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1 **Comparative Functional Genomic Analysis of Pasteurellaceae Adhesins using Phage Display**

2

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13 **Running head:** Functional genomic analysis of the Pasteurellaceae

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24

25 **Abstract**

26 The Pasteurellaceae contain a number of important animal pathogens. Although related, the various
27 members of this family cause a diversity of pathology in a wide variety of organ systems. Adhesion is
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*, *A. pleuropneumoniae*, *P. multocida* and *Aggregatibacter*
32 *actinomycetemcomitans*, were prepared in the phage display vector pG8SAET. The libraries were
33 screened against human or porcine fibronectin, serum albumin or a commercial extracellular matrix
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.*
36 *influenzae*; (ii) a 12.8 kDa fibronectin-binding protein from *Pasteurella multocida*; (iii) a 13.7 kDa
37 fibronectin-binding protein from *A. actinomycetemcomitans*; and (iv) a 9.5kDa serum albumin-binding
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
40 novel adhesins in this family of bacteria are discussed.

41

42 **Key words:** Phage display; Pasteurellaceae; adhesins; fibronectin;

43 Introduction

44 The family Pasteurellaceae currently contains 61 taxa in 12 genera (www.the-
45 icsp.org/taxa/Pasteurellaceaelist.htm). This family of bacteria contain a significant number of
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
49 *Pasteurella multocida* (Hunt et al., 2000). These organisms cause a range of diverse diseases such as
50 meningitis, otitis media, chancroid, pneumonia, periodontitis and cat cuddlers cough, to name but a
51 few. Animal pathogens include *Pasteurella multocida* (Hunt et al., 2000), *Actinobacillus*
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.
55 The ability of these related bacteria to cause such a diverse range of pathology is interesting and must
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
62 phage display with the fragmented genomic DNA from four members of this family: *Haemophilus*
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.*
69 *multocida* NCTC 10322/ATCC 43137 (pig isolate) were purchased from the National Collection of
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 10µg/ml hemin and 2µg/ml β-NAD (Sigma-Aldrich Co. Ltd. Poole, United Kingdom) in the case
73 of *H. influenzae*. *A. pleuropneumoniae* Serotype 1, strain 4074 was routinely cultured on chocolate
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.

77 The phagemid pG8SAET (a kind gift from Lars Frykberg, Department of Microbiology, Swedish
78 University of Agricultural Sciences, Uppsala, Sweden) and the *Escherichia coli* host TG1 [*supE hsd Δ5*
79 *thi Δ (lac-proAB) F'(traD36 proAB⁺ lacI^q lacZΔM15)*] were used in the construction of the phage
80 display library. *E. coli* was grown in nutrient broth No. 2 (NB-2, Oxoid Ltd.). The medium was
81 supplemented when appropriate with 200 µg/ml of ampicillin to maintain the phagemid. All cultures of
82 *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,

91 at pH 7.4). The isolated fibronectin was analysed by SDS-PAGE to ensure it was at least 95%
92 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 *Sna*BI-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- μ m-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).

128 **Amplification of eluted phage:** Suspensions of eluted phage were added to 8ml of log-phase *E. coli*
129 TG1 cells and incubated at 37°C for 1 h. These infections were then superinfected with the helper
130 phage R408 at a multiplicity of infection (MOI) of 20 and incubated at 37°C for a further 30 min.
131 Cultures were then added to 190 ml of NB-2 containing ampicillin and grown overnight at 37°C. Phage
132 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.

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

141 and plasminogen, all of which were purchased from Sigma-Aldrich Co. Ltd.; mouse laminin
142 (Collaborative Biomedical Products, Bedford, Mass.); and the 120-kDa α -chymotryptic fragment of
143 fibronectin (Chemicon International). The wells were each blocked with 0.1 ml of 1% BSA in PBS for
144 1 h. After the wells were rinsed three times with PBS containing 0.05% Tween 20 and then with PBS,
145 0.1 ml of a 1×10^9 -CFU/ml phage solution was added to each well and incubated for 1.5 h. The
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 μ l of 1 M Tris-HCl buffer (pH 8.0).

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

152 **Bioinformatic analysis:** Phage insert DNA sequences were analysed using Basic Local Alignment
153 Search Tool (BLAST) searches of the bacterial genomes databases on NCBI
154 (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) and also using the Pedant database
155 (<http://pedant.gsf.de>), which contains complete and partial genome sequences of the chosen bacteria.
156 Pedant was also the source of the numbering for the *A. actinomycetemcomitans* and *M. haemolytica*
157 open reading frames (ORFs). Predicted protein sequences for each ORF were used to predict the
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/>).

161

162 **Results**

163 *Construction, size and complexity of phage display libraries*

164 Phage display libraries were made in the phagemid vector pG8SAET from genomic DNA of *H.*
165 *influenzae*, *P. multocida*, *A. pleuropneumoniae* and *A. actinomycetemcomitans* as described in the
166 materials and methods section. To assess the ratio of clones that harbored phagemids containing insert
167 DNA, 20 clones were chosen randomly and the plasmids isolated using the Qiagen miniprep kit and
168 digested with NcoI and EcoRI. As shown in Table 1, the libraries have complexities of between $5.33 \times$
169 10^6 and 3.97×10^7 (i.e. the number of transformants containing phagemids with insert DNA), and
170 phage titres of 1.4×10^{12} to 2.6×10^{12} phage/ml.

171 *Isolation of phage displaying putative bacterial adhesins*

172 Initial panning experiments involved the addition of 1 ml of each phage library to immobilised
173 fibronectin (Fn) or immobilised BSA to control for any binding to the BSA used as a blocking agent
174 (see materials and methods). The *A. pleuropneumoniae* library was panned against porcine fibronectin
175 and the other three libraries against human fibronectin. The total number of phage particles bound after
176 the first round of panning (1st round) were recorded, and bound phage were then amplified to a titre of
177 approximately 1×10^{11} phage/ml before undertaking a second round of panning. This procedure was
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
180 BSA) undertaken resulted in successive increases in the number of phage bound from the 1st to the 3rd
181 round of panning (Fig. 1A). The number of bound phage in five of the pannings (*H. influenzae* vs ECM
182 proteins, *P. multocida* vs BSA, *P. multocida* vs ECM proteins, *A. actinomycetemcomitans* vs BSA and
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
188 least 5 clones contained either identical inserts or inserts with overlapping sequences after the 2nd round
189 of panning (data not shown), while after the 3rd round of panning, at least 8/10 of the clones were
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
199 phagemid clones containing the *hi0367* gene were amplified to a phage titre of 1×10^{11} cfu/ml and used
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.

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

210 indicated that only the sequence corresponding to *orf10* could be expressed on the surface of the phage
211 as this sequence was in frame with the phage coat protein. Bioinformatic analyses indicate that *orf10*
212 has previously been identified as a human-matrix binding protein (accession number AY560112), is
213 part of a four-gene operon and codes for a putative 13.8kDa protein containing a signal sequence.
214 BLASTp searches of the microbial genomes databases did not find any homologues of the protein
215 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
226 the gene coding for HI0367 is a gene from *P. multocida* (predicted to code for PM2009) which shares
227 51% sequence identity.

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

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

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

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

250

251 **Discussion**

252 Phage display, in its many variants, is a much used technique for determining protein and peptide-
253 interactions (Wrighton et al., 1996; Trepel et al., 2002; Ladner et al., 2004; Wang and Yu, 2004;
254 Rosander et al., 2002). This methodology can also be used as a functional genomic screen to identify
255 genes coding for proteins that interact with a specified target. While, this is in theory, a very powerful
256 interactomic methodology, it has only been employed with a small number of individual bacterial
257 (mainly Gram-positive-) genomes to identify potential adhesins (Mullen et al., 2006). No one has yet
258 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
262 rationale for a comparison of the adhesins present in four members of the Pasteurellaceae is to identify
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
267 libraries of genomic DNA from four members of the Pasteurellaceae: *H. influenzae*, *P. multocida*, *A.*
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
273 phage expressing a particular protein sequence can be increased by up to 10^7 fold after amplification.
274 Therefore, a substantial increase in the numbers of phage binding to the ligand of interest in successive
275 rounds of panning is expected if the recombinant phage are expressing a true adhesin for the ligand that
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*.

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

284 not shown). DNA sequencing and BLAST searches using the sequence of these clones revealed an orf
285 with 99% similarity predicted to code for a hypothetical protein HI0367 in the completed *H. influenzae*
286 Rd KW20 genome sequence. So-called 'hypothetical' genes have not been experimentally
287 characterized and functions cannot be deduced solely on the basis of sequence comparisons (Kolker et
288 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
292 and St. Geme, 1996; St. Geme and Cutter, 2000; Laarmann et al., 2002), Hap (Fink et al., 2002, 2003),
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.

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

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

309 binding proteins from *P. multocida*, none of which appear to correspond to the predicted protein
310 product of *pm1665*, based on apparent molecular masses. Hence, it appears that this is the first
311 indication that *pm1665* may code for a fibronectin-binding protein in members of the Pasteurellaceae.
312 The presence of homologues of both *hi0367* and *pm1665* in the other members of the Pasteurellaceae
313 studied here raises the question of why these genes were only identified in *H. influenzae* and *P.*
314 *multocida* respectively, but were not found in all of the libraries panned against BSA and Fn. There is
315 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
317 libraries were constructed and some from the way in which the panning experiments were done. In this
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.

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

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

340 It is also interesting that three of the successful pannings, *H. influenzae* and *A. pleuropneumoniae*
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 membrane-
350 associated proteins. In contrast to the clones selected by panning the *H. influenzae* and *P. multocida*
351 libraries against BSA and fibronectin respectively, the clones selected after the third round of panning
352 contained overlapping 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,
366 rather to a region of intergenic DNA. These results are indicative of some of the limitations of the
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
374 or on their conformation. On the other hand, small DNA fragments such as those selected for in the *H.*
375 *influenzae* library panned against Fn, could adopt a conformation that selects for this clone to the
376 exclusion of all others.

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

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.

385

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389

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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
512 containing 200µg of ampicillin and counting the number of amp^R colonies. **A:** Panning of phage
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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519 **Figure 2.** Binding of recombinant phage displaying the protein product of gene *pm1665* in fusion with
520 coat protein VIII to a variety of immobilised proteins found in the extracellular matrix or in plasma.
521 The data are presented as the mean \pm SEM for triplicate wells. The data shown are representative of
522 three separate experiments.

523

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

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533 **Table 1.** Construction of phage display libraries from genomic DNA of *Haemophilus influenzae*,
 534 *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *Actinobacillus actinomycetemcomitans* in
 535 the phagemid vector pG8SAET. Library size is dependent upon the number of *E.coli* TG1
 536 transformants after electroporation with chromosomal DNA ligated to the phagemid vector. The
 537 complexity of the library was determined by analyses of twenty randomly chosen clones to determine
 538 the ratio of transformants containing the phagemid with insert DNA, rather than phagemid alone.
 539 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 transformants	Complexity of library	Titre of phage library (phage /mL)
<i>H. influenzae</i>	8.2 x 10 ⁹	5.33 x 10 ⁶	1.46 x 10 ¹²
<i>P. multocida</i>	3.52 x 10 ⁷	2.99 x 10 ⁷	1.82 x 10 ¹²
<i>A. actinomycetemcomitans</i>	3.27 x 10 ⁷	2.6 x 10 ⁷	2.6 x 10 ¹²
<i>A. pleuropneumoniae</i>	5.3 x 10 ⁷	3.97 x 10 ⁷	1.4 x 10 ¹²

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

Phage display library of DNA from:	Ligand	Phage inserts		Gene	Predicted protein product
		Size	No in 3 rd round		
<i>H. influenzae</i>	Fibronectin	52bp	9/10	No ORF (Intergenic DNA)	None
<i>H. influenzae</i>	BSA	633bp	10/10	<i>hi0367</i>	Hypothetical protein
<i>P. multocida</i>	Fibronectin	611bp	10/10	<i>pm1665</i>	Hypothetical protein
<i>A. actinomycetemcomitans</i>	Fibronectin	283-750bp	8/10	<i>orf10</i>	Hypothetical protein
<i>A. pleuropneumoniae</i>	BSA	87bp	5/10	<i>orf16</i>	Na ⁺ -transporting methylmalonyl- 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
<i>P. multocida</i>	<i>PM2009</i>	51	65	318aa
<i>H. somnus</i>	<i>orf 80</i>	50	64	318aa
<i>A. actinomycetemcomitans</i>	<i>orf 67</i>	47	62	315aa
<i>M. succiniciproducens</i>	<i>MS1918</i>	47	66	324aa
<i>H. ducreyi</i>	<i>orf 813</i>	36	52	323aa
<i>A. pleuropneumoniae</i>	<i>orf 42</i>	35	54	322aa
<i>M. haemolytica</i>	<i>orf 13</i>	33	51	332aa

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580 **Table 4.** Homologues of gene *pm1665* identified in members of the Pasteurellaceae by NCBI BLAST
 581 searches or in the cases of *A. actinomycetemcomitans* and *M. haemolytica* BLAST searches on the
 582 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
<i>H. somnus</i> 129PT	<i>orf 22</i>	57	84	111aa
<i>A. actinomycetemcomitans</i>	<i>orf 1426</i>	50	67	109aa
<i>M. succiniciproducens</i>	<i>ComEA</i>	53	75	161aa
<i>H. influenzae</i>	<i>hi1008</i>	60	87	112aa
<i>H. ducreyi</i>	<i>orf507</i>	45	60	119aa
<i>M. haemolytica</i>	<i>orf 35</i>	41	60	211aa
<i>A. pleuropneumoniae</i>	<i>orf 4</i>	52	73	114aa

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