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Research article

Outer membrane proteomics of *Pasteurella multocida* isolates to identify putative host-specificity determinants

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Pasteurella multocida is a Gram-negative bacterium responsible for diseases affecting a broad range of farm-reared animals. Although there is an association between capsular serotype and disease, the molecular basis of host specificity is poorly understood. Outer membrane proteins (OMPs) are at the interface of bacterium and host and are likely to play important roles in host specificity and disease. Two classes are of particular importance—adhesins that are adapted for colonization of specific host niches and iron-acquisition proteins that allow pathogens to acquire iron from host-specific iron complexes. A comparative analysis of the outer membrane (OM) proteome of eight *P. multocida* isolates associated with disease of avian, bovine, ovine and porcine species was performed to identify putative host-specificity determinants. Isolates were cultured in iron-replete media, and also in iron-limited conditions to mimic the iron-limited host environment, and induce expression of iron-regulated OMPs expressed *in vivo*. The OMP-rich sarcosyl-insoluble cell fraction was isolated and the OMPs were separated by SDS-PAGE and identified by matrix-assisted light desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). The expressed proteome was compared with the *in silico* predicted proteome from the genome sequence of *P. multocida* strain Pm70, using PSORTb and Proteome Analyst subcellular localization software. In iron-rich conditions isolates were clustered into three groups based on high molecular weight (HMW) OMP similarity. Isolates responsible for invasive disease were clustered into a single group. Putative colonization OMPs were present in isolates recovered from different host species, but showed molecular weight heterogeneity. Such proteins are good candidates for further study as disease or host-specificity determinants, as variation between these proteins may be a consequence of adaptation to different host niches. HMW OMPs were identified as being involved in iron-uptake. However, isolates associated with different diseases and host species expressed different iron-uptake proteins, or regulated expression differently, suggesting adaptation to specific host niches.

Key words: *Pasteurella multocida*, outer membrane protein, host specificity, iron acquisition.

Introduction

The bacterium *Pasteurella multocida* is a Gram-negative facultative anaerobe commonly found as a commensal in the upper respiratory tract of a diverse range of mammalian and avian species.[1] Many strains are opportunistic agents of primary or secondary disease, resulting in economically significant outbreaks affecting farm-reared animals. Disease syndromes include fowl cholera of poultry, progressive atrophic rhinitis (PAR) in pigs, haemorrhagic septicaemia of cattle in Asia and Africa, and pneumonic pasteurellosis in cattle, pigs and sheep in western countries, as well as cases of meningitis, abortion, localized infections and mastitis.[2–4] Asymptomatic carriers of *P. multocida* act as a reservoir of infection, and transmission is thought to occur by direct contact with infected animals, or dissemination in water supplies.[5]

The association of particular strains of *P. multocida* with specific disease syndromes is complex. Strains can be grouped serologically into 5 capsular types (A, B, D, E and F) and 16 somatic lipopolysaccharide-types (1–16).[1] Previous studies have reported an association between the

capsular serogroup and disease, although certain serogroups are associated with a range of diseases in different host species.[6] *P. multocida* strains have also been characterized by outer membrane protein (OMP)-type and 16S rRNA-type.[6] 16S rRNA-typing revealed that the majority of clinical isolates belong to a single lineage containing seven 16S-types. However a range of capsular types, OMP-types and host species were represented, indicating significant heterogeneity between closely related strains. Disease progression is therefore thought to depend on a complex interaction of host factors including species, age and immune status, and strain-dependent virulence factors such as production of toxins, adhesins and mechanisms for acquiring nutrients from the host.[7]

OMPs are key mediators of bacterial interaction with the host environment.[8] Until recently, characterized OMPs included the major proteins OmpA and OmpH and a limited number of minor OMPs that were investigated as virulence factors or immunogens.[9] Completion of the genome sequence of an avian serogroup A:3 strain, *P. multocida* strain Pm70, in conjunction with the development of *in silico* proteome prediction tools, has facilitated a bioinformatics-based approach to the study of OMPs.[10] OMP expression can now be considered within the context of the full OM-proteome complement. Recent studies have taken advantage of this technology to investigate *in vivo* and *in vitro* OMP expression via mass spectrometry (MS) and microarrays, and to identify candidate immunogens for reverse vaccine development.[9, 11, 12] However, OMP expression of isolates from different host species has not been investigated.

This study aimed to characterize the OM proteome of *P. multocida* isolates from different host species and to identify OMPs that may contribute to host specificity. OMPs present in isolates from different host species are likely to have an essential function for bacterial survival. A comparative analysis of the OM proteome of eight *P. multocida* isolates recovered from avian, bovine, ovine and porcine hosts was performed. OMPs were isolated by sarcosyl extraction and OMP profiles compared by SDS-PAGE. OMPs of interest were identified and compared by MALDI-TOF-MS, a rapid, high throughput method capable of measuring peptide mass to 0.01% accuracy.[13] OMPs expressed by isolates from different host species were compared against the predicted OM proteome of *P. multocida* strain Pm70 using Proteome Analyst (PA) and PSORTb software. PA predicts protein features such as subcellular localization from SWISS-PROT gene annotation key words of sequence homologues, providing high coverage of Gram-negative proteomes, whereas PSORTb is a high precision tool combining multiple primary sequence features to predict protein features.[14, 15] Isolates were also cultured in iron-replete conditions and iron-limited conditions to replicate host *in vivo* conditions and facilitate the identification of iron-regulated OMPs.

Materials and methods

Bacterial isolates and culture conditions

Eight *P. multocida* isolates commonly associated with invasive or pneumonic disease syndromes in avian, bovine, ovine or porcine hosts, and representing a range of serotypes and OMP-types, were included in this study (Table 1). Pure *P. multocida* cultures were obtained by plating 10 μl bacterial suspensions from frozen glycerol stocks [50% (v/v) glycerol in brain-heart infusion broth (BHIB); Oxoid] onto blood agar plates [5% defibrinated sheep's blood in brain-heart infusion agar (BHIA); Oxoid] and incubating at 37°C for 24 h.

Optimization of iron-limited culture conditions

Isolates were grown in BHIB containing the iron-chelating agent 2,2′-dipyridyl to reduce the concentration of iron available in the growth media. Dipyridyl concentrations capable of inducing observable expression of iron-uptake OMPs without completely inhibiting growth were determined by growth curves conducted in 100 ml BHIB containing 0, 50, 100, 150 or 200 μM 2,2′-dipyridyl. Growth curves were repeated over a range of dipyridyl concentrations where small changes in dipyridyl concentration produced a rapid decline in growth. The final concentrations of 2,2′-dipyridyl selected for batch culture of each isolate were PM144, 100 μM; PM246, 140 μM; PM564, 120 μM; PM632, 100 μM; PM684, 100 μM; PM734, 60 μM; PM966, 100 μM; PM982, 100 μM.

Sarcosyl extraction of OMPs

OMP fractions were prepared using the sarcosyl extraction method described previously.[16, 17] Inocula were prepared by inoculating five to six colonies into 15 ml BHIB and

Table 1. Properties of *Pasteurella multocida* isolates

Isolate designation	Host origin	Serotype[a]	OMP type[a]	16S type[a]	Associated disease[a]
PM144	Avian	A	1.1	2	Septicaemia
PM246	Avian	F	2.2	2	Septicaemia
PM564	Bovine	A	2.1	3	Pneumonia
PM632	Bovine	A	4.1	1	Pneumonia
PM684	Porcine	A	6.1	2	Suspected PAR[b]
PM734	Porcine	A	1.1	2	Pneumonia
PM966	Ovine	A	1.1	1	Pneumonia
PM982	Ovine	D	3.1	1	Pneumonia

[a]Data taken from a previous study by Davies.[6]
[b]PAR, Porcine atrophic rhinitis.

incubating overnight at 37°C with shaking at 120 rpm. For batch culture, 400 ml of pre-warmed BHIB were inoculated with 800 μl (1:500) of overnight culture and incubated at 37°C with shaking at 120 rpm, until cultures reached mid-log phase equivalent to an $OD_{600\,nm}$ of ~1.0 (6–8 h of growth). For iron-limited growth conditions, the appropriate concentration of sterile 2,2′-dipyridyl was added. Growth curves were generated by removing 1 ml of culture every 1–2 h and measuring $OD_{600\,nm}$ with a WPA Biowave CO8000 cell density meter. On achieving mid-log phase, cultures were placed on ice water for 5 min to stop growth. Purity checks were performed by plating out one drop of culture onto blood agar and incubating for 24 h at 37°C. Cultures were harvested by centrifugation at 10 000*g* for 30 min at 4°C. The bacterial pellet was resuspended in 50 ml ice cold 20 mM Tris-HCl (pH 7.2) and centrifugated at 10 000*g* for 30 min at 4°C. The pellet was resuspended in ~8.0 ml of ice-cold 20 mM Tris-HCl (pH 7.2) and the cells were lysed by sonication for 5 min in ice water using a Soniprep sonicator at 12 μm amplitude. Unbroken cells were removed by centrifugation at 4°C for 30 min at 10 000*g*. The supernatants were carefully transferred to 10 ml ultracentrifuge tubes and cell envelopes pelleted by centrifugation at 50 000*g* for 1 h at 4°C. The gelatinous pellets were fully resuspended in 10 ml 0.5% sodium *N*-lauroylsarcosine (sarcosyl) using long-form Pasteur pipettes, and the sarcosyl-insoluble OM fraction was pelleted by centrifugation at 50 000*g* for 1 h at 4°C. The OM pellet was resuspended in 10 ml 20 mM Tris-HCl (pH 7.2) and centrifugated again at 50 000*g* for 1 h at 4°C. The final OM pellet was carefully resuspended in a small volume (less than 1 ml) of 20 mM Tris-HCl (pH 7.2) to achieve a final concentration greater than 2.0 mg/ml and stored at −20°C.

Modified Lowry assay for the determination of OMP concentration

The modified assay improves solubilization of membrane proteins.[18] Fifty microlitre volumes of OM samples were made up to 1 ml with distilled water in a test tube. Three millilitres of alkaline copper reagent (2.0% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate, 1.0% SDS, 0.04% $CuSO_4.5H_2O$) were added and the tubes vortexed and incubated at room temperature for 60 min. Three hundred microlitres of 1:1 dilute Folin-Ciocalteu phenol reagent were added and the tubes vortexed and incubated for a further 45 min at room temperature. Absorbance was measured at 660 nm on a Unicam UV/VIS spectrophotometer. A standard curve was generated from triplicate bovine serum albumin (BSA) protein standards in the range 0.0–1.0 mg/ml. OMP stocks were then adjusted to a final concentration of 2.0 mg/ml.

SDS-PAGE of OMPs

OMP samples were adjusted to 1.0 mg/ml in 2× sample buffer [0.125 M Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-β-mercaptoethanol, 0.5% (w/v) bromophenol blue] and heated in a boiling water bath for 5 min. SDS-PAGE was carried out using a Protean I Dual Slab apparatus (Bio-Rad, Richmond, CA94804). The gel comprised a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. A Tris-glycine reservoir buffer (25 mM Tris Base, 192 mM glycine and 0.1% SDS; pH 8.3) was used. Twenty micrograms of OMP samples were loaded into each lane and electrophoresis conducted using a constant current of 20 mA per gel through the stacking gel and 30 mA per gel through the resolving gel. Protein bands were visualized by staining with Coomassie blue. Gels were stored in 5% (v/v) acetic acid.

Preparation of proteins for MALDI-TOF-MS

Thirty-six protein bands expressed in iron-rich culture were selected for identification and 38 bands were selected from isolates grown in iron-limited media. Protein bands were excised for *in situ* trypsin digestion. Gel pieces were washed in 100 mM ammonium bicarbonate for 1 h and washed again with acetonitrile/100 mM ammonium bicarbonate (1:1). Cysteine reduction was achieved by incubating samples in 160 μl 45 mM DTT/100 mM ammonium bicarbonate (1:15) at 60°C for 30 min. Samples were cooled to room temperature and alkylated by adding 10 μl of 100 mM iodoacetamide and incubating in darkness at room temperature for 30 min. Gel slices were washed for 1 h in acetonitrile/100 mM ammonium bicarbonate (1:1), dehydrated in acetonitrile for 10 min and dried in a Speedvac vacuum centrifuge. Gel pieces were fully rehydrated at 4°C in 25 mM ammonium bicarbonate containing 0.2 mg/ml sequence grade modified porcine trypsin (Promega, V111). Sufficient 25 mM ammonium bicarbonate was added to cover the gel pieces. Proteins were digested overnight at 37°C. Trypsin digested peptides were extracted by adding an equal volume of acetonitrile to the digest, incubating at room temperature for 20 min and transferring the eluate to a 96-well plate. The extraction was repeated using 1% formic acid. Pooled peptide extracts were precipitated by vacuum centrifugation at 45°C. The precipitate was resuspended in 4 μl of 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid (TFA) and 1 μl mixed with an equal volume of matrix comprising a saturated solution of cyano-4-hydroxycinnamic acid (CHCA; Bruker Daltonics GmBH, Germany) in 50% (v/v) acetonitrile, 0.1% (v/v) TFA on a polished stainless steel target plate (MTP 384 target plate, Bruker Daltonics GmBH, Germany) and allowed to air dry at room temperature.

Peptide mass fingerprinting by MALDI-TOF-MS

OMPs were analysed using a Voyager DEpro MALDI-TOF-MS (Applied Biosystems). Mass spectra were internally calibrated against autologous trypsin peptide peaks. Data were collected over the mass range 800–4000 Da, using a minimum signal-to-noise ratio filter of 10. OMPs were

identified from the peptide mass fingerprinting (PMF) output by the MASCOT (Matrix Science) search engine.[19] The database search was restricted to the phylum Proteobacteria and allowed for a maximum of one missed cleavage, modification by carbaridomethylation, variable modification of methionine residues by oxidation and a positive peptide charge of 1.

Prediction of OMPs from genome sequence

OMPs identified by MS were compared with proteins predicted to have an OM subcellular localization from the complete genome sequence of *P. multocida* strain Pm70 (GenBank accession no. AE004439).[10] To maximize coverage of the OM proteome, OMP predictions were generated from the combined results of analysis of the Pm70 genome using PA v.3.0 (http://www.cs.ualberta.ca/~bioinfo/PA/) and PSORTb v.2.0 (http://www.psort.org/psortb) software. All PSORTb OM subcellular localization predictions had a confidence value >9.0.

Results

SDS-PAGE and MALDI-TOF-MS of OMPs from iron-rich culture

OMP profiles comprised two major proteins (OmpA and OmpH) and 11–14 minor proteins (Fig. 1). Positive controls from lane 5 (PM684) and lane 8 (PM982) were correctly identified as OmpA and OmpH by PMF. It was noted that proteins in the HMW OMP region (85–100 kDa) formed three groups, comprising either three weakly expressed OMPs (Fig. 1, lanes 1, 2 and 5), two weakly expressed OMPs and one lower MW, strongly expressed OMP (Fig. 1, lanes 3, 4 and 8) or five moderately expressed OMPs (Fig. 1, lanes 6 and 7). When isolates were clustered by HMW OMP homology, there was an association between HMW OMP-type and disease-type (Fig. 2). Isolates responsible for invasive diseases (avian PM144 and PM246, fowl cholera; porcine PM684, PAR) formed a single distinct cluster. The remaining isolates formed two clusters associated with pneumonic pasteurellosis.

MALDI-TOF-MS identified that OMPs in the HMW region were mainly involved in iron acquisition. The two groups of isolates associated with pneumonic disease expressed different iron-acquisition proteins depending on the HMW OMP-type. The group comprising both bovine isolates (PM564 and PM632) and ovine isolate PM982 expressed a haemoglobin-binding protein (HgbA) and transferrin-binding protein (TbpA). The second pneumonic group, comprising porcine isolate PM734 and ovine isolate PM966, expressed haem-acquisition receptors HemR and PfhR. The OMP profiles of PM734 and PM966 were

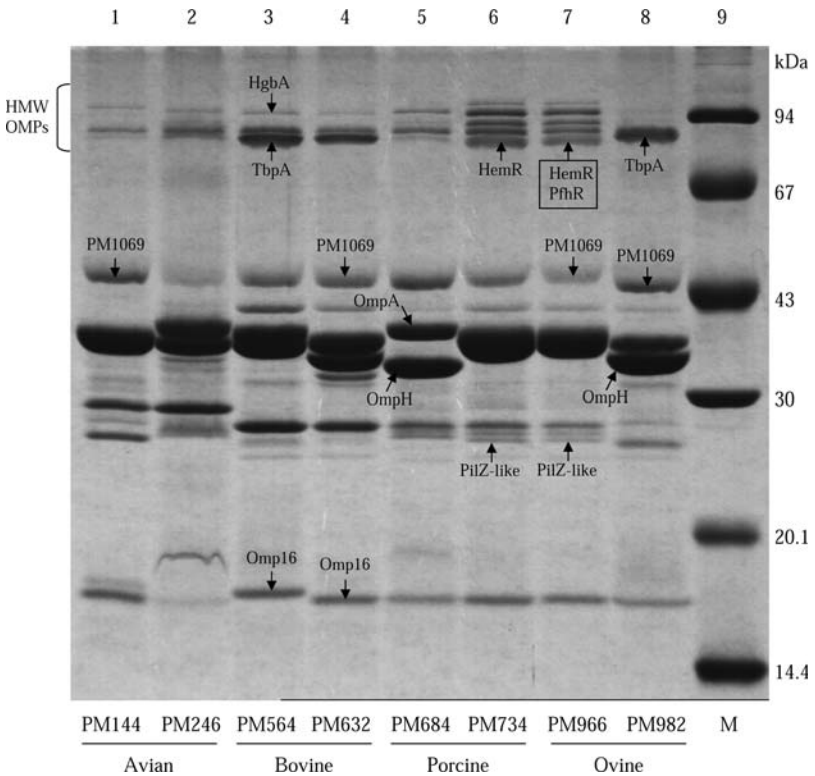

Figure 1. Coomassie blue-stained SDS-PAGE gel showing OMP profiles of *Pasteurella multocida* isolates of avian, bovine, porcine and ovine origin. Isolates were grown in iron-rich media. Lane numbers are given at the top of the gel. Isolate designation and host of origin is stated at the bottom. Lane 9 shows the molecular weight (MW) standards (kDa). Labelled bands correspond to OMPs of interest discussed in the main text and were identified by MALDI-TOF-MS.

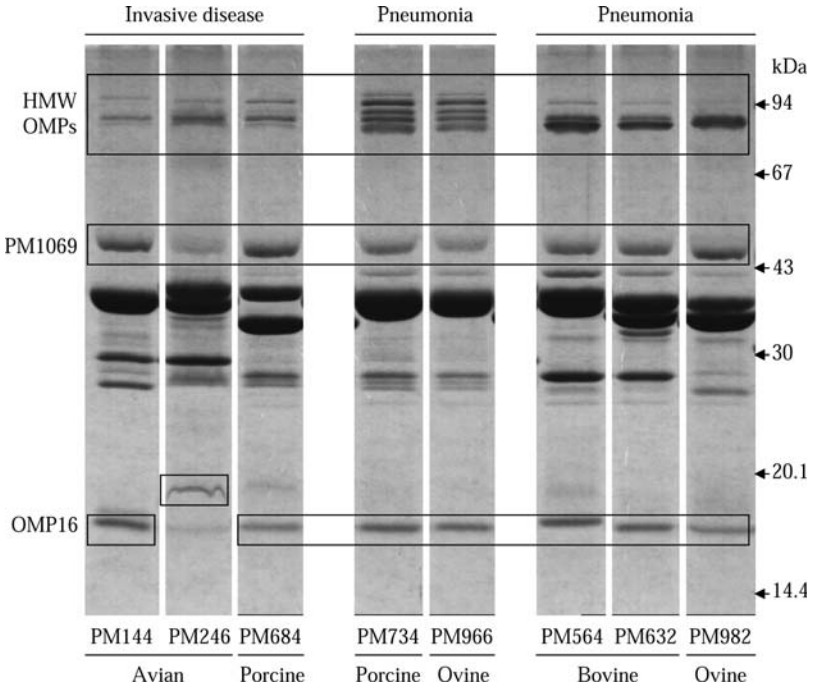

Figure 2. SDS-PAGE profiles of *Pasteurella multocida* isolates grown in iron-rich media, clustered by homology of HMW OMPs. Isolates associated with invasive diseases clustered into a single group. Pneumonic isolates formed two distinct clusters. Disease type is indicated at the top of the gel. Isolate designation and host of origin is stated at the bottom. MW standards (kDa) are indicated. The HMW OMP region is highlighted, as are proteins PM1069 and OMP16 that were present in all isolates but demonstrated MW variation.

generally very similar, and both also expressed a protein with a moderate degree of homology to a *Shewanella pealeana* type IV pilus biogenesis protein PilZ (Fig. 1). Only one HMW OMP of the invasive disease cluster was successfully identified by MS at this point and was an organic solvent tolerance (Ost) protein.

SDS-PAGE and MALDI-TOF-MS of OMPs from iron-limited culture

Although invasive disease isolates PM144, PM246 and PM684 and pneumonic isolates PM734 and PM966 formed distinct groups by HMW OMP similarity following iron-rich culture, expression of HMW OMPs by these groups was altered in response to iron-limitation (Fig. 3). Isolates that were previously similar by HMW OMP-type had different HMW OMP profiles depending on the host of origin (Fig. 4, Tables 2 and 3). In contrast, OMP expression by the pneumonic disease cluster comprising bovine isolates PM564, PM632 and ovine isolate PM982 was unaffected by iron limitation. In particular, there was no significant change in expression of iron-acquisition proteins TbpA or HgbA.

MS of proteins in the HMW region revealed that several iron-acquisition OMPs were expressed by isolates from the invasive disease group and the PM734/PM966 pneumonic group. These included haem-acquisition OMPs HasR, HemR, PfhR and hypothetical protein PM0741, a putative iron-transport OMP (Fig. 3). HemR and PfhR were common to all invasive disease isolates. Porcine isolate PM684 expressed an additional OMP, HasR, which was not identified from the invasive avian isolates, but was expressed by the other porcine isolate PM734, which was associated with pneumonic disease.

HMW OMP expression by porcine isolate PM734 increased in response to iron limitation, and six bands were visible compared with five bands in iron-rich culture. Expressed iron-uptake OMPs identified were HasR, HemR, PfhR and PM0741 (Fig. 4, Tables 2 and 3). Ovine isolate PM966, which had a very similar OMP profile to PM734 in iron-rich culture, had reduced HMW OMP expression (four bands visible), although expression of HemR, PfhR and PM0741 was confirmed (Fig. 4, Tables 2 and 3).

OMPs demonstrating MW variation

MS of proteins expressed following iron-rich or iron-limited culture identified four proteins that were present in isolates from different host species but in variant MW forms. A conserved band of ~47.3 kDa was identified as hypothetical protein PM1069 (Fig. 1). MW variation of PM1069 was observed for isolates from different hosts and was most notable for ovine isolate PM982 which possessed a 44.2 kDa MW variant (Fig. 2). A 17.9 kDa conserved protein was identified as OMP16 (Fig. 1). PM564 expressed an 18.1 kDa form, and PM246 a 19.2 kDa form (Fig. 2). A 40.7 kDa OMP, HexD, was conserved across species but was

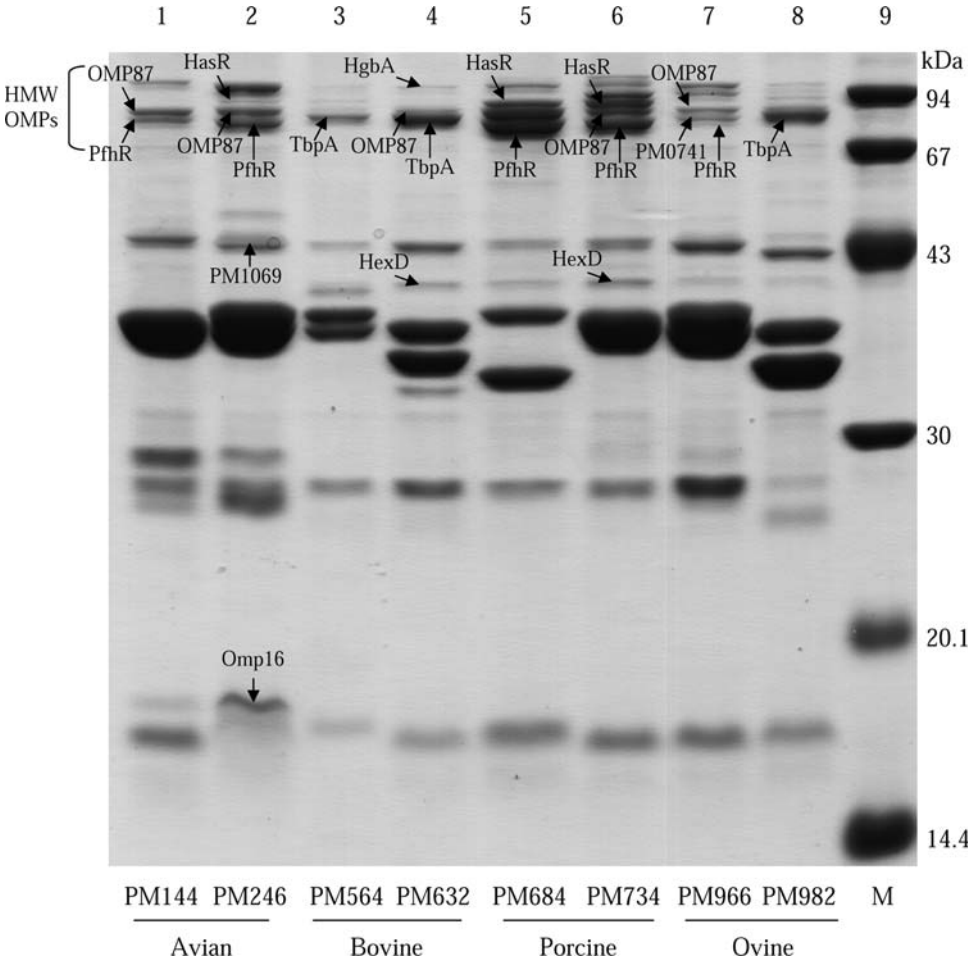

Figure 3. Coomassie blue-stained SDS-PAGE showing OMP profiles of *Pasteurella multocida* isolates of avian, bovine, porcine and ovine origin, following growth in iron-limited conditions. Lane numbers are given at the top of the gel. Isolate designation and host of origin is stated at the bottom. Lane 9 shows the MW standards (kDa). Labelled bands denote OMPs of interest discussed in the main text and were identified by MALDI-TOF-MS.

only weakly expressed by avian isolates and porcine isolate PM684 (Fig. 3). Expression of PM1069, OMP16 and HexD was not affected by iron limitation. MS of HMW OMPs revealed that OMP87, which is not associated with iron-uptake, was conserved across isolates from all four hosts (Fig. 3). However, expression levels were difficult to compare due to the tight clustering of bands and altered expression of proteins in the HMW region under iron-rich or iron-limited conditions.

The *in silico* predicted OM proteome

Analysis of the *P. multocida* strain Pm70 genome sequence by PSORTb and PA software identified a total of 79 proteins predicted to have an OM localization. Thirty-nine OMPs were identified by both prediction algorithms, whereas 11 OMPs were predicted only by PSORTb and 29 by PA alone. Predicted OMPs represented 12 functional classes by clusters of orthologous groups (COGs) (Fig. 5). The major groups identified were cell envelope biogenesis (31 OMPs), inorganic ion and metabolic trafficking (12 OMPs), and secretion and intracellular transport (8 OMPs). A quarter of predicted OMPs were of unknown function.

Comparison of expressed and predicted *P. multocida* OM proteome

In total, 22 different OMPs were shown to be expressed by *P. multocida* isolates from different hosts under iron-rich or iron-limited growth conditions. A small number of proteins identified by MS were not identified by the proteome prediction software, including the major protein OmpA and OMP16. This may have been due to differences in nomenclature. Prediction software identified multiple occurrences of OmpH (three occurrences), Hsf (two occurrences) and LspB (two occurrences). Expression of all definitively identified iron-uptake OMPs predicted from the Pm70 genome was confirmed in different isolates. However, whereas only three definitive iron-acquisition OMPs were predicted from the Pm70 genome (HasR, HemR and PfhR), MS identified five

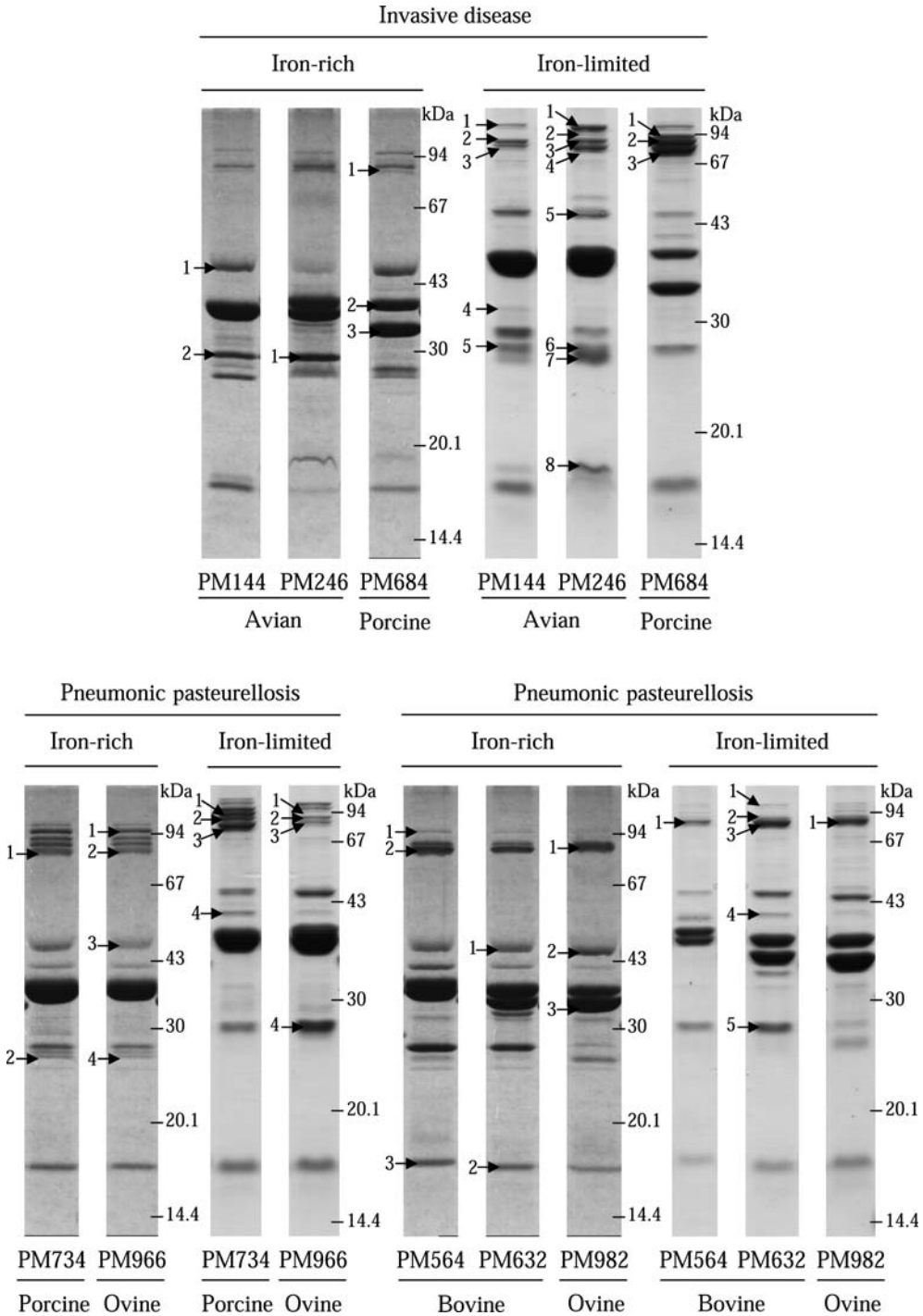

Figure 4. OMP profiles of *Pasteurella multocida* isolates cultured in iron-rich or iron-limited media resolved by Coomassie blue-stained SDS-PAGE. Isolates are grouped by HMW OMP similarity under iron-rich culture conditions. Isolate ID, host species, disease type, iron availability and MW standards (kDa) are indicated. Numbered bands denote OMPs identified by MALDI-TOF-MS. Corresponding band identities are shown in Tables 2 (iron-rich culture) and 3 (iron-limited culture).

iron-uptake OMPs (HasR, HemR, PfhR, HgbA and TbpA). HgbA and TbpA were not predicted from the avian Pm70 genome, but were expressed by bovine isolates PM564 and PM632 and ovine isolate PM966.

Eight hypothetical proteins were predicted to have iron-uptake functions. Expression of hypothetical OMPs PM0300, PM0336 and PM0741 (putative iron-uptake OMPs by COGs classification) was confirmed (Fig. 4,

Table 2. Identification of selected *Pasteurella multocida* OMPs from iron-rich culture, determined by Mascot and BLASTP analysis

Band identity[a]	Accession No.	Protein name	Molecular weight	Protein score	Matched peptides	Coverage (%)
PM144.1	15214145	47kDa OMP precursor	46 032.7	219	19	56
	15602934	PM1069	47 831.6	217	19	54
PM144.2	49182350	OmpA	37 785.4	85	17	61
PM246.1	15602651	PM0786	38 121.6	129	25	73
	49182354[b]	**OmpA**	**38 125.6**	**102**	**22**	**67**
PM564.1	15602165	PM0300	110 044.6	153	42	47
	33340606[b]	**HgbA**	**110 664.9**	**125**	**37**	**44**
PM564.2	13027667	TbpA	89 377.0	547	33	47
PM564.3	6977946	OMP 16 protein	14 207.0	180	6	73
PM632.1	15214145	47 kDa OMP precursor	46 032.7	108	18	60
	15602934	PM1069	47 831.6	106	18	58
PM632.2	6977946	OMP 16 protein	14 207.0	140	8	82
PM684.1	25008882	LPS-assembly precursor (Ost)	90 819.4	88	26	40
PM684.2	15602651	PM0786	38 121.6	114	20	66
	49182354[b]	**OmpA**	**38 125.6**	**85**	**16**	**54**
PM684.3	2853254	OmpH	34 656.9	105	18	54
PM734.1	15602441	HemR	85085.8	330	26	39
PM734.2	157962851	PilZ [*S. pealeana* ATCC 700345]	92 441.9	78	28	41
PM966.1	7716525	Putative TonB-dependent receptor	110 287.5	79	33	35
PM966.2	15602441	HemR	85 085.8	187	22	33
	15601905	PfhR	81 510.1	182	24	30
PM966.3	15214145	47 kDa OMP precursor	46 032.7	84	20	60
	15602934	PM1069	47 831.6	82	20	58
PM966.4	157962851	PilZ [*S. pealeana* ATCC 700345]	92 441.9	76	27	40
PM982.1	110725170	TbpA	89 399.2	329	36	53
PM982.2	15214145	47 kDa OMP precursor	46 032.7	78	18	57
	15602934	PM1069	47 831.6	76	18	55
PM982.3	99083546	OMP	38 821.3	123	15	40
	2853254[b]	**OmpH**	**34 656.9**	**83**	**13**	**39**

[a]Band identities correspond to Fig. 4.
[b]Bands in bold are the nearest characterized protein identification when the highest scoring OMP was a poorly characterized or hypothetical protein (HP).

Tables 2 and 3). A further five hypothetical proteins with probable iron receptor functions were predicted from the Pm70 genome (PM0337, PM0745, PM1081, PM1282, PM1428), but expression was not confirmed.

Discussion

Candidate adhesins of *P. multocida*

It has been proposed that the broad host range of *P. multocida* is due to colonization by adherence to extracellular matrix (ECM) components that are ubiquitous between animal species.[20] However, the role of specific adhesins in host colonization and disease has not been well characterized.[21, 22] Study of a bovine *P. multocida* strain implicated fibronectin (Fn) as a specific receptor for *P. multocida* and identified five OMPs involved in binding to host Fn.[20] Fn is a dimer that occurs in different isoforms and has eight functional domains that may be exploited as receptors for bacterial colonization. Two classes of Fn have been identified—a soluble form present in body fluids and an ECM form.[23] Dabo *et al.*[20] showed that bovine strains preferentially bound the *N*-terminal Hep 1 domain. In the current study, three of the OMPs identified previously,

Table 3. Identification of selected *Pasteurella multocida* OMPs from iron-limited culture, determined by Mascot and BLASTP analysis

Band ID[a]	Accession No.	Protein name	Molecular weight	Protein score	Matched peptides	Coverage (%)
PM144.1	145632020	CGSHi22421_00657 [*H. influenzae*]	90 430	90	13	21
PM144.2	15603857	HP PM1992	87 822	250	28	45
	27527748[b]	**Omp87**	**87688**	**222**	**26**	**41**
PM144.3	15601905	PfhR	81 510	179	24	39
PM144.4	15603595	PlpB	30 276	110	12	59
PM144.5	15602863	HP PM0998	30 915	89	9	44
PM246.1	15602201	HP PM0336	114 285	224	29	35
PM246.2	15603487	HasR	96 079	331	35	50
PM246.3	15603857	HP PM1992	87 822	132	20	32
	27527748[b]	**Omp87**	**87 688**	**120**	**19**	**29**
PM246.4	15601905	PfhR	81 510	264	29	51
PM246.5	15214145	47 kDa OMP precursor	46 033	212	21	69
	15602934	HP PM1069	47 832	209	21	67
PM246.6	15602651	HP PM0786	38 122	172	21	51
	49182354[b]	**OmpA**	**38 126**	**147**	**18**	**45**
PM246.7	15603408	HP PM1543	26 864	97	11	60
PM246.8	6977946	OMP 16 protein	14 207	115	8	90
PM564.1	13027667	TbpA	89 377	254	28	45
PM632.1	33340606	HgbA	110 665	148	22	29
PM632.2	15603857	HP PM1992	87 822	307	33	53
	27527748[b]	**Omp87**	**87 688**	**293**	**32**	**53**
PM632.3	13027667	TbpA	89 377	350	36	54
PM632.4	15602643	HexD	43 034	123	14	52
PM632.5	15602863	HP PM0998	30 915	86	9	41
	15214146	24 kDa OMP precursor	28 719	78	8	35
PM684.1	15603487	HasR	96 079	369	37	51
PM684.2	15602668	HP PM0803	91 050	147	22	32
PM684.3	15601905	PfhR	81 510	271	30	55
PM734.1	15603487	HasR	96 079	248	29	47
PM734.2	27527748	Omp87	87 688	119	18	32
PM734.3	15601905	PfhR	81 510	268	30	50
PM734.4	15602643	HexD	43 034	131	15	60
PM966.1	15603857	HP PM1992	87 822	302	32	50
	27527748[b]	**Omp87**	**87 688**	**288**	**31**	**51**
PM966.2	15602606	HP PM0741	89 716	114	19	30
PM966.3	15601905	PfhR	81 510	182	24	40
PM966.4	15602863	HP PM0998	30 915	107	9	40
	15214146[b]	**24 kDa OMP precursor**	**28 719**	**98**	**8**	**34**
PM982.1	110725170	TbpA	89 399	250	30	48

[a]Band identities correspond to Fig. 4.
[b]Bands in bold are the nearest characterized protein identification when the highest scoring OMP was a poorly characterized or hypothetical protein (HP).

PM1069 (P80603), OMP87 and OMP16,[20] were conserved between isolates from different host species, providing evidence of the importance of these OMPs to the broad host range of *P. multocida*. OMPs PM1069 and OMP16 were present across different isolates but displayed MW heterogeneity. OMPs that display MW heterogeneity across isolates from different hosts are of particular interest, as the observed variation may have arisen due to selective pressures acting on

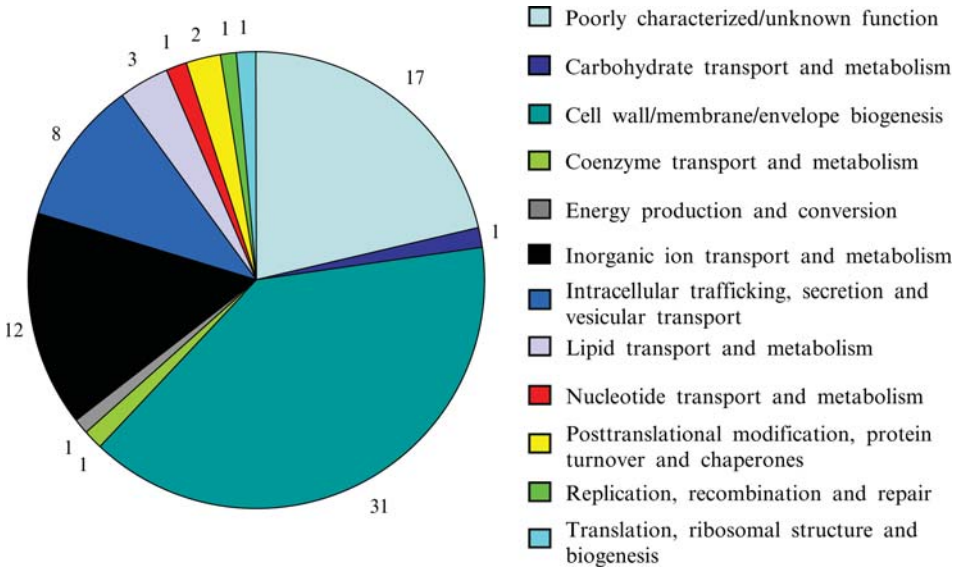

Figure 5. COGs classification of OMPs predicted from the OM proteome of *Pasteurella multocida* strain Pm70. A total of 79 OMPs were predicted using PSORTb and PA software.

these OMPs within different host niches. These proteins are therefore useful candidates for further study as host-specificity determinants.[24] For example, MW heterogeneity between these OMPs could reflect recognition of Fn-types encountered in different host niches during invasive or pneumonic disease, variation in the abundance of Fn isoforms found within particular host species, or recognition of different Fn domains.

Pilus structures expressed by *P. multocida* have been implicated as the primary mechanism for colonization of nasal epithelia, but there are few studies to support this hypothesis.[25] A protein with homology to the type IV pilus assembly protein PilZ of *S. pealeana* was present in the OMP profile of PM734 and PM966. However, other potential pilus components, such as those comprising the OM integral O-ring, were not identified. In addition, putative filamentous adhesin Hsf and haemagglutinins PfhB1 and PfhB2 were predicted from the Pm70 genome and may be important virulence determinants, but were not detected in the present study.[10] This could have been due to incomplete identification of the OM proteome, lack of expression *in vitro* or because the vigorous methods involved in sarcosyl extraction are inappropriate for the isolation of fragile filamentous surface structures.

Expression of type IV pili by *P. multocida* has been characterized for serogroups A, B and D, and is responsible for twitching motility.[21] Twitching motility is an important step in biofilm formation, for example during lung infections by *Pseudomonas aeruginosa* in cystic fibrosis patients.[26] It is feasible that biofilm formation is also important during lung infections by *P. multocida*, which would link expression of type IV pili to the ability of isolates to cause pneumonic disease. Other authors have previously speculated that biofilm formation by *P. multocida* may be important in establishing disease, but this area has not been well studied.[27]

HMW OMP profile and host specificity

When isolates were cultured in iron-rich media, they clustered into three groups by HMW OMP homology and there was an association between HMW OMP-type and disease, as all invasive disease isolates clustered into a single group. It is possible that homology of HMW OMPs expressed in a nutrient-rich environment reflects an epidemiological relationship between these isolates. Nutrient-rich culture may elicit a basal state of OMP expression, as there is no pressure for increased expression of a particular class of uptake system. This would explain why isolates from different host species had similar HMW OMP profiles in a nutrient-rich environment, but had dissimilar HMW OMP profiles under iron-limitation stress.

The results also suggested that expression or regulation of iron-uptake OMPs may be adapted for survival within particular host species or specific host niches. Avian isolates PM144 and PM246 were both associated with septicaemia and had very similar OMP profiles under iron-limited conditions. However, porcine isolates PM684 and PM734 were associated with diseases (PAR and pneumonia respectively) that occur at different sites within the host, and had different HMW OMP profiles under iron limitation.

Haem is the most abundant iron source in birds and mammals.[28] Avian isolates PM144, PM246, porcine isolates PM684, PM734 and ovine isolate PM966 expressed an array of OMPs involved in the acquisition of iron via haem-uptake

(HasR, HemR and PfhR). HasR is a haemophore receptor. Haemophore systems typically involve type I secretion of the haemophore HasA into the extracellular environment, binding of host haem and delivery to the transmembrane channel HasR. However, less efficient haem-uptake via direct binding of haem by HasR may also occur.[28] BLAST analysis revealed that the Pm70 genome does not contain the *hasA* gene suggesting that at least some *P. multocida* isolates utilize a HasA-independent mechanism. HemR and PfhR are thought to be haem-transport porins which bind different haem-containing compounds directly. The presence of multiple haem-uptake mechanisms may broaden the range of haem sources accessible by these isolates.

Haem-uptake systems are often associated with haemolysins that liberate haem from host erythrocytes and other cell types. *P. multocida* is described as non-haemolytic under aerobic conditions.[29] However, recent studies have reported production of an inner membrane-bound haemolysin AhpA, expressed under anaerobic conditions *in vitro* and is most active against avian red blood cells.[30, 31] Furthermore, a haemolysin secretion and activation protein LspB was predicted from the Pm70 genome by both PSORTb and PA. The role of the putative AhpA virulence factor in host pathogenesis is unknown, but it is conceivable that haemolysin production could enhance haem acquisition under certain *in vivo* conditions and could be associated with increased virulence of strains.

Bovine isolates PM564, PM632 and ovine isolate PM982 expressed a unique set of iron-acquisition OMPs, HgbA and TbpA, which were not expressed by avian or porcine isolates and were not predicted from the avian Pm70 genome. Acquisition of iron from host transferrin (Tf) typically involves two components, a lipoprotein TbpB, which is a high-specificity receptor for host Tf, and a 100 kDa tonB-dependent iron transport channel TbpA. TbpB-independent Tf binding can occur, but is less efficient. The present study concurs with previous reports that *P. multocida* expresses an 82 kDa form of TbpA and does not express TbpB at all, and furthermore, that TbpA is expressed only by bovine and ovine isolates.[32] In this study, TbpA was constitutively expressed in iron-rich and iron-limited conditions. This may be because the *P. multocida tbp* locus lacks the Fur-regulated promoter which is usually upstream of *tbpB* and is therefore not regulated by iron availability.[33] The presence of a neighbouring insertion sequence suggests that the *tbpA* gene may have been acquired by horizontal transmission.

Comparison of *in vitro* and *in silico* OM proteomics

The OMPs identified in this study represented around one-quarter of the OMPs predicted from the Pm70 genome. This proportion may have been higher if all protein bands had been subjected to MS. A similar study by Boyce *et al.*[9] suggested that around one-third of the predicted OM proteome was expressed. While the expressed OM proteome is expected to differ from the complete OM proteome due to environmental and temporal regulation of expression, it has been suggested that the majority of OMPs could fail detection due to low levels of expression.[34] OMPs may also be mistakenly predicted from genes with unusual localization signal sequences or from pseudogenes.[9] In this study, three OmpH proteins (OmpH_1, OmpH_2, OmpH_3) were predicted. However, comparison against the OmpH_1 amino acid sequence by BLAST analysis revealed that OmpH_2 and OmpH_3 contain sequence gaps and are potentially non-functional pseudogenes.

In conclusion, 22 OMPs were identified from the OM proteome of eight *P. multocida* isolates recovered from different host species. Expression of several hypothetical OMPs predicted from the *P. multocida* Pm70 proteome was confirmed by PMF. A variety of potential host colonization factors were identified, some of which were present in isolates from different host species and occurred as MW variants (e.g. OMP16, PM1069). HMW OMPs were mainly involved in iron acquisition. In iron-replete conditions, isolates clustered into three groups by HMW OMP profile, and there was an association between these groups and disease. However within these clusters, isolates from different host species regulated iron-acquisition OMPs differently in response to iron limitation, suggesting that similar isolates may have evolved alternate mechanisms for regulating expression of iron-acquisition OMPs. This may reflect adaptation for survival within different host niches.

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References

1. Rimler RB, Rhoades KR (1989) Pasteurella multocida. *In* CF Adlam, JM Rutter, eds, *Pasteurella and Pasteurellosis.* London: Academic Press, pp. 37–73.
2. Chanter N, Rutter JM (1989) Pasteurellosis in pigs and the determinants of virulence of toxigenic *Pasteurella multocida*. In CF Adlam, JM Rutter, eds, *Pasteurella and Pasteurellosis*. London: Academic Press, pp. 161–195.
3. Frank GH (1989) Pasteurellosis of cattle. In CF Adlam, JM Rutter, eds, *Pasteurella and Pasteurellosis*. London: Academic Press, pp. 197–222.
4. Rhoades KR, Rimler RB (1989) Fowl cholera. In CF Adlam, JM Rutter, eds, *Pasteurella and Pasteurellosis*. London: Academic Press, pp. 95–113.

5. Carter GR, de Alwis MCL (1989) Haemorrhagic septicaemia. In CF Adlam, JM Rutter, eds, *Pasteurella and Pasteurellosis*. London: Academic Press, pp. 131–160.
6. Davies RL (2004) Genetic diversity among *Pasteurella multocida* strains of avian, bovine, ovine and porcine origin from England and Wales by comparative sequence analysis of the 16S rRNA gene. *Microbiology* 150: 4199–4210.
7. Boyce JD, Adler B (2006) How does *Pasteurella multocida* respond to the host environment? *Curr Opin Microbiol* 9: 117–122.
8. Lin J, Huang S, Zhang Q (2002) Outer membrane proteins: key players for bacterial adaptation in host niches. *Microbes Infect* 4: 325–331.
9. Boyce JD, Cullen PA, Nguye V *et al*. (2006) Analysis of the *Pasteurella multocida* OM sub-proteome and its response to the *in vivo* environment of the natural host. *Proteomics* 6: 870–880.
10. May BJ, Zhang Q, Li L *et al*. (2001) Complete genomic sequence of *Pasteurella multocida*, Pm70. *Proc Natl Acad Sci* 98: 3460–3465.
11. Al-Hasani K, Boyce J, McCarl V *et al*. (2007) Identification of novel immunogens in *Pasteurella multocida*. *Microb Cell Fact* 6: 3.
12. Paustian ML, May BJ, Kapur V (2001) *Pasteurella multocida* gene expression in response to iron limitation. *Infect Immun* 69: 4109–4115.
13. Blackstock WP, Weir MP (1999) Proteomics: quantitative and physical mapping of cellular proteins. *Trends Biotechnol* 17: 121–127.
14. Szafron D, Lu P, Greiner R *et al*. (2004) Proteome Analyst: custom predictions with explanations in a web-based tool for high-throughput proteome annotations. *Nucleic Acids Res* 32 (Web-Server issue): W365–W371.
15. Gardy JL, Laird MR, Chen F *et al*. (2005) PSORTb v.2.0: expanded prediction of bacterial protein subcellular localization and insights gained from comparative proteome analysis. *Bioinformatics* 21: 617–623.
16. Davies RL, Donachie W (1996) Intra-specific diversity and host-specificity within *Pasteurella haemolytica* based on variation of capsular polysaccharide, lipopolysaccharide and outer-membrane proteins. *Microbiology* 142: 1895–1907.
17. Davies RL, Parton R, Coote JG *et al*. (1992) Outer membrane protein and lipopolysaccharide variation in *Pasteurella haemolytica* serotype A1 under different growth conditions. *J Gen Microbiol* 138: 909–922.
18. Markwell MAK, Haas SM, Bieber LL *et al*. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 87: 206–210.
19. Perkins DN, Pappin DJC, Creasy DM *et al*. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20: 3551–3567.
20. Dabo SM, Confer AW, Hartson SD (2005) Adherence of *Pasteurella multocida* to fibronectin. *Vet Microbiol* 110: 265–275.
21. Harper M, Boyce JD, Adler B (2006) *Pasteurella multocida* pathogenesis: 125 years after Pasteur. *FEMS Microbiol Lett* 265: 1–10.
22. Ruffolo CG, Tennet JM, Michaelski WP *et al*. (1997) Identification, purification and characterization of the type 4 fimbriae of *Pasteurella multocida*. *Infect Immun* 65: 339–343.
23. Potts JR, Campbell ID (1994) Fibronectin structure and assembly. *Curr Opin Cell Biol* 6: 648–655.
24. Davies RL, MacCorquodale R, Baillie S *et al*. (2003) Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis. *J Med Microbiol* 52: 59–67.
25. Glorioso JC, Jones GW, Rush HG *et al*. (1981) Adhesion of type A *Pasteurella multocida* to rabbit pharyngeal cells and its possible role in rabbit respiratory tract infections. *Infect Immun* 35: 1103–1109.
26. Gomez MI, Prince A (2007) Opportunistic infection in lung disease: *Pseudomonas* infections in cystic fibrosis. *Curr Opin Pharm* 7: 244–251.
27. Ross RF (2007) *Pasteurella multocida* and its role in porcine pneumonia. *Anim Health Res Rev* 7: 13–29.
28. Andrews SC, Robinson AK, Rodriguez-Quiñones F (2003) Bacterial iron homeostasis. *FEMS Microbiol Rev* 27: 215–237.
29. Smith JE (1974) *Bergey's Manual of Determinative Bacteriology*, 8th edn. Baltimore, MD: Williams & Wilkins.
30. Cox AJ, Hunt ML, Ruffolo CG *et al*. (2000) Cloning and characterisation of the *Pasteurella multocida ahpA* gene responsible for a haemolytic phenotype in *Escherichia coli*. *Vet Microbiol* 72: 135–152.
31. Diallo IS, Frost AJ (2000) Characteristics of a haemolytic extract from avian *Pasteurella multocida*. *Vet Microbiol* 72: 37–45.
32. Ogunnariwo JA, Schryvers AB (2001) Characterization of a novel transferring receptor in bovine strains of *Pasteurella multocida*. *J Bacteriol* 183: 890–896.
33. Cornelissen CN (2003) Transferrin-iron uptake by Gram-negative bacteria. *Front Biosci* 8: 836–847.
34. Pedersen SK, Harry JL, Sebastian L *et al*. (2002) Unseen proteome: mining below the tip of the iceberg to find low abundance and membrane proteins. *J Proteome Res* 2: 303–311.

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