

Comparative Functional Genomic Analysis of Pasteurellaceae Adhesins using Phage Display

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Lisa M. Mullen, Sean P. Nair, John M. Ward, Andrew N. Rycroft, Rachel J. Williams, et al.. Comparative Functional Genomic Analysis of Pasteurellaceae Adhesins using Phage Display. Veterinary Microbiology, 2007, 122 (1-2), pp.123. 10.1016/j.vetmic.2006.12.022 . hal-00532189

HAL Id: hal-00532189 https://hal.science/hal-00532189

Submitted on 4 Nov 2010

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Accepted Manuscript

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Please cite this article as: Mullen, L.M., Nair, S.P., Ward, J.M., Rycroft, A.N., Williams, R.J., Henderson, B., Comparative Functional Genomic Analysis of Pasteurellaceae Adhesins using Phage Display, *Veterinary Microbiology* (2006), doi:10.1016/j.vetmic.2006.12.022

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25 Abstract

26 The Pasteurellaceae contain a number of important animal pathogens. Although related, the various members of this family cause a diversity of pathology in a wide variety of organ systems. Adhesion is 27 28 an important virulence factor in bacterial infections. Surprisingly little is known about the adhesins of 29 the Pasteurellaceae. To attempt to identify the genes coding for adhesins to some key components of 30 the hosts extracellular matrix molecules, phage display libraries of fragmented genomic DNA from 31 Haemophilus influenzae. pleuropneumoniae. Р. multocida and Aggregatibacter А. actinomycetemcomitans, were prepared in the phage display vector pG8SAET. The libraries were 32 screened against human or porcine fibronectin, serum albumin or a commercial extracellular matrix 33 34 containing type IV collagen, laminin and heparin sulphate. Four genes encoding putative adhesins 35 were identified. These genes code for: (i) a 34kDa human serum albumin binding protein from H. influenzae; (ii) a 12.8 kDa fibronectin-binding protein from Pasteurella multocida; (iii) a 13.7 kDa 36 fibronectin-binding protein from A. actinomycetemcomitans; and (iv) a 9.5kDa serum albumin-binding 37 38 protein from A. pleuropneumoniae. None of these genes have previously been proposed to code for 39 adhesins. The applications of phage display with whole bacterial genomes to identify genes encoding novel adhesins in this family of bacteria are discussed. 40

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Key

Phage

words:

display; P

Pasteurellaceae;

fibronectin;

adhesins;

43 Introduction

The 44 family Pasteurellaceae currently contains 61 12 (www.thetaxa in genera This family of bacteria contain a significant number of 45 icsp.org/taxa/Pasteurellaceaelist.htm). 46 opportunistic pathogens and pathogens of humans and domesticated animals. Human pathogens 47 include Haemophilus influenzae (St. Geme, 2000; 2002; Rodriguez et al., 2003), Haemophilus ducreyi 48 (Spinola et al., 2002), Aggregatibacter actinomycetemcomitans (Henderson et al., 2003) and Pasteurella multocida (Hunt et al., 2000). These organisms cause a range of diverse diseases such as 49 meningitis, otitis media, chancroid, pneumonia, periodontitis and cat cuddlers cough, to name but a 50 51 Animal pathogens include Pasteurella multocida (Hunt et al., 2000), Actinobacillus few. 52 pleuropneumoniae (Bosse et al., 2002), Actinobacillus equuli (Rycroft and Garside, 2000) and 53 Mannheimia haemolytica (Frank, 1989), which cause, among other diseases, atrophic rhinitis, fowl 54 cholera, porcine pleuropneumonia, equine fatal septicaemia and bovine pneumonic pasteurellosis.

The ability of these related bacteria to cause such a diverse range of pathology is interesting and must 55 56 reflect a diversity of virulence behaviors. One of the basic elements in bacterial pathogenesis is 57 adhesion. There is now an enormous literature on the adhesins of a range of microorganisms (Ofek and 58 Doyle, 1994) from Gram-negative enteric bacteria (Korhonen, 1982) to Gram-positive organisms such 59 as the staphylococci (Peacock et al., 1999). However, relatively little is known about the adhesins used 60 by the Pasteurellaceae to colonise their hosts and cause disease. In an attempt to identify, and to 61 compare and contrast, the adhesins used by the Pasteurellaceae to colonise their hosts we have used phage display with the fragmented genomic DNA from four members of this family: Haemophilus 62 63 influenzae, Pasteurella multocida, Actinobacillus pleuropneumoniae and Aggregatibacter 64 actinomycetemcomitans. The genomes of these organisms have been or are in the process of being 65 sequenced so enabling the full-length open reading frames associated with the gene fragments 66 identified by phage display to be determined.

67 Materials and Methods

68 Bacterial strains and plasmids: H. influenzae NCTC 8470/ATCC 9332 Pittman type D and P. multocida NCTC 10322/ATCC 43137 (pig isolate) were purchased from the National Collection of 69 70 Type Cultures (London, UK) and cultured on chocolate agar or grown in Brain Heart Infusion (BHI) 71 broth (Oxoid Ltd., Basingstoke, United Kingdom) aerobically at 37°C. BHI broth was supplemented 72 with 10 μ g/ml hemin and 2 μ g/ml β -NAD (Sigma-Aldrich Co. Ltd. Poole, United Kingdom) in the case of H. influenzae. A. pleuropneumoniae Serotype 1, strain 4074 was routinely cultured on chocolate 73 74 agar, then grown in BHI broth supplemented with 2µg/ml NAD, aerobically at 37°C. A. 75 actinomycetemcomitans strain HK1651 (JP2 clone) was maintained on blood agar or grown in BHI 76 broth at 37°C in a 5% CO₂ atmosphere. All of these strains are clinical isolates.

The phagemid pG8SAET (a kind gift from Lars Frykberg, Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden) and the *Escherichia coli* host TG1 [*supE hsd* Δ 5 *thi* Δ (*lac-proAB*) F'(*traD36 proAB*⁺ *lacI*^q *lacZ* Δ M15)] were used in the construction of the phage display library. *E. coli* was grown in nutrient broth No. 2 (NB-2, Oxoid Ltd.). The medium was supplemented when appropriate with 200 µg/ml of ampicillin to maintain the phagemid. All cultures of *E. coli* TG1 were grown at 37°C under aerobic conditions.

83 Purification of porcine fibronectin from pig serum: Pig serum was purchased from Invitrogen-Gibco. 84 Serum was diluted 1:5 in stabilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA at pH 85 7.4) and applied to a 10ml gelatin-Sepharose (Sigma-Aldrich) column equilibrated with stabilisation 86 buffer. Bound proteins were eluted from the column with 20 ml of elution buffer (50 mM Tris-HCl, 87 250 mM NaCl, 1 mM EDTA, 3 M urea at pH 7.4). The eluant was then diluted 1:1 in stabilization 88 buffer and applied to a 10 ml heparin-Sepharose (Sigma-Aldrich) column that had been extensively 89 washed with stabilization buffer. After first washing the column with 40 ml of stabilisation buffer, 90 bound proteins were eluted in 40 ml of elution buffer (50 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA,

at pH 7.4). The isolated fibronectin was analysed by SDS-PAGE to ensure it was at least 95%
homogenous.

93 *Construction of genomic DNA phage display libraries:* To construct the phage display libraries, 94 chromosomal DNA from each of the four bacteria was extracted from cells by treatment with 95 lysozyme, 10% SDS, RNase and pronase, followed by spooling of the DNA by the addition of 5M 96 NaCl and 2 volumes of ethanol. Spooled DNA was then applied to a CsCl₂ gradient containing 97 ethidium bromide to visualize the DNA and centrifuged at 40,000 rpm in a Beckman-Coulter Optima 98 L100-XP ultracentrifuge, using a 70.1 Ti angle rotor for 48 h. EtBr was removed by the addition of 99 saturated isopropanol, the DNA precipitated by salt/ethanol precipitation and resuspended in TE buffer 100 (10 mM Tris-Cl pH 7.5, 1 mM EDTA). Isolated chromosomal DNA was sheared by sonication to 101 obtain fragments of between 0.5 and 3 kb, as assessed by agarose gel electrophoresis. Without further 102 size fractionation, the chromosomal fragments were blunt ended by using the Klenow fragment (New 103 England Biolabs) and T4 DNA polymerase (New England Biolabs). Fragments were then ligated to 104 SnaBI-digested and dephosphorylated phagemid vector pG8SAET purified from agarose gels using a 105 Ready-to-Go T4 DNA ligase kit (GE Healthcare, Buckinghamshire, United Kingdom). Repeated 106 ligations of this material failed to produce transformants. It appears that the gel purification of the 107 digested pG8SAET inhibited ligation and this purification step had to be abandoned and the digested 108 and dephosphorylated vector had to be purified on spin-columns (Qiagen PCR purification kit). 109 Purified DNA was introduced, by electroporation, into E. coli TG1. The electroporated cells were 110 allowed to recover for 2 h at 37°C in 10 ml of NB-2 prior to infection with helper phage R408 111 (Promega, Southampton, United Kingdom) at a multiplicity of infection of 20. The infected cells were 112 grown overnight in 190 mL of NB-2 containing ampicillin. The phage were recovered from the culture 113 supernatant by precipitation with a solution containing 20% (wt/vol) polyethylene glycol 8000 (Sigma-114 Aldrich Co. Ltd.,) and 1 M NaCl, resuspended in phosphate-buffered saline (PBS) pH 7.4 (Sigma-115 Aldrich Co. Ltd.), and sterilized by passage through a 0.45-µm-pore-size filter.

116 Panning of the phage display libraries against immobilised ligands: A 5-ml Nunc Maxisorb 117 immunotube (Invitrogen-Gibco, Paisley, United Kingdom) was coated overnight at 4°C with 1 ml of a 118 solution containing 0.5 mg of ligand (fibronectin from human (Sigma-Aldrich Co. Ltd) or porcine 119 serum (purified as described above), or a mixture of human extracellular matrix proteins (from BD 120 Biosciences, Oxford, UK), consisting of type IV collagen, laminin and heparin sulphate. The tube was 121 blocked with 4 ml of 2% bovine serum albumin (BSA) in PBS for 2 h at room temperature. Control 122 tubes were coated overnight with 2% BSA, then blocked with a further 2% BSA. After the tube had 123 been extensively washed with PBS containing 0.05% Tween 20 and then with PBS, 1 ml of the relevant 124 phage display library was added to the tube and incubated for 2 h at room temperature. All unbound 125 phage were removed by 10 washes with 4 ml of PBS containing 0.05% Tween 20, followed by 10 126 washes with 4 ml of PBS. The bound phage were eluted in 1 ml of 0.1 M glycine buffer (pH 2.1) for 10 127 min at room temperature, which was then neutralized with 0.5 ml of 1 M Tris-HCl buffer (pH 8.0).

Amplification of eluted phage: Suspensions of eluted phage were added to 8ml of log-phase *E. coli*TG1 cells and incubated at 37°C for 1 h. These infections were then superinfected with the helper
phage R408 at a multiplicity of infection (MOI) of 20 and incubated at 37°C for a further 30 min.
Cultures were then added to 190 ml of NB-2 containing ampicillin and grown overnight at 37°C. Phage
were recovered from the culture supernatant by precipitation as described above.

133 *Titration of phage:* The number of phage were determined indirectly as the number of colony forming 134 units per millilitre (CFU/ml) of *E. coli* after infection with phage. *E coli* TG1 were grown to the mid-135 exponential phase and then incubated with the phage for 30 min at 37°C, before the bacteria were 136 plated onto NB-2 containing ampicillin and grown overnight at 37°C.

Binding of recombinant phage: Wells of Nunc Maxisorp microtiter plates (Invitrogen) were coated
overnight at 4°C with 0.1-ml aliquots of 0.1mg/ml solutions of the following test ligands dissolved in
PBS: fibronectin, the 30-kDa N-terminal and 45-kDa α-chymotryptic fragments of fibronectin, collagen
(type I, acid soluble, from human placenta), fibrinogen (fraction I from human plasma), hyaluronate,

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and plasminogen, all of which were purchased from Sigma-Aldrich Co. Ltd.; mouse laminin 141 142 (Collaborative Biomedical Products, Bedford, Mass.); and the 120-kDa a-chymotryptic fragment of 143 fibronectin (Chemicon International). The wells were each blocked with 0.1 ml of 1% BSA in PBS for 1 h. After the wells were rinsed three times with PBS containing 0.05% Tween 20 and then with PBS, 144 0.1 ml of a 1 x 109-CFU/ml phage solution was added to each well and incubated for 1.5 h. The 145 146 unbound phage were removed by five washes with 0.2 ml of PBS containing 0.05% Tween 20, 147 followed by five washes with 0.2 ml of PBS. The bound phage were eluted with 0.1 ml of 0.1 M 148 glycine buffer (pH 2.1) for 10 min and neutralized with 50 µl of 1 M Tris-HCl buffer (pH 8.0).

DNA sequencing: Insert DNA was sequenced using dye terminator chemistry and cycle sequencing
using the BigDye terminator kit according to the manufacturer's instructions (ABI Perkin Elmer, UK).
The reactions were run on an ABI 310 genetic analyser.

152 Bioinformatic analysis: Phage insert DNA sequences were analysed using Basic Local Alignment 153 Tool bacterial Search (BLAST) searches of the genomes databases on **NCBI** 154 (http://www.ncbi.nlm.nih.gov/sutils/genom table.cgi) and also using the Pedant database (http://pedant.gsf.de), which contains complete and partial genome sequences of the chosen bacteria. 155 156 Pedant was also the source of the numbering for the A. actinomycetemcomitans and M. haemolytica open reading frames (ORFs). Predicted protein sequences for each ORF were used to predict the 157 158 presence of signal sequences using the SignalP program (http://www.expasy.ch/tools/), and to predict 159 transmembrane domains using TMPred, TMHMM and SMART on the Expasy tools site 160 (http://www.expasy.ch.tools/).

162 **Results**

163 Construction, size and complexity of phage display libraries

Phage display libraries were made in the phagemid vector pG8SAET from genomic DNA of *H. influenzae, P. multocida, A. pleuropneumoniae and A. actinomycetemcomitans* as described in the materials and methods section. To assess the ratio of clones that harbored phagemids containing insert DNA, 20 clones were chosen randomly and the plasmids isolated using the Qiagen miniprep kit and digested with NcoI and EcoRI. As shown in Table 1, the libraries have complexities of between 5.33 x 10^{6} and 3.97×10^{7} (i.e. the number of transformants containing phagemids with insert DNA), and phage titres of 1.4×10^{12} to 2.6×10^{12} phage/ml.

171 Isolation of phage displaying putative bacterial adhesins

Initial panning experiments involved the addition of 1 ml of each phage library to immobilised 172 fibronectin (Fn) or immobilised BSA to control for any binding to the BSA used as a blocking agent 173 (see materials and methods). The A. pleuropneumoniae library was panned against porcine fibronectin 174 175 and the other three libraries against human fibronectin. The total number of phage particles bound after the first round of panning (1st round) were recorded, and bound phage were then amplified to a titre of 176 approximately 1 x 10^{11} phage/ml before undertaking a second round of panning. This procedure was 177 178 repeated until three rounds of panning were complete. Five of the ten pannings (H. influenzae vs Fn, H. 179 influenzae vs BSA, P. multocida vs Fn, A. actinomycetemcomitans vs Fn and A. pleuropneumoniae vs BSA) undertaken resulted in successive increases in the number of phage bound from the 1st to the 3rd 180 181 round of panning (Fig. 1A). The number of bound phage in five of the pannings (H. influenzae vs ECM proteins, P. multocida vs BSA, P. multocida vs ECM proteins, A. actinomycetemcomitans vs BSA and 182 183 A. pleuropneumoniae vs Fn) showed no consistent increase in the number of phage bound on 184 successive rounds of panning (Fig. 1B).

185 DNA sequencing of 20 randomly chosen phagemids (10 phagemids from the second round of panning 186 and 10 phagemids from the third round of panning) from each of the 10 pannings revealed that in the 187 five pannings where a sequential increase in the number of bound phage was observed (see Fig. 1A), at least 5 clones contained either identical inserts or inserts with overlapping sequences after the 2nd round 188 of panning (data not shown), while after the 3rd round of panning, at least 8/10 of the clones were 189 190 identical or contained overlapping inserts (Table 2). Sequencing of clones from the five pannings that 191 did not show a sequential increase in the numbers of phage bound (see Fig. 1B) showed that the inserts 192 contained unrelated sequences.

193 Binding specificity of recombinant phage: Of the recombinant phage inserts listed in Table 2, those 194 selected from panning of H influenzae vs Fn, H. influenzae vs BSA, P. multocida vs Fn and A. 195 pleuropneumoniae vs BSA contained identical inserts to each other, while those from panning the A. 196 actinomycetemcomitans library against immobilized Fn contained inserts with overlapping sequences. 197 As a true selection is generally believed to be manifested by clones containing overlapping inserts 198 (Jacobson et al., 2003), one of the phagemid clones containing the *pm1665* gene and one of the phagemid clones containing the hi0367 gene were amplified to a phage titre of 1 x 10¹¹ cfu/ml and used 199 200 in studies to confirm that the recombinant phage had the capacity to bind to fibronectin or BSA 201 respectively. These phage stocks were also panned against a range of other proteins immobilized on 202 microtiter plate wells (Figures 2 and 3). The recombinant phage containing *pm1665* showed significant 203 binding to fibronectin, the 120kDa fragment of fibronectin and to human type I collagen. The 204 recombinant phage containing almost two thirds of hi0367 showed significant binding to BSA and 205 plasminogen.

The inserts with overlapping sequences encompassed approximately 752bp of DNA, with a 261-bp region being present in all of the clones. BLAST searches of the NCBI sequence database from the ongoing *A. actinomycetemcomitans* (HK1651) sequencing project revealed that these overlapping inserts mapped to a locus coding for two ORFs designated *orf9* and *orf10*. Further analysis of the insert DNA

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indicated that only the sequence corresponding to *orf10* could be expressed on the surface of the phage
as this sequence was in frame with the phage coat protein. Bioinformatic analyses indicate that *orf10*has previously been identified as a human-matrix binding protein (accession number AY560112), is
part of a four-gene operon and codes for a putative 13.8kDa protein containing a signal sequence.
BLASTp searches of the microbial genomes databases did not find any homologues of the protein
predicted to be coded by *orf10*.

216 BLAST searches of the NCBI sequence database which contains the complete genome of *H. influenzae* 217 (strain KW20) showed that the phage selected by panning of the H. influenzae library against Fn 218 contained inserts which mapped not to an ORF but to an intergenic region of DNA. Panning of the 219 same library against BSA resulted in the selection of phage clones containing a partial ORF with 99% 220 homology to an orf, hi0367, which is, to date, uncharacterised and is therefore referred to as a 221 'hypothetical protein'. The predicted protein HI0367 has a molecular mass of 34kDa, and while it does 222 not appear to have a standard signal sequence, it does contain a predicted transmembrane region. The 223 inserts within the phage encompassed approximately two thirds of the gene predicted to code for 224 HI0367. This gene is part of a four-gene operon, and both the gene and its location within this operon 225 are conserved among several other members of the Pasteurellaceae (Table 3). The closest homologue to the gene coding for HI0367 is a gene from P. multocida (predicted to code for PM2009) which shares 226 227 51% sequence identity.

Similar mining of the NCBI sequence database which contains the completed *P.multocida* genome revealed that the identical clones selected by panning of the *P. multocida* library against fibronectin mapped to a gene predicted to code for a hypothetical protein, designated PM1665. The inserts within the phage contained a 630 bp fragment of a gene which is predicted to code for almost the complete orf of PM1665 (with the exception of 6 bp from the 3' end of the gene) and a 270 bp intergenic region upstream of the predicted start codon for this gene. The predicted protein PM1665 has a molecular mass of 12.7 kDa and is predicted to have a typical signal sequence. BLAST searches with the

predicted protein sequence indicated that this protein has homology to proteins involved in DNA uptake that are highly conserved across a wide range of bacterial species including several members of the Pasteurellaceae (Table 4) and showed the greatest identity with a gene annotated as coding for a ComEA protein of '*M. succiniciproducens*', although the ComEA proteins in the *Mannheimia* species are larger than the predicted protein product of *pm1665*.

The panning of the *A. pleuropneumoniae* library against BSA resulted in the selection of a small DNA fragment, present in 5 out of 10 clones, after the 3rd round of panning (see Table 2). This insert DNA was identical to a gene in *A. pleuropneumoniae* predicted to code for a small protein of 10kDa annotated as a Na⁺-transporting methylmalonly-CoA/oxaloacetate decarboxylase, gamma subunit in the A. *pleuropneumoniae* genome database. Homologues of this gene were found in two other members of the Pasteurellaceae, *P. multocida* and *M. succiniproducens*.

In contrast to the positive results obtained with screening against fibronectin or BSA, screening the *H. influenzae* and *P. multocida* libraries against a commercial source of human extracellular matrix proteins containing type IV collagen, laminin and heparin sulphate failed to identify any binding clones.

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251 **Discussion**

Phage display, in its many variants, is a much used technique for determining protein and peptideinteractions (Wrighton et al., 1996; Trepel et al., 2002; Ladner et al., 2004; Wang and Yu, 2004; Rosander et al., 2002). This methodology can also be used as a functional genomic screen to identify genes coding for proteins that interact with a specified target. While, this is in theory, a very powerful interactomic methodology, it has only been employed with a small number of individual bacterial (mainly Gram-positive-) genomes to identify potential adhesins (Mullen et al., 2006). No one has yet employed this technique for comparative functional genomic analysis of a range or family of bacteria.

259 Almost of the Pasteurellaceae are pathogenic. What is so interesting about this family of bacteria is 260 their host range. Some members of the family are host specific, such as A. pleuropneumoniae in pigs or 261 H. influenzae in humans. Others, such as P. multocida, are pathogens of a wide range of species. The rationale for a comparison of the adhesins present in four members of the Pasteurellaceae is to identify 262 263 putative novel adhesins and to consider whether the adhesins expressed by different bacteria of this 264 family could be related to host specificity. As the first step in infection is colonization of host tissue, 265 the presence of adhesins that are specific for particular host molecules may contribute to pathogenic 266 ability. In this study we have employed phage display to identify adhesin-encoding genes by preparing libraries of genomic DNA from four members of the Pasteurellaceae: H. influenzae, P. multocida, A. 267 268 pleuropneumoniae and A. actinomycetemcomitans. Panning of these libraries against individual 269 purified ECM components resulted in the identification of genes that code for potential bacterial 270 adhesins. Sequential increases in the numbers of phage binding to the ligand after each round of 271 panning is deemed to be indicative of true binding events (Jacobsson et al., 2003) and this proved to be 272 the case. The amplification of bound phage after each round of panning is such that the numbers of phage expressing a particular protein sequence can be increased by up to 10^7 fold after amplification. 273 274 Therefore, a substantial increase in the numbers of phage binding to the ligand of interest in successive rounds of panning is expected if the recombinant phage are expressing a true adhesin for the ligand that 275 276 is being panned on. Using purified Fn as a ligand, two genes were identified that code for proteins 277 which could bind Fn from P. multocida and A. actinomycetemcomitans. Panning on BSA identified 278 genes coding for putative proteins with the capacity to bind this molecule from H. influenzae and A. 279 pleuropneumoniae.

Panning of the *H. influenzae* library against BSA resulted in the selection of identical clones after the third round of panning. As *H. influenzae* is not known to infect any host other than humans, the capacity of the recombinant phage, isolated on panning against BSA, to bind to human serum albumin (HSA) was investigated; these phage exhibited identical binding characteristics to BSA and HSA (data

not shown). DNA sequencing and BLAST searches using the sequence of these clones revealed an orf
with 99% similarity predicted to code for a hypothetical protein HI0367 in the completed *H. influenzae*Rd KW20 genome sequence. So-called 'hypothetical' genes have not been experimentally
characterized and functions cannot be deduced solely on the basis of sequence comparisons (Kolker et al., 2004).

289 A number of adhesins have been identified in H. influenzae, in both encapsulated (Rodriguez et al., 290 2003) and non-typeable (St. Geme, 2002) strains. These include the high-molecular weight (HMW) 291 proteins 1 and 2 (St. Geme et al., 1998; Buscher et al., 2004), the well-characterised Hia (Barenkamp and St. Geme, 1996; St. Geme and Cutter, 2000; Laarmann et al., 2002), Hap (Fink et al., 2002, 2003), 292 293 Hsf autotransporters (St. Geme et al., 1996; Cotter et al., 2005), and the haemagglutinating pili 294 identified in H. influenzae type b strains (Van Ham et al., 1994; Virkola et al., 2000). However, none of 295 these adhesins are known to bind to serum albumin. Experiments are now underway with the 296 recombinant HI0367 to investigate the biological importance of this protein. Bioinformatic analyses 297 reveal that the closest homologue to HI0367 is a gene in *P. multocida* predicted to code for PM2009.

Panning of the *P. multocida* library against Fn resulted in the selection of clones containing inserts which mapped to a locus in the genome of the strain PM70 which is predicted to code for a small hypothetical protein designated PM1665. This predicted protein (a relatively small 12.7kDa protein) shows homology with the ComEA DNA uptake proteins. Bioinformatic analysis indicated the presence of homologues of PM1665 in all other sequenced members of the Pasteurellaceae.

Although most of the known adhesins in the Pasteurellaceae have been identified and characterized in *H. influenzae* (as mentioned above), the adherence of *P. multocida* to fibronectin has been investigated. *P. multocida* outer membrane proteins (OMPs) have been shown to bind to various host components (Jaques et al., 1993; Al-Haddawi et al., 2000), but there is a dearth of identified adhesins in this organism, apart from OmpA which Dabo and colleagues demonstrated binds to fibronectin (Dabo et al., 2003). A more recent study by Dabo et al., (2005) also identified a further five putative fibronectin-

binding proteins from *P. multocida*, none of which appear to correspond to the predicted protein product of *pm1665*, based on apparent molecular masses. Hence, it appears that this is the first indication that *pm1665* may code for a fibronectin-binding protein in members of the Pasteurellaceae.

The presence of homologues of both *hi0367* and *pm1665* in the other members of the Pasteurellaceae studied here raises the question of why these genes were only identified in *H. influenzae* and *P. multocida* respectively, but were not found in all of the libraries panned against BSA and Fn. There is also the question of why the known adhesins were not found in any of the libraries.

316 There are a number of potential reasons for these observations, some arising from the way in which the libraries were constructed and some from the way in which the panning experiments were done. In this 317 318 study, construction of the phage display libraries involves ligating bacterial DNA to the phagemid 319 vector so that bacterial recombinant proteins are made as fusions to phage coat protein VIII. However, 320 the presence of insert DNA in the phagemid does not guarantee that a recombinant protein will be 321 displayed on the phage surface. Even though before amplification the libraries are theoretically large 322 enough to give full coverage (several times) of the individual bacterial genomes only a proportion of 323 phage will actually display a bacterial protein. It is estimated that only 1 in 18 phage will contain an 324 insert that is in the correct orientation and in-frame with the promoter and with gene VIII and so 325 display a recombinant protein (Jacobsson et al., 2003). This can be taken into account when calculating 326 the size of a library that would be necessary to ensure full genome coverage.

The other important parameter that will affect the numbers of phage displaying unique bacterial proteins is the ease with which the recombinant protein can be produced, folded and exported from the *E. coli* cell during phage synthesis. Proteins encoded by phagemid insert DNA that are toxic to *E. coli* or that cannot be folded correctly during assembly of the phage in the periplasm will not be represented in the final phage library. The ease with which the recombinant proteins are synthesized during phage assembly in *E. coli* is also important during the panning procedure. After each round of panning, the bound phage are eluted and amplified in *E. coli*, so that phage containing DNA coding for bacterial

proteins that are easily produced in *E. coli* are likely to be over-represented in the amplified phage stock. On the other hand, phage containing DNA coding for bacterial proteins that are toxic to *E.coli* are unlikely to be represented in the initial library. The panning procedure can also result in a bias towards clones that can withstand the harsh elution conditions. Clearly, there are a number of factors that need investigation to determine why the known adhesins or the homologues of the clones found were not selected after 3 rounds of panning.

It is also interesting that three of the successful pannings, H. influenzae and A. pleuropneumoniae 340 341 against BSA and P. multocida against fibronectin, resulted in the selection of identical clones. This is 342 in contrast to most of the published work in this area (Jacobsson and Frykberg, 1995; 1996; Bjerktorp 343 et al., 2002; Williams et al., 2002; Jacobsson, 2003; Jonsson et al., 2004), where clones containing 344 overlapping fragments of a particular gene are selected by panning. This may be a reflection of the 345 relative ease with which these inserts can be translated in E. coli or even a function of the number of 346 fusion proteins formed on the phage surface, which would increase the avidity of binding to the ligand. 347 Another gene encoding a putative adhesin identified in this study is orf10 from A. 348 actinomycetemcomitans. This gene codes for a predicted 13.8kDa protein with no homologues in the 349 databases and appears to be part of a four-gene operon containing genes coding for several membraneassociated proteins. In contrast to the clones selected by panning the H. influenzae and P. multocida 350 351 libraries against BSA and fibronectin respectively, the clones selected after the third round of panning 352 contained over lapping inserts rather than identical DNA sequences. Searches for genes coding for 353 similar proteins in the genomes of other members of the Pasteurellaceae revealed similar operons in 354 Haemophilus somnus, P. multocida and Mannheimia haemolytica but these proteins had much higher 355 molecular masses than that coded by orf10. A number of adhesins have been identified in A. 356 actinomycetemcomitans including the major pilus subunit Flp-1 protein (Inoue et al., 1998), an inner 357 membrane protein encoded by the *impA* gene (Mintz and Fives-Taylor, 2000), the autotransporter Aae

358 (Rose et al., 2003) and the collagen-binding protein EmaA (Mintz, 2004), but none of these known 359 adhesins were identified by panning of the phage display library in the current study.

360 The original hypothesis for this study was that panning of individual phage display libraries from 361 members of the Pasteurellaceae would result in the identification of identical or similar genes that are 362 important in adhesion to host molecules. However, this did not turn out to be the case. None of the ten 363 pannings undertaken with the four libraries identified similar genes and half of the panning experiments 364 failed to identify putative adhesins. In one of the pannings, that of the H. influenzae library against Fn, 365 the clones identified after the third round of panning did not map to an ORF in the sequenced genome, rather to a region of intergenic DNA. These results are indicative of some of the limitations of the 366 367 phage display technique. The identification of genes coding for adhesins requires that whole or partial 368 proteins displayed on the phage surface are folded in the correct conformation to retain their native 369 binding capacity. This capacity is influenced by the random nature by which fragmented DNA is fused 370 to the upstream and downstream regions of the gene coding for the phage coat proteins. Additionally, 371 protein products encoded by DNA fragments, rather than complete genes, could fail to adopt the 372 tertiary structure necessary to exhibit binding capacity. The fact that the proteins being displayed on the 373 surface of the phage are foreign to E. coli could also impact on the ability of this host to express them or on their conformation. On the other hand, small DNA fragments such as those selected for in the H. 374 375 influenzae library panned against Fn, could adopt a conformation that selects for this clone to the 376 exclusion of all others.

The results presented here demonstrate the advantages, as well as the limitations, of this functional genomic approach and the adaptability of phage display technology. We have demonstrated that libraries can be screened against a range of ligands and comparisons between sequenced genomes of the Pasteurellaceae can be used to identify genes coding for common proteins or motifs that may be important in adhesion. These results also show the existence of as yet uncharacterized adhesins in the Pasteurellaceae. Further work is underway to clone and express the four putative adhesins identified in

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383	this study to confirm their status as novel binding proteins and to determine if their homologues in
384	other members of this family of bacteria may serve similar functions.
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386	Acknowledgements
387	The authors (BH, ANR, SPN, JMW) acknowledge the support of the BBSRC (Grant no BBS/B/03238)
388	for financial support of LMM.
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509 Legends to Figures:

510 Figure 1. Numbers of bound phage after rounds 1, 2 and 3 of panning. Numbers of phage were 511 determined by infection of E. coli TG1 cells with the eluted phage solution, plating of E. coli on NB-2 containing 200µg of ampicillin and counting the number of amp^R colonies. A: Panning of phage 512 513 display libraries against Fn or BSA showing successive increases in the numbers of bound phage with 514 successive rounds of panning. These pannings resulted in the enrichment of specific clones after three 515 rounds of panning. B: Panning of phage display libraries against ECM proteins, Fn or BSA showing no 516 consistent increases in the numbers of bound phage with successive rounds of panning. These pannings 517 did not result in the enrichment of specific clones after three rounds of panning.

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Figure 2. Binding of recombinant phage displaying the protein product of gene pm1665 in fusion with coat protein VIII to a variety of immobilised proteins found in the extracellular matrix or in plasma. The data are presented as the mean \pm SEM for triplicate wells. The data shown are representative of three separate experiments.

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Figure 3. Binding of recombinant phage displaying the protein product of partial ORF *hi0367* in fusion with coat protein VIII to a variety of immobilised proteins found in the extracellular matrix or in plasma. The data are presented as the mean \pm SEM for triplicate wells. The data shown are representative of three separate experiments.

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Table 1. Construction of phage display libraries from genomic DNA of *Haemophilus influenzae*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *Actinobacillus actinomycetemcomitans* in the phagemid vector pG8SAET. Library size is dependent upon the number of *E.coli* TG1 transformants after electroporation with chromosomal DNA ligated to the phagemid vector. The complexity of the library was determined by analyses of twenty randomly chosen clones to determine the ratio of transformants containing the phagemid with insert DNA, rather then phagemid alone. Superinfection of *E.coli* transformants with helper phage yielded the final phage display library.

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DNA from:	No. of <i>E.coli</i> amp ^r	Complexity of	Titre of phage library	
	transformants	library	(phage /mL)	
H. influenzae	8.2 x 10 ⁶	5.33 x 10 ⁶	1.46 x 10 ¹²	
P. multocida	3.52×10^7	2.99 x 10 ⁷	1.82×10^{12}	
A. actinomycetemcomitans	3.27 x 10 ⁷	2.6 x 10 ⁷	2.6 x 10 ¹²	
A. pleuropneumoniae	5.3×10^7	3.97×10^7	1.4×10^{12}	

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Table 2. Summary of results from the panning of phage display libraries against immobilized fibronectin or BSA. Chromosomal DNA inserts from 10 randomly chosen phage clones from the 3^{rd} round of panning were determined by sequencing. BLAST searches of the bacterial genomes databases were used to map these DNA sequences to specific loci.

Phage display library of	Ligand	Phage inserts		Gene	Predicted protein product	
DNA from:		Size	No in 3 rd round	6	5	
H. influenzae	Fibronectin	52bp	9/10	No ORF (Intergenic DNA)	None	
H. influenzae	BSA	633bp	10/10	hi0367	Hypothetical protein	
P. multocida	Fibronectin	611bp	10/10	pm1665	Hypothetical protein	
A. actinomycetemcomitans	Fibronectin	283-750bp	8/10	orf10	Hypothetical protein	
A. pleuropneumoniae	BSA	87bp	5/10	orf16	Na ⁺ -transporting methylmalonly- CoA/oxaloacetate decarboxylase, gamma subunit	

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566 **Table 3.** Homologues of gene *hi0367* identified in members of the Pasteurellaceae by NCBI BLAST

567 searches or in the cases of A. actinomycetemcomitans and M. haemolytica BLAST searches on the

568 Pedant database. Sizes of predicted proteins are given by the number of amino acids (aa). Gene *hi0367*

569 is as yet uncharacterised and encodes a predicted protein of 303 amino acids.

Organism	Gene	Identity (%)	Similarity (%)	Size of predicted protein
P. multocida	PM2009	51	65	318aa
H. somnus	orf 80	50	64	318aa
A. actinomycetemcomitans	orf 67	47	62	315aa
M. succiniciproducens	MS1918	47	66	324aa
H. ducreyi	orf 813	36	52	323aa
A. pleuropneumoniae	orf 42	35	54	322aa
M. haemolytica	orf 13	33	51	332aa

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Table 4. Homologues of gene *pm1665* identified in members of the Pasteurellaceae by NCBI BLAST
searches or in the cases of A. *actinomycetemcomitans and M. haemolytica* BLAST searches on the

Pedant database. Sizes of predicted proteins are given by the number of amino acids (aa). Gene *pm1665*

583 is as yet uncharacterised and encodes a predicted protein of 115 amino acids.

Organism	Gene	Identity (%)	Similarity (%)	Size of predicted
				protein
H. somnus 129PT	orf 22	57	84	111aa
A. actinomycetemcomitans	orf 1426	50	67	109aa
M. succiniciproducens	ComEA	53	75	161aa
H. influenzae	hi1008	60	87	112aa
H. ducreyi	orf507	45	60	119aa
M. haemolytica	orf 35	41	60	211aa
A. pleuropneumoniae	orf 4	52	73	114aa





