°C) was pre-equilibrated with 10 mM NaCl, 10 mM Tris-HCl, pH 8.0, and the dialysate was applied to it. The non-adsorbed material was collected and concentrated by ultrafiltration (Diaflo Ultrafiltration Membrane, PM 30, 43 mm diameter; Amicon Corp., Danvers, MA). The concentrate was then dialysed for 2 h against 10 mM sodium citrate, pH 5.2 with HCl (4 l, with one change; 4°C) and then overnight under the same conditions. A column of CM-Sepharose CL-6B (Pharmacia; 2.4 cm x 30 cm; 4°C) was pre-equilibrated with 10 mM sodium citrate, pH 5.2, and the dialysate was applied to it. Transferrin was eluted by a gradient obtained by mixing 500 ml each of 10 mM and 75 mM sodium citrate, pH 5.2 with HCl; elution was monitored by recording the absorbance of the eluate at 280 nm. Fractions containing transferrin were pooled, dialysed overnight against H_2O (4 l, with one change; 4°C), and lyophilised.

Ovine transferrin was obtained similarly except that the non-adsorbed material from the DEAE-Sepharose column was discarded, and the material to be concentrated and dialysed for chromatography using CM-Sepharose was eluted from the DEAE-Sepharose column using a gradient obtained by mixing 500 ml each of 20 mM and 200 mM NaCl, each in 10 mM Tris-HCl, pH 8.0.

The addition of 9 μg Fe^{3+} as $FeCl_3$ to a 3-ml solution of porcine or ovine transferrin (3 mg of transferrin in 3 ml of 20 mM $NaHCO_3$, 200 mM Tris, pH 7.4;

adjusted to pH 8.0) resulted in an increase in the absorbance of the transferrin solution at 465 nm confirming that the transferrin had conserved its activity. The purity of the transferrins was confirmed by SDS-PAGE using the method of Lutgenberg *et al.* (1975).

3.3.4 Isolation and purification of porcine and ovine lactoferrins

The methods used were based on those described by Roberts and Boursnell (1975). Porcine or ovine milk, as appropriate (250 ml), was filtered through cheesecloth to remove extraneous particulate matter. The milk was defatted by centrifugation (10,000 x g, 20 min, 4°C) followed by filtration through cheesecloth. The filtrate (whey) was adjusted to pH 7.0 with 400 mM Na_2HPO_4 and then diluted with an equal volume of 150 mM NaCl, 20 mM Na_2HPO_4-HCl, pH 7.0. The diluted whey was added to CM-Sepharose CL-6B (sufficient to fill a 2.4 cm x 30 cm column) which had been pre-washed, on Whatman No. 1 filter paper, with water (three washes of 50 ml each) followed by 150 mM NaCl, 20 mM Na_2HPO_4-HCl, pH 7.0 (50 ml), and the mixture was stirred gently at room temperature for 4 h. The CM-Sepharose was then collected on Whatman No. 1 filter paper and following two washes with 150 mM NaCl, 20 mM Na_2HPO_4-HCl, pH 7.0 (50 ml; 4°C), used to form a column (2.4 cm x 30 cm; 4°C). The column was washed with 150 mM NaCl, 20 mM Na_2HPO_4-HCl, pH 7.0 until protein was no longer detected in the eluate, and then with 500 mM NaCl, 20 mM Na_2HPO_4-HCl, pH 7.0 (500 ml); elution was monitored by recording the absorbance of the eluate at 280 nm. Protein-containing

fractions resulting from the latter wash were pooled and the proteins were concentrated by ultrafiltration (Diaflo Ultrafiltration Membrane, PM 10, 43 mm diameter; Amicon Corp., Danvers, MA). The concentrate was dialysed overnight against 150 mM NaCl, 20 mM Na_2HPO_4-HCl, pH 7.0 (4 l; 4°C) and the dialysate was then applied to another column of CM-Sepharose CL-6B (2.4 cm x 30 cm; 4°C) pre-equilibrated with 150 mM NaCl, 20 mM Na_2HPO_4-HCl, pH 7.0. The column was washed with equilibration buffer (250 ml) and the lactoferrin was then eluted from the column using a gradient obtained by mixing 500 ml each of 150 mM and 750 mM NaCl, each in 20 mM Na_2HPO_4-HCl, pH 7.0. Fractions containing lactoferrin were pooled, dialysed overnight against H_2O (4 l, with one change; 4°C), and lyophilised.

Conservation of lactoferrin iron-binding ability and the purity of the lactoferrins were confirmed as for transferrin (section 3.3.3).

3.3.5 Preparation of iron sources

The transferrins (bovine, human, ovine and porcine) were loaded (to the required saturation levels) with Fe^{3+} using $FeCl_3$/sodium citrate in 2 mM $NaHCO_3$, 40 mM Tris-HCl, pH 7.4 (Caldwell and Archibald, 1987). The iron-loaded transferrins were stirred at room temperature for 20 minutes and then allowed to stand at room temperature for 20 minutes. The transferrins were dialysed as described by Caldwell and Archibald (1987) using dialysis bags (Spectrum Medical Industries, Inc., Houston, TX) with a molecular

mass cut-off of approximately 12 to 14 kDa. The lactoferrins (porcine and ovine) and haemoglobins (bovine, human and porcine) were dialysed similarly. Following dialysis, all preparations were filter-sterilised (0.2 μm pore size) and the protein concentrations and, where appropriate, iron-saturation levels, were then verified using the methods of Peterson (1977) and Mazurier and Spik (1980), respectively. Haemin stock solution (8.2 mg ml^{-1} in 20 mM NaOH, 50 % ethanol) was prepared as described by Lascelles (1979) and then filter-sterilised (0.2 μm pore size).

3.3.6 Growth conditions and preparation of membrane fractions

For the growth of *A. pleuropneumoniae*, the basic medium was the HEPES-buffered tryptone/yeast extract medium (TYE-H) described earlier (section 2.3.3); all media were supplemented with NAD (5 μM) and $NaHCO_3$ (10 mM), and the inoculum was 1% (v/v). Incubation was at 37°C, with agitation on a gyratory shaker (200 rpm), in an atmosphere not enriched with CO_2. Growth was monitored turbidimetrically at 660 nm (model 240 spectrophotometer, Gilford Instrument Laboratories, Inc., Oberlin, OH) using diluted samples as appropriate.

The procedures used to investigate the capacities of the *A. pleuropneumoniae* strains to obtain iron from the various iron-containing proteins were based on those described by Morton and Williams (1990). For control cultures, TYE-H plus NAD and $NaHCO_3$ (20-ml volumes) was contained in acid-washed 125-ml screw-capped flasks

(Nalgene); all experiments were performed in triplicate. For test cultures, the medium also contained deferrated EDDA (100 μM) (to inhibit growth completely) or EDDA plus 5 mg (transferrins and haemoglobins) or 2-5 mg (lactoferrins) of the protein to be tested as an iron source; in some experiments, the protein was contained in a dialysis bag (Spectrum Medical Industries, Inc., Houston, TX; molecular mass cut-off of approximately 12 to 14 kDa) and was released 9-12 h post-inoculation by piercing the bag with a sterile needle. The results given for the dialysis bag experiments are the means of duplicates. The reason for this is that it was only very seldom that no problem was encountered with at least one of the triplicates. The dialysis bags had to be autoclaved and then filled, tied and rinsed under aseptic conditions, and they were then subject to vigorous shaking (200 rpm) for the duration of the experiment. Problems that were encountered ranged from suspected leakage/ineffective rinsing of the bags, to obvious contamination, most usually after the bags were pierced. For EDDA control cultures, TYE-H plus NAD, $NaHCO_3$ and EDDA was supplemented with $FeCl_3$ (100 μM). When positive results (good growth) were obtained using a particular protein, outer membranes were prepared from the organisms as described previously (section 2.3.4). For control purposes only, *A. pleuropneumoniae* strains were grown under both iron-replete and iron-restricted conditions and outer membranes were prepared from these organisms, as described previously by Niven *et al.* (1989) (section 2.3.3).

To confirm that the iron contained in the bovine, human and ovine transferrins, and ovine and porcine lactoferrins, was available for microbial growth, *E. coli* K12 and

deferrated minimal salts medium (M-9) containing glucose (50 mM) and $MgCl_2$ (98 μM) (Braun *et al.*, 1976) were used as a test system. The deferrated medium was either used *per se* or supplemented with either $MgCl_2$ (98 μM), $FeCl_3$ (100 μM) or the protein under investigation (2-5 mg); inoculation and other conditions were as described above.

The ability of *A. pleuropneumoniae* strain K17 to use haemin as an iron source was investigated using the methods described above except that the EDDA concentration was 300 μM; media were supplemented, as appropriate, with 0.82 mg haemin.

Protein concentrations were estimated using the method of Peterson (1977) and bovine serum albumin as a standard.

3.3.7 SDS-PAGE

Proteins were separated by SDS-PAGE as described in section 2.3.4. The silver staining method of Harlow and Lane (1988) was used to visualize the separated polypeptides.

3.4 Results

Of the four *A. pleuropneumoniae* strains investigated, only the type strain (ATCC 27088) and the reference strain of biotype 2 (Bertschinger 2008/76) were able to use porcine transferrin as an iron source for growth (Figs. 3.1A, 3.2A, 3.3A and 3.4A); with strains BC181 and K17, the OD_{660} values remained unchanged even after 24 h incubation. No growth was observed if the transferrin was contained in a dialysis bag (Figs. 3.5 and 3.6). It should be noted that strains ATCC 27088 and Bertschinger 2008/76 were both capable of growth if the dialysis bags were pierced at 9-12 h post-inoculation (Figs. 3.5 and 3.6). In keeping with previous studies (Niven *et al.*, 1989; Gonzalez *et al.*, 1990), none of the strains could use human or bovine transferrin (~50% iron-saturated) as an iron source (Figs. 3.1A, 3.2A, 3.3A and 3.4A) and, in addition, none could use ovine transferrin (~50% iron-saturated) or ovine or porcine lactoferrin (>50% iron-saturated) (Figs. 3.1B, 3.2B, 3.3B and 3.4B); in each of these experiments the OD_{660} values remained constant during 24 h incubation.

To confirm that the latter results accurately reflected the capabilities of the organisms rather than some problem with the transferrin and lactoferrin preparations, *E. coli* K12 was used as a positive control. In all cases, the growth of *E. coli* K12 in a deferrated minimal salts medium (see Methods) was enhanced when the organisms were

FIG. 3.1. Growth of *A. pleuropneumoniae* ATCC 27088 in (A), TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and 250 μg ml^{-1} porcine transferrin (50% iron-saturated) (▼), 100 μM EDDA and 250 μg ml^{-1} bovine transferrin (50% iron-saturated) (♦), or 100 μM EDDA and 250 μg ml^{-1} human transferrin (50% iron-saturated) (●), and (B), TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA and 250 μg ml^{-1} ovine transferrin (50% iron-saturated) (○), 100 μM EDDA and 250 μg ml^{-1} porcine lactoferrin (>50% iron-saturated) (□), or 100 μM EDDA and 250 μg ml^{-1} ovine lactoferrin (>50% iron-saturated) (△). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

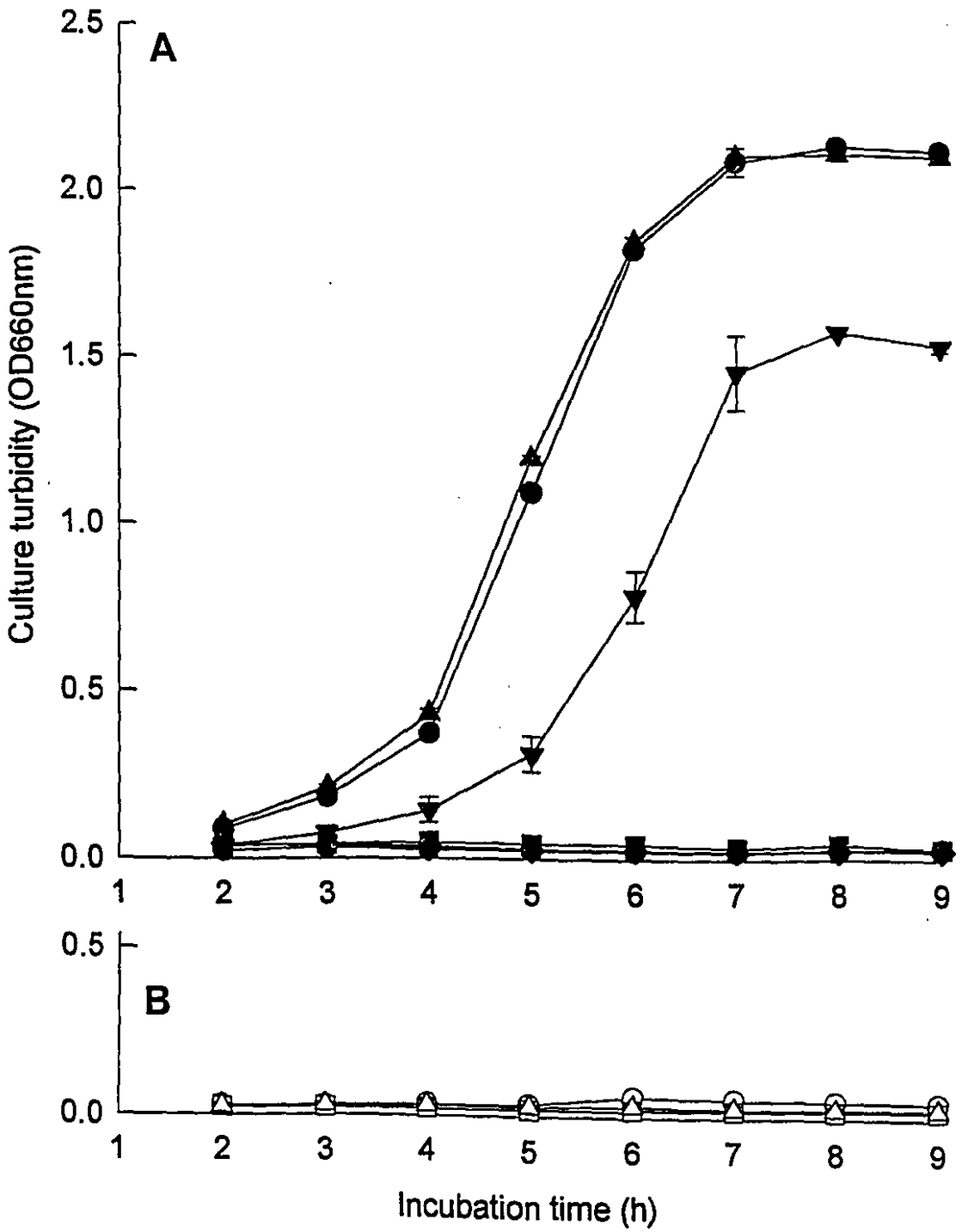

A
B
2.5
2.0
1.5
1.0
0.5
0.0
0.5
0.0
1
2
3
4
5
6
7
8
9
Culture turbidity (OD660nm)
Incubation time (h)

FIG. 3.2. Growth of *A. pleuropneumoniae* Bertschinger 2008/76 in (A), TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and 250 μg ml^{-1} porcine transferrin (50% iron-saturated) (▼), 100 μM EDDA and 250 μg ml^{-1} bovine transferrin (50% iron-saturated) (♦), or 100 μM EDDA and 250 μg ml^{-1} human transferrin (50% iron-saturated) (⬣), and (B), TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA and 250 μg ml^{-1} ovine transferrin (50% iron-saturated) (○), 100 μM EDDA and 250 μg ml^{-1} porcine lactoferrin (>50% iron-saturated) (□), or 100 μM EDDA and 250 μg ml^{-1} ovine lactoferrin (>50% iron-saturated) (△). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

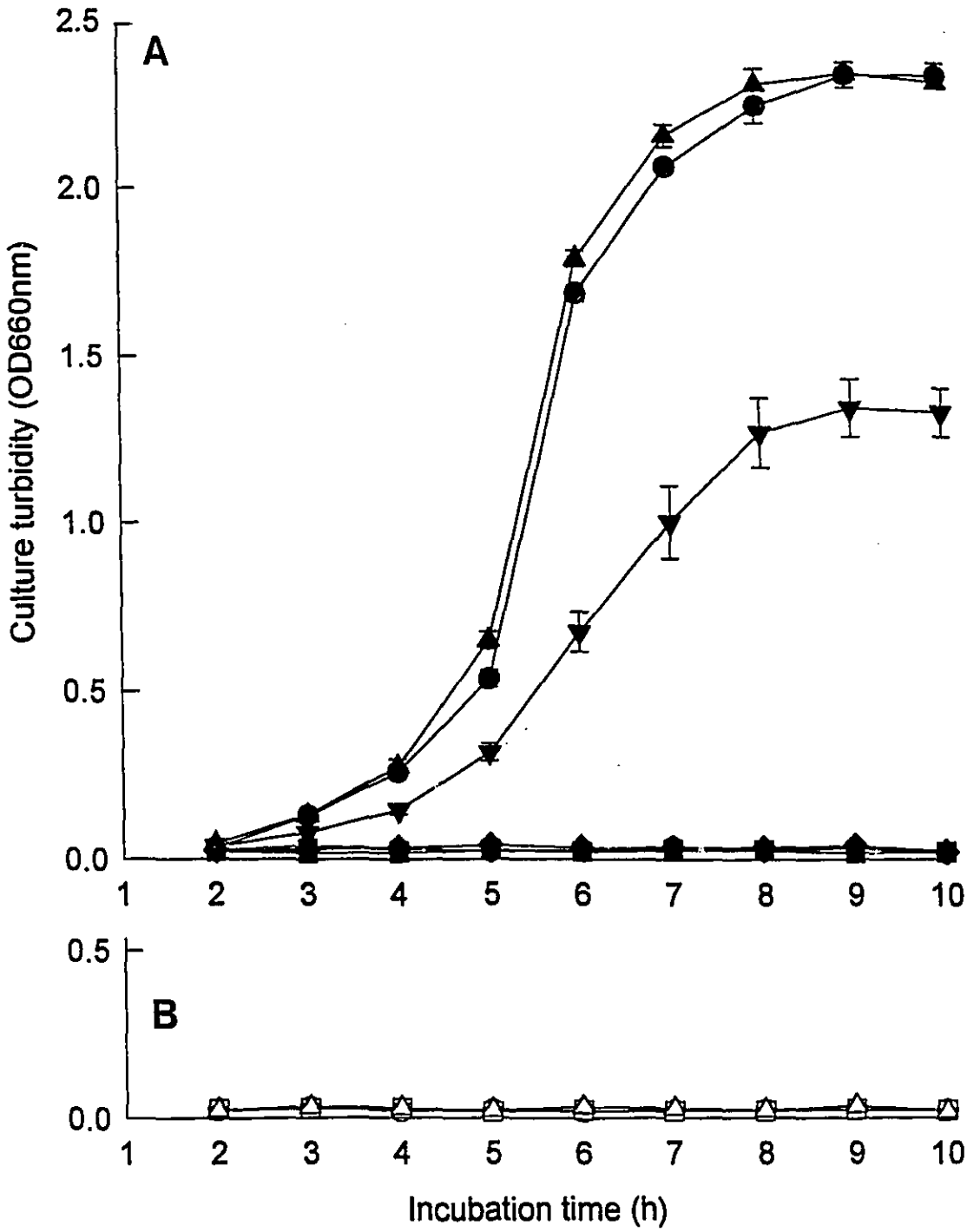

A
B
Culture turbidity (OD660nm)
Incubation time (h)
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
9
10

FIG. 3.3. Growth of *A. pleuropneumoniae* BC181 in (A), TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and 250 μg ml^{-1} porcine transferrin (50% iron-saturated) (▼), 100 μM EDDA and 250 μg ml^{-1} bovine transferrin (50% iron-saturated) (♦), or 100 μM EDDA and 250 μg ml^{-1} human transferrin (50% iron-saturated) (⬬), and (B), TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA and 250 μg ml^{-1} ovine transferrin (50% iron-saturated) (○), 100 μM EDDA and 250 μg ml^{-1} porcine lactoferrin (>50% iron-saturated) (□), or 100 μM EDDA and 250 μg ml^{-1} ovine lactoferrin (>50% iron-saturated) (△). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

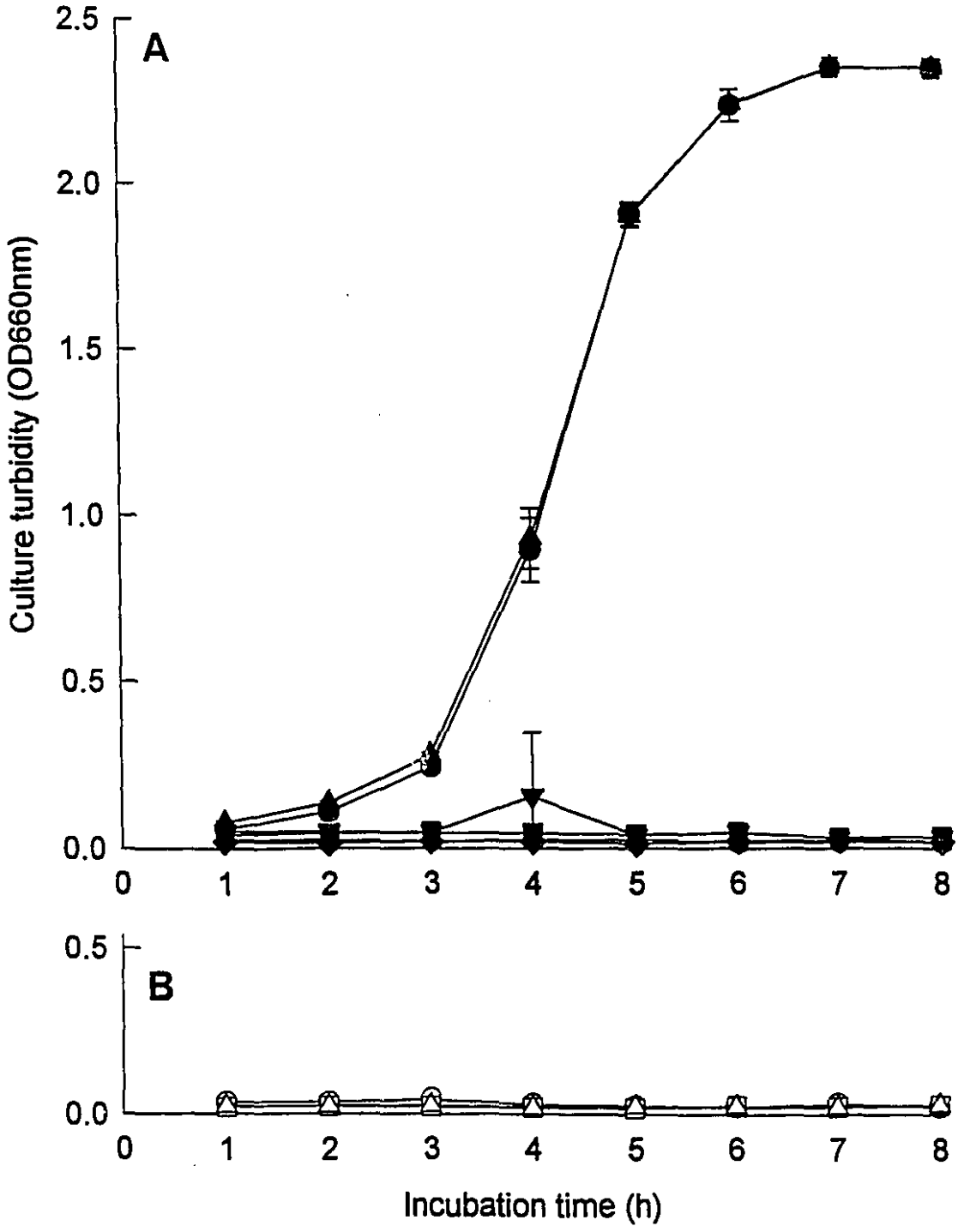

A
B
Culture turbidity (OD660nm)
Incubation time (h)
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
7
8

FIG. 3.4. Growth of *A. pleuropneumoniae* K17 in (A), TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and 250 μg ml^{-1} porcine transferrin (50% iron-saturated) (▼), 100 μM EDDA and 250 μg ml^{-1} bovine transferrin (50% iron-saturated) (♦), or 100 μM EDDA and 250 μg ml^{-1} human transferrin (50% iron-saturated) (⬣), and (B), TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA and 250 μg ml^{-1} ovine transferrin (50% iron-saturated) (○), 100 μM EDDA and 250 μg ml^{-1} porcine lactoferrin (>50% iron-saturated) (□), or 100 μM EDDA and 250 μg ml^{-1} ovine lactoferrin (>50% iron-saturated) (△). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

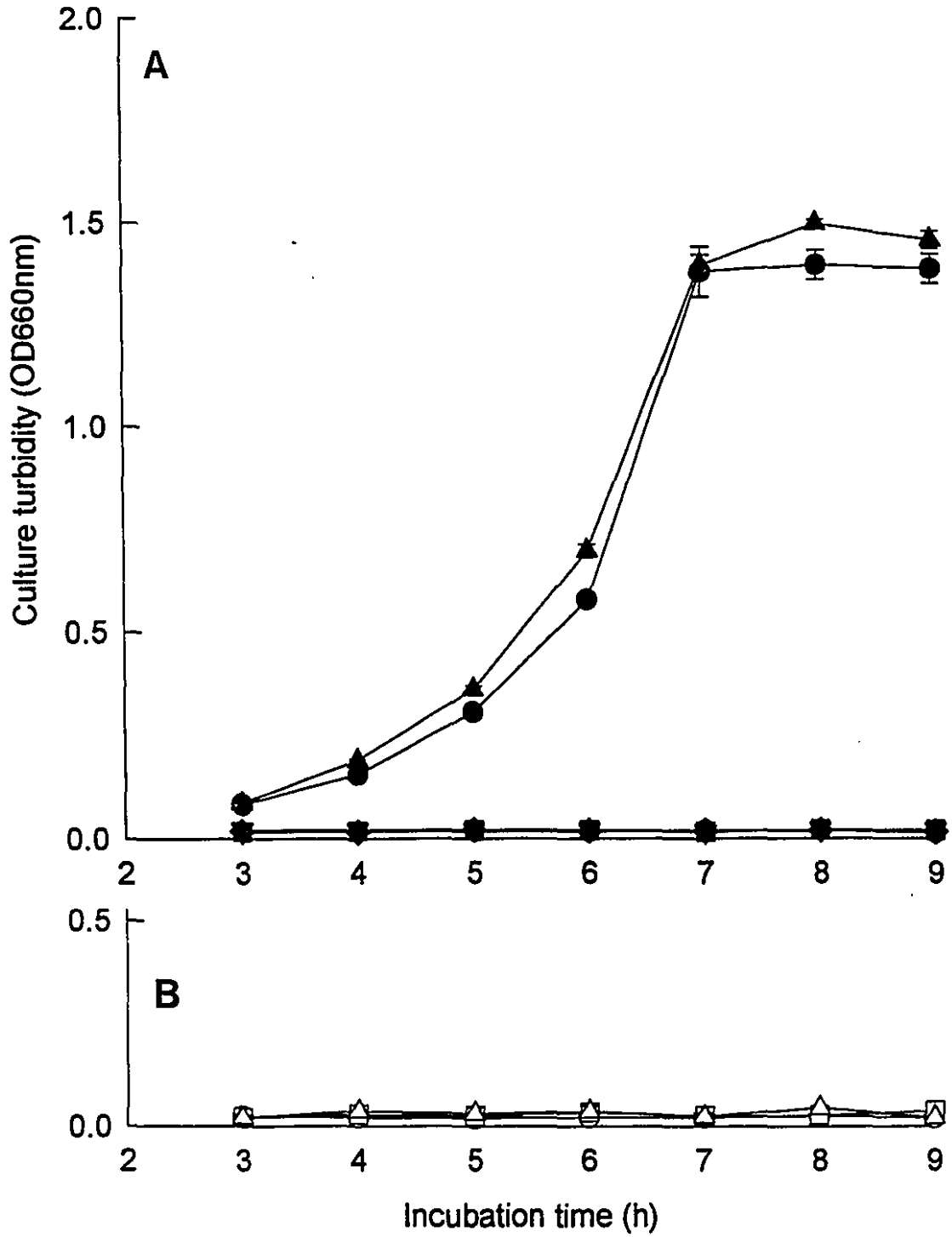

2.0
A
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
2
3
4
5
6
7
8
9
0.5
B
0.0
2
3
4
5
6
7
8
9
Incubation time (h)

FIG. 3.5. Growth of *A. pleuropneumoniae* ATCC 27088 in TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and a dialysis bag containing 5 mg porcine transferrin (50% iron-saturated) (▼), 100 μM EDDA and a dialysis bag containing 5 mg porcine haemoglobin (♦), 100 μM EDDA and a dialysis bag containing 5 mg bovine haemoglobin (◓), or 100 μM EDDA and a dialysis bag containing 5 mg human haemoglobin (○). The dialysis bags were pierced 9 h post-inoculation. Culture turbidity values at 2-9 h are the means of triplicates and those at 24-30 h are the means of duplicates (see text). Standard error bars were plotted for all data points and may be hidden within the symbols.

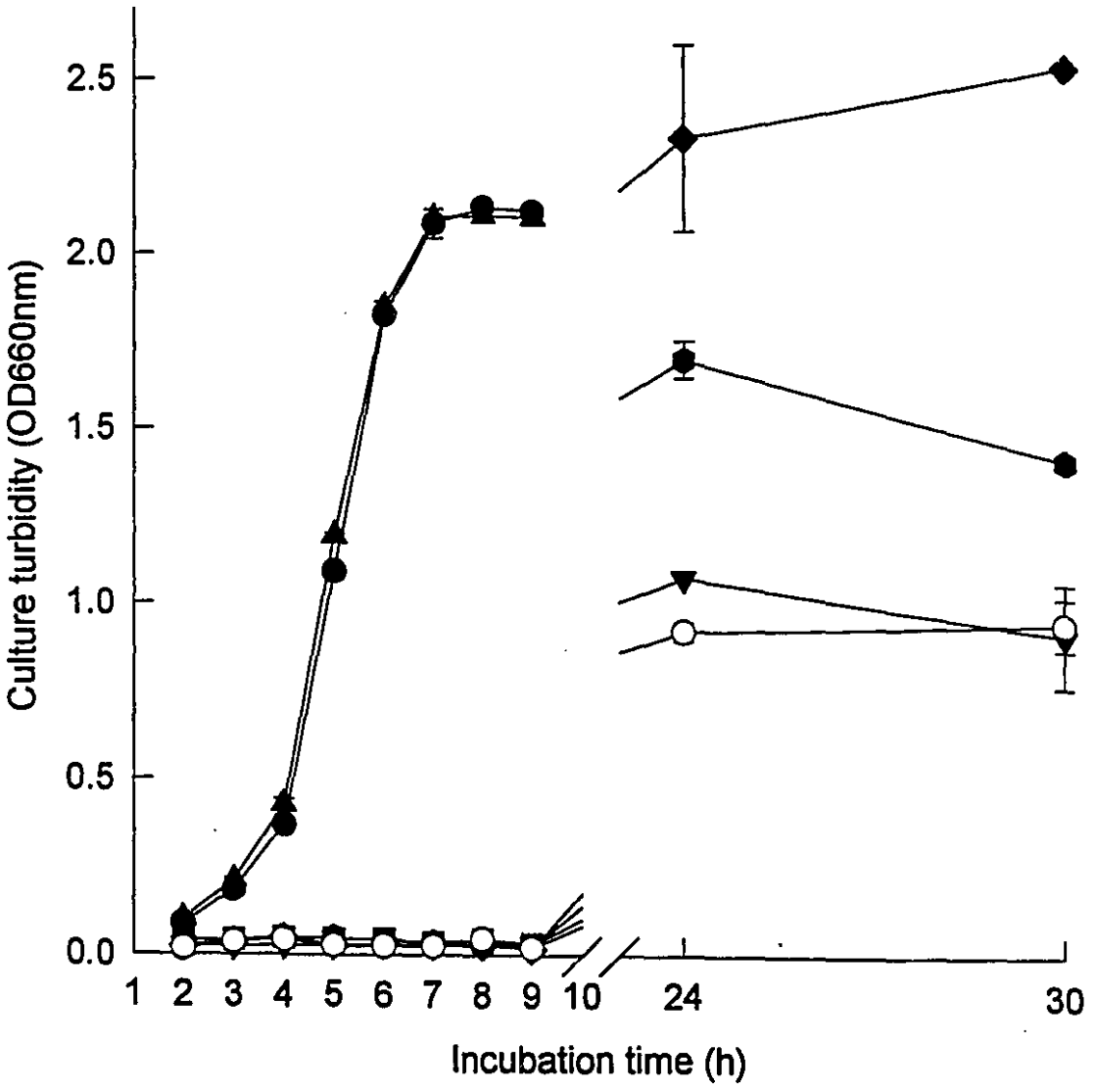
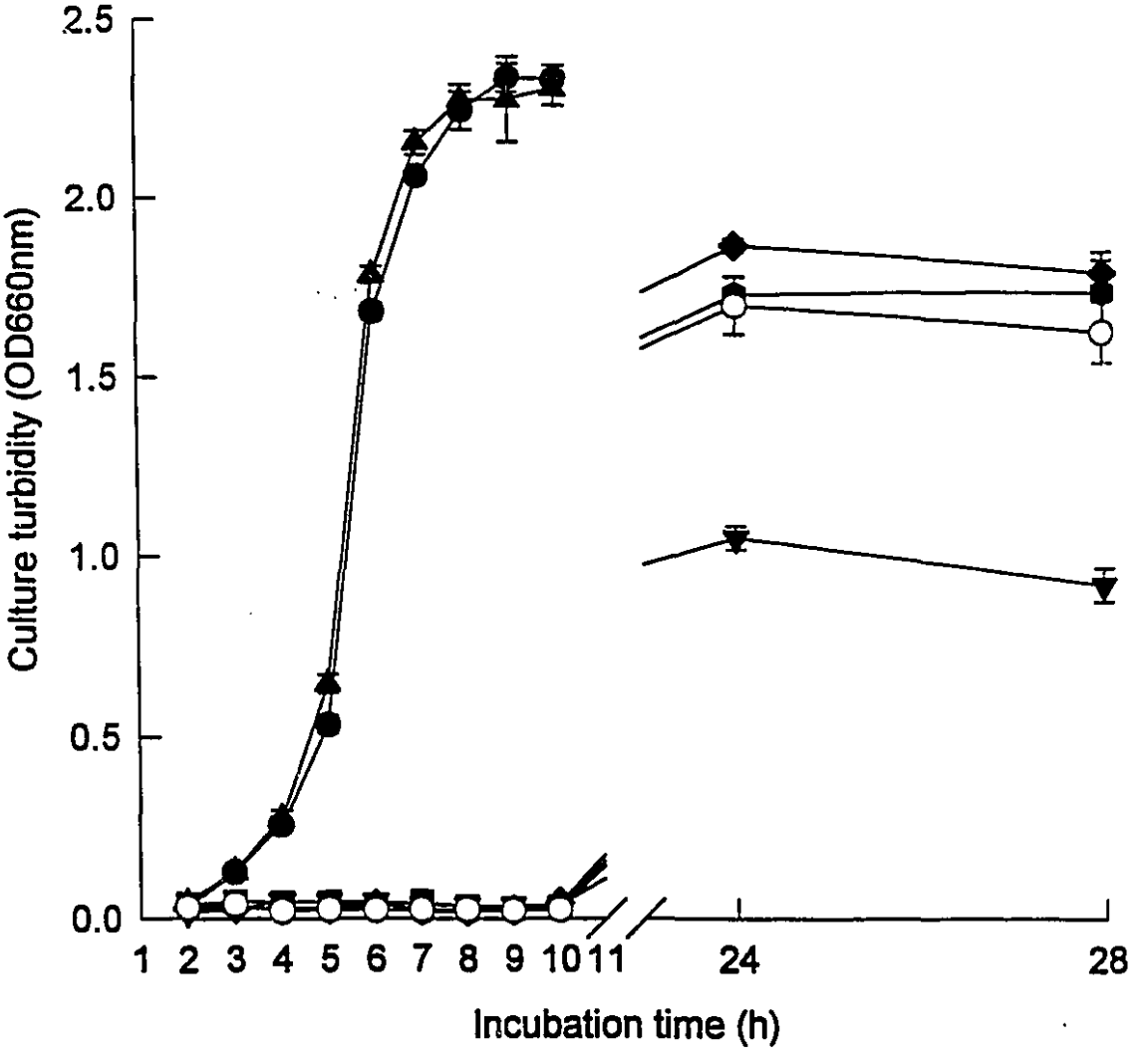

Culture turbidity (OD660nm)
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10
24
30
Incubation time (h)

FIG. 3.6. Growth of *A. pleuropneumoniae* Bertschinger 2008/76 in TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and a dialysis bag containing 5 mg porcine transferrin (50% iron-saturated) (▼), 100 μM EDDA and a dialysis bag containing 5 mg porcine haemoglobin (♦), 100 μM EDDA and a dialysis bag containing 5 mg bovine haemoglobin (◓), or 100 μM EDDA and a dialysis bag containing 5 mg human haemoglobin (○). The dialysis bags were pierced 10 h post-inoculation. Culture turbidity values at 2-10 h are the means of triplicates and those at 24-28 h are the means of duplicates (see text). Standard error bars were plotted for all data points and may be hidden within the symbols.


2.5
2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
1 2 3 4 5 6 7 8 9 10 11
24
28
Incubation time (h)

provided with the protein in question (Fig. 3.7).

A. pleuropneumoniae strains ATCC 27088, Bertschinger 2008/76, and BC181 could also use porcine haemoglobin as an iron source (Figs. 3.8, 3.9 and 3.10). Strain K17, however, could use porcine haemoglobin to only a very limited extent, if at all (Fig. 3.11); the OD_{660} values remained constant from hour 5 to 24 h incubation. Similar results were obtained with human and bovine haemoglobins (Figs. 3.8, 3.9, 3.10 and 3.11) and no growth was observed when porcine, bovine or human haemoglobin was contained in a dialysis bag (Figs. 3.5, 3.6 and 3.12). Strain K17 could acquire iron from neither ovine transferrin nor ovine lactoferrin (Fig. 3.4B) and failed to use haemin (Fig. 3.13); the OD_{660} values did not increase even after 24 h incubation.

Outer membranes prepared from the strains of *A. pleuropneumoniae* that exhibited growth under iron-restricted conditions in the presence of iron-containing proteins were analyzed by SDS-PAGE (Figs. 3.14, 3.15 and 3.16). For comparison purposes only, outer membranes prepared from organisms grown under iron-replete and iron-restricted conditions were also analyzed (Figs. 3.14, 3.15 and 3.16). As with the results reported in Chapter 2, growth under iron-restricted conditions resulted in comparable changes in the outer membrane protein (OMP) profiles of each of the organisms and several polypeptides could be identified as IRMPs (Figs. 3.14, 3.15 and 3.16). For example, iron-restriction enhanced the production of the 101 and 99 kDa polypeptides, and hence, IRMPs, of strain ATCC 27088 (Fig. 3.14, lanes A and B), the 98 and 93 kDa polypeptides of strain Bertschinger 2008/76 (Fig. 3.15, lanes A and

FIG. 3.7. Growth of *E. coli* K12 in deferrated M-9 minimal medium (●), or deferrated M-9 minimal medium supplemented with 98 μM $MgCl_2$ (■), 100 μM Fe as $FeCl_3$ (▲), 98 μM $MgCl_2$ and 2 mg porcine lactoferrin (100% iron-saturated) (▼), 98 μM $MgCl_2$ and 2 mg ovine transferrin (50% iron-saturated) (♦), or 98 μM $MgCl_2$ and 2 mg ovine lactoferrin (100% iron-saturated) (⬬). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

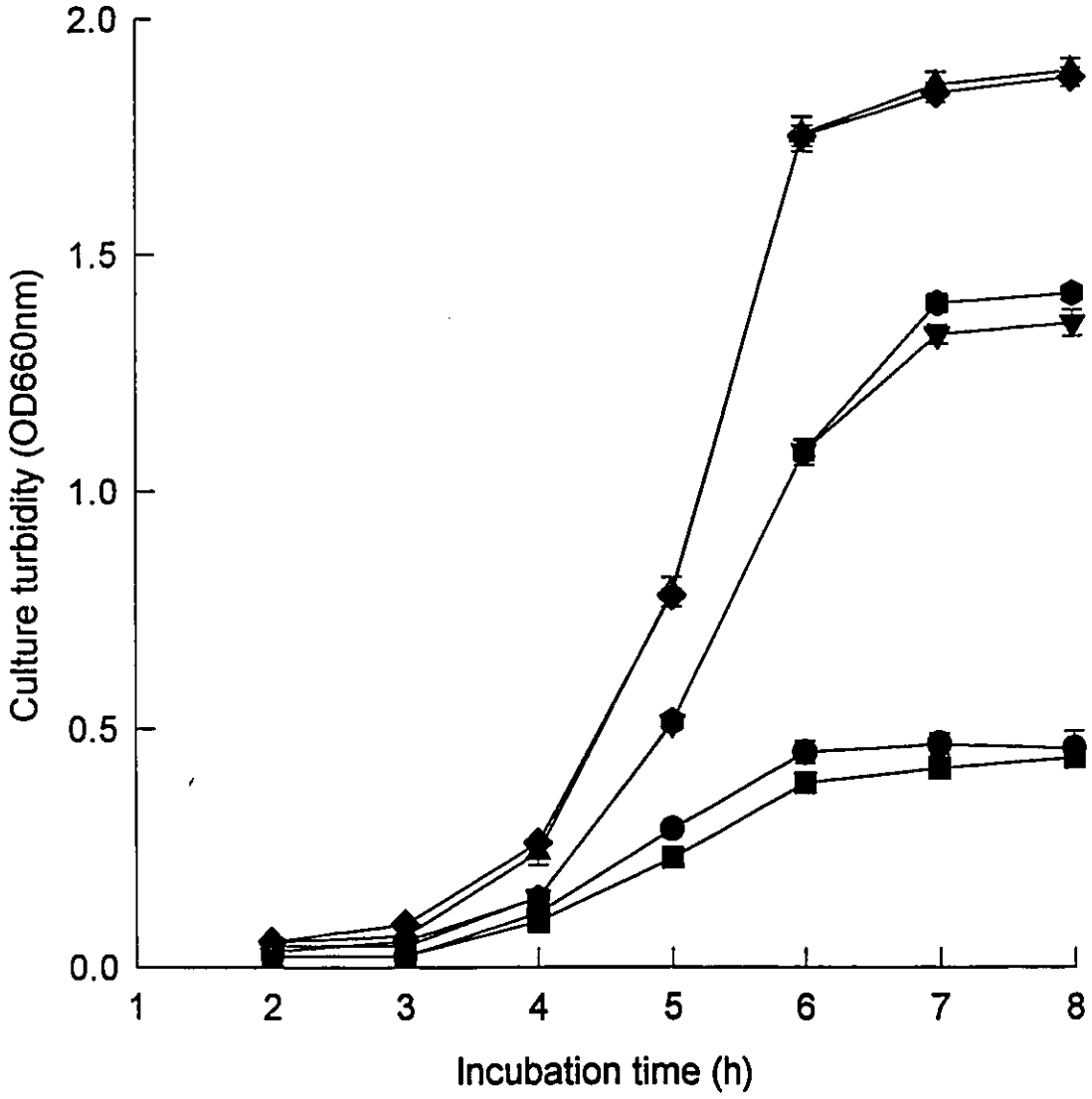

2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
1
2
3
4
5
6
7
8
Incubation time (h)

FIG. 3.8. Growth of *A. pleuropneumoniae* ATCC 27088 in TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and 250 μg ml^{-1} porcine haemoglobin (▼), 100 μM EDDA and 250 μg ml^{-1} bovine haemoglobin (♦), or 100 μM EDDA and 250 μg ml^{-1} human haemoglobin (⬬). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

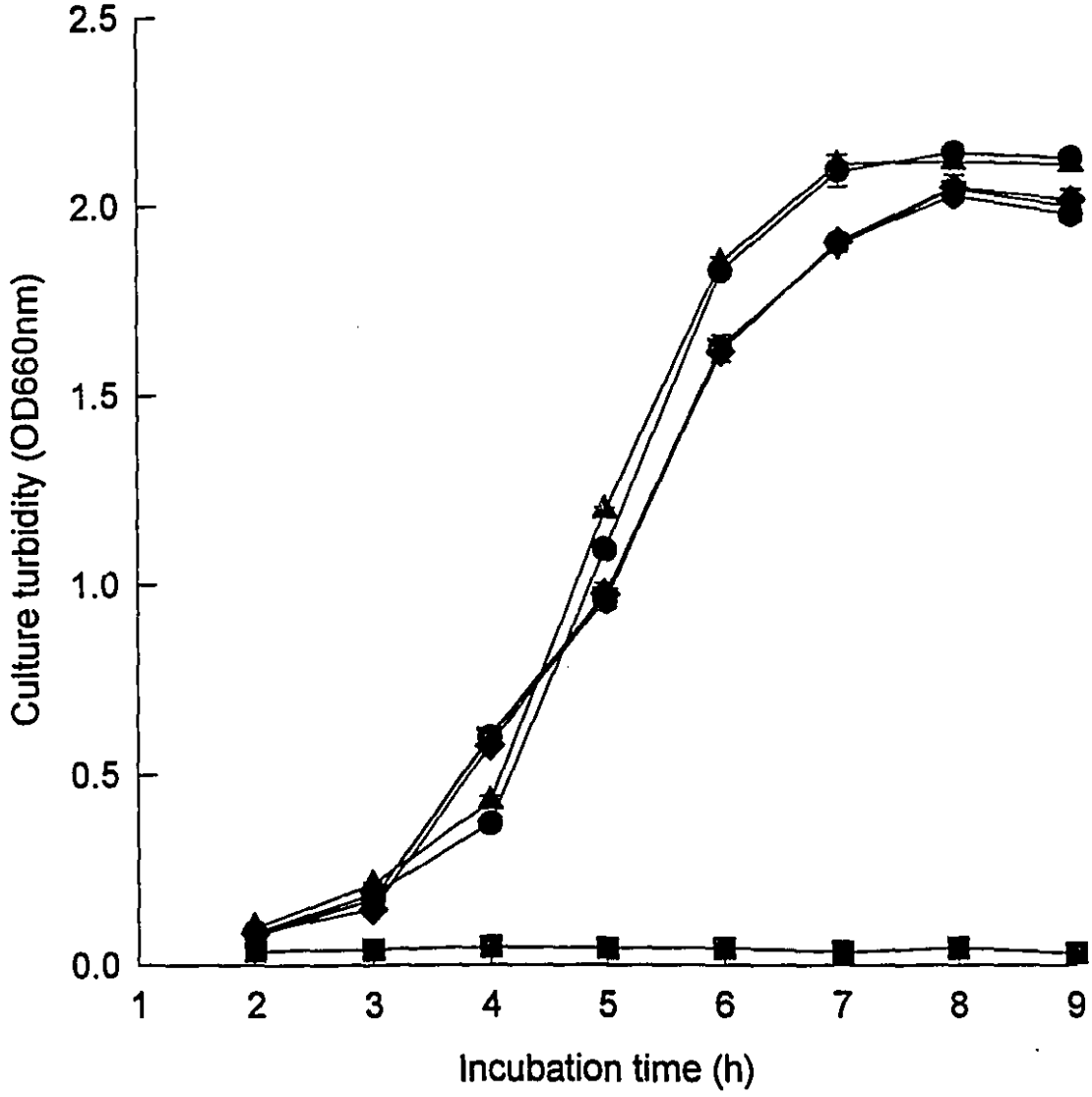

2.5
2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
1
2
3
4
5
6
7
8
9
Incubation time (h)

FIG. 3.9. Growth of *A. pleuropneumoniae* Bertschinger 2008/76 in TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and 250 μg ml^{-1} porcine haemoglobin (▼), 100 μM EDDA and 250 μg ml^{-1} bovine haemoglobin (♦), or 100 μM EDDA and 250 μg ml^{-1} human haemoglobin (⬬). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

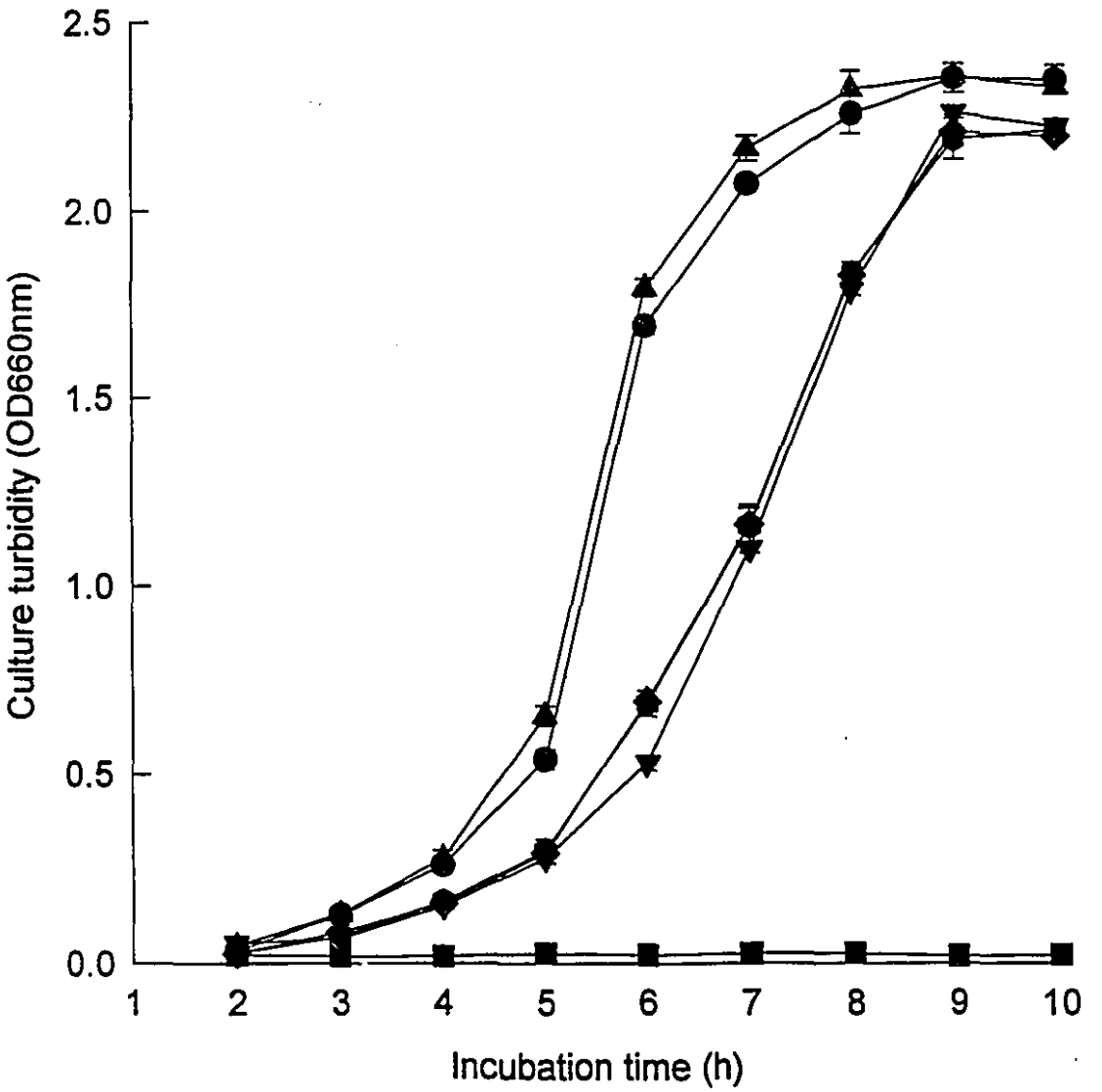

2.5
2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
1
2
3
4
5
6
7
8
9
10
Incubation time (h)

FIG. 3.10. Growth of *A. pleuropneumoniae* BC181 in TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and 250 μg ml^{-1} porcine haemoglobin (▼), 100 μM EDDA and 250 μg ml^{-1} bovine haemoglobin (♦), or 100 μM EDDA and 250 μg ml^{-1} human haemoglobin (●). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

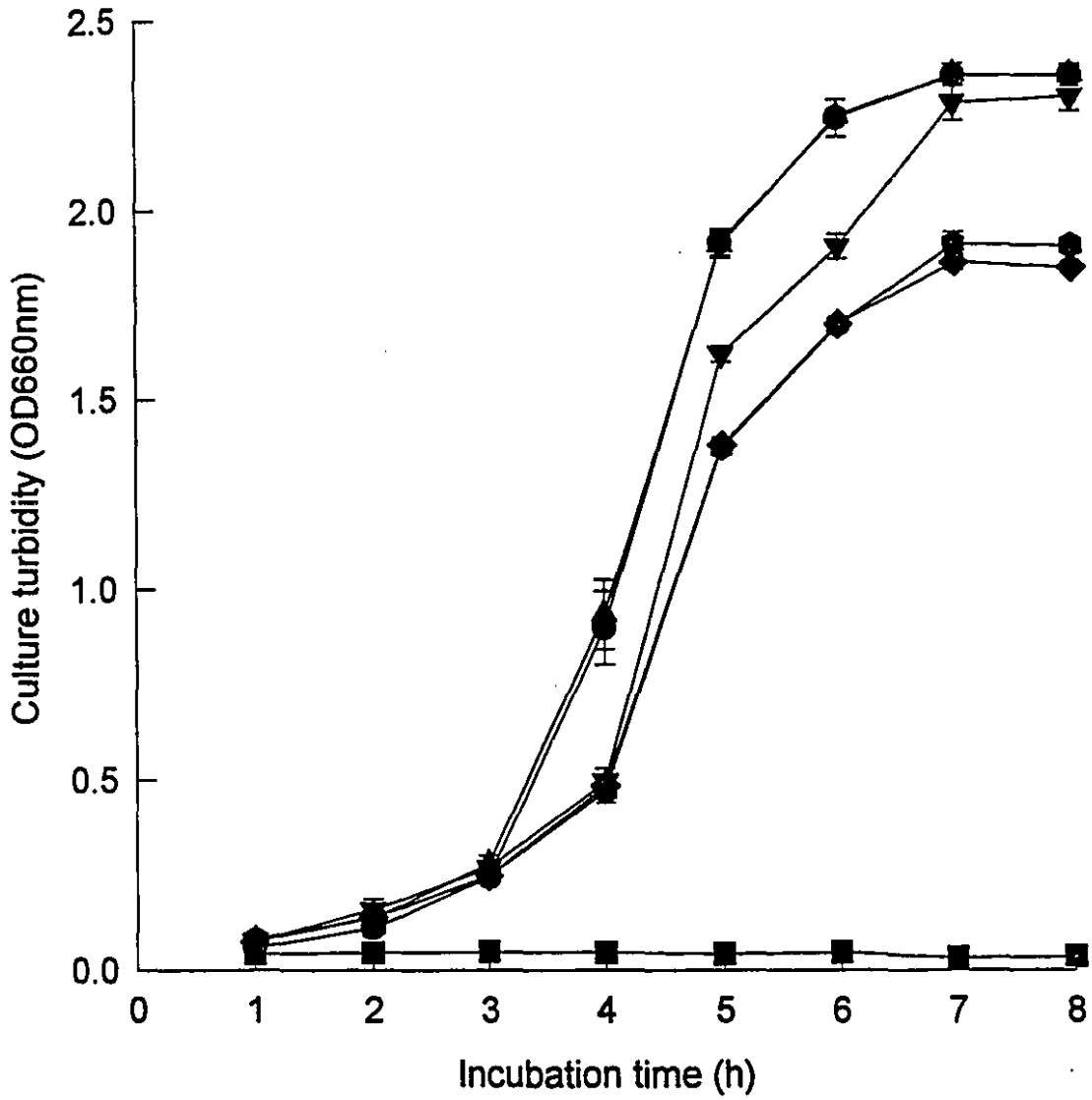

2.5
2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
0
1
2
3
4
5
6
7
8
Incubation time (h)

FIG. 3.11. Growth of *A. pleuropneumoniae* K17 in TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and 250 μg ml^{-1} porcine haemoglobin (▼), 100 μM EDDA and 250 μg ml^{-1} bovine haemoglobin (♦), or 100 μM EDDA and 250 μg ml^{-1} human haemoglobin (●). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

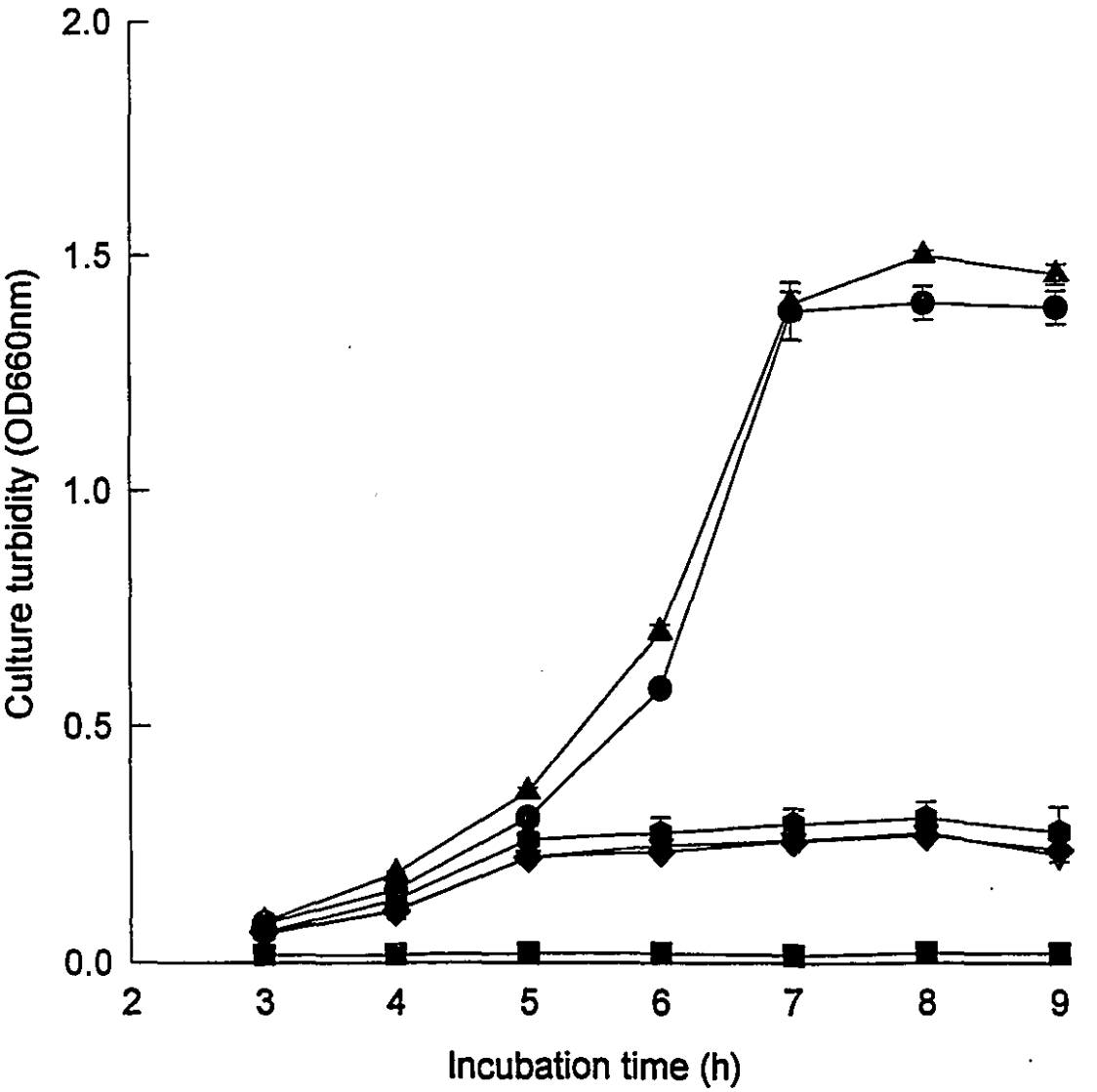

2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
2
3
4
5
6
7
8
9
Incubation time (h)

FIG. 3.12. Growth of *A. pleuropneumoniae* BC181 in TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 100 μM EDDA (■), 100 μM EDDA and 100 μM $FeCl_3$ (▲), 100 μM EDDA and a dialysis bag containing 5 mg porcine haemoglobin (▼), 100 μM EDDA and a dialysis bag containing 5 mg bovine haemoglobin (♦), or 100 μM EDDA and a dialysis bag containing 5 mg human haemoglobin (●). The dialysis bags were pierced 9 h post-inoculation. Culture turbidity values at 1-8 h are the means of triplicates and those at 24-28 h are the means of duplicates (see text). Standard error bars were plotted for all data points and may be hidden within the symbols.

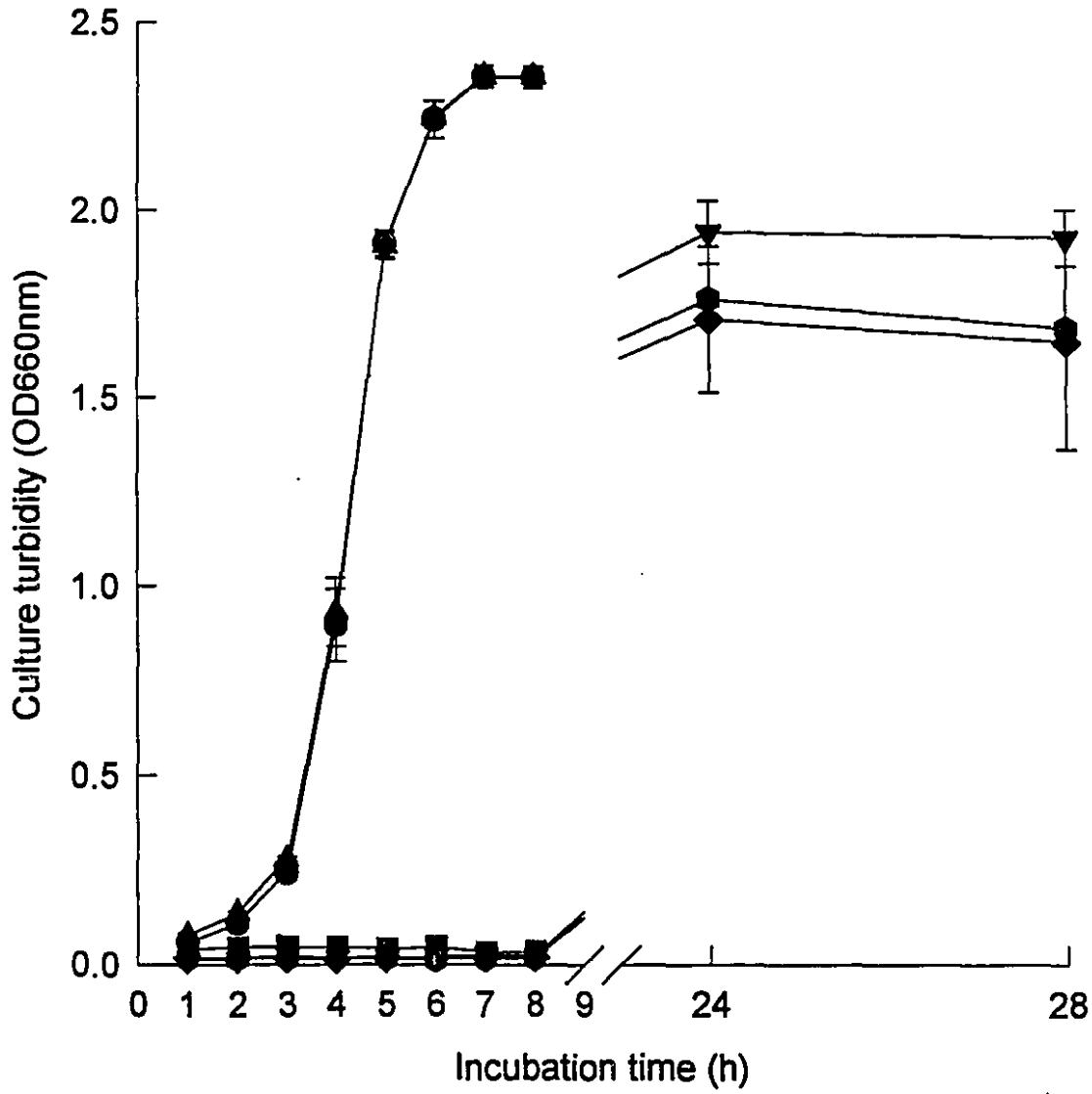

2.5
2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
0 1 2 3 4 5 6 7 8 9
24
28
Incubation time (h)

FIG. 3.13. Growth of *A. pleuropneumoniae* K17 in TYE-H plus NAD and $NaHCO_3$ (●), or TYE-H plus NAD and $NaHCO_3$ supplemented with 300 μM EDDA (■), 300 μM EDDA and 300 μM $FeCl_3$ (▲), or 300 μM EDDA and 0.82 mg haemin (▼). Culture turbidity values are the means of triplicates. Standard error bars were plotted for all data points and may be hidden within the symbols.

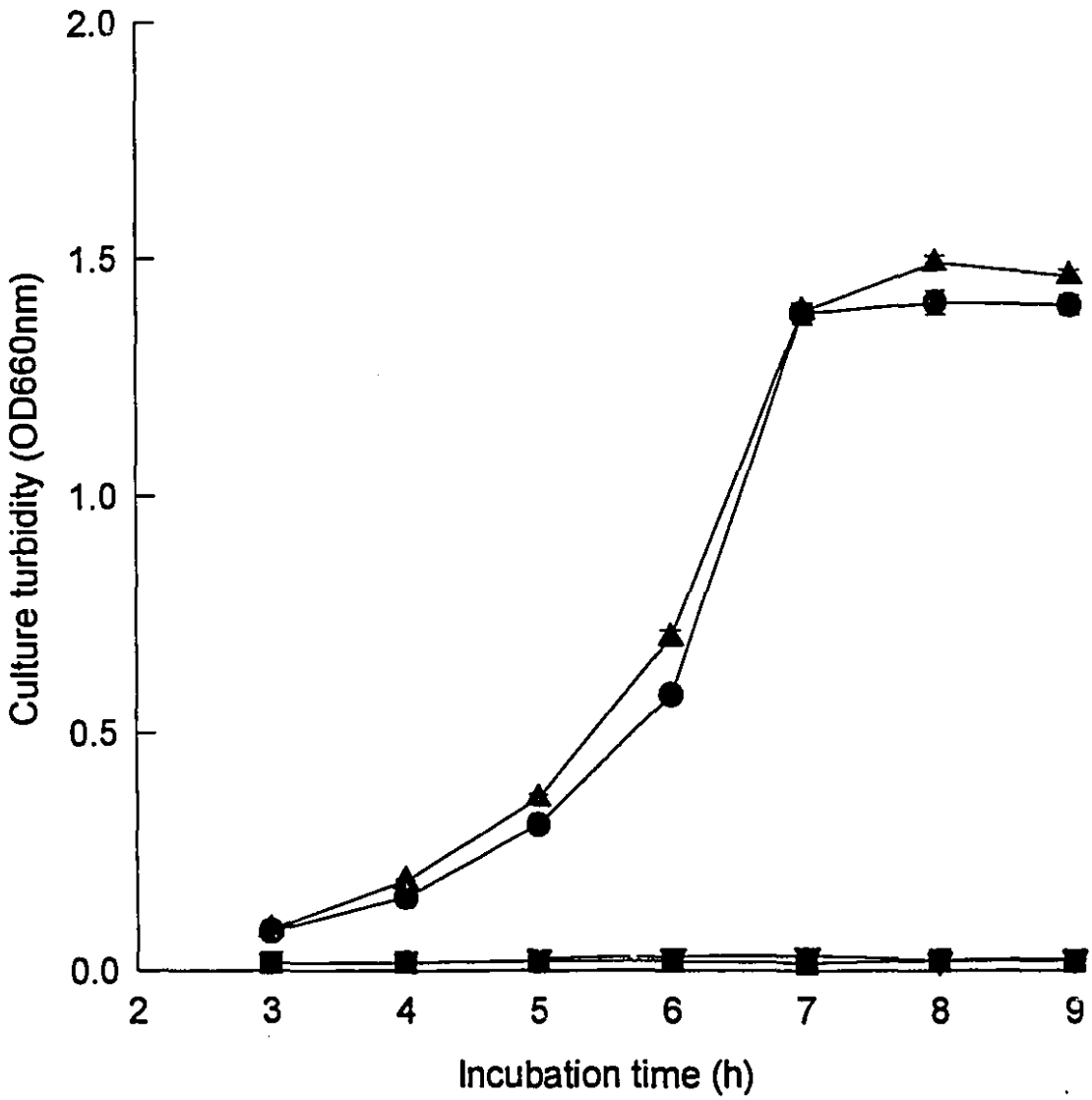

2.0
1.5
1.0
0.5
0.0
Culture turbidity (OD660nm)
2
3
4
5
6
7
8
9
Incubation time (h)

FIG. 3.14. Protein profiles of outer membrane preparations derived from *A. pleuropneumoniae* strain ATCC 27088 grown under iron-replete and iron-restricted conditions. Outer membrane proteins were separated by SDS-PAGE and visualized by silver staining. The outer membrane preparations were from organisms grown in TYE-H (iron-replete; lane A), TYE-H plus 50-60 μM EDDA (iron-restricted; lane B), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} porcine transferrin (50% iron-saturated) (lane C), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} porcine haemoglobin (lane D), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} human haemoglobin (lane E), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} bovine haemoglobin (lane F). The numbers refer to the relative molecular masses (kDa) of the indicated polypeptides.

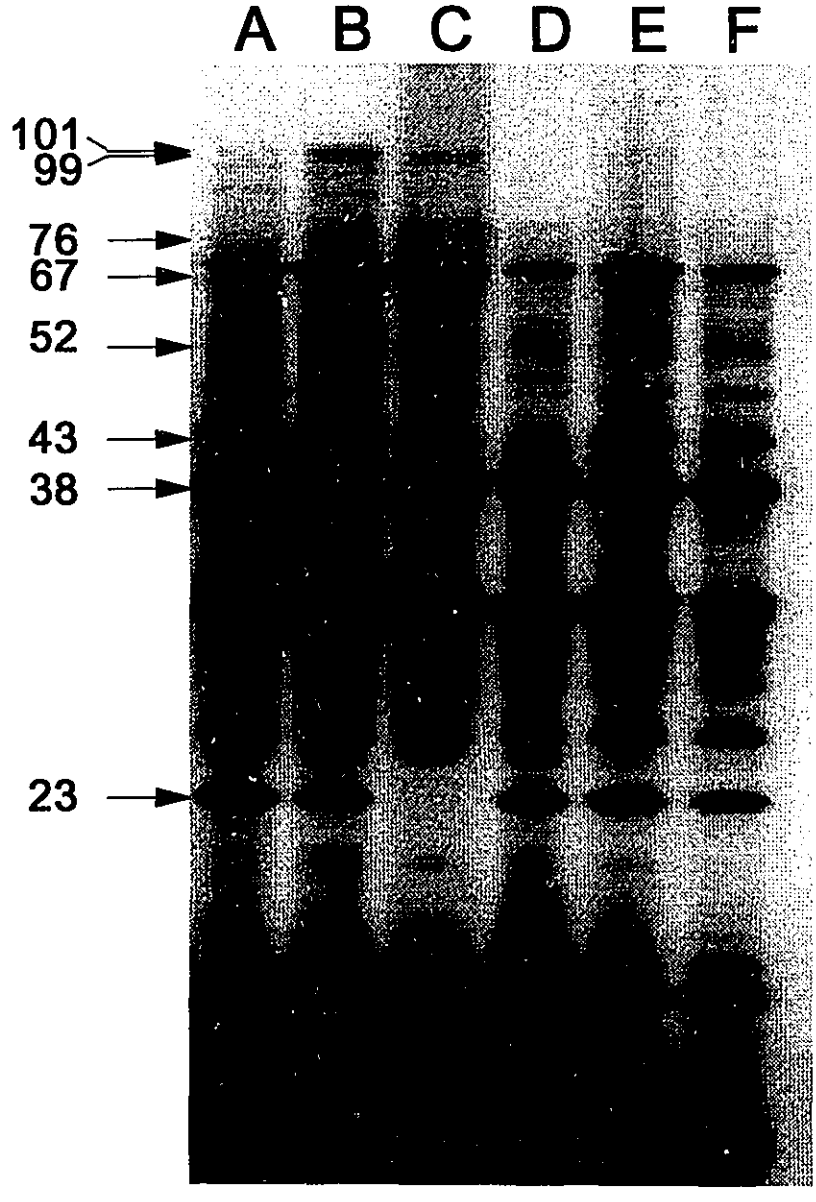

A B C D E F
101
99
76
67
52
43
38
23

FIG. 3.15. Protein profiles of outer membrane preparations derived from *A. pleuropneumoniae* strain Bertschinger 2008/76 grown under iron-replete and iron-restricted conditions. Outer membrane proteins were separated by SDS-PAGE and visualized by silver staining. The outer membrane preparations were from organisms grown in TYE-H (iron-replete; lane A), TYE-H plus 50-60 μM EDDA (iron-restricted; lane B), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} porcine transferrin (50% iron-saturated) (lane C), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} porcine haemoglobin (lane D), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} human haemoglobin (lane E), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} bovine haemoglobin (lane F). The numbers refer to the relative molecular masses (kDa) of the indicated polypeptides.

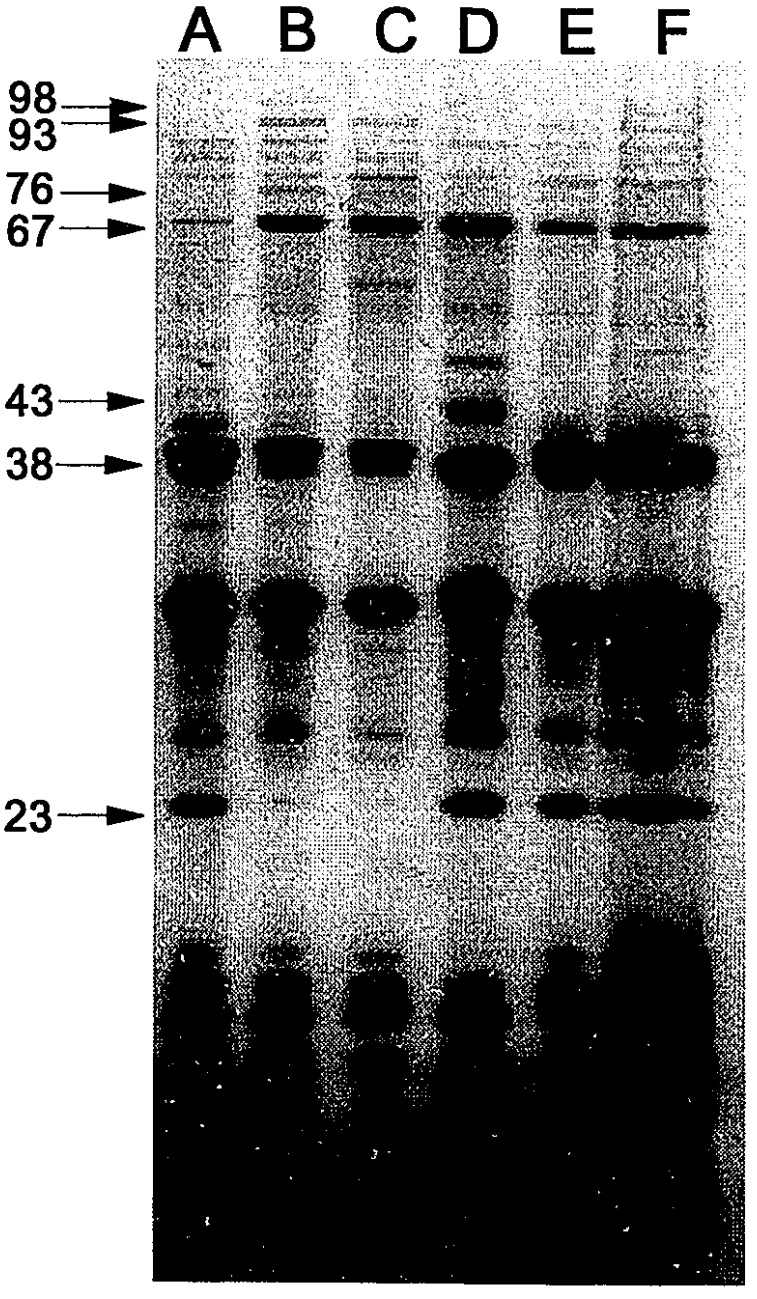
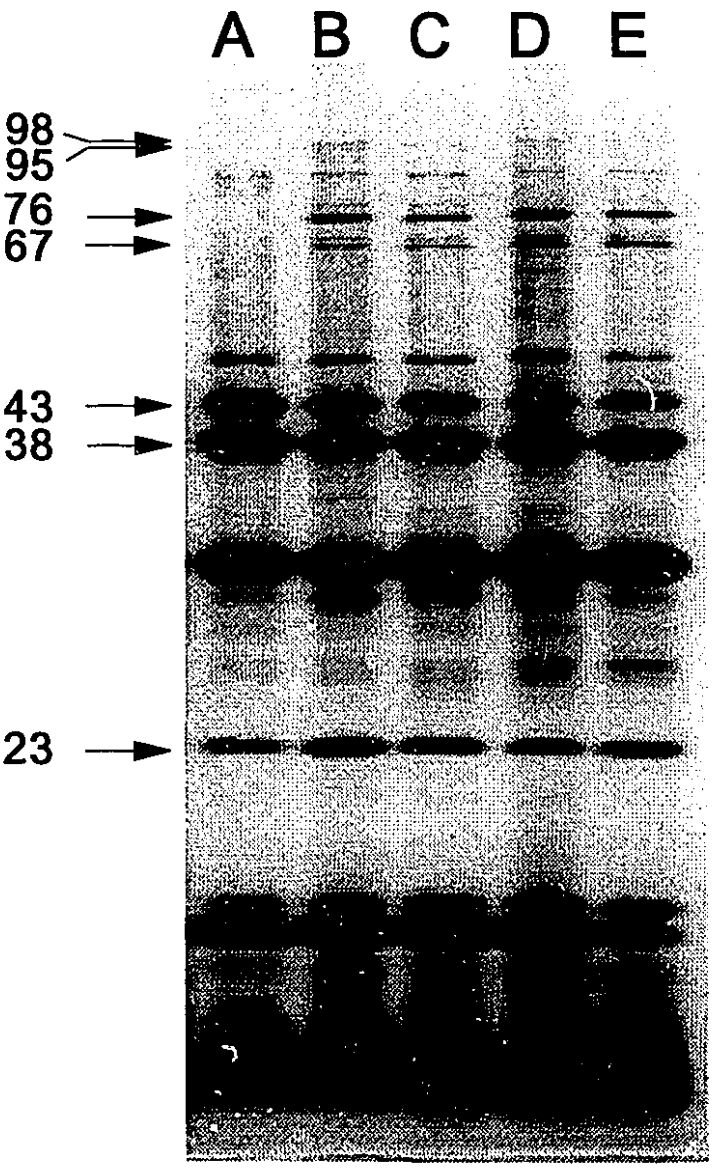

A
B
C
D
E
F
98
93
76
67
43
38
23

FIG. 3.16. Protein profiles of outer membrane preparations derived from *A. pleuropneumoniae* strain BC181 grown under iron-replete and iron-restricted conditions. Outer membrane proteins were separated by SDS-PAGE and visualized by silver staining. The outer membrane preparations were from organisms grown in TYE-H (iron-replete; lane A), TYE-H plus 50-60 μM EDDA (iron-restricted; lane B), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} porcine haemoglobin (lane C), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} human haemoglobin (lane D), TYE-H plus 100 μM EDDA and 250 μg ml^{-1} bovine haemoglobin (lane E). The numbers refer to the relative molecular masses (kDa) of the indicated polypeptides.


A
B
C
D
E
98
95
76
67
43
38
23

B), and the 98 and, barely detectable, 95 kDa polypeptides of strain BC181 (Fig. 3.16, lanes A and B).

Although differences, where apparent, between the resulting profiles (Fig. 3.14, lanes C-F; Fig. 3.15, lanes C-F; Fig. 3.16, lanes C-E) and the controls (Figs. 3.14, 3.15 and 3.16, lanes B), are for the most part, difficult to rationalize, it is notable that the 99 kDa polypeptide of strain ATCC 27088 was identified in the OMP profile when porcine transferrin was the iron source (Fig. 3.14, lane C) but was barely detectable when the iron source was a haemoglobin (Fig. 3.14, lanes D-F). Similarly, the 93 kDa polypeptide of strain Bertschinger 2008/76 was present when the organisms were grown with porcine transferrin (Fig. 3.15, lane C) but was barely detectable at least when the iron source was porcine haemoglobin (Fig. 3.15, lanes D-F).

3.5 Discussion

In the present experiments, interest was centered on the acquisition of iron, from various transferrins, lactoferrins and haemoglobins, by four strains of *A. pleuropneumoniae* that differ with respect to virulence and/or animal source. Initially, the transferrin specificities of the organisms were investigated; the results of these experiments showed that like the type strain (ATCC 27088) (Niven *et al.*, 1989; this study), and all other biotype 1 strains examined to date (Gonzalez *et al.*, 1990; Morton and Williams, 1990), the reference strain of biotype 2 (Bertschinger 2008/76) could acquire iron only from porcine transferrin (Fig. 3.2A). In contrast, strain BC181 (biotype 1), which is less virulent than the type strain (Rosendal *et al.*, 1985), and strain K17 (biotype 1), which was isolated from a lamb (Biberstein *et al.*, 1977), failed to use porcine transferrin as an iron source for growth (Figs. 3.3A and 3.4A). None of the strains under study could use ovine, bovine or human transferrin, or porcine or ovine lactoferrin (Figs. 3.1, 3.2, 3.3 and 3.4; A and B). Since siderophores are non-specific with respect to iron acquisition from transferrin and lactoferrin (see e.g., Griffiths, 1987*b*), these results indicate that none of these strains produces a conventional siderophore. When grown under iron-restricted conditions *in vitro*, several pathogens do not produce siderophores but can still acquire iron from host iron-binding/containing proteins (section 1.3.2). *A. pleuropneumoniae* ATCC 27088 produces neither hydroxamate nor phenolate siderophores (Niven *et al.*, 1989). Moreover, strains ATCC 27088 and Bertschinger

2008/76 could not acquire iron from porcine transferrin when the transferrin was contained in a dialysis bag (Figs. 3.5 and 3.6). These results also indicate that iron acquisition from porcine transferrin requires direct contact between the organisms and the protein, and this, in turn, suggests the existence of specific receptors. It would appear that similar receptors may also be involved in iron acquisition from haemoglobin since none of the organisms could use the haemoglobin if it was contained in a dialysis bag (Figs. 3.5, 3.6 and 3.12). However, the haemoglobin receptors appear to be less specific than the transferrin receptors since good growth of the organisms was observed with porcine, bovine or human haemoglobin.

While strains ATCC 27088 and Bertschinger 2008/76 appear to possess a similar complement of iron acquisition mechanisms, strain BC181 and K17, both of which can seemingly cause disease in pigs (Biberstein *et al.*, 1977; Rosendal *et al.*, 1985), are clearly less capable of obtaining iron from host proteins. To what extent this might account for the lower virulence of strain BC181, with respect to the type strain (Rosendal *et al.*, 1985), is difficult to evaluate, however, since virulence is typically multifactorial in nature. On the other hand, these results do suggest that the ability to use porcine transferrin (or lactoferrin) as an iron source may not be essential for a strain of *A. pleuropneumoniae* to induce disease in swine.

Several biotype 1 strains of *A. pleuropneumoniae* are known to produce IRMPS in response to growth under iron-restricted conditions (Deneer and Potter, 1989*b*, Niven *et al.*, 1989; Gonzalez *et al.*, 1990) and some of these IRMPs have been identified as

porcine transferrin-binding polypeptides (González *et al.*, 1990; Ricard *et al.*, 1991). To determine if the ability of *A. pleuropneumoniae* to obtain iron from porcine transferrin and the haemoglobins reflected the ability to produce particular IRMPs, outer membranes from organisms grown under iron-restricted conditions in the presence of iron-containing proteins (Figs. 3.14, 3.15 and 3.16) were analyzed by means of SDS-PAGE. Note that strains ATCC 27088 and Bertschinger 2008/76 could utilize porcine transferrin and haemoglobin while strain BC181 could use haemoglobin but not transferrin and strain K17 could use neither transferrin nor haemoglobin nor haemin. Despite these differences, all four strains produced IRMPs that could, potentially, serve as receptor components (Figs. 3.14, 3.15, 3.16 and 2.10). In particular, strain ATCC 27088 produced a 99 kDa IRMP; this polypeptide is known to represent the transferrin-binding protein 1 (TBP1) of this organism (Ricard *et al.*, 1991) and by analogy, it would appear that the 93, 95 (Figs. 3.15 and 3.16) and 92 kDa (Fig. 2.10) IRMPs produced by strains Bertschinger 2008/76, BC181 and K17, respectively, may also represent TBPs1. Note that the existence of the 95 and 92 kDa polypeptides and the lack of transferrin utilization are not mutually exclusive since the acquisition and subsequent utilization of transferrin-bound iron presumably involves several surface-located proteins (Williams and Griffiths, 1992); in effect, the inability of a strain to use porcine transferrin as an iron source could reflect the lack of, or a defect in, any component of the uptake system and not just a deficiency in a receptor component.

The 101 kDa (strain ATCC 27088), 98 kDa (Bertschinger 2008/76 and BC181)

and 96 kDa (strain K17) IRMPs (Figs. 3.14, 3.15, 3.16 and 2.10) are also intriguing, especially in light of recent studies on the existence of haemin- and/or haemoglobin-binding proteins in the pathogenic neisseriae and *H. influenzae* (Lee, 1992 *a*,*b*; Lee and Hill, 1992; Martel and Lee, 1994); these proteins appear to be IRMPs that recognize the haem moiety, and affinity procedures have allowed the isolation of a 39.5 kDa polypeptide from *H. influenzae* (Lee 1992*b*), 97 kDa and 44 kDa polypeptides from *N. gonorrhoeae* (Lee 1992*a*) and 97 kDa and 50 kDa polypeptides from *N. meningitidis* (Lee, 1994) (cited by Martel and Lee, 1994). It is tempting to speculate, therefore, that the 101 kDa, 98 kDa and 96 kDa polypeptides of the *A. pleuropneumoniae* strains may also represent haemin and/or haemoglobin receptor components, again, functional or otherwise, since strain K17 essentially failed to use haemoglobin, or even haemin, as an iron source.

Chapter 4. Isolation and identification of porcine transferrin-binding polypeptides from *A. pleuropneumoniae*

Rationale

In chapter 2, strains ATCC 27088, Bertschinger 2008/76, BC181 and K17 of *A. pleuropneumoniae* were shown to produce IRMPs. In chapter 3, iron acquisition from iron-binding/containing proteins by the four strains was investigated and it was observed that only strains ATCC 27088 and Bertschinger 2008/76 could use porcine transferrin. These results would suggest that the 95 kDa and 92 kDa IRMPs produced by strains BC181 and K17 are not involved in the uptake of iron from porcine transferrin. On the other hand, it is possible that these IRMPs in strains BC181 and K17 represent defective transferrin receptors or alternatively, it is possible that some other components of the iron-uptake systems of these organisms are defective or absent. With these points in mind, the purpose of this study was to attempt the isolation of transferrin-binding polypeptides from all four strains of *A. pleuropneumoniae*.

Currently, there is no effective vaccine against swine pleuropneumonia and the prospect of using TBPs as vaccine components shows promise. Hence, an additional purpose of this study was to determine the N-terminal amino acid sequence of TBP1 of strain ATCC 27088 with a view to the commercial synthesis of an oligonucleotide probe

to be used in subsequent cloning and characterization of receptor genes.

This chapter is a component of the research publication:

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. In press.

4.1 Summary

Total membranes derived from strains ATCC 27088, Bertschinger 2008/76, BC181 and K17 of *Actinobacillus pleuropneumoniae* grown under iron-replete and iron-restricted conditions were subjected to an affinity isolation technique based on biotinylated porcine transferrin plus streptavidin-agarose. The following polypeptides were isolated: 99 kDa and 64 kDa from strain ATCC 27088; 93 kDa from strain Bertschinger 2008/76; 95 kDa (trace amounts) and 60 kDa from strain BC181; none from strain K17. From these results it can be concluded that these polypeptides represent porcine transferrin-binding polypeptides (TBPs). The 99 kDa polypeptide from the type strain was purified by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and subjected to N-terminal amino acid sequence analysis. The N-terminal amino acid sequence was identified as Glu-Gln-Ala-Val-Gln-Leu-Asn-Asp-Asp-Tyr-Gly-Thr-Thr-.

4.2 Introduction

A variety of host-specific pathogens (see e.g., Williams and Griffiths, 1992), including, most recently, *Moraxella catarrhalis* (Campagnari *et al.*, 1994), have been shown to acquire iron from specific transferrins and/or lactoferrins by mechanisms independent of siderophore production, and with many of these organisms, affinity methods have allowed the isolation of IRMPs that appear to function as specific receptor components (Ogunnariwo and Schryvers, 1992; Williams and Griffiths, 1992).

Several biotype 1 strains of *A. pleuropneumoniae* can acquire iron from, specifically, porcine transferrin (Morton and Williams, 1989; Niven *et al.*, 1989; Morton and Williams, 1990; Schryvers and Gonzalez, 1990; Gonzalez *et al.*, 1990). Iron acquisition has been shown to involve two IRMPs, one of high molecular mass (99-105 kDa) and one of lower molecular mass (56-64 kDa), referred to often as TBP1 (porcine transferrin-binding protein 1) and TBP2, respectively.

Like *A. pleuropneumoniae* strain ATCC 27088, strains Bertschinger 2008/76, BC181 and K17 have been shown to produce IRMPs that have the potential to serve as transferrin receptors (Chapter 2). However, of the four strains studied (Chapter 3), only strains ATCC 27088 and Bertschinger 2008/76 could use porcine transferrin as an iron source and strain BC181 could utilize it to only a very limited extent if at all. The purpose of the present study was to attempt the isolation and identification of TBPs from all four strains and to determine whether the IRMPs (Chapter 2) were involved in the acquisition

of transferrin-bound iron.

The TBP1 of strain ATCC 27088 (Ricard *et al.*, 1991; this study) has been isolated using affinity chromatography and is the 99 kDa polypeptide. This polypeptide is of interest because of its potential as a vaccine component. With this in mind, an additional purpose of this study was to determine the N-terminal amino acid sequence of the TBP1 of strain ATCC 27088 followed by synthesis of an oligonucleotide probe, in order to initiate molecular biological studies pertaining to the cloning and sequencing of receptor genes.

4.3 Materials and Methods

4.3.1 Organisms

A. pleuropneumoniae strains ATCC 27088, Bertschinger 2008/76, BC181 and K17, were stored, and used to prepare frozen inocula, as described previously (section 2.3.1).

4.3.2 Chemicals

Porcine serum albumin (fraction V powder) was from Sigma. Biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (biotin-X-NHS) and *N*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate (Zwittergent 3-14) were purchased from Calbiochem. Streptavidin-agarose was from BRL. CNBr-activated Sepharose 4B was from Pharmacia. The sources of all other chemicals were as described previously (sections 2.3.2 and 3.3.2).

4.3.3 Growth conditions and preparation of membrane fractions

The growth of *A. pleuropneumoniae* under iron-replete and iron-restricted conditions and the preparation of outer membrane and total membrane fractions were as

described previously (section 2.3.3).

4.3.4 Isolation and purification of porcine transferrin

Porcine transferrin was isolated and purified from porcine serum according to the method of Niven *et al.* (1989), as described in section 3.3.3.

4.3.5 Preparation of iron-binding proteins

Porcine and human transferrins were saturated with iron and then dialysed according to the method of Caldwell and Archibald (1987), as described in section 3.3.5.

4.3.6 Preparation of biotinylated proteins

Porcine transferrin, human transferrin and porcine albumin were biotinylated according to the method described for lactoferrin by Schryvers and Morris (1988*b*) and modified by Ricard *et al.* (1991). Iron-saturated transferrins (5 mg protein ml^{-1}) and porcine albumin (5 mg ml^{-1} in 50 mM Tris-HCl, pH 7.5) were dialysed against 50 mM Tris-HCl, pH 7.5 (2 l; 4°C; overnight). 160 µl of biotin-X-NHS solution (7.2 mg dissolved in 500 µl dimethylformamide) [16 µl $(mg\ protein)^{-1}$] was added to each dialysate. The resulting mixtures were incubated for 2 h at 4°C (with gentle agitation). Reactions were

terminated by the addition of 1 ml glycine (10 mg ml^{-1}) to each mixture, and incubation was continued for a further 2 h. The biotinylated proteins were then dialysed overnight against 10 mM NaCl, 50 mM Tris-HCl, pH 7.5 (1 l, with three changes; 4°C) and stored as necessary, at -20°C.

4.3.7 Isolation of transferrin-binding polypeptides using biotinylated transferrin

Transferrin-binding polypeptides were isolated using the batch affinity procedure and wash system 3 developed by Schryvers and Morris (1988*b*), as modified by Ricard *et al.* (1991). Briefly, total membranes (750 µg protein) from *A. pleuropneumoniae* grown under iron-replete or iron-restricted conditions and 200 µg of biotinylated protein (porcine or human transferrin, or porcine albumin) were mixed and the final volume brought to 1 ml with 100 mM NaCl, 50 mM Tris-HCl, pH 8.0; no biotinylated proteins were added to the control samples. Samples were incubated (1 h; 37 °C; gentle agitation) and then centrifuged (16,000 x g, 10 min; Eppendorf microcentrifuge). The supernatant fractions were discarded and each pellet was resuspended in 1 ml 0.75% (w/v) Sarkosyl in 100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0. 100 µl of streptavidin-agarose (diluted 2-fold with distilled water) was added to each tube and the samples were mixed well and incubated (1 h, 20 °C). The samples were then centrifuged (750 x g, 3 min, 20 °C) and the supernatant fractions were carefully removed. Affinity resin pellets were washed (by successive cycles of resuspension in buffer, incubation for 10 min at 20 °C, and

centrifugation) using the wash system of Schryvers and Morris (1988*b*) and consisting of three washes in 0.5% Sarkosyl, 1 M NaCl, 10 mM EDTA, 250 mM guanidine-HCl, 50 mM Tris-HCl, pH 8.0, followed by one wash in 1 M NaCl, 250 mM guanidine-HCl, 50 mM Tris-HCl, pH 8.0 and a final wash in 100 mM NaCl, 50 mM Tris-HCl, pH 8.0.

To identify the affinity-isolated polypeptides, the washed affinity resin pellets were resuspended in 200 µl of a sample buffer containing 2% (w/v) SDS, 30% (v/v) glycerol and 0.1% (w/v) bromophenol blue in 200 mM Tris-HCl, pH 6.8. The samples were boiled for 5 min, cooled on ice, and centrifuged (750 x g, 3 min). The supernatant fractions were recovered and transferred to clean tubes. The supernatant was mixed with 2-mercaptoethanol (to 1.4 M), and following incubation (15 min, 20 °C), the samples were mixed well and 50-µl aliquots were subjected to SDS-PAGE (as previously; section 2.3.4). The separated polypeptides were visualised by silver staining according to the method of Harlow and Lane (1988). In all other cases, SDS-PAGE was performed as described earlier (section 2.3.4) except that protein loading was compatible with silver staining and separated polypeptides were visualised accordingly (Harlow and Lane, 1988).

4.3.8 Preparation of Sepharose-coupled porcine transferrin

Sepharose-coupled porcine transferrin was prepared according to the instructions supplied with the CNBr-activated Sepharose 4B. Briefly, CNBr-activated Sepharose 4B (3.2 g) was rehydrated and washed of additives in a sintered glass funnel using 1 mM HCl

(800 ml). The rehydrated gel was rinsed with 60 ml of 500 mM NaCl, 100 mM $NaHCO_3$, pH 8.3 with NaOH (coupling buffer) and transferred quickly to a 50-ml flask containing 95 mg of iron-saturated porcine transferrin in 24 ml coupling buffer. This resulting mixture was incubated for 2 h at 20°C, with gentle agitation on a gyratory shaker, and unbound ligand was removed by rinsing the Sepharose-coupled porcine transferrin with 60 ml of coupling buffer. The Sepharose-coupled porcine transferrin was transferred to 24 ml of blocking buffer (500 mM NaCl, 200 mM glycine, pH 8.0 with NaOH), and incubated for a further 2 h. The slurry was used to pack a small column (1 x 10 cm) and excess unbound ligand was removed by three cycles of washing, each consisting of 30 ml of 500 mM NaCl, 100 mM sodium acetate, pH 4.0 with HCl, followed by 30 ml of 500 mM NaCl, 100 mM Tris-HCl, pH 8.0. Three bed vols of a solution of $FeCl_3$ (5 μg Fe ml^{-1}) in 200 mM Tris, 20 mM $NaHCO_3$, pH 7.4 was passed through the column to resaturate the porcine transferrin with iron.

4.3.9 Isolation of transferrin-binding polypeptides using Sepharose-coupled porcine transferrin

The procedure was essentially as described by Ricard *et al.* (1991) with some modifications. Total membranes (60 mg protein in 2 ml of 10 mM HEPES, pH 7.4) from organisms grown under iron-replete or iron-restricted conditions were mixed with 41 ml of 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, containing 1.8 g (wet weight) of Sepharose-

coupled porcine transferrin, and the mixture was incubated for 1 h at 37°C, with gentle agitation. Zwittergent (3-14) and EDTA (final concentrations of 2% (w/v) and 10 mM respectively) were added to the mixture and incubation was continued for 1 h at 20°C. The beads were collected in a Poly-Prep chromatography column (10 ml; Bio-Rad) and rinsed with 3 bed vols of 100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, containing 0.5% (w/v) Zwittergent (100 mM NaCl-rinse buffer). Bound proteins were eluted with 6 bed volumes of 3 M NaCl-rinse buffer. Fractions (1 ml) were collected and elution was monitored by recording the absorbance of each fraction at 280 nm; protein-containing fractions were pooled and dialysed overnight against 50 mM Tris-HCl, pH 6.8, containing 0.05% (w/v) Zwittergent (2 l; 4°C). The beads of Sepharose-coupled porcine transferrin (from which proteins had been eluted using 3 M NaCl-rinse buffer) were transferred to Eppendorf tubes and washed 3-4 times using distilled water (to remove NaCl) followed by centrifugation (6,000 x g, 3 min, 4°C).

To identify the isolated polypeptides, the above fractions (pooled/dialysed samples) were mixed with sample buffer (section 2.3.4) and appropriate volumes subjected to SDS-PAGE (as previously; section 2.3.4); when the Sepharose preparation was the sample to be analyzed, sample buffer was added to the beads and the resulting suspension was then boiled for 5 min and centrifuged (6,000 x g, 3 min, 4°C). The supernatant was analyzed by SDS-PAGE (as above). The separated polypeptides were visualized by means of silver staining according to the method of Harlow and Lane (1988).

Pooled/dialysed fractions that contained the desired polypeptides were

concentrated by lyophilisation (48 h) and then resuspended in a small volume (100 µl) of 0.001% SDS; this material and the material recovered from the washed and boiled Sepharose beads were subjected to SDS-PAGE (~60 h; 12%, w/v, acrylamide separating gels), essentially as described earlier (section 2.3.4). The separated polypeptides were transferred electrophoretically onto PVDF membrane as described below.

4.3.10 Electroblotting

The procedure followed was that described by Niven *et al.* (1989). Following electrophoretic separation, polypeptides were transferred electrophoretically to PVDF membrane (0.2 µm pore size, Bio-Rad Labs, Richmond, CA) which was pre-soaked for 15 min in transfer buffer (4°C) containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. Gels were also pre-soaked in the transfer buffer (4°C) for 15 min and transfer was accomplished using a Transblot cell (Bio-Rad; 75 V for 2 h and then 50 V for 18 h) packed on ice.

Following electroblotting, the PVDF membrane was washed twice with transfer buffer (4°C), and stained for 2 min with 0.1% Coomassie Brilliant Blue R250 in 50% methanol. The membrane was de-stained with 50% methanol, 10% acetic acid, followed by 2-3 washes with distilled water (4°C). The membrane was stored in distilled water (overnight, 4°C), and submitted for commercial N-terminal amino acid sequence analysis. The analysis was conducted by the Sheldon Biotechnology Centre, McGill University.

4.4 Results and Discussion

4.4.1 Isolation and identification of porcine transferrin-binding polypeptides

Strains ATCC 27088, Bertschinger 2008/76, BC181 and K17 of *A. pleuropneumoniae* produce IRMPs (Chapter 2). The 99 kDa polypeptide of strain 27088 is known to represent the TBP1 of this organism, and by analogy, it would seem reasonable to conclude that the 93, 95 and 92 kDa polypeptides of strains Bertschinger 2008/76, BC181 and K17, respectively, may also represent TBPs1. These polypeptides could, potentially, serve as transferrin receptor components (Figs. 4.1, 4.2, 4.3, and 4.4; lanes A and B), despite the differences in the abilities of the four strains to obtain iron from porcine transferrin (Chapter 3).

Total membranes from the four strains of *A. pleuropneumoniae* were subjected to an affinity isolation procedure based on biotinylated porcine transferrin plus streptavidin-agarose (Ricard *et al.*, 1991). In keeping with the results of Ricard *et al.* (1991), two putative TBPs (99 kDa and 64 kDa) were isolated from total membranes of strain ATCC 27088 grown under iron-restricted conditions (Fig. 4.1, lane C). Neither of these two polypeptides was isolated in the absence of biotinylated porcine transferrin (Fig. 4.1, lane F) nor were they when the biotinylated porcine transferrin

FIG. 4.1. Identification of transferrin-binding polypeptides isolated from total membranes of *A. pleuropneumoniae* ATCC 27088. Outer membrane proteins, prepared from either iron-replete cells (lane A) or iron-restricted cells (lane B), and affinity-isolated polypeptides (lanes C-G) were separated by SDS-PAGE and visualised by silver staining. The latter polypeptides were affinity-isolated from total membranes prepared from iron-restricted cells (lanes C-F) or iron-replete cells (lane G) using either biotinylated porcine transferrin (lanes C and G), biotinylated porcine albumin (lane D), biotinylated human transferrin (lane E) or no biotinylated protein (lane F). The numbers refer to the relative molecular masses (kDa) of the indicated polypeptides.

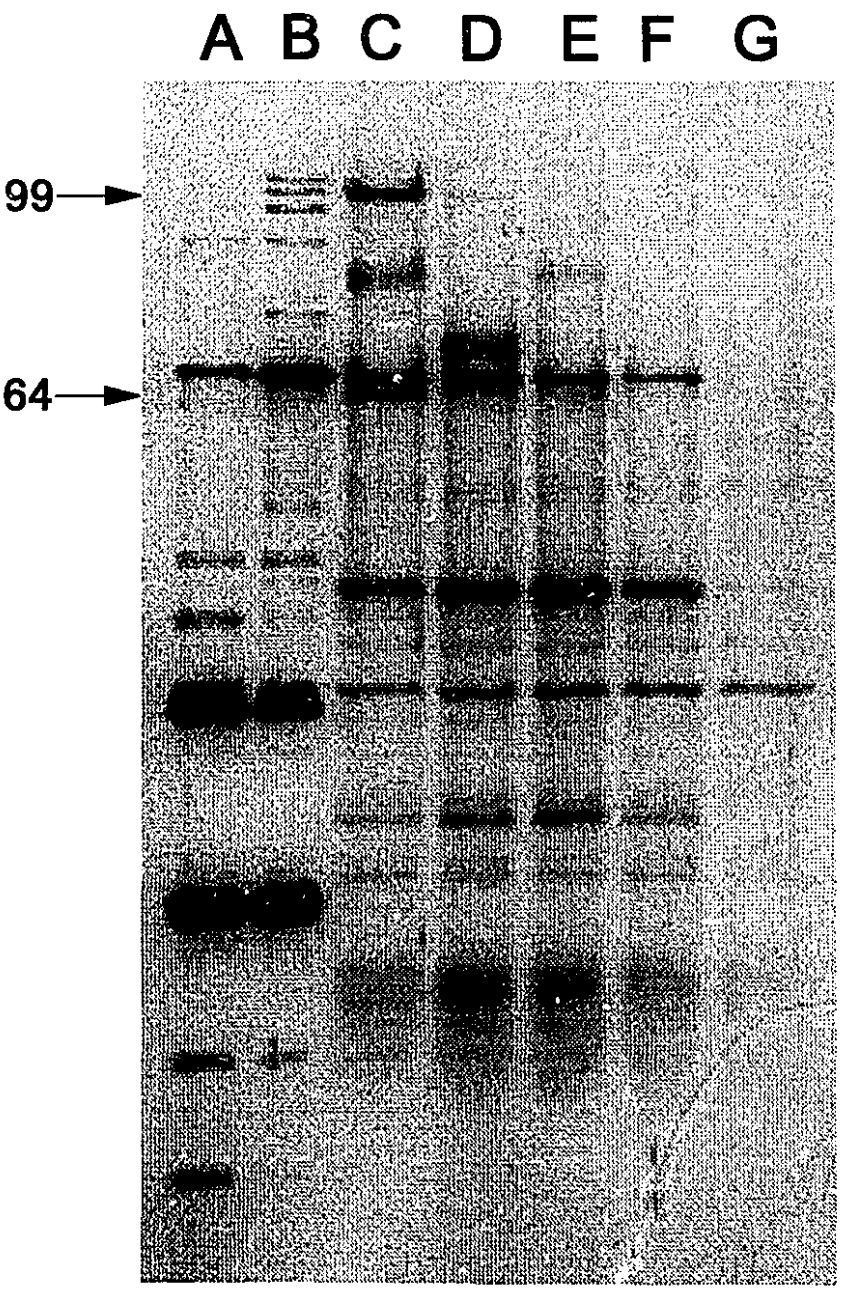

A B C D E F G
99
64

FIG. 4.2. Identification of transferrin-binding polypeptides isolated from total membranes of *A. pleuropneumoniae* Bertschinger 2008/76. Outer membrane proteins, prepared from either iron-replete cells (lane B) or iron-restricted cells (lane A), and affinity-isolated polypeptides (lanes C-G) were separated by SDS-PAGE and visualised by silver staining. The latter polypeptides were affinity-isolated from total membranes prepared from iron-restricted cells (lanes C-F) or iron-replete cells (lane G) using either biotinylated porcine transferrin (lanes C and G), biotinylated porcine albumin (lane D), biotinylated human transferrin (lane E) or no biotinylated protein (lane F). The number refers to the relative molecular mass (kDa) of the indicated polypeptide.

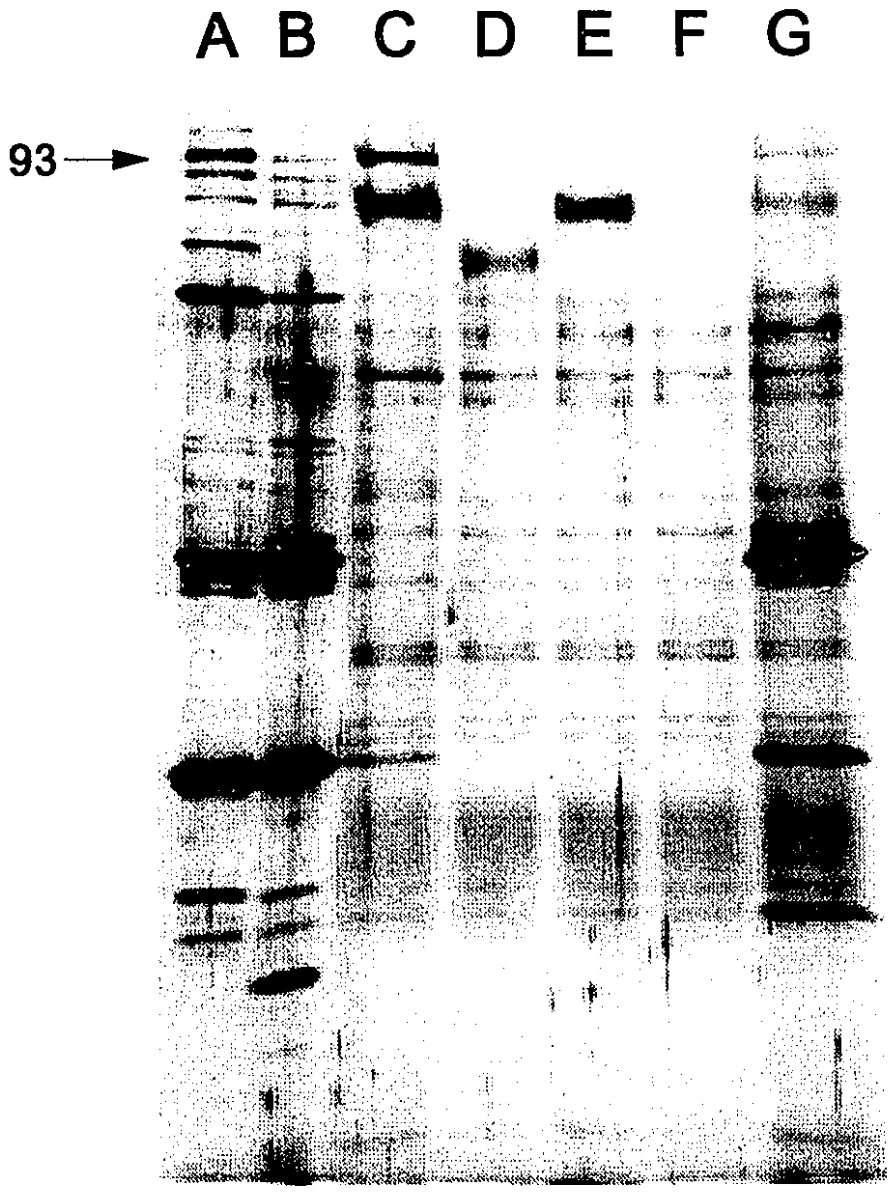

A B C D E F G
93

FIG. 4.3. Identification of transferrin-binding polypeptides isolated from total membranes of *A. pleuropneumoniae* BC181. Outer membrane proteins, prepared from either iron-replete cells (lane A) or iron-restricted cells (lane B), and affinity-isolated polypeptides (lanes C-G) were separated by SDS-PAGE and visualised by silver staining. The latter polypeptides were affinity-isolated from total membranes prepared from iron-restricted cells (lanes C-F) or iron-replete cells (lane G) using either biotinylated porcine transferrin (lanes C and G), biotinylated porcine albumin (lane D), biotinylated human transferrin (lane E) or no biotinylated protein (lane F). The numbers refer to the relative molecular masses (kDa) of the indicated polypeptides.

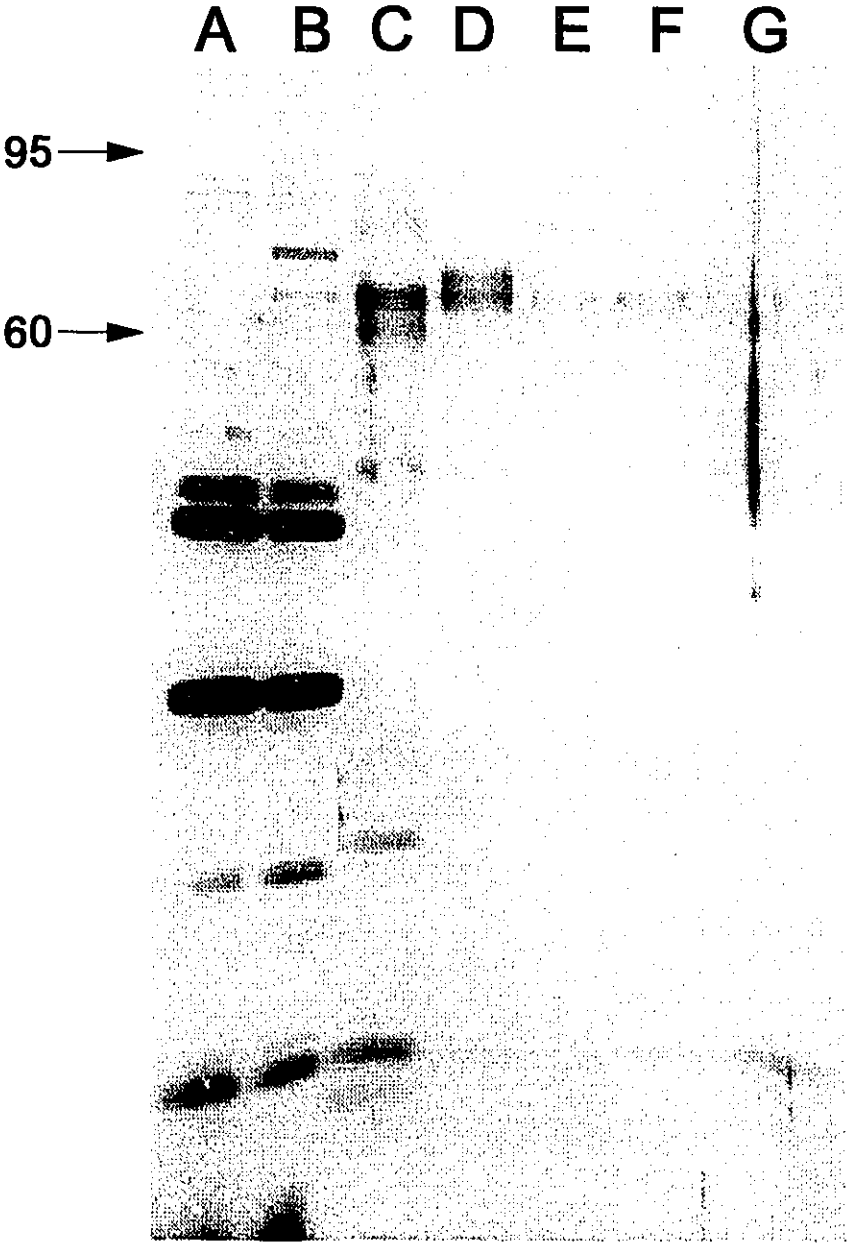
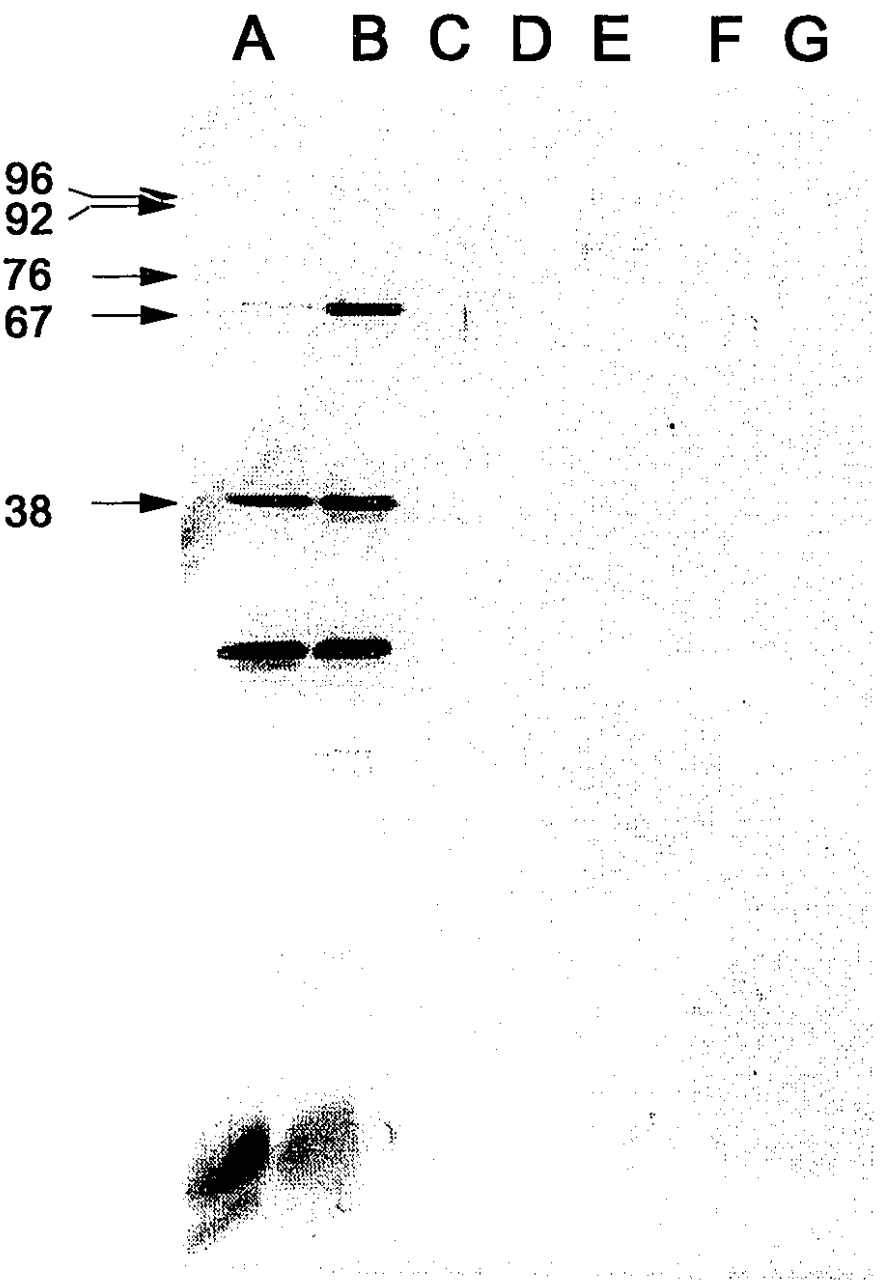

A B C D E F G
95
60

FIG. 4.4. Identification of transferrin-binding polypeptides isolated from total membranes of *A. pleuropneumoniae* K17. Outer membrane proteins, prepared from either iron-replete cells (lane A) or iron-restricted cells (lane B), and affinity-isolated polypeptides (lanes C-G) were separated by SDS-PAGE and visualised by silver staining. The latter polypeptides were affinity-isolated from total membranes prepared from iron-restricted cells (lanes C-F) or iron-replete cells (lane G) using either biotinylated porcine transferrin (lanes C and G), biotinylated porcine albumin (lane D), biotinylated human transferrin (lane E) or no biotinylated protein (lane F). The numbers refer to the relative molecular masses (kDa) of the indicated polypeptides.


A B C D E F G
96
92
76
67
38

was replaced with biotinylated human transferrin (Fig. 4.1, lane E). Although trace amounts of the 99 kDa polypeptide were affinity-isolated using biotinylated porcine albumin (Fig. 4.1, lane D), this result may be attributable to the porcine albumin preparations being contaminated with trace amounts of porcine transferrin (Ricard *et al.*, 1991). The 99 kDa polypeptide was not isolated from total membranes of organisms grown under iron-replete conditions (Fig. 4.1, lane G). These results indicate that the 99 kDa polypeptide is iron-repressible and appears to be a component of the transferrin receptor in strain ATCC 27088. The 99 kDa polypeptide is clearly an IRMP (Fig. 4.1, lanes A-C; Ricard *et al.*, 1991). Although the 64 kDa polypeptide would appear to be iron-repressible in that it was not isolated from total membranes from iron-replete organisms (Fig. 4.1, lane G; Ricard *et al.*, 1991), it was absent from Sarkosyl-extracted outer membranes of the organisms (Fig. 4.1, lane B). While the *in situ* location of the 64 kDa polypeptide is therefore uncertain, the comparable polypeptides from three other biotype 1 strains of *A. pleuropneumoniae* are known to be IRMPs (Gonzalez *et al.*, 1990). It would appear that these polypeptides are also easily solubilized during the Sarkosyl-extraction procedure (Gonzalez *et al.*, 1990), and hence, by analogy, it would seem reasonable to conclude, as previously (Ricard *et al.*, 1991), that the 64 kDa polypeptide of strain ATCC 27088 is associated, somehow, with the outer membrane of the organism. Gerlach *et al.* (1992*b*) have demonstrated that the TBPs2 (TfbA proteins) and encoding genes (*tfbA* genes) of strain ATCC 27088 and another serotype 1 strain of *A. pleuropneumoniae* (strain AP37), are highly homologous, and notably, that the nucleotide

sequence of the *tfbA* gene of strain AP37 specifies a TBP2 with a predicted molecular mass of 65 kDa.

The application of the affinity isolation procedure, using biotinylated porcine transferrin as the binding ligand, to iron-restricted total membranes from the three other strains, allowed the isolation of putative TBPs of 93 kDa from strain Bertschinger 2008/76 (Fig. 4.2, lane C), 95 kDa (trace amounts) and 60 kDa from strain BC181 (Fig. 4.3, lane C), but none from strain K17 (Fig. 4.4, lane C). As in the case of strain ATCC 27088, the TBPs of strains Bertschinger 2008/76 and BC181 were not isolated when biotinylated porcine transferrin was omitted (Figs. 4.2 and 4.3, lanes F), nor were they isolated when biotinylated porcine transferrin was replaced with biotinylated human transferrin (Figs. 4.2 and 4.3, lanes E) or biotinylated porcine albumin (Figs. 4.2 and 4.3, lanes D). Although the 93 kDa polypeptide of strain Bertschinger 2008/76 was isolated from iron-replete total membranes by means of biotinylated porcine transferrin (Fig. 4.2, lane G), it is clearly an IRMP (Fig. 4.2, lanes A-C). The affinity isolation experiment provides strong evidence to the effect that the 93 kDa polypeptide of strain Bertschinger 2008/76, does, as suspected (above), represent the TBP1 of this organism. No TBP2 was isolated from strain Bertschinger 2008/76. This result, however, does not preclude the existence of TBP2 in this strain. A similar problem, related, apparently, to the stringency of the washing procedures, has been encountered with the TBP2 of *Pasteurella haemolytica* (Ogunnariwo and Schryvers, 1990).

The 95 kDa and 60 kDa polypeptides of strain BC181 (Fig. 4.3, lane G) were not

isolated from iron-replete total membranes of the organism, indicating that both of these polypeptides are iron-repressible. Like the 64 kDa polypeptide of strain ATCC 27088, the 60 kDa of strain BC181, while lacking an outer-membrane counterpart (Fig. 4.3, lane B), is almost certainly outer-membrane-associated. The TBPs2 and *tfbA* genes of several *A. pleuropneumoniae* serotypes, including the reference strain of serotype 3 (strain ATCC 27090), were shown to have a high degree of homology to those of a serotype 7 strain (strain AP205) which produces a TBP2 with a predicted molecular mass of 60 kDa (Gerlach *et al.*, 1992*b*). Since strain BC181 is a serotype 3 strain (Rosendal *et al.*, 1985), it would seem reasonable to conclude that the 95 kDa and 60 kDa polypeptides of strain BC181 represent TBP1 and TBP2, respectively. Regarding the inability of strain BC181 to utilize porcine transferrin, it may be that the low levels of TBP1 are incompatible with effective iron acquisition; alternatively, the organism may lack, or possess a defect in, some other component involved in the overall iron-acquisition process, but note that such a deficiency, if it exists, does not compromise the acquisition of iron from the haemoglobins.

Strain K17 could use neither porcine transferrin, any of the haemoglobins tested, nor even haemin as an iron source for growth. Neither the putative TBP1 (92 kDa) nor any other polypeptide was affinity-isolated from iron-restricted total membranes from this strain. This suggests that the 92 kDa IRMP does not bind transferrin or is a component of the transferrin receptor that may be defective. It is possible that strain K17 possesses a defective receptor/receptor complex which is common for transferrin and

haemoglobin. Further information pertaining to the nature of the haemoglobin and/or haemin receptors of *A. pleuropneumoniae* is needed before conclusions can be advanced regarding the presence or absence of TBP1 (and, perhaps, TBP2) in strain K17.

It was suggested in Chapter 3 that IRMPs of 101 kDa (strain ATCC 27088) (Fig. 3.14, lane B), 98 kDa (strains Bertschinger 2008/76 and BC 181) (Figs. 3.15 and 3.16, lanes B) and 96 kDa (strain K17) (Fig. 2.10, lane E) may represent haemin and/or haemoglobin receptor components, functional or otherwise since strain K17 could use neither haemoglobin nor haemin as an iron source. Affinity procedures, utilizing haemin-agarose, have allowed the isolation of 97 kDa and 44 kDa polypeptides from *N. gonorrhoeae* (Lee, 1992*a*), and 97 kDa and 50 kDa polypeptides from *N. meningitidis* (Lee, 1994) (cited by Martel and Lee, 1994). It has been suggested that *A. pleuropneumoniae* possesses a common mechanism for the acquisition of iron from transferrin and haemin (Gerlach *et al.*, 1992*a*) since it appears that the 60 kDa TBP2 of strain AP205 can bind both porcine transferrin and haemin. On the other hand, the haemin- and/or haemoglobin-binding proteins of *H. influenzae* and the pathogenic neisseriae do not appear to bind transferrin (Lee, 1992*a,b*; Lee and Hill, 1992). Moreover, each of the haemin-binding polypeptides that has been affinity-isolated from *H. influenzae* and *N. gonorrhoeae* is distinct from that affinity-isolated with human transferrin (Lee, 1992*a,b*).

The existence of specifically host transferrin (and lactoferrin)-binding polypeptides has important implications for explanations of host specificity as well as interstrain

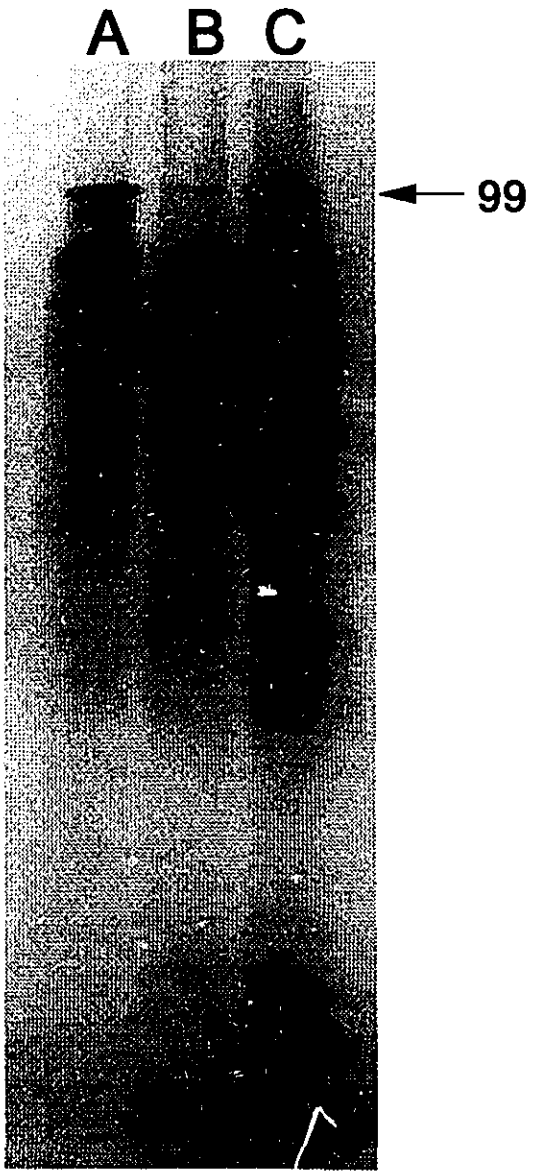
differences in virulence. For example, transferrin and lactoferrin of human, but not bovine, origin can enhance meningococcal infections in mice (Schryvers and Gonzalez, 1989) suggesting that an otherwise identical strain that lacks, or possesses a defect in, an appropriate receptor, may exhibit reduced virulence. In this respect, it is notable that unlike the parent, a mutant of *N. gonorrhoeae*, defective in the uptake of iron from human transferrin and haemoglobin, is avirulent in mouse subcutaneous chambers (Genco *et al.*, 1991). Similarly, in the case of the strains under investigation, the fact that only trace amounts of TBP1 were affinity-isolated from total membranes of strain BC181 could explain its involvement only in chronic infections and its lower virulence compared to the type strain.

In summary, the experiments conducted in this study have allowed the isolation and/or identification of putative porcine transferrin-binding polypeptides from strains of *A. pleuropneumoniae* that both can, and can not, use porcine transferrin (and various haemoglobins; Chapter 3) as an iron source for growth. While the inability of particular strain(s) to use porcine transferrin, and/or the haemoglobins, may reflect defects in the corresponding receptor components, it is also possible that other components involved in the overall iron-acquisition process may be defective or absent.

4.4.2 N-terminal amino acid sequence analysis

The TBP1 of strain ATCC 27088 was isolated using Sepharose-coupled porcine transferrin, and identified by SDS-PAGE (Fig. 4.5). While several attempts were made to isolate the TBP1 free of any contaminating polypeptides, 3 M NaCl rinse-buffer appeared to allow the isolation of the protein only in the presence of other, smaller molecular mass polypeptides. Consequently, prior to submission for N-terminal amino acid analysis, the smaller molecular mass polypeptides were well separated from the TBP1 by electrophoresing the TBP1-containing samples for extended periods of time (~60 h). Subsequent electroblotting of the polypeptides onto PVDF membrane followed by commercial sequence analysis allowed the N-terminal amino acid sequence to be identified as Glu-Gln-Ala-Val-Gln-Leu-Asn-Asp-Asp-Tyr-Gly-Thr-Thr-. No homology was noted to any known TBP when the amino acid sequence data were computer-analyzed using Swissprot.sequence data base. Little is known about codon usage in *A. pleuropneumoniae*. Based on the N-terminal amino acid sequence and the codon usage in the *hlyX* gene of *A. pleuropneumoniae* strain CM5 (biotype 1, serotype 1; MacInnes *et al.*, 1990), an oligonucleotide probe (to be used in molecular biological studies) was synthesized commercially.

FIG. 4.5. Identification of transferrin-binding polypeptides isolated from total membranes of *A. pleuropneumoniae* ATCC 27088 by means of Sepharose-coupled porcine transferrin and Zwittergent. The bound proteins were eluted from the affinity resin using 3 M NaCl-rinse buffer (lanes A and B). Elution was monitored by recording the absorbance of each fraction at 280 nm. Lane A represents lyophilised protein sample, while lane B represents lyophilised sample prepared from pooled fractions that were collected subsequent to the fractions represented in lane A. Lane C represents proteins released from Sepharose beads that had been washed with 6 bed volumes of 3 M NaCl rinse-buffer and then subjected to boiling. The number refers to the relative molecular mass (kDa) of the indicated polypeptide.


A B C
99

Chapter 5. Cloning of the gene encoding the porcine transferrin-binding protein 1 of the type strain of *Actinobacillus pleuropneumoniae.*

Rationale

Porcine transferrin-binding polypeptides of 99 kDa and 64 kDa have been isolated from total membranes of the type strain of *A. pleuropneumoniae* (Chapter 4). It appears that the 99 kDa polypeptide is the TBP1 of the organism.

Since the TBPs1 and TBPs2 of *A. pleuropneumoniae* may prove to be useful as components of subunit vaccines, the aims of this study were to construct an oligonucleotide probe corresponding to the N-terminus of TBP1 from the type strain and to initiate studies pertaining to the cloning and sequencing of the gene encoding TBP1.

5.1 Summary

The genomic DNA of *A. pleuropneumoniae* ATCC 27088 was completely digested with *Hind*III and analyzed on an agarose gel. A Southern blot of the digested DNA revealed strong hybridization (under conditions of low stringency) to three DNA fragments of approximately 3.1 kb, 5.6 kb and 19 kb in size, when probed with the commercially-synthesized oligonucleotide corresponding to the amino terminus of the 99 kDa polypeptide (TBP1) of the organism. Fragments of ca. 5.6 kb were isolated and ligated to pUC9 plasmid DNA that was digested with *Hind*III. The ligation mix was used to transform competent *E. coli* DH5α cells. Transformants were isolated on LB-Amp-X-gal agar plates (white colonies) and plasmid DNA was isolated from them by the alkaline lysis method. Digestion of the plasmid DNA with *Hind*III allowed the identification of the 5.6 kb DNA insert on agarose gels. Subsequent Southern blotting and probing of the nylon filter with the oligonucleotide corresponding to the TBP1 revealed the presence of only two clones containing insert DNA that hybridized to the probe under conditions of low stringency. Attempts were made to sub-clone the insert DNA from these two clones for eventual nucleotide sequencing.

5.2 Introduction

Specific polypeptides of several pathogens including *H. somnus* (Ogunnariwo *et al.*, 1990), *N. meningitidis* (Schryvers and Morris, 1988*a*,*b*; Schryvers and Lee, 1989; Ala'Aldeen *et al.*, 1990; Griffiths *et al.*, 1990), *N. gonorrhoeae* (Lee and Bryan, 1989; Schryvers and Lee, 1989), *H. influenzae* (Schryvers, 1989; Morton and Williams, 1990; Holland *et al.*, 1992; Stevenson *et al.*, 1992) and *P. haemolytica* (Ogunnariwo and Schryvers, 1990) have been identified as components of transferrin or lactoferrin receptors and many of these polypeptides have been affinity-isolated specifically with host transferrin or lactoferrin (Schryvers and Morris, 1988*a*,*b*; Lee and Bryan, 1989; Schryvers, 1989; Schryvers and Lee, 1989; Ogunnariwo and Schryvers, 1990). The polypeptides isolated with transferrin have included one of high molecular mass (94-106 kDa) and one of low molecular mass (58-86 kDa) and it has been suggested that these polypeptides exist *in situ* in the form of iron acquisition complexes (Lee and Bryan, 1989; Schryvers, 1989; Schryvers and Lee, 1989; Ogunnariwo and Schryvers, 1990). These polypeptides have been designated transferrin-binding proteins 1 (TBP1) and 2 (TBP2), respectively. It is still not known whether TBP1 and TBP2 constitute a single receptor.

Porcine TBPs with molecular sizes of 105 kDa and 56 kDa have been isolated from serotypes 1, 2 and 7 of *A. pleuropneumoniae* (all biotype 1 strains) and both of these polypeptides have been shown to bind transferrin (Gonzalez *et al.*, 1990). TBPs with molecular sizes of 99 kDa and 64 kDa have also been affinity-isolated from the type strain

(ATCC 27088; serotype 1) of *A. pleuropneumoniae* (Ricard *et al.*, 1991). While no information is available regarding the gene encoding TBP1 of *A. pleuropneumoniae*, the gene (designated *tfbA*) encoding TBP2 (60 kDa) of a serotype 7 strain has been isolated and Southern blotting and immunoblot analysis have shown that a highly homologous gene and gene product are present in serotypes 2, 3, 4, 8, 9, 10 and 11 (Gerlach *et al.*, 1992*b*). The N-terminus of this 60 kDa protein has a high degree of homology to the TBP2 of *N. meningitidis* and common antigenic domains have been identified in the TBPs2 of *H. influenzae*, *N. meningitidis* and *N. gonorrhoeae* (Stevenson *et al.*, 1992).

The *tbp1* or *tbpA* and *tbp2* or *tbpB* genes, coding for the TBP1 and TBP2, respectively, of *N. meningitidis* have been isolated (Legrain *et al.*, 1993). Isogenic *tbp1* and *tbp2* mutants, deficient in TBP1 and TBP2 respectively, were constructed from *N. meningitidis* strain B16B6 (Irwin *et al.*, 1993). Mutants deficient in either TBP1 or TBP2 showed reduced transferrin binding activity, and neither mutant was capable of using transferrin for growth. These results suggest that both TBP1 and TBP2 are needed for iron acquisition from transferrin.

The *tbpA* and *tbpB* genes have been identified in *N. gonorrhoeae* (Cornelissen *et al.*, 1992). The gene encoding gonoccoccal TBP1 (*tbpA*) predicts a protein sequence that is homologous to the TonB-dependent outer membrane receptors of *E. coli* and *P. putida*. The gene encoding TBP2 (*tbp*B) has been cloned and sequenced (Anderson *et al.*, 1994). *tbpA* is located immediately downstream from *tbpB*. Transferrin has been shown to bind to both TBP1 and TBP2 of *N. gonorrhoeae*. However, it appears that

although TBP2 facilitates binding to transferrin, it is not essential for the acquisition of transferrin-bound iron since mutants lacking TBP2 showed normal growth on transferrin-containing medium (Anderson *et al.*, 1994).

The mechanism by which iron levels control the expression of the *tbp1* and *tbp2* genes in bacteria has not been elucidated.

5.3 Materials and Methods

5.3.1 Organisms

A. pleuropneumoniae ATCC 27088 was stored and used to prepare frozen inocula as described earlier (section 2.3.1).

5.3.2 Chemicals

DNA from *A. pleuropneumoniae* strains Bertschinger 2008/76 and CM5 (biotype 1, serotype 1) were a generous gift from J. MacInnes, University of Guelph. Proteinase K was from Sigma. Agarose (high melting, electrophoretic grade) was from Fisher Scientific. Restriction enzymes, DNA ligase, Salmon testes DNA, T7 QuickPrime kit and Sephadex G-50 Nick columns (DNA grade) were from Pharmacia. Alkaline phosphatase was from Promega. [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from ICN. Standard

Lambda *Hind*III markers and positively charged nylon membrane were from Boehringer Mannheim. The Geneclean kit was purchased from Bio/Can Scientific. Purified pUC9 plasmid DNA and *E. coli* DH5α competent cells were a generous gift from T.C. Charles, McGill University, Macdonald Campus.

5.3.3 Isolation of genomic DNA

DNA was isolated based on the method of Marmur as described by Smart (1987) (cited by Smart *et al.*, 1988). Briefly, cells from a 24-h plate culture of *A. pleuropneumoniae* ATCC 27088 were washed with normal saline (NS; 0.15 M). A bent pasteur pipette was used to detach cells from the plate. Cells were transferred to Eppendorf tubes and harvested by centrifugation (12,000 x g, 2 min). The cells were then washed in 1 ml NS, centrifuged and resuspended thoroughly in 1 ml NS, and disrupted by the addition of 50 µl of 10 mg ml^{-1} proteinase K and 200 µl of 10% (w/v) SDS. The cell suspension was incubated for 3 h at 37°C.

The contents of the Eppendorf tubes were pooled in a centrifuge tube. An equal volume (25 ml) of chloroform solution (24 ml chloroform + 1 ml amyl alcohol) was added to the disrupted cell supension and the mixture was agitated for 2-3 min. The contents of the tube were centrifuged at 16,000 x g for 5 min. The viscous supernatant was carefully removed with a pipette. The supernatant was treated with the chloroform solution, as above (until no white precipitate was visible at the interface; 10-12 times), to get rid of

any protein present.

The DNA present in the lysate was precipitated by the addition of NaCl (to 0.3 M) and 2 vols of 95% ethanol. The precipitated DNA was recovered from the solution using a bent pasteur pipette and transferred to an Eppendorf tube containing 70% ethanol (1 ml). The DNA was removed from the ethanol with a bent pasteur pipette, allowed to air dry for 10 min at 37°C and then dissolved in 1ml TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA).

The A_{260}/A_{280} ratio was used to determine the purity of the DNA preparation. The ratio was found to be ~1.8 indicating that the DNA preparation was free of protein contamination (Sambrook *et al.*, 1989).

5.3.4 Preparation and electrophoresis of restriction digests

50-µg aliquots of genomic DNA from *A. pleuropneumoniae* ATCC 27088 were digested with 100 units of the restriction enzymes, *Eco*RI, *Hind*III, *Pst*I and *Pvu*II, according to the instructions provided by the enzyme suppliers. Genomic DNA (50 µg) from *A. pleuropneumoniae* strains Bertschinger 2008/76 and CM5 were each digested with 100 units of *Hind*III. All genomic DNA samples were incubated for 16 h at 37°C (to ensure that digestion was complete). The digested DNA samples were analyzed by agarose gel electrophoresis. The gel was run at a constant current of 55 mA for ~9 h. The gel was stained with ethidium bromide (5 mg ml^{-1}) to observe the pattern of DNA

digestion (Fig. 5.1).

5.3.5 Southern blotting

Each gel was rinsed with distilled water and incubated in a solution containing 1.5 M NaCl, 0.5 N NaOH (45 min, room temperature, gentle agitation). The gel was rinsed with distilled water and further incubated in buffer containing 1.5 M NaCl, 1 M Tris-HCl, pH 7.4 (30 min, room temperature, gentle agitation, with one change). The DNA fragments were transferred from the gel to nylon membrane by Southern blotting (~20 h, room temperature) using 10X SSC (A 1 l solution of 20X SSC contains 175.3g NaCl, 88.2g Na-citrate, pH 7.0) as transfer buffer, according to the procedure of Sambrook *et al.* (1989).

5.3.6 Pre-hybridization of nylon membranes

On completion of Southern blotting, the DNA on the nylon membrane was cross-linked using an automated UV Stratalinker (Stratagene). Following cross-linking, the nylon membrane was pre-hybridized for 24 h at 50°C in buffer (25 ml) containing 20X SSC (6.25 ml), 10% (w/v) SDS (0.5 ml), 100X Denhardt's medium (Sambrook *et al.*, 1989) (0.25 ml), distilled water (17.5 ml) and 0.5 ml of salmon testes DNA (10 mg in 1ml TE, pH 8.0 which was boiled for 5 min and cooled rapidly on ice, before addition to the

pre-hybridization buffer).

5.3.7 [α-^{32}P]dCTP labelling of the oligonucleotide probe

The oligonucleotide (corresponding to the 99 kDa polypeptide (TBP1)) probe was synthesized commercially and then labelled with [α-^{32}P]dCTP according to the instructions provided with the ^{T7}QuickPrime kit. This procedure uses [α-^{32}P]dCTP (3000 Ci/mmol) to label between 25 and 50 ng DNA to a specific activity of 1-2 x 10^9 dpm μg^{-1}. Briefly, 50 ng DNA (oligonucleotide; 34 µl) was mixed with 10 µl of reagent mix containing a buffered aqueous solution of dATP, dGTP, dTTP, and random oligodeoxyribonucleotides, primarily 9-mers. 5 µl of [α-^{32}P]dCTP (3000 Ci/mmol) (50 µCi) was added to the mixture followed by ~4-8 units of T7 DNA polymerase. The mixture was gently agitated and incubated for 2 h at 37°C. The labelled oligonucleotide was purified using Nick columns (Sephadex G-50, DNA grade, Pharmacia) and 200 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 as elution buffer according to the instructions provided with the columns. The labelled and purified oligonucleotide probe was boiled for 5 min and cooled rapidly on ice before being used for hybridization to the DNA on the nylon membrane.

5.3.8 Hybridization of DNA on the nylon membranes

For hybridization of DNA on the nylon membrane, the buffer (25 ml) was the same as that used for pre-hybridization except that the salmon testes DNA was omitted. Labelled and purified oligonucleotide probe (400 µl) was added to the hybridization buffer and the nylon filter was hybridized for 24 h at 50°C.

5.3.9 Post-hybridization washing

The nylon filter was rinsed with two changes of 5X SSC (100 ml), at room temperature. The filter was washed with 5X SSC, 0.1% (w/v) SDS (250 ml) for 15 min at room temperature, with one change. The washed filter was exposed to X-ray film (Kodak) (-80°C, 48 h; Fig. 5.2).

5.3.10 Isolation of specific-size DNA fragments

The 3.1 kb, 5.6 kb and 19 kb genomic DNA fragments (to which the oligonucleotide probe hybridized in the previous gel) were cut from an agarose gel (on which *Hind*III-digested genomic DNA of *A. pleuropneumoniae* ATCC 27088 had been electrophoresed). The fragments were isolated using the Geneclean kit according to the instructions provided with the kit. The purity of the isolated DNA fragments was

determined by analyzing the fragments on a 0.7% agarose gel (Fig 5.3).

5.3.11 Ligation of vector and fragment DNA

The procedure used was adapted from Ausubel *et al.* (1991). 20 µg pUC9 plasmid DNA in an Eppendorf tube was digested with 100 units *Hind*III for 2 h at 37°C. The digested plasmid DNA sample was heated for 5 min at 65°C. Alkaline phosphatase (1 unit) was added to the digested DNA and the mixture was incubated for 30 min at 37°C. The dephosphorylated plasmid DNA mixture was heated for 15 min at 85°C. The plasmid DNA preparation was diluted 1:4 with distilled water. 1 µl of this diluted DNA was mixed with 6.5 µl insert DNA (5.6 kb fragment isolated with the Geneclean kit) and the samples were heated for 5 min at 65°C. Ligation was accomplished by the addition of 1 µl ATP (10 mM) and 0.5 µl (0.5 units) of T4 DNA ligase. The samples were incubated for 16 h at 15°C.

5.3.12 Transformation

The protocol followed was essentially as described by Ausubel *et al.* (1991). Briefly, *E. coli* DH5α competent cells (100 µl) were thawed on ice. 5 µl ligation mixture was mixed with 50 µl of ice-cold 100 mM $CaCl_2$ and the competent cells. The sample was mixed carefully with a pipette tip and kept on ice for 30 min. The mixture was transferred

to a water bath (37°C) for 2 min (heat shock) and immediately thereafter, to ice for 2 min. LB broth (0.5 ml) was added to the mixture which was then incubated for 30 min at 37°C. Competent cells were recovered by centrifugation (12,000 x g, 1 min) and the pellet was resuspended with 120 µl LB broth. 100-, 10- and 1-µl aliquots were plated on LB-Ampicillin-X-gal agar plates. The plates were incubated for ~16 h at 37°C. Transformants (white colonies) were transferred individually to 5 ml LB broth containing 500 µg ampicillin. The tubes were incubated at 37°C for 16 h.

5.3.13 Plasmid minipreps: Alkaline lysis method

The procedure was as described by Birnboim (1983). Transformants (1.5 ml) grown in LB broth containing ampicillin were sedimented by centrifugation (12,000 x g, 1 min). The pellet was suspended in 100 µl TEG (20 mM EDTA, 1% glucose, 50 mM Tris-HCl, pH 8.0). The suspended cells were mixed with 200 µl alkaline lysis solution (ALS; 0.2 M NaOH, 1% SDS) followed by 150 µl 3 M sodium acetate (pH 4.8). The mixture was cooled on ice (2 min) and then centrifuged (12,000 x g; 5 min). The supernatant fraction was transferred to a clean tube and 1 ml of 70% ethanol was added. The mixture was mixed well followed by centrifugation (12,000 x g; 1 min). The supernatant fraction was discarded and the pellet was then suspended in distilled water (50 µl) and incubated for 15 min at 65°C. The suspended plasmid DNA was stored at 4°C.

5.3.14 Confirmation of positive clones

To confirm the presence of the 5.6 kb insert DNA fragment in the plasmid DNA which was extracted from the positive clones, restriction digests were prepared as described earlier (5.3.4) except that for each DNA sample to be analyzed, 20 µl of solution of plasmid DNA was digested with 20 units *Hind*III. The DNA samples from positive clones (showing the presence of the 5.6 kb insert DNA fragment) were transferred to nylon membrane and hybridized with the oligonucleotide probe corresponding to TBP1, as described earlier (sections 5.3.5-5.3.8).

5.4 Results and Discussion

Oligonucleotide probes have been used extensively over the past 10 years in the identification and isolation of genes that were previously inaccessible (Sambrook *et al.*, 1989). The oligonucleotide probe used in the DNA hybridization experiments in this study was synthesized commercially based on the N-terminus of the 99 kDa polypeptide (TBP1) using the codon usage of the *hlyX* gene of *A. pleuropneumoniae* CM5. Although in *A. pleuropneumoniae*, the *hlyX* gene is not highly expressed, the codon usage for this gene in strain CM5 was chosen, since at the time this study was initiated, the information on gene sequences and codon usage in *A. pleuropneumoniae* was very limited. The sequence of the 25-mer oligonucleotide was 5'G A I C A I G C I G T I C A I C/T T I A A T/C G

A T/C G3'. Inosine was chosen as the base at positions 3, 6, 9, 12, 15 and 18 since there was a choice of two or more bases at these positions. Inosine forms stable base pairs with all four conventional bases, and the strength of pairing is approximately equal in each case (Martin *et al.*, 1985*a*; Ohtsuka *et al.*, 1985; Takahashi *et al.*, 1985). Consequently, Martin *et al.* (1985*a*) suggest that it is better to use inosine rather than a conventional base at positions of three- or four-fold ambiguity.

"Oligolabelling" was developed as a method for labelling DNA fragments to high specific activity for use as hybridization probes (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984). The ^{T7}QuickPrime kit was chosen for labelling the oligonucleotide since previous attempts at end-labelling using [γ-^{32}P]ATP (Sambrook *et al.*, 1989), or by using a non-radioactive method (Digoxigenin), gave unsatisfactory results. The DNA to be labelled using the ^{T7}QuickPrime kit is mixed with oligonucleotides of random sequence. These "random oligomers" anneal to random sites on the DNA and then serve as primers for DNA synthesis by T7 DNA polymerase. Since labelled nucleotide is present during this synthesis, highly labelled DNA is generated. This method is of great advantage in that it can be used to label very small quantities of DNA and permits probes of very high specific activity to be produced.

The experiments conducted in this study involved the cloning of the gene encoding the TBP1 of *A. pleuropneumoniae* ATCC 27088. The oligonucleotide probe hybridized to 5.6 kb insert DNA fragments from two positive clones (white colonies). In order to identify the presence of TBP1 in the positive clones, cells of *E. coli* DH5α (positive

clones) were fixed onto nitrocellulose membrane which was then treated with biotinylated porcine transferrin. The filter was then probed with Streptavidin-horse radish peroxidase and finally developed using 4-chloro-1-naphthol reagent. Neither of the two clones gave a positive reaction. This tends to suggest that the whole gene may not have been cloned in *E. coli* or that the functional domain or domains may not have been cloned or that the gene may not be expressed in *E. coli*. On the other hand, the positive control (iron-restricted cells of *A. pleuropneumoniae* ATCC 27088) did not give a positive reaction either. These results indicate a technical problem with the experiment (only a single attempt was made). Hence, the protocol needs to be investigated before any conclusions can be made. More work is required before the gene encoding TBP1 can be sequenced and the amino acid sequence deduced. Subsequently, it would be possible to compare the deduced amino acid sequence of the receptor gene(s) from *A. pleuropneumoniae* ATCC 27088 with those from other pathogens and to determine the degree of homology between the TBP genes from *A. pleuropneumoniae* and other organisms (the N-terminus of TBP2 of *A. pleuropneumoniae* has a high degree of homology to TBP2 of *N. meningitidis* and common antigenic domains have been identified in the TBPs2 of *H. influenzae*, *N. meningitidis* and *N. gonorrhoeae* (Stevenson *et al.*, 1992)).

The prospect of using TBPs as vaccines has received considerable attention in recent years. TBP1 and TBP2 have been found to be immunogenic and to be expressed *in vivo* by several pathogens (Donachie and Gilmour, 1988; Deneer and Potter, 1989*b*; Sutherland *et al.*, 1990; Holland *et al.*, 1992; Gerlach *et al.*, 1992*a*) including *A.*

pleuropneumoniae (Niven *et al.*, 1989; Ogunnariwo and Schryvers, 1990). TBPs may be important virulence determinants and hence are potential targets for immunoprophylaxis (Bhanerjee-Bhatnagar and Frasch, 1990; Griffiths *et al.*, 1990; Stevenson *et al.*, 1992; Ala'Aldeen *et al.*, 1994). Intraperitoneal injection of the meningococcal TBP1-TBP2 complex in mice results in a broadly cross-reactive antibody response to TBP1 and a less cross-reactive antibody response to TBP2 (Ala'Aldeen *et al.*, 1994).

Detailed knowledge of the antigenic structures as well as detailed information about TBP molecules is still required before a safe and efficacious vaccine against swine pleuropneumonia can be developed.

FIG. 5.1. Analysis of restriction digests of genomic DNA of *A. pleuropneumoniae* strains Bertschinger 2008/76 (lane A), CM5 (lane B) and ATCC 27088 (lanes C-F) on a 0.7% agarose gel in 0.5 X TBE. The DNA was digested with *Hind*III (lanes A, B and E), *Pvu*II (lane C), *Pst*I (lane D) and *Eco*RI (lane F). Lane G represents standard λ *Hind*III markers. The numbers refer to the relative molecular sizes (kb) of some of the separated DNA fragments.

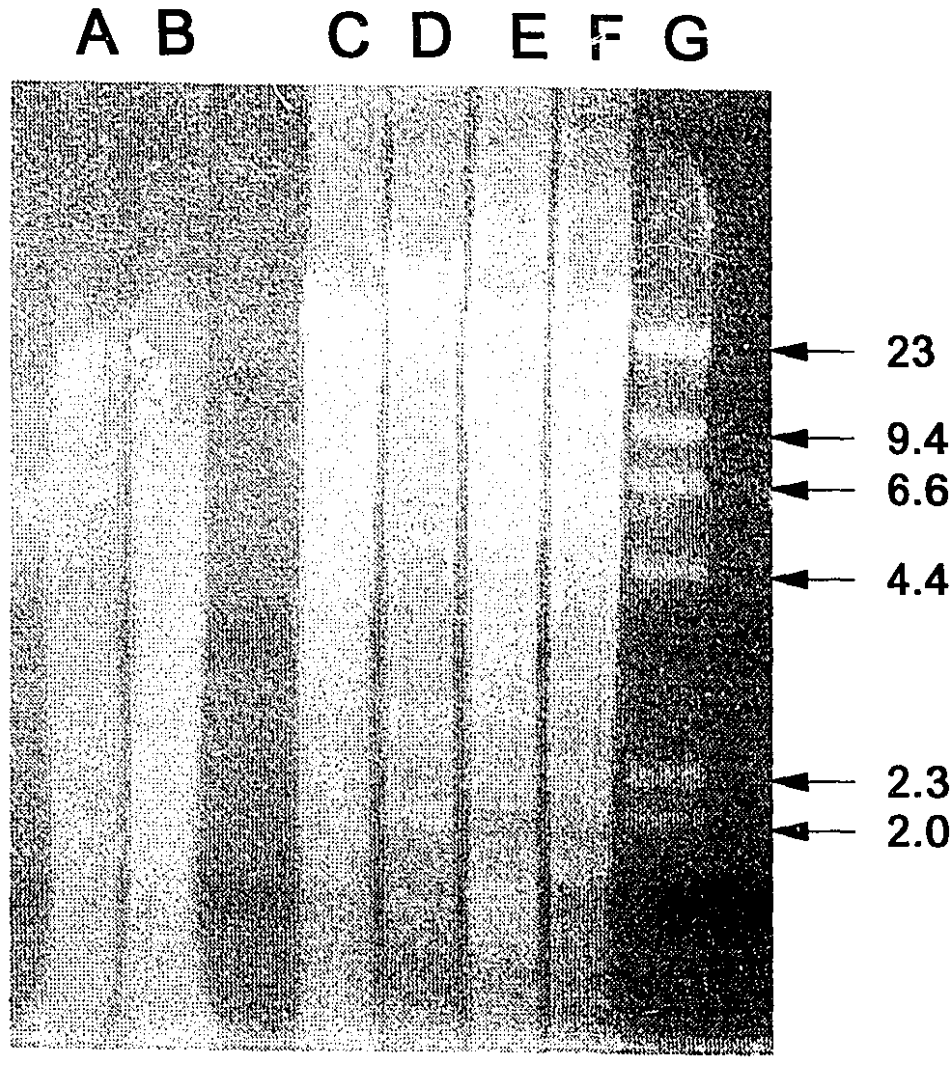

A B C D E F G
23
9.4
6.6
4.4
2.3
2.0

FIG. 5.2. Autoradiogram of restriction digests of genomic DNA of *A. pleuropneumoniae* strains Bertschinger 2008/76 (lane A), CM5 (lane B) and ATCC 27088 (lanes C-F). The DNA was digested with *Hind*III (lanes A, B and E), *Pvu*II (lane C), *Pst*I (lane D) and *Eco*RI (lane F). Lane G represents standard λ *Hind*III markers. The restriction digests were analyzed on an agarose gel and transferred to positively charged nylon membrane (by Southern blotting). The nylon membrane was hybridized with [α-^{32}P]dCTP-labelled oligonucleotide probe to the N-terminus of TBP1 and exposed to an X-ray film. The numbers refer to the relative molecular sizes (kb) of some of the separated DNA fragments.

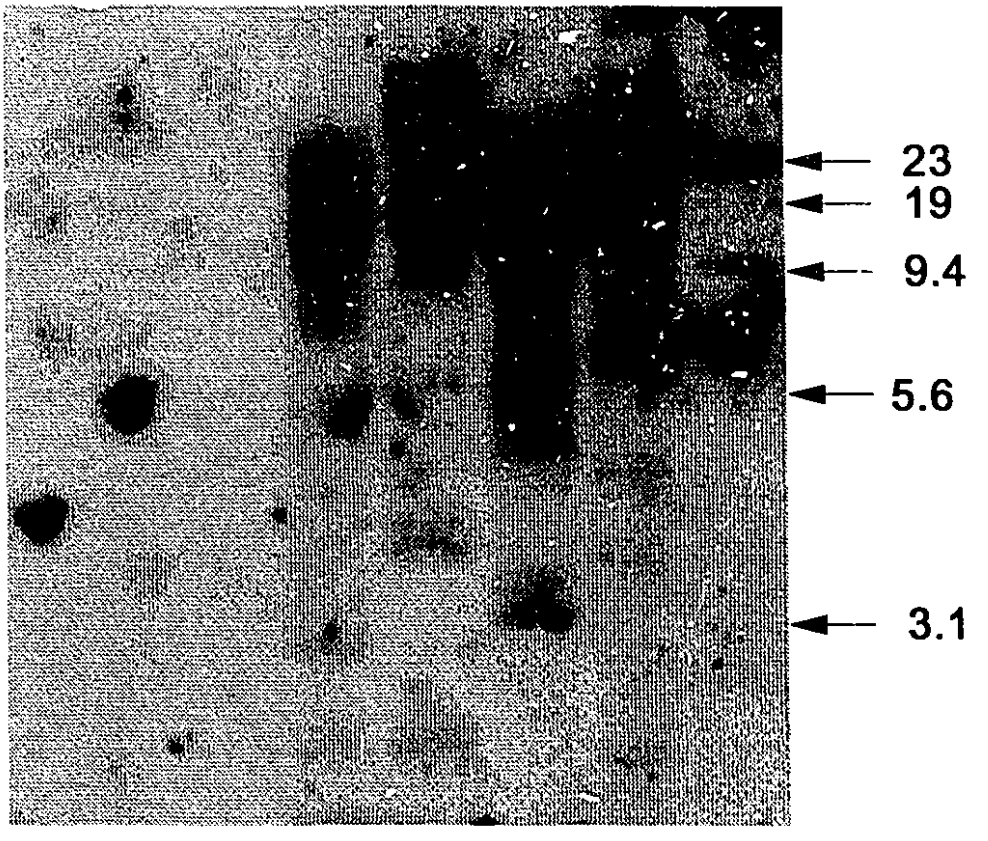
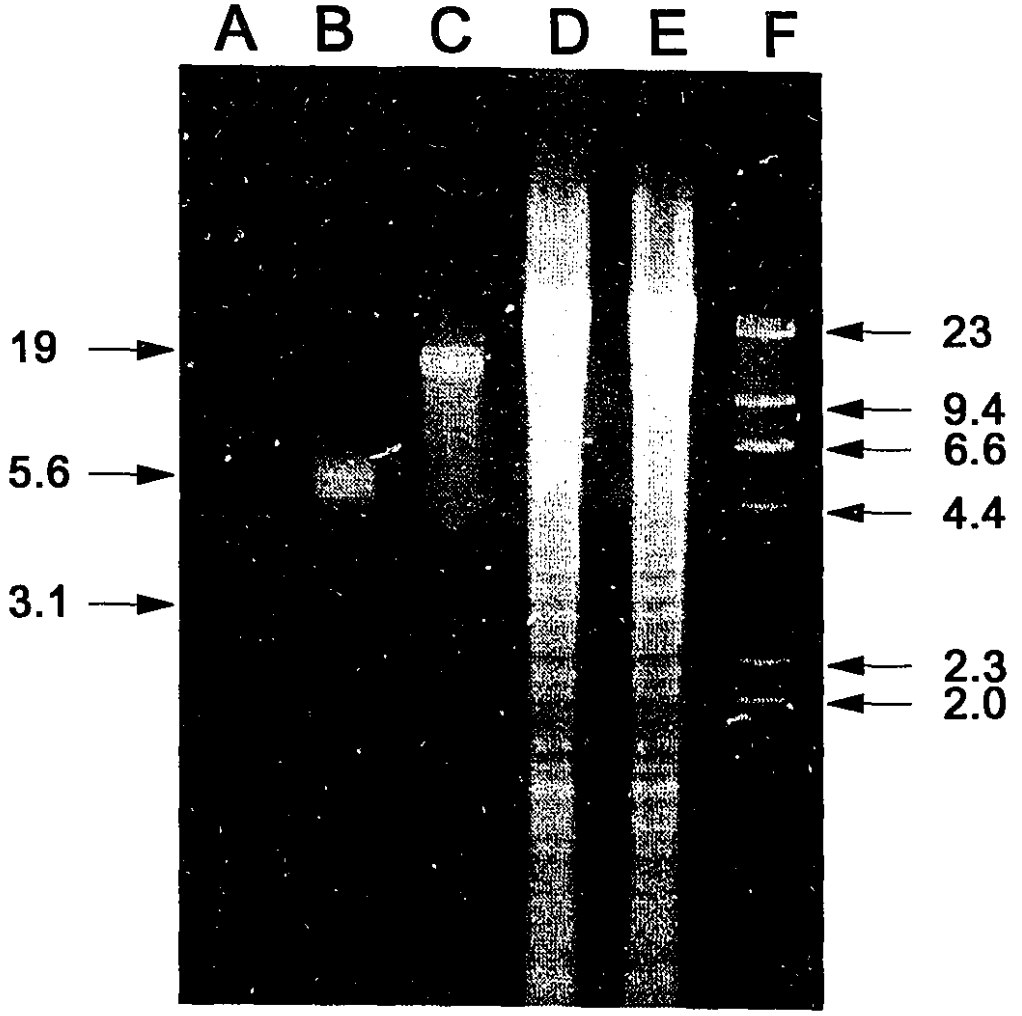

A B C D E F G
23
19
9.4
5.6
3.1

FIG. 5.3. Agarose gel electrophoresis of DNA fragments. In lanes A-C, the DNA fragments were isolated from *Hind*III-digested genomic DNA of *A. pleuropneumoniae* ATCC 27088 using the Geneclean kit. Lanes D and E represent *Hind*III-digested genomic DNA from *A. pleuropneumoniae* ATCC 27088. Lane F represents standard λ *Hind*III markers. The numbers refer to the relative molecular sizes (kb) of some of the separated DNA fragments.


A
B
C
D
E
F
19
5.6
3.1
23
9.4
6.6
4.4
2.3
2.0

Chapter 6. Conclusions and general discussion

EDDA is reported to have a high specificity for iron (Miles and Khimji, 1975). Except when Fe was the metal ion supplement, the growth yields obtained with the type strain of *A. pleuropneumoniae* were significantly lower for the test cultures (TYE-H supplemented with EDDA and either Fe, Mg, Mn, Cu, Ca, Zn or Ni) than they were when the organisms were grown under iron-replete conditions. When iron was the added metal ion, the growth yield was essentially the same as when the culture was grown in TYE-H alone. The OMP profiles of the type strain grown in an iron-restricted medium supplemented with either Fe, Mg, Mn, Cu, Ca, Zn or Ni (some of the important metal ions required by most bacteria) demonstrated that except for Fe, none of the metals could reverse, even to a small extent, the effect of EDDA, and the OMP profiles were very similar to those obtained with outer membranes from organisms grown under iron-restricted conditions (TYE-H plus EDDA). These results indicate that the effect of EDDA on the OMP profile of *A. pleuropneumoniae* is due specifically to its iron-chelating capacity.

EDDA and α,α'-dipyridyl are used commonly as iron chelators. On comparing the OMP profiles of *A. pleuropneumoniae* ATCC 27088 grown under iron-replete and iron-restricted conditions (using either EDDA or α,α'-dipyridyl), it was observed that these chelators have the same or very similar effects on the production of OMPs by the organisms. Both chelating agents caused the appearance of new polypeptides with

molecular masses of 101 kDa and 99 kDa, as well as increased synthesis of the 76 kDa, 67 kDa and 52 kDa polypeptides and decreased synthesis of the 43 kDa, 38 kDa and 23 kDa polypeptides. Hence, either EDDA or α,α'-dipyridyl can be used as iron chelators to study the effects of iron restriction on the OMP profile of *A. pleuropneumoniae* ATCC 27088.

The TMP profiles demonstrated that all four strains of *A. pleuropneumoniae* produced increased amounts of high molecular mass polypeptides in the 92-101 kDa range, as well as the 76 kDa and 67 kDa polypeptides in response to iron-restriction.

The OMP profile of strain ATCC 27088 exhibited noticeable increases in the amounts of IRMPs of 101 kDa and 99 kDa, as well as those of 76 kDa, 67 kDa and 52 kDa in response to iron restriction. Strain Bertschinger 2008/76 produced a novel IRMP of 98 kDa in response to iron restriction. The OMP profile also showed increases in the amounts of the 93 kDa, 76 kDa and 67 kDa polypeptides. Strain BC181 synthesized a novel IRMP of 98 kDa in response to iron restriction. Furthermore, there were increases in the amounts of the 95 kDa, 76 kDa and 67 kDa polypeptides. Two novel IRMPs of 96 kDa and 92 kDa were produced by strain K17. In addition, there were increased amounts of polypeptides of 76 kDa and 67 kDa.

The four strains of *A. pleuropneumoniae*, differing with respect to virulence and/or animal source, were investigated with respect to iron acquisition from various transferrins, lactoferrins and haemoglobins. The results of these experiments demonstrated that like the type strain (ATCC 27088) (Niven *et al.*, 1989; this study), the reference strain of

biotype 2 (Bertschinger 2008/76) could acquire iron from porcine transferrin. In contrast, strain BC181 (biotype 1), which is less virulent than the type strain (Rosendal *et al.*, 1985), and strain K17 (biotype 1), which was isolated from a lamb (Biberstein *et al.*, 1977), were unable to use porcine transferrin as an iron source for growth. None of the four strains could acquire iron from ovine, bovine or human transferrin, or porcine or ovine lactoferrin. Siderophores are known to be non-specific with respect to iron acquisition from transferrin and lactoferrin (see e.g., Griffiths, 1987*b*). Hence, these results indicate that none of these strains produces a conventional siderophore. Strains ATCC 27088 and Bertschinger 2008/76 could not use porcine transferrin when the transferrin was contained in a dialysis bag indicating that iron acquisition required direct contact between the organisms and the protein. This, in turn, suggested the existence of specific receptors. Strains ATCC 27088, Bertschinger 2008/76 and BC181 could acquire iron from haemoglobin but not if it was contained in a dialysis bag. It is possible therefore that receptors may also be involved in iron acquisition from haemoglobin. Considering the fact that good growth of the organisms was observed with porcine, bovine or human haemoglobin, the putative haemoglobin receptors would appear to be less specific than the transferrin receptors.

Strains ATCC 27088 and Bertschinger 2008/76 possess a similar complement of iron acquisition systems. On the other hand, strains BC181 and K17, both of which can cause disease in swine (Biberstein *et al.*, 1977; Rosendal *et al.*, 1985), are clearly less capable of obtaining iron from host proteins. To what extent this might account for the

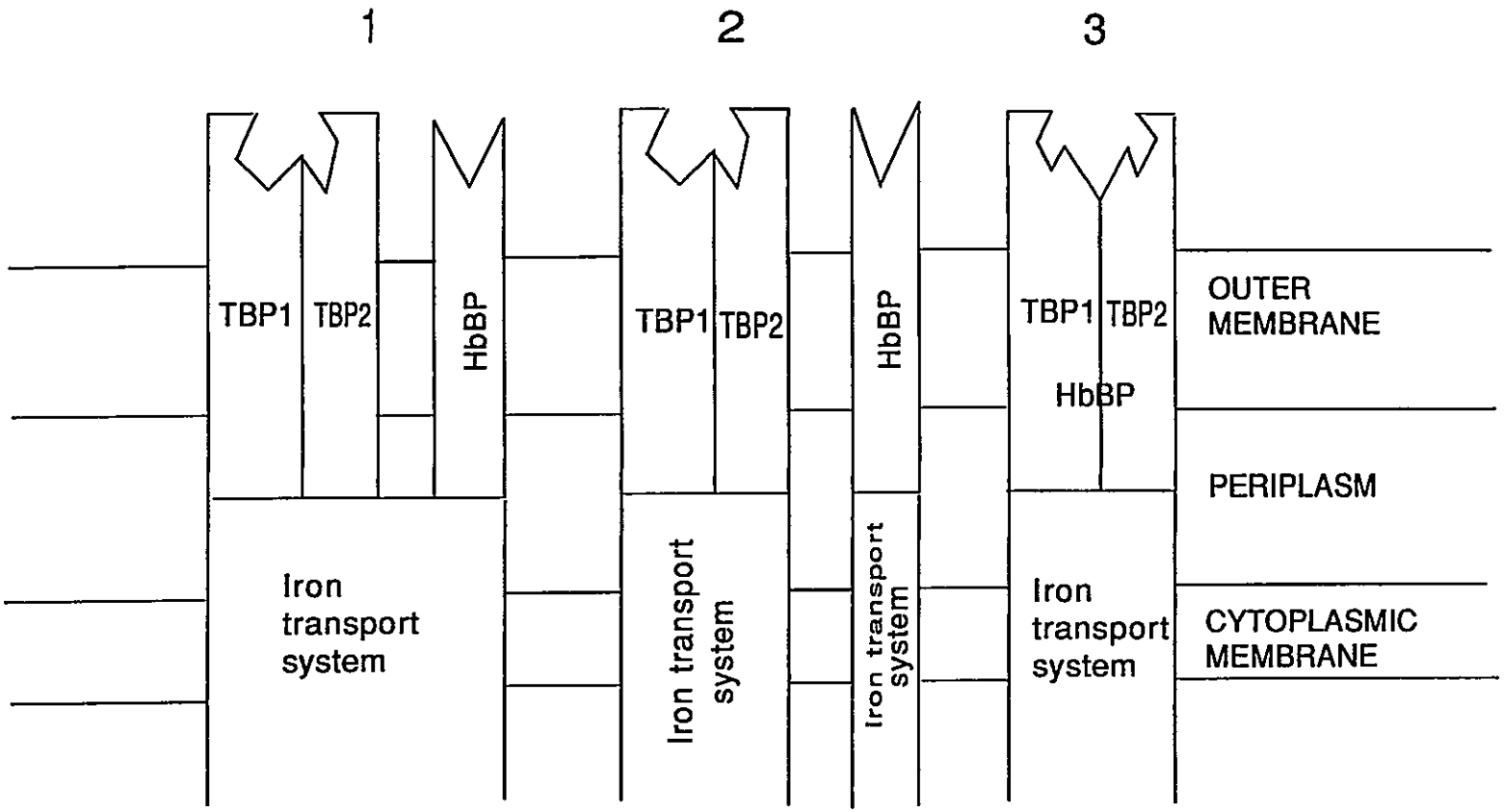
lower virulence of strain BC181, with respect to the type strain (Rosendal *et al.*, 1985), is difficult to evaluate, however, since virulence is typically multifactorial in nature. Only strains ATCC 27088 and Bertschinger 2008/76 could acquire iron from, specifically, porcine transferrin. While this specificity for transferrin reflects the host specificity of the organisms, the fact that strains BC181 and K17 did not use porcine or ovine transferrin indicate that transferrin specificity is not the only factor involved in determining host specificity and moreover, that the ability of a strain of *A. pleuropneumoniae* to use porcine transferrin (or lactoferrin) as an iron source is not essential for the strain to induce disease in swine.

Some of the IRMPs produced by some biotype 1 strains of *A. pleuropneumoniae* in response to growth under iron-restricted conditions have been identified as TBPs (Gonzalez *et al.*, 1990; Ricard *et al.*, 1991). All four strains of *A. pleuropneumoniae* used in the present study produced IRMPs that could, potentially, serve as receptor components. Two putative TBPs (99 kDa and 64 kDa) were isolated from total membranes of strain ATCC 27088 grown under iron-restricted conditions (in keeping with the results of Ricard *et al.* (1991)). The 99 kDa IRMP is known to represent the TBP1 of the type strain and by analogy, it was reasoned that the 93 kDa, 95 kDa and 92 kDa polypeptides of strains Bertschinger 2008/76, BC181 and K17 may also represent TBPs1. In this respect, a putative TBP of 93 kDa was affinity-isolated from strain Bertschinger 2008/76 and it was reasoned that this polypeptide represents TBP1; although no TBP2 was isolated, this does not preclude its existence. Despite the inability of strain

BC181 to use porcine transferrin, IRMPs of 95 kDa and 60 kDa, which may represent TBP1 and TBP2, respectively, could be affinity-isolated from iron-restricted total membranes of this organism. However, with strain K17, which, like strain BC181, cannot acquire iron from transferrin, neither the putative TBP1 (92 kDa) nor any other polypeptide could be affinity-isolated from iron-restricted total membranes of this organism.

It would appear that haemin- and/or haemoglobin-binding proteins of *H. influenzae* and the pathogenic neisseriae do not bind transferrin (Lee, 1992*a*, *b*; Lee and Hill, 1992). Moreover, each of the haemin-binding polypeptides that has been affinity-isolated from *H. influenzae* and *N. gonorrhoeae* is distinct from those that can be affinity-isolated with human transferrin (Lee, 1992*a*, *b*; Martel and Lee, 1994). On the other hand, Gerlach *et al.* (1992*a*) suggest a common mechanism for the acquisition of iron from transferrin and haemin by *A. pleuropneumoniae* since it appears that the 60 kDa TBP2 of strain AP205 can bind both porcine transferrin and haemin. The fact that these two sets of results are contradictory makes it (that much more) difficult to interpret the results obtained in the present study. However, to attempt an explanation, three different models were drawn up (based on the results of Lee, 1992*a*,*b*, Lee and Hill, 1992 and Gerlach *et al.*, 1992*a*) to accomodate the possible mechanisms involved in the acquisition of iron from host iron-binding/containing proteins by *A. pleuropneumoniae* (Fig. 6.1). The results of the experiments relating to iron acquisition from transferrin and haemoglobin by strains ATCC 27088 and Bertschinger 2008/76 can be explained by all

FIG. 6.1. Putative mechanisms for the acquisition of transferrin- and haemoglobin-bound iron by *A. pleuropneumoniae*. Model 1: The transferrin receptor (comprising TBP1 and TBP2) is distinct from the haemoglobin receptor (HbBP) and a common system exists for the transfer of the iron to the cytoplasm. Model 2: The transferrin and haemoglobin receptors are distinct from each other and each receptor has a distinct iron transport system associated with it. Model 3: Transferrin and haemoglobin bind to a common receptor/receptor complex and a common system is responsible for the transfer of the iron to the cytoplasm.


1
2
3
TBP1
TBP2
HbBP
TBP1
TBP2
HbBP
TBP1
TBP2
HbBP
OUTER MEMBRANE
PERIPLASM
CYTOPLASMIC MEMBRANE
Iron transport system
Iron transport system
Iron transport system
Iron transport system

three models. However, with strain BC181, the results can be best explained on the basis of models 1 and 2. According to model 1, low levels of TBP1 (only trace amounts of the 95 kDa IRMP were affinity-isolated from iron-restricted total membranes) may compromise iron acquisition from transferrin and, in addition, TBP1 may have an unusually low affinity for transferrin. Moreover, the existence of distinct receptors for transferrin and haemoglobin would explain the ability of strain BC181 to use haemoglobin despite its inability to use transferrin. Based on model 2, low levels of TBP1, and perhaps, the low affinity of TBP1 for transferrin, would explain the inability of strain BC181 to use porcine transferrin. In addition, it is possible that some other component involved in iron transport from transferrin to the cytoplasm may be defective or absent. Although it is "difficult" to explain the results obtained with strain BC181 based on model 3, it is possible that the transferrin-binding site of the receptor is defective while the haemoglobin-binding site is fully functional. With strain K17, the results can be explained by all three models. According to model 1, a defect in, or absence of, TBP1 and/or TBP2 and one or more haemoglobin-binding polypeptides (HbBPs) could account for the inability of strain K17 to use transferrin and haemoglobin. On the other hand, a defect in, or absence of, one or more of the other components involved in iron acquisition (TBP1 and perhaps, TBP2 may also be defective or absent) could also account for the results. Based on model 2, at least four explanations can be advanced to explain the ability of strain K17 to utilize neither transferrin nor haemoglobin. In the first, TBP1 and/or TBP2 and one or more HbBPs may be defective or absent. In the second, there may be

defects in one or more components (or they may be absent) of the iron transport system associated with the transferrin receptor and the HbBP(s) may be defective or absent. The third would represent the reverse scenario, with one or more components of the iron transport system associated with the haemoglobin receptor being defective or absent and TBP1 and/or TBP2 may be defective or absent. In the fourth, the transport components associated with both the transferrin and haemoglobin receptors may be defective or absent. According to model 3, the results obtained with strain K17 can be explained relatively easily. The ability to isolate neither TBP1 nor TBP2 from strain K17 suggests that either one or both of these polypeptides is defective or absent in this strain and this could account for the inability of strain K17 to use porcine transferrin. Alternatively, or in addition, it may be that the other components involved in iron transport, from transferrin or haemoglobin, to the cytoplasm are defective or absent. While model 3 best explains the results obtained with strain K17, the results obtained with strain BC181 can be best explained by models 1 and 2. It would appear, therefore, that more information is needed regarding the nature of the haemoglobin receptors and also on the existence of a common system for the acquisition of transferrin- and haemoglobin-bound iron before conclusions of any kind can be made about the mechanism(s) of iron acquisition by *A. pleuropneumoniae*.

Interestingly, preliminary experiments with the type strain have shown that the 101 kDa IRMP can be affinity-isolated with Sepharose-coupled haemoglobin (M. Estey and D.F. Niven, unpublished data) indicating that the transferrin and haemoglobin receptors

are, in fact, distinct. It would appear, therefore, that model 3 at least, is not appropriate to describe the receptor-mediated uptake of transferrin- and haemoglobin-bound iron.

Commercial sequence analysis allowed the N-terminal amino acid sequence of the TBP1 of strain ATCC 27088 to be identified as Glu-Gln-Ala-Val-Gln-Leu-Asn-Asp-Asp-Tyr-Gly-Thr-Thr-. No homology was noted to any known TBP when the amino acid sequence data were computer-analyzed using Swissprot.sequence data base. An oligonucleotide probe (to be used in molecular biological studies) was synthesized commercially based on the N-terminal amino acid sequence of TBP1 of the type strain and, since little was known about codon usage in *A. pleuropneumoniae*, the codon usage for the *hlyX* gene of strain CM5 was employed (biotype 1, serotype 1; MacInnes *et al.*, 1990). The codon usage for the *tfbA* gene (encoding TBP2) of *A. pleuropneumoniae* is now known and a comparison of the *hlyX* gene and the *tfbA* gene revealed that the codon usage for both genes is essentially the same.

The sequence of the 25-mer oligonucleotide synthesized was 5'G A I C A I G C I G T I C A I C/T T I A A T/C G A T/C G3'. Inosine was chosen as the base at positions 3, 6, 9, 12, 15 and 18 since there was a choice of two or more bases at these positions and studies pertaining to production of oligonucleotide probes indicate that it is better to use inosine rather than a conventional base at positions of three- or four-fold ambiguity (Martin *et al.*, 1985*a*).

The experiments conducted in this study involved the cloning of the gene encoding the TBP1 of *A. pleuropneumoniae* ATCC 27088. In an attempt to demonstrate

transferrin-binding by positive clones and thereby confirm that the gene encoding TBP1 had been cloned and expressed in *E. coli* DH5α, cells of positive clones were fixed to nitrocellulose membrane. Biotinylated transferrin and streptavidin-horseradish peroxidase were then used to investigate transferrin binding; unfortunately, however, no reaction was observed with any of the samples. While these results, obtained in a single trial, would suggest that the gene encoding TBP1 is not expressed in *E. coli* DH5α cells and/or that the whole gene may not have been successfully cloned in *E. coli*, it should be noted that even the positive control (iron-restricted cells of *A. pleuropneumoniae* ATCC 27088) failed to give any reaction. This tends to suggest a technical problem with the experiment and hence, the protocol needs to be thoroughly investigated before any conclusion can be made regarding the success of the cloning experiment. In effect, more work is required before the gene encoding TBP1 can be sequenced.

TBP1 and TBP2 have been found to be antigenic and to be expressed *in vivo* by several pathogens including *A. pleuropneumoniae* (Niven *et al.*, 1989; Ogunnariwo and Schryvers, 1990) and hence the prospect of incorporating TBP1 and TBP2 into vaccines is of special interest at the present time. Results of recent experiments have demonstrated that intraperitoneal injection of the meningococcal TBP1-TBP2 complex in mice results in a broadly cross-reactive antibody response to TBP1 and a less cross-reactive antibody response to TBP2 (Ala'Aldeen *et al.*, 1994). While TBP1 and TBP2 appear to be potential targets for immunoprophylaxis, a detailed understanding of the antigenic structures as well as more information in general about the TBP molecules is still required before an

effective and safe vaccine against swine pleuropneumonia can be developed.

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