Isolation and identification of a putative porcine transferrin receptor from *Actinobacillus pleuropneumoniae* biotype 1

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Each of two affinity isolation methods, the first based on biotinylated porcine transferrin plus streptavidin-agarose, and the second on Sepharose-coupled porcine transferrin, followed by SDS-PAGE, allowed the isolation and identification of two potential porcine-transferrin-binding polypeptides (~64 kDa and 99 kDa) from total membranes of *Actinobacillus pleuropneumoniae* grown under iron-restricted conditions. Both polypeptides were iron-repressible and were identified as potential receptor candidates as they were not isolated when biotinylated human transferrin was used instead of biotinylated porcine transferrin. The 64 kDa polypeptide was the more easily removed from Sepharose-coupled porcine transferrin and only the 99 kDa polypeptide appeared to be an outer-membrane protein. While these results suggest that the 99 kDa polypeptide represents the porcine transferrin receptor of *A. pleuropneumoniae*, and that the 64 kDa polypeptide represents an associated protein serving an accessory role, other interpretations are also possible.

Introduction

The growth of pathogens extracellularly *in vivo* occurs under conditions of iron restriction due to the presence of host iron-binding proteins such as transferrin and lactoferrin (e.g. Martínez *et al.*, 1990). In response to iron-restricted growth conditions, both *in vitro* and *in vivo*, many pathogens develop high-affinity iron transport systems comprising siderophores, which are secreted and can remove iron from transferrin and lactoferrin, and iron-repressible outer-membrane proteins (IRMPs) which serve as receptors for the siderophore-iron complexes (e.g. Brown & Williams, 1985; Griffiths, 1987). In contrast, when grown under iron-restricted conditions *in vitro*, *Neisseria meningitidis* (Archibald & DeVoe, 1980), *Neisseria gonorrhoeae* (Norrod & Williams, 1978; West & Sparling, 1985), *Pasteurella haemolytica* (Ogunnariwo & Schryvers, 1990), *Haemophilus influenzae* (Pidcock *et al.*, 1988; Morton & Williams, 1989) and also *Haemophilus haemolyticus* (Morton & Williams, 1989) do not produce siderophores. Despite

this, all of these organisms can still acquire iron from specific transferrins (Archibald & DeVoe, 1979; Mickelsen & Sparling, 1981; Herrington & Sparling, 1985; Morton & Williams, 1989; Ogunnariwo & Schryvers, 1990), and, in some cases, from lactoferrin (Mickelsen *et al.*, 1982; Schryvers, 1988), haemoglobin (Mickelsen & Sparling, 1981; Pidcock *et al.*, 1988), and haemoglobin-haptoglobin complexes (Dyer *et al.*, 1987; Pidcock *et al.*, 1988). Apparently, iron acquisition from transferrin and lactoferrin requires direct contact between the organisms and the proteins (Archibald & DeVoe, 1979; Simonson *et al.*, 1982; McKenna *et al.*, 1988; Morton & Williams, 1990), and involves iron-regulated receptors that exhibit specific recognition properties (Lee & Schryvers, 1988; Schryvers, 1988; Schryvers & Morris, 1988a, b; Tsai *et al.*, 1988; Morton & Williams, 1990; Ogunnariwo & Schryvers, 1990); indeed, the specificities of these receptors for transferrin or lactoferrin from particular animal species afford possible explanations for the host specificities of the organisms and also for the capacities of human transferrin and lactoferrin to enhance meningococcal infections in mice (Schryvers & Gonzalez, 1989). Such receptors are probably IRMPs, and in keeping with this suggestion, IRMPs are known to be produced *in vitro* by all of the above organisms (Norqvist *et al.*, 1978; Brener *et al.*, 1981; Mietzner *et al.*, 1984;

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Abbreviations: OMP, outer-membrane protein; IRMP, iron-repressible outer-membrane protein; HRP, horseradish peroxidase.

Herrington & Sparling, 1985; Williams & Brown, 1986; Pidcock *et al.*, 1988; Morton & Williams, 1989; Deneer & Potter, 1989a) and also *in vivo*, by at least *N. meningitidis* (Black *et al.*, 1986). Because of the implications for vaccine development, IRMP-receptor relationships have recently received considerable attention and as a result of such studies, specific proteins of *N. meningitidis* (Schryvers & Morris, 1988a, b; Schryvers & Lee, 1989; Ala'Aldeen *et al.*, 1990; Griffiths *et al.*, 1990), *N. gonorrhoeae* (Lee & Bryan, 1989; Schryvers & Lee, 1989), *P. haemolytica* (Ogunnariwo & Schryvers, 1990) and *H. influenzae* (Schryvers, 1989; Morton & Williams, 1990) have now been tentatively identified as components of transferrin or lactoferrin receptors.

Actinobacillus pleuropneumoniae biotype 1, known previously as *Haemophilus pleuropneumoniae* (e.g. Pohl *et al.*, 1983), is an important respiratory tract pathogen of swine (Sebunya & Saunders, 1983), and like the above organisms, *A. pleuropneumoniae* produces IRMPs in response to iron restriction (Deneer & Potter, 1989b; Niven *et al.*, 1989), and does not produce siderophores (Morton & Williams, 1989; Niven *et al.*, 1989). It can obtain iron from various haem compounds, including haemoglobin (Deneer & Potter, 1989b; Niven *et al.*, 1989), and produces one or more haemolysins (e.g. Frey & Nicolet, 1988) suggesting possible mechanisms for iron acquisition. Furthermore, and perhaps more important in the initial stages of infection, *A. pleuropneumoniae* can use transferrin as an iron source and indeed, exhibits a transferrin specificity that not only correlates with host specificity but also indicates the existence of a specific transferrin receptor (Morton & Williams, 1989; Niven *et al.*, 1989; Morton & Williams, 1990; Schryvers & Gonzalez, 1990). Gonzalez *et al.* (1990) have recently confirmed the existence of such receptors in three field isolates of *A. pleuropneumoniae* and demonstrated that these receptors are IRMPs of molecular mass 105 kDa. The purpose of the present study was to establish the existence and identity of such a receptor in the type strain of *A. pleuropneumoniae* which is known to produce at least three IRMPs that are potential receptor candidates (Niven *et al.*, 1989).

Methods

Organism. *A. pleuropneumoniae* biotype 1 (ATCC 27088) was stored and frozen inocula were prepared as described previously (O'Reilly *et al.*, 1984).

Chemicals. 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. Porcine serum albumin (fraction V powder) was from Sigma, CNBr-activated Sepharose 4B was from Pharmacia, and streptavidin-agarose was from BRL. The sources of other chemicals used, and the methods

used for the isolation and purification of porcine transferrin, were as described previously (Niven *et al.*, 1989).

Growth conditions and preparation of membranes. The growth of *A. pleuropneumoniae* under iron-replete and iron-restricted conditions has been described previously (Niven *et al.*, 1989). Briefly, iron-replete cells were cultured in a HEPES-buffered tryptone/yeast extract medium (TYE-H) to which filter-sterilized NAD had been added to 5 μ M. For the growth of iron-restricted cells, TYE-H was supplemented with deferrated EDDA (ethylenediamine di-*o*-hydroxyphenylacetic acid). Cultures were incubated at 37 °C, with agitation on a gyratory shaker (200 r.p.m.), and 1 h after the onset of stationary phase, the organisms were harvested by centrifugation, washed with saline (0.85% NaCl), resuspended in 10 mM-HEPES, pH 7.4 with NaOH, and frozen at -20 °C.

For the preparation of total membranes, frozen suspensions were thawed, and the bacteria were either disrupted by sonication (Niven *et al.*, 1989), or, when large quantities of membranes were required, concentrated by centrifugation (20000 *g*, 25 min, 5 °C) and disrupted by three passages through a French pressure cell operating at 12000 lb in⁻² ($\sim 8.3 \times 10^4$ kPa). Unbroken cells and large debris were sedimented by centrifugation (16000 *g*, 10 min, 5 °C) and the top 80% of each supernatant fraction was carefully removed and subjected to high-speed centrifugation (180000 *g*, 1 h, 5 °C). The resulting pellets (total membranes) were resuspended with small volumes of 10 mM-HEPES, pH 7.4. Outer membranes were prepared from total membranes by means of Sarkosyl (sodium *N*-lauroyl sarcosine) extraction (Niven *et al.*, 1989).

Preparation of iron-binding proteins. Porcine and human transferrins were saturated with iron and then dialysed as described by Caldwell & Archibald (1987).

Preparation of biotinylated proteins. Porcine transferrin, human transferrin and porcine albumin were biotinylated as described for lactoferrin by Schryvers & Morris (1988b) but with some minor modifications. The 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 overnight against 50 mM-Tris/HCl, pH 7.5 (2 l, 4 °C). Biotin-X-NHS (7.2 mg) was dissolved in 500 μ l dimethylformamide and 160 μ l of this solution [16 μ l (mg protein)⁻¹] were added to each dialysate. The resulting mixtures were incubated for 2 h at 4 °C (with gentle agitation), the reactions were stopped by addition of 1 ml glycine (10 mg ml⁻¹) to each solution, 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.

Isolation of transferrin-binding polypeptides using biotinylated porcine transferrin. Transferrin-binding polypeptides were isolated by the batch affinity procedure described by Schryvers & Morris (1988b) but using 200 μ g of biotinylated protein for each extraction. Briefly, total membranes (750 μ g protein) from *A. pleuropneumoniae* grown under iron-replete or iron-restricted conditions were mixed with 200 μ g of biotinylated protein (porcine or human transferrin, or porcine albumin) and the volume brought to 1 ml with 100 mM-NaCl, 50 mM-Tris/HCl, pH 8.0; for control samples, the biotinylated proteins were omitted. The samples were incubated (1 h, 37 °C, gentle agitation) and then centrifuged (16000 *g*, 10 min; Eppendorf microcentrifuge). The supernatant fractions were removed carefully 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. Streptavidin-agarose (diluted 2-fold with water; 100 μ l) was added to each tube and the samples were mixed and incubated (1 h, 20 °C). The samples were then centrifuged (750 *g*, 3 min, 20 °C) and the affinity resin pellets were washed (by successive cycles of resuspension in buffer, incubation for 10 min at 20 °C, and

centrifugation) using one of three wash systems. Wash system 1 consisted of three washes in 0.5% Sarkosyl, 100 mM-NaCl, 10 mM-EDTA, 50 mM-Tris/HCl, pH 8.0, followed by two washes in 100 mM-NaCl, 50 mM-Tris/HCl, pH 8.0. Wash system 2 consisted of three washes in 0.5% Sarkosyl, 1 M-NaCl, 10 mM-EDTA, 50 mM-Tris/HCl, pH 8.0, followed by one wash in 1 M-NaCl, 50 mM-Tris/HCl, pH 8.0, and a final wash in 100 mM-NaCl, 50 mM-Tris/HCl, pH 8.0. Wash system 3 consisted 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 as in wash system 2.

To identify the 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 sample tubes were immersed in boiling water for 5 min, cooled on ice, and centrifuged (750 g, 3 min). The supernatant fractions were then transferred to clean tubes, 2-mercaptoethanol was added (to 1.4 M), and following incubation (15 min, 20 °C), the samples were mixed thoroughly and 50 µl aliquots were subjected to SDS-PAGE (~16 h; 10%, w/v, acrylamide separating gels) essentially as described by Niven *et al.* (1989). The gels were fixed for 24 h in 10% (v/v) acetic acid, 50% (v/v) ethanol and the separated polypeptides visualized by means of silver staining according to the method of Oakley *et al.* (1980) except that development of the stain was stopped with 0.35% (v/v) acetic acid.

For SDS-PAGE, outer membranes were prepared similarly, and electrophoresis (1 µg protein per lane) and staining were done as described above.

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 800 ml 1 mM-HCl. Following a rinse with 60 ml 500 mM-NaCl, 100 mM-NaHCO₃, pH 8.3 with NaOH (coupling buffer), the swelled gel was transferred quickly to a 50 ml flask containing 95 mg of iron-saturated porcine transferrin in 24 ml of coupling buffer. This mixture was incubated for 2 h at 20 °C, with gentle agitation on a gyratory shaker, and the resulting Sepharose-coupled porcine transferrin was rinsed of unbound ligand with 60 ml of coupling buffer, 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 then used to pack a small column (1 × 10 cm) and excess unbound ligand was removed by three cycles of washing, each consisting of 30 ml 500 mM-NaCl, 100 mM-sodium acetate, pH 4.0 with HCl, followed by 30 ml 500 mM-NaCl, 100 mM-Tris/HCl, pH 8.0. The porcine transferrin was then resaturated with iron by passing through the column, 3 bed vols of a solution of FeCl₃ (5 µg Fe ml⁻¹) in 200 mM-Tris, 20 mM-NaHCO₃, pH 7.4.

Isolation of transferrin-binding polypeptides using Sepharose-coupled porcine transferrin. Total membranes (60 mg protein in 2 ml 10 mM-HEPES, pH 7.4) from organisms grown under iron-replete or iron-restricted conditions were added to 41 ml 100 mM-NaCl, 50 mM-Tris/HCl, pH 8.0, containing 1.8 g (wet wt) of Sepharose-coupled porcine transferrin, and the mixture was incubated for 1 h at 37 °C, with gentle agitation. Zwittergent (3-14), or Sarkosyl, and EDTA were added to give final concentrations of 2% (w/v), 0.75% (w/v) and 10 mM, respectively, 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 100 mM-NaCl, 10 mM-EDTA, 50 mM-Tris/HCl, pH 8.0, containing 0.5% (w/v) of either Zwittergent or Sarkosyl (100 mM-NaCl-rinse buffer), as appropriate. Bound proteins were eluted with either 3 bed vols 2.5 M-NaCl-rinse buffer or with 3 bed vols of each of a series of NaCl (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 M)-rinse buffers followed by 3 bed vols of each of a series of urea solutions (2, 4, 6, 8 M;

each pH 7.0 with HCl) containing 0.5% (w/v) of either Zwittergent or Sarkosyl, as appropriate. Fractions (1 ml) were collected and elution was monitored by recording the absorbance of each fraction at 280 nm; protein-containing fractions were then pooled, as appropriate, and fractions containing NaCl at concentrations of 2 M or greater were dialysed overnight against 50 mM-Tris/HCl, pH 6.8, containing 0.05% (w/v) of either Zwittergent or Sarkosyl (2 l; 4 °C).

To identify the isolated proteins, the above samples were mixed with sample buffer (Niven *et al.*, 1989), and appropriate volumes were subjected to SDS-PAGE (~16 h; 10%, w/v, acrylamide separating gels), essentially as described by Niven *et al.* (1989). Outer membranes (1 µg protein per lane) were treated similarly, and in all cases, the separated polypeptides were visualized by means of silver staining (Harlow & Lane, 1988).

In some experiments, the method of Blake & Gotschlich (1982) was used to solubilize total membranes prior to their contact with Sepharose-coupled porcine transferrin. Total membranes (200 mg protein in 5 ml 10 mM-HEPES, pH 7.4) were mixed with 5 ml 500 mM-NaCl, 10 mM-EDTA, 50 mM-Tris/HCl, pH 8.0, containing 5% (w/v) Zwittergent (extraction buffer) and incubated, with agitation, for 1 h at 37 °C. The mixture was centrifuged (16000 g, 10 min, 5 °C), the supernatant was retained, and the pellet was re-extracted (as above) with 10 ml of extraction buffer. The two supernatant fractions were then pooled and dialysed overnight against 0.05% (w/v) Zwittergent in 10 mM-EDTA, 50 mM-Tris/HCl, pH 8.0 (Tris-Z3,14 buffer; 2 l; 4 °C). The dialysate was applied to a column of Sepharose-coupled porcine transferrin (1 × 10 cm; 4 °C) pre-equilibrated with Tris-Z3,14 buffer, and proteins were eluted by a gradient obtained by mixing 500 ml each of Tris-Z3,14 buffer and 0.3 M-NaCl in Tris-Z3,14 buffer, followed by a second gradient obtained by mixing 500 ml each of 0.3 M-NaCl in Tris-Z3,14 buffer and 3 M-NaCl in Tris-Z3,14 buffer, and finally by the passage of 60 ml 6 M-urea (pH 7.0 with HCl) containing 0.05% (w/v) Zwittergent. Elution was monitored as described above, and protein-containing fractions were pooled, as appropriate, dialysed as necessary to reduce the NaCl concentration, and then analysed by means of SDS-PAGE.

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

Results

Our initial attempts at the isolation and identification of a transferrin receptor from *A. pleuropneumoniae* were based on methods described by Schryvers & Morris (1988a) and involved the separation of outer membrane, and also total membrane polypeptides by means of SDS-PAGE followed by electroblot analysis with horseradish peroxidase (HRP)-conjugated porcine transferrin. However, the results from these studies were inconclusive due to non-specific binding reactions and consequently, this approach was abandoned in favour of receptor isolation by means of affinity chromatography followed by identification by means of SDS-PAGE.

Two affinity isolation procedures were used. The first was based on that used by Schryvers & Morris (1988b) to isolate the lactoferrin-binding protein from *N. meningitidis*. Briefly, total membranes from *A. pleuropneumoniae* grown under iron-restricted or iron-replete conditions

Fig. 1. Identification of transferrin-binding polypeptides isolated from *A. pleuropneumoniae* by means of biotinylated porcine transferrin. Lanes A–J, affinity-isolated polypeptides from total membranes prepared from iron-restricted cells; lane K, affinity-isolated polypeptides from total membranes prepared from iron-replete cells; lane L, outer-membrane polypeptides from iron-restricted cells. The biotinylated proteins and the wash systems were as follows: lanes A and K, porcine transferrin, wash 1; lane B, porcine transferrin, wash 2; lane C, porcine transferrin, wash 3; lane D, no biotinylated protein, wash 1; lane E, porcine albumin, wash 1; lane F, porcine albumin, wash 2; lane G, porcine albumin, wash 3; lane H, human transferrin, wash 1; lane I, human transferrin, wash 2; lane J, human transferrin, wash 3. See Methods and Results for additional details; the numbers refer to the relative molecular sizes (kDa) of the indicated polypeptides.

were incubated in the presence or absence of a biotinylated protein. The detergent Sarkosyl was then added followed by streptavidin–agarose. The resulting complexes, consisting of streptavidin–agarose, biotinylated protein and bound proteins, were harvested by centrifugation, washed according to one of three protocols, and then boiled to release the bound proteins for analysis by means of SDS-PAGE. Typical results are presented in Fig. 1 (lanes A–K). Lanes A–C (Fig. 1) illustrate that irrespective of the wash protocol, the use of biotinylated porcine transferrin as binding ligand allowed the isolation of several polypeptides from membranes derived from iron-restricted organisms. Of these polypeptides, the 64 and 99 kDa polypeptides were of particular interest as these polypeptides were not isolated when the biotinylated porcine transferrin was omitted (Fig. 1, lane D), they were not isolated when the biotinylated porcine transferrin was replaced with

biotinylated human transferrin (Fig. 1, lanes H–J), and they were not isolated when membranes from iron-replete organisms were used (Fig. 1, lane K). While these results indicated that the 64 and 99 kDa polypeptides were specific, iron-repressible, porcine-transferrin-binding polypeptides, it was appreciated that our porcine transferrin preparations are occasionally contaminated with trace amounts of porcine serum albumin. To ensure that the 64 and 99 kDa polypeptides were indeed transferrin-binding polypeptides rather than albumin-binding polypeptides, control experiments were done using biotinylated porcine albumin as binding ligand. The resulting polypeptide profiles (Fig. 1, lanes E–G) revealed that while biotinylated porcine albumin (Fig. 1, lanes E–G) and biotinylated porcine transferrin (Fig. 1, lanes A–C) were associated with the isolation of the same polypeptides, substantially smaller amounts of the 64 and 99 kDa polypeptides were isolated when the binding

ligand was biotinylated porcine albumin. While these results *per se* indicated strongly that the 64 and 99 kDa polypeptides were transferrin-binding polypeptides rather than albumin-binding polypeptides, the albumin-associated isolation of these polypeptides remained somewhat enigmatic. However, just as our porcine transferrin preparations are occasionally contaminated with porcine albumin, iron-loading studies (not presented) revealed that, conversely, the commercial porcine serum albumin preparation was contaminated with an iron-binding protein, presumably porcine transferrin; in effect, it would appear that the isolation of the 64 and 99 kDa polypeptides was indeed due to a specific interaction(s) with porcine transferrin.

Fig. 1 also allows the affinity-isolated polypeptides (lanes A–K) to be compared with the outer-membrane polypeptides (OMPs) from iron-restricted organisms (lane L). Interestingly, the 99 kDa polypeptide (Fig. 1, lanes A–C), which is iron-repressible (above), co-migrated with an OMP (Fig. 1, lane L) which is known to be an IRMP (Fig. 2, lanes A and B; Niven *et al.*, 1989). While it is tempting to speculate that these polypeptides are identical, and an outer-membrane location would be compatible with a transferrin-binding capability, the 64 kDa polypeptide (Fig. 1, lanes A–C), which is also iron-repressible (above), did not appear to have an outer-membrane counterpart (Fig. 1, lane L). This, however, may only indicate that the 64 kDa polypeptide represents a peripheral OMP that is released during Sarkosyl-extraction of total membranes; in effect, the transferrin-associated isolation of the 64 kDa polypeptide and its absence from Sarkosyl-extracted outer membranes are not mutually exclusive.

Of the other polypeptides isolated (Fig. 1, lanes A–K), the 67 kDa polypeptide is also notable. Although it was isolated under all conditions (Fig. 1, lanes A–K), the relative intensities of the polypeptide bands indicate that while the 67 kDa polypeptide can bind to streptavidin-agarose (Fig. 1, lane D), it may also be capable of binding to some component(s) present in the biotinylated transferrin (Fig. 1, lanes A and H) and biotinylated albumin (Fig. 1, lane E) preparations. While this component(s) remains to be identified, it is interesting to note that the 67 kDa polypeptide co-migrated with an OMP (Fig. 1, lane L) and moreover, that the production of both appeared to be iron-regulated (Fig. 1, cf. lanes A and K; Fig. 2, cf. lanes A and B). On the other hand, previous studies with the same strain of *A. pleuropneumoniae* have demonstrated that the production of a 69 kDa OMP (equivalent to our 67 kDa OMP) is enhanced not only by reducing the supply of iron to the organism (Niven *et al.*, 1989) but also by reducing the pyridine nucleotide supply or the supply of glucose (T. O'Reilly, D. F. Niven & M. R. W. Brown, unpublished).

Fig. 2. Identification of transferrin-binding polypeptides isolated from *A. pleuropneumoniae* by means of Sepharose-coupled porcine transferrin. Lane A, outer-membrane polypeptides from iron-replete cells; lane B, outer-membrane polypeptides from iron-restricted cells; lanes C–F, affinity-isolated materials from iron-restricted total membranes that were either (lanes C, E and F) treated with Sepharose-transferrin and then with Sarkosyl, or (lane D) solubilized with Zwittergent and then applied to a column of Sepharose-transferrin. The bound proteins were eluted from the affinity resins as follows: lane C, when a solution containing 2 M-urea was followed by one containing 4 M-urea; lane D, by a gradient of NaCl (0.3–3.0 M) in buffer; lane E, when rinse buffer was followed by a buffer solution containing 0.5 M-NaCl; lane F, when rinse buffer was followed by a buffer solution containing 2.5 M-NaCl. See Methods and Results for additional details; the numbers refer to the relative molecular sizes (kDa) of the indicated polypeptides.

In effect, if the 67 kDa affinity-isolated polypeptide and the 67 kDa OMP are identical, it is highly probable that the 67 kDa polypeptide, rather than being involved specifically in iron acquisition from transferrin, or from some other iron source, plays a somewhat general role in the outer membrane of *A. pleuropneumoniae*.

In the second affinity isolation procedure, total membranes from *A. pleuropneumoniae* grown under iron-

restricted or iron-replete conditions were incubated with Sepharose-coupled porcine transferrin followed by Sarkosyl or Zwittergent (3-14). The reaction mixtures were then transferred to small chromatography columns where the Sepharose materials were rinsed and the bound proteins were eluted for subsequent analysis by means of SDS-PAGE. Fig. 2 (lanes C, E and F) illustrates results that were obtained when the membranes were from iron-restricted organisms and Sarkosyl was used as detergent. The use of a single elution buffer containing 2.5 M-NaCl allowed the elution of polypeptides of 38 and 67 kDa along with relatively smaller amounts of several other polypeptides including a polypeptide of 64 kDa (Fig. 2, lane F). Interestingly, when a step gradient of NaCl, rather than a single elution buffer, was used to wash the affinity column, many of the above polypeptides were found to elute at NaCl concentrations of less than 2.5 M (e.g. Fig. 2, lane E); on the other hand, complete elution of the 6 kDa polypeptide required the passage of a buffer solution containing 3 M-NaCl (not shown) and when the NaCl gradient was followed by one of urea, a 99 kDa polypeptide was also isolated (Fig. 2, lane C). Results that were similar to those depicted in Fig. 2, lanes C, E and F, were also obtained when Zwittergent, rather than Sarkosyl, was used to treat the Sepharose-transferrin-treated membranes and interestingly, while the reverse procedure does not appear to work effectively when Sarkosyl or Triton X-100 is used as detergent (Schryvers & Morris, 1988b; Schryvers, 1989), all of the above polypeptides were also isolated (see Methods for elution procedures) when membranes from *A. pleuropneumoniae* were treated with Zwittergent prior to their contact with the Sepharose-coupled porcine transferrin (e.g. Fig. 2, lane D); it is therefore conceivable that by using Zwittergent as the membrane-solubilizing agent, it may be possible to use conventional affinity chromatography, routinely, for the isolation and purification of bacterial receptor proteins. Finally, the 64 and 99 kDa polypeptides (Fig. 2) were found to be iron-repressible in that they, unlike the 67 kDa polypeptide, were not isolated when the membranes were from iron-replete organisms (not shown), and based on this information, as well as the molecular sizes and binding characteristics of the isolated polypeptides, it was concluded that the 64 and 99 kDa polypeptides that were isolated by means of Sepharose-coupled porcine transferrin, and those that were isolated by means of biotinylated porcine transferrin, were the same, porcine-specific, transferrin-binding polypeptides.

Discussion

Affinity isolation procedures have recently allowed the successful isolation of iron-repressible, specifically host-

transferrin-binding polypeptides from a variety of strains of *N. meningitidis*, *N. gonorrhoeae*, *H. influenzae* and *P. haemolytica* (Schryvers & Morris, 1988b; Lee & Bryan, 1989; Schryvers, 1989; Schryvers & Lee, 1989; Ogunnariwo & Schryvers, 1990). Typically, the isolated polypeptides 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 may exist *in situ* in the form of iron acquisition complexes (Lee & Bryan, 1989; Schryvers, 1989; Schryvers & Lee, 1989; Ogunnariwo & Schryvers, 1990). Regarding the capacities of these polypeptides to function as transferrin receptors, considerable attention has been focussed on the affinity-isolated polypeptides from *N. meningitidis* B16B6 and *H. influenzae* KC548 (Schryvers, 1989; Schryvers & Lee, 1989). Each of these organisms yielded two such polypeptides and following SDS-PAGE and electoblotting, the low molecular mass polypeptides, but not the high molecular mass polypeptides, were found to be capable of binding HRP-transferrin. Interestingly, however, it would appear that the high molecular mass polypeptides are also capable of direct binding to transferrin as these polypeptides were the last polypeptides to be eluted from the transferrin-affinity resins (Schryvers, 1989; Schryvers & Lee, 1989). Although it would appear, therefore, that the transferrin receptors of *N. meningitidis* and *H. influenzae* may comprise both low molecular mass and high molecular mass polypeptides, the capacity to bind HRP-transferrin after SDS-PAGE and electoblotting is exhibited by a low molecular mass polypeptide from each of only some strains of these organisms (Schryvers, 1989; Schryvers & Lee, 1989), and to further complicate the issue, the results of Tsai *et al.* (1988) indicate that such a polypeptide may not be necessary for receptor function. In effect, the nature of the transferrin receptors of these organisms remains uncertain.

In the present study, each of two affinity isolation methods allowed the isolation of two potentially transferrin-binding polypeptides (~99 kDa and 64 kDa) from the type strain of *A. pleuropneumoniae* biotype 1 (Figs 1 and 2). This organism acquires transferrin-bound iron by means of a non-siderophore-mediated, porcine-transferrin-specific mechanism (Niven *et al.*, 1989) and in keeping with this fact, both polypeptides were isolated specifically with porcine transferrin (Fig. 1). These results are similar to those obtained recently in similar studies with three other strains of *A. pleuropneumoniae* (Gonzalez *et al.*, 1990); each of these strains also yielded two, specifically porcine-transferrin-binding polypeptides. There are, however, slight differences in the relative molecular sizes of the low molecular mass polypeptides isolated from the different strains, the values ranging from 56 kDa in strain AP7 (Gonzalez *et al.*, 1990) to 64 kDa in the type strain (this study).

Interestingly, such interstrain molecular size heterogeneity has also been observed with the analogous low molecular mass polypeptides affinity-isolated with human transferrin from strains of *N. gonorrhoeae* and *N. meningitidis* (Lee & Bryan, 1989; Schryvers & Lee, 1989). On the other hand, note that the high molecular mass transferrin-binding polypeptides from *N. gonorrhoeae* and *N. meningitidis* are very similar in size (Lee & Bryan, 1989; Schryvers & Lee, 1989) and it would appear that this is also the case in strains of *A. pleuropneumoniae*; although the high molecular mass polypeptides from strains AP7, AP78 and H49 were reported to have molecular masses of 105 kDa (Gonzalez *et al.*, 1990), the data presented by Gonzalez *et al.* (1990) and the fact that the polypeptides in question co-migrated with the high molecular mass transferrin-binding polypeptide from *N. meningitidis* B16B6 (Gonzalez *et al.*, 1990) indicate a molecular mass of closer to 98 kDa (see Schryvers & Lee, 1989) and close to that reported here (99 kDa).

Regarding the capacities of these polypeptides to function as transferrin receptors in *A. pleuropneumoniae*, all of the polypeptides that were affinity-isolated from strains AP7, AP78 and H49 were shown to be IRMPs, and following SDS-PAGE and electroblotting, the low molecular mass polypeptides were shown to be capable of binding HRP-transferrin (Gonzalez *et al.*, 1990). While the 99 kDa polypeptide from the type strain is almost certainly also an IRMP, the *in situ* location of the 64 kDa polypeptide, which is also iron-repressible, is much less obvious as this polypeptide was absent from Sarkosyl-extracted outer membranes (Figs 1 and 2). However, it would appear that the low molecular mass, affinity-isolated IRMPs from strains AP7, AP78 and H49 are also easily solubilized during the Sarkosyl-extraction procedure (Gonzalez *et al.*, 1990) and hence, it would seem reasonable to suggest that in the type strain, the 64 kDa polypeptide is also associated, somehow, with the outer membrane. Although we do not know if the 64 kDa polypeptide can bind transferrin, the 99 kDa polypeptide was the last polypeptide to be eluted from the Sepharose-coupled porcine transferrin (Fig. 2) indicating that it, at least, is capable of direct binding to the host protein.

In summary, the available data (this study; Gonzalez *et al.*, 1990) indicate that while the polypeptides that were affinity-isolated from *A. pleuropneumoniae* could have been isolated in the form of a complex, both are capable of binding porcine transferrin and hence, both have to be considered as receptor candidates. While it is possible that the affinity-isolated polypeptides function as a receptor unit, it is also possible that only one is involved in the initial binding of the transferrin. For example, it is tempting to speculate that in the type strain, the 99 kDa polypeptide represents the porcine transferrin receptor and that the 64 kDa polypeptide

represents an associated protein serving an accessory role. Clearly, however, other interpretations are also possible and further studies are required to determine to what extent each of these polypeptides is involved in receptor activity.

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