Comparative analysis of shared and unique mechanisms important for diverse strains of *Pasteurella multocida* to cause systemic infection in mice

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Running title: TraDIS investigation of *P. multocida* pathogenesis

Abstract

Pasteurella multocida is a Gram-negative bacterium that causes a range of distinct diseases in livestock animals. Different *P. multocida* diseases are associated with different capsule and lipopolysaccharide (LPS) types, but little else is known about what underpins this disease specificity. In this study, we utilised transposon-directed insertion site sequencing (TraDIS) to identify genes in two diverse *P. multocida* strains that are required for growth in rich media, and genes important for survival during systemic infections in BALB/c mice. Analysis of growth in heart infusion broth showed that the fowl cholera isolate VP161 (capsule type A and LPS type L1) and the bovine haemorrhagic septicaemia isolate M1404 (capsule type B and LPS type L2) shared 461 genes essential for growth in rich media, with 95% of these present in all publicly available closed *P. multocida* genomes. *In vivo* fitness analysis identified 63 and 94 genes important for VP161 and M1404 survival in BALB/c mice, respectively. Only 35 homologs were identified as important for survival in both strains, showing that conserved

biological systems can be differentially important for different *P. multocida* strains. Investigation of proteins involved in the catabolite response showed that an active cyclic-adenosine monophosphate (cAMP) receptor protein (CRP) was required for maximal fitness in M1404. Furthermore, disrupting CRP or cAMP production also reduced capsule production in M1404, but increased capsule production in VP161, demonstrating that these *P. multocida* strains have different regulatory systems for crucial virulence factors.

Author Summary

Pasteurella multocida is an important livestock pathogen, causing several distinct severe diseases in many different livestock animals. *P. multocida* can spread rapidly throughout animal populations, with peracute infections causing death within 48 h, resulting in large outbreaks with high mortality. Host predilection and disease presentation often correlate with the capsule and lipopolysaccharide type produced by the causative strain. However, the processes that allow certain strains to cause a particular disease are not well understood. In this study, we have comprehensively identified genes required for two *P. multocida* strains to cause systemic infection in mice, showing that these diverse strains have differential requirements for survival during a systemic infection. This information is crucial for understanding *P. multocida* diseases and for the development of new strategies to combat infection.

Introduction

Pasteurella multocida is an upper respiratory tract commensal of many mammal and bird species [1-3], but the bacterium can also cause serious disease, including fowl cholera in birds, haemorrhagic septicaemia in ungulates, atrophic rhinitis in pigs, and pneumonia in most livestock animals [4]. Strains of *P. multocida* are commonly classified based on capsule type (namely A, B, D, E and F) and/or lipopolysaccharide (LPS) genotype (namely L1-L9) [5-7]. *P. multocida* disease presentation and host predilection often correlates with genotype of surface structures produced by the infecting strain [4]. For instance, fowl cholera is caused primarily by strains with capsule type A or F, and LPS type L1, L3 or L6 [5, 8-10], while

53 haemorrhagic septicaemia is only caused by strains with capsule type B or E, and LPS type
54 L2 or L5 [11-13]. Apart from capsule and LPS type, very little is known about the pathogenic
55 mechanisms that are unique to strains causing these different diseases or what pathogenic
56 mechanisms are shared by such strains.

57 *P. multocida* disease often progresses rapidly and death can occur within 48 h of infection [14,
58 15], making antibiotic treatment in these cases rarely successful. *P. multocida* is readily
59 transmitted between animals within a population, either directly or via environmental
60 contamination. Large *P. multocida* disease outbreaks occur in both wild and livestock animal
61 populations [16-20], with increased susceptibility linked to environmental stresses such as
62 increased temperature and humidity, as well as animal crowding [21]. Outbreaks of
63 *P. multocida* disease in livestock result in high mortality and large economic losses.
64 Vaccination is an important preventative measure against *P. multocida* disease. However,
65 while several *P. multocida* vaccines are currently available [22], most only provide protection
66 against strains producing the same capsule and LPS structures as the vaccine strain and the
67 duration of immunity is generally short [23]. Currently, there is no vaccine available that
68 protects against the full range of diseases in different hosts. We predict that to improve the
69 breadth of protection of *P. multocida* vaccines, it is important to understand what pathogenic
70 mechanisms are shared or unique across *P. multocida* strains that cause distinct diseases.

71 In this study, we investigated the pathogenic mechanisms shared between two different
72 *P. multocida* strains, VP161, a chicken fowl cholera isolate belonging to capsule serotype A
73 and LPS genotype L1, and M1404, a bison haemorrhagic septicaemia isolate belonging to
74 capsule serotype B and LPS genotype L2. These strains are genetically diverse, but both can
75 cause lethal systemic infection in mice [24-26]. We performed transposon-directed insertion
76 site sequencing (TraDIS, also called Tn-Seq) in both strains to identify all genes in each strain
77 that were essential for growth on rich media, and those genes important for *in vivo* fitness in
78 a mouse systemic infection model. The TraDIS analysis identified a core-set of 447 genes that
79 we predict are essential for the *P. multocida* species. We also identified shared pathways and

80 processes important for both strains VP161 and M1404 to survive *in vivo* (35 genes), and
81 processes important only for strain VP161 (28 genes) or only for strain M1404 (59 genes) to
82 survive *in vivo*. These data identify multiple unique requirements for each of these strains to
83 cause disease in mice.

84 **Results**

85 **Production of a *P. multocida* strain M1404 *Himar1* mutant library, systemic infections**
86 **in mice, and TraDIS library production**

87 We previously constructed a *Himar1* transposon mutant library in the *P. multocida* strain
88 VP161 (capsule type A and LPS type L1) [27], which was also used in this study. For TraDIS
89 analysis of strain M1404 (capsule type B and LPS type L2), we generated a comparable
90 *Himar1* transposon mutant library. A total of ~1.12 x 10^6 M1404 *Himar1* transposon mutants
91 were pooled to produce the final library. To generate a high-quality M1404 reference genome
92 for TraDIS analysis, we used a combination of Nanopore and Illumina sequencing that allowed
93 for the assembly of a 2.42 Mbp genome that contained two contigs (see S1_Text for full
94 assembly methodology).

95 The M1404 and VP161 *Himar1* mutant libraries were each used to infect BALB/c mice at a
96 dose of ~2 x 10^7 CFU via intraperitoneal injection (project overview described in S1 Fig and
97 infectious doses are given in S1 Table; note that all Supplemental Tables, Supplemental
98 Figures and Supplemental Figure Legends are in S1_Text). All mice succumbed to systemic
99 infection, reaching humane endpoints at five hours (S2A Fig). Surviving *P. multocida* mutants
100 were recovered from the blood, liver, and spleen by plating *in vivo*-derived samples onto rich
101 media. Greater than 4 x 10^6 CFU of *P. multocida* were recovered from each site for all
102 infections (S2B Fig). Four TraDIS library replicates were generated using genomic DNA
103 isolated from the recovered mutants representing each strain (VP161 and M1404) and
104 condition (rich media input culture, recovered mutants from each tissue following infection in
105 mice). Each library was sequenced on an Illumina NextSeq and analysed using the Bio-TraDIS
106 pipeline and related scripts (full TraDIS data in S1_Data). Combined, the four TraDIS library

107 replicates generated for each strain and condition had high transposon-DNA junction read
108 coverage across the genome and a small bp distance between unique insertion sites (UIS)
109 (S3 Fig); the VP161 libraries had between 31 and 33 bp distance between each UIS, and the
110 M1404 libraries had between 40 and 43 bp between each UIS (S2 Table in S1_Text). Principal
111 component analysis using UIS or reads per gene showed that in all conditions the rich media
112 TraDIS libraries clustered together and separated away from the mouse TraDIS libraries (S4
113 Fig).

114 **Identification of core genes essential for growth in rich media**

115 We used the input mutant libraries, grown on solid heart infusion media overnight, to identify
116 genes essential for growth in rich media for both *P. multocida* strain VP161 and M1404.
117 Essential genes were identified by comparing normalised UIS counts per gene (Fig 1A, S5
118 Fig, see S2_Data for the list of all essential genes for growth on rich media). There were 585
119 genes, of which 542 were coding sequences (CDS), identified as essential for strain VP161.
120 There were also 585 genes, of which 540 were CDS, essential for strain M1404. Although a
121 similar number of genes were identified as essential in both strains, only 461 were homologs
122 (Fig 1B), as determined using eggNOG or BlastP. The majority of these 461 shared essential
123 proteins were involved in housekeeping processes. Analysis using the STRING database
124 identified several processes and pathways that were enriched in the essential protein set,
125 including: DNA replication (false discovery rate [FDR] = 4×10^{-4}), transcription (FDR = 0.047),
126 translation (FDR = 2.04×10^{-16}) including ribosomal structural proteins (FDR = 2.7×10^{-5}) and
127 tRNA amino acylation (FDR = 7.83×10^{-5}), cell division and regulation of cell shape (FDR =
128 0.015), fatty acid biosynthesis (FDR = 4×10^{-6}), peptidoglycan (FDR = 0.004) and lipid A
129 biosynthesis (FDR = 0.019), glycolysis (FDR = 0.026), purine nucleoside biosynthesis (FDR =
130 0.0026), heme/porphyrin biosynthesis (FDR = 0.027), isoprenoid biosynthesis (FDR = 0.027),
131 and ATP production (FDR = 0.0033).

132 We then assessed if these shared essential genes were also conserved across the entire
133 *P. multocida* species. We analysed 167 publicly available complete *P. multocida* genomes,

134 together with VP161 and M1404, and identified a total of 1,564 genes present in all strains
135 (the 100% core-genome). A core-genome phylogeny generated from this set of 169 complete
136 genomes was highly similar to a previously constructed *P. multocida* core-genome phylogeny
137 generated using 402 *P. multocida* strains that included complete and incomplete genomes
138 (S6 Fig) [7], indicating that this subset of 169 strains is representative of the entire *P. multocida*
139 species. Of the 461 essential genes identified in both strain VP161 and strain M1404, 447
140 (97%) were present in the 100% core-genome (Fig 1B). We also compared the 100% core-
141 genome and conserved rich media essential genes to our curated *P. multocida*-specific
142 virulence factor and antibiotic resistance database (PastyVRDB) [7]. This analysis identified
143 53 genes associated with virulence that are present in all *P. multocida* strains, and included
144 the genes *hpbA*, *oma87*, *spoT*, *fur*, Pm0442, and *kdtA*, which were all essential in both strains
145 (S3 Table in S1_Text).

146 There were 75 genes essential in VP161 that had a non-essential homolog in M1404, and 65
147 genes essential in M1404 that had a non-essential homolog in VP161 (Fig 1B). Genes
148 identified as essential in VP161 but not M1404 included *crp*, *crr* and *relA*, and genes identified
149 as essential in M1404 but not VP161 included several genes involved in the Tol-Pal system
150 (*tolBQR* and *pal*), DNA repair (*ruvBC* and *aroQ*), and sugar transport (*ptsH*). There were also
151 six essential VP161 genes that had no homolog in M1404, and 14 essential genes in M1404
152 that had no homolog in VP161 (Fig 1B). Most of the unique essential M1404 genes encoded
153 hypothetical proteins; however, M1404_00858 and M1404_00859 contained antitoxin-like
154 domains, and M1404_01327 contained a putative peptidase toxin domain.

155 **_P. multocida_ strains VP161 and M1404 have both shared and unique genes required for**
156 **virulence in mice**

157 To identify genes that were important for *P. multocida* fitness during murine systemic
158 infections, we compared the read count per gene between the mouse and rich media TraDIS
159 libraries. Any gene with a four-fold decrease in *Himar1* insertions and a *q*-value < 0.01 was
160 identified as resulting in decreased *in vivo* fitness when disrupted, and any gene with a four-

161 fold increase in *Himar1* insertions and a *q*-value < 0.01 was identified as showing increased
162 *in vivo* fitness when disrupted (see S3_data and S4_data for full fitness-associated data).
163 Overall, 134 genes were *in vivo* fitness-associated in at least one type of tissue and one strain
164 (Table 1, Fig 2A). There were 63 and 94 genes that resulted in decreased *in vivo* fitness of
165 strain VP161 and M1404, respectively (Fig 2B, S4 Table in S1_Text). For both VP161 and
166 M1404, most of these genes were identified as important for *in vivo* fitness in all three tissues
167 (Fig 2B). The genes were then compared to identify homologs between the two strains. Of the
168 genes that had a homolog in the other strain, 35 were important for fitness in both strains, 23
169 were important for fitness only in VP161, and 47 were important for fitness only in M1404 (Fig
170 2C). Furthermore, there were five genes important for fitness in VP161 that did not have a
171 homolog in M1404, and 12 genes important for fitness in M1404 that did not have a homolog
172 in VP161 (Fig 2C). In total, there were seven genes that increased fitness when disrupted in
173 either VP161 or M1404, with only two genes, *yeeZ* and *speB*, common to both strains (S5
174 Table in S1_Text).

175 Common pathways and processes were identified as important for both VP161 and M1404
176 during infection in mice (Fig 3A, S7 Fig and S8 Fig). In both strains, disruption of almost all
177 capsule biosynthesis genes had a severe fitness-cost. Both *fis* and *hfq*, which encode
178 transcriptional regulators known to positively regulate capsule biosynthesis genes in strain
179 VP161, were important for fitness in VP161 but not in M1404, suggesting these strains used
180 different regulatory mechanisms. Several genes encoding proteins involved in LPS core-
181 polysaccharide synthesis were identified as important for *in vivo* fitness. Genes from the ADP-
182 L-glycero-β-D-manno-heptose synthesis pathway (*hldDE-gmhAB*), that encode proteins that
183 synthesise a modified heptose that is added to the LPS core-polysaccharide, were either
184 important for *in vivo* fitness or essential in both strains. The gene *hptA*, which encodes an LPS
185 inner-core heptosyltransferase, was identified as important for fitness in both strains.
186 Additionally, genes encoding the LPS inner-core heptosyltransferase genes *hptC* and *hptD*,
187 the LPS inner-core glucosyltransferase *gtcD*, and the LPS outer-core galactosyltransferase

188 *gatD* were important for fitness of strain M1404. Several genes identified as important for *in*
189 *vivo* fitness in both strains encoded proteins involved in amino acid synthesis and transport,
190 or protein translation (Fig 3). These included an aminotransferase involved in aspartate,
191 phenylalanine, and tyrosine synthesis (*aspC*), an asparagine synthase (*asnA*) and regulator
192 of asparagine synthase expression (*asnC*), a putative alanine transporter (*alsT_1*), and
193 proteins involved in translation efficiency (*prmC* and *epmA*) (Fig 3). Two genes in the L-serine
194 biosynthesis pathway (*serAB*) and a putative tyrosine transporter (*tyrP_1*) were important for
195 the *in vivo* fitness of VP161 but not M1404. Conversely, elongation factor Tu subunit genes
196 (*tufB_1* and *tufB_2*) were important for the *in vivo* fitness of strain M1404 but not strain VP161.
197 Other systems important for *in vivo* fitness in both strains included the purine biosynthesis
198 pathway, the Ton-B energy transduction system (*tonB-exbBD*), methionine uptake (*plpB-*
199 *metPN*), and *aroA* from the chorismate pathway.

200 Several pathways were identified as important for *in vivo* fitness of M1404 but not VP161 (Fig
201 3A). This included genes encoding two components of the sialic acid uptake system (*nanP*
202 and *nanU*), sialic acid metabolism (*nanE*) and the N-acetylneuraminate CMP transferase
203 (*neuA_1*), a sensor histidine kinase (*cpxA*), the sorbitol-specific EIIA (*srlB*), and genes
204 encoding the Mla phospholipid transport system (*mlaBDEF*). The genes encoding adenylate
205 cyclase (*cyaA*) and cAMP receptor protein (*crp*) were also identified as important for *in vivo*
206 fitness of M1404 but not VP161; however, *crp* was identified as essential for strain VP161 to
207 grow in rich media. The gene M1404_01811, which encodes a *P. multocida* capsule type B-
208 and E-specific surface lipoprotein (SLP) PmSLP-3 [28], and the adjacent gene M1404_01812
209 that encodes a surface lipoprotein assembly modulator (Slam) required for PmSLP-3 export,
210 were both identified as important for M1404 *in vivo* fitness. Comparatively, other putative SLPs
211 (PlpE and PmVP161_1666 in strain VP161, M1404_01814 in strain M1404), and the Slam
212 exporter in VP161 (PmVP161_1663) were not important for *in vivo* fitness. Most of the genes
213 important for *in vivo* fitness of M1404 that did not have a homolog in VP161 were required for
214 production of the type B capsule polymer (Fig 3B). Genes required for *in vivo* fitness of VP161

215 but not for M1404 included genes encoding components of the ubiquinone biosynthesis
216 pathway (*ubiGHE_3X*), a heme utilisation protein (*hutZ_1*), components of the
217 aminodeoxychorismate synthase complex (*pabAB*), a putative sodium proton antiporter
218 (PmVP161_0183), a putative tyrosine importer (*tyrP_1*), and *aroE* from the chorismate
219 pathway (Fig 3A). All of the genes important for *in vivo* fitness in VP161 that did not have a
220 homolog in M1404 were required for production of the type A capsule polymer (Fig 3C).

221 **The genes *alsT_1*, *crp* and *cyaA* are important for strain M1404 fitness *in vivo***

222 We performed time to euthanasia experiments with selected individual *P. multocida* directed
223 mutants to confirm that a sub-set of genes identified by TraDIS were individually important for
224 *in vivo* survival. ClosTron mutagenesis was used to generate individual *alsT_1*, *crp* and *cyaA*
225 mutants in strain M1404. Wild-type M1404 and each mutant strain was used to inoculate
226 separate groups of BALB/c mice via intraperitoneal injection on two different days (see S1
227 Table in S1_Text for infectious doses). Signs of systemic infection were monitored and used
228 to determine time to euthanasia. Mice infected with wild-type M1404 reached the humane
229 endpoint on average 6.5 hours post infection (hpi) (Fig 4). Mice infected with the *crp* or *cyaA*
230 mutants reached the humane endpoint at an average 11 and 10.3 hpi, respectively (Fig 4).
231 Mice infected with the *alsT_1* mutants did not reach the humane endpoint and showed low
232 total clinical scores over the 12-hour infection period (Fig 4). All strains had a significant
233 increase in time to euthanasia when compared to wild-type M1404, confirming the TraDIS
234 data that *alsT_1*, *crp* and *cyaA* are important for *in vivo* fitness of strain M1404.

235 **The carbon catabolite repression system controls capsule production differently in**
236 ***P. multocida* strains VP161 and M1404**

237 Carbon catabolite repression was important for *P. multocida* fitness and is controlled by the
238 cyclic-adenosine monophosphate (cAMP) receptor protein (CRP, also called catabolite
239 activator protein (CAP) or virulence factor regulator (VFR) in other species). CRP was
240 important for *in vivo* fitness of strain M1404, and essential for strain VP161 to grow in rich
241 media. While CRP has not been well characterised in *P. multocida*, in other bacterial species

242 CRP family proteins are global transcriptional regulators that have increased affinity for DNA
243 targets when bound to cAMP [29]. Thus, CRP activity is modulated by the intracellular levels
244 of cAMP. The level of intracellular cAMP is controlled by the adenylate cyclase CyaA, which
245 synthesises cAMP from ATP [30], and the cAMP phosphodiesterase CpdA, which degrades
246 cAMP to AMP [31]. As shown above, both *crp* and *cyaA* were identified as important for *in*
247 *vivo* fitness of M1404 by TraDIS analysis and individual mutants in these genes showed
248 reduced virulence in mice. Furthermore, the TraDIS data showed that inactivation of *cpdA* in
249 M1404 slightly improved fitness during *in vivo* growth (average $\log_2$ fold-change of between
250 0.93 and 1.34, see S1_data). Collectively, these data suggest that cAMP-dependent activation
251 of CRP is important for M1404 *in vivo* fitness. CRP family proteins are known to regulate a
252 wide range of targets in different bacterial species [32-34], and transcriptomic analysis of a
253 *P. multocida* strain 0818 *crp* mutant identified reduced capsule biosynthesis gene expression
254 [35]. To determine if inactivating the catabolite repression system affected capsule production
255 in M1404, capsule production was assessed using cells grown overnight on solid media. Both
256 the M1404 *crp* and M1404 *cyaA* mutants harbouring empty vector produced barely detectable
257 absorbance readings, indicating that these strains are essentially acapsular (Fig 5A).
258 Providing the *crp* mutant with a wild-type copy of *crp in trans* led to a modest, but significant
259 increase in absorbance readings, compared to the *crp* mutant harbouring empty vector (Fig
260 5A). Providing the *cyaA* mutant with the *cyaA* complementation plasmid restored absorbance
261 readings, and therefore capsule production, to wild-type levels (Fig 5A). These data confirm
262 that adenylate cyclase is required for capsule production in M1404 and strongly suggest that
263 this is a CRP-dependent mechanism.

264 The role of active CRP and catabolite repression in VP161 was different from that observed
265 in M1404. While *crp* was essential for strain VP161 growth in rich media, the TraDIS data
266 showed that inactivation of *cyaA* had no impact on *in vivo* fitness of VP161, and inactivation
267 of *cpdA* slightly reduced fitness (average $\log_2$ fold-change of between -1.75 and -1.93, see
268 S1_Data). In our previous TraDISort study, *cpdA* was identified as important for *in vitro*

269 capsule production in strain VP161 [27]. Collectively, these data suggest that active CRP-
270 cAMP may act to repress capsule production in strain VP161. As CRP was essential in VP161,
271 we attempted to inactivate *cpdA* and *cyaA* using ClosTron mutagenesis; however, only a *cyaA*
272 mutant was successfully generated. Capsule absorbance assays were performed using wild-
273 type VP161 and the *cyaA* mutant harbouring empty vector, and the complemented *cyaA*
274 mutant. Capsule was extracted from cells during early stationary phase, when cAMP levels
275 would be expected to be high. The VP161 *cyaA* mutant produced significantly more capsule
276 than wild-type VP161, with complementation restoring normal levels of capsule production
277 (Fig 5). These data indicate that inactivation of adenylate cyclase, which should reduce the
278 level of cAMP and therefore CRP activity, de-represses capsule production in VP161. This
279 strongly suggests that an active CRP-cAMP represses capsule in strain VP161, which is the
280 opposite of the role of CRP-cAMP in M1404.

281 **Discussion**

282 In this study, we performed TraDIS analysis of two distinct *P. multocida* strains, the poultry
283 isolate VP161 (A:L1) and the bovine isolate M1404 (B:L2). These strains produce different
284 capsule and LPS structures. Initially, we identified genes required for both VP161 and M1404
285 to grow in rich media, with 585 genes identified as essential in both strains. We have previously
286 reported that 509 genes were essential for VP161 to grow in rich media [27], 95% of which
287 were also identified as essential in this study. The increase in rich media essential genes is
288 likely due to the mutant libraries being grown for ~48 h longer than the previous study, allowing
289 for further reduction of mutants with reduced viability from the population. Previous analysis
290 of random transposon mutant viability has shown that the time required for a mutation to lead
291 to a replication defect determines at what time point the gene is identified as important for
292 survival [36]. Strains VP161 and M1404 shared a set of 461 protein-encoding genes that were
293 essential for growth in rich media, most of which were involved in housekeeping processes.
294 Pan-genome analysis of 169 complete *P. multocida* genomes identified 1,564 genes present
295 in all genomes. Of these 1,564 core genes, 447 were identified as essential in both strain

296 VP161 and strain M1404 for growth in rich media. Thus, 97% of the 461 essential genes
297 identified in strains VP161 and M1404 are present in all *P. multocida* genomes examined and
298 are likely to be required for all *P. multocida* strains to grow in rich media. While most of these
299 genes encode housekeeping genes involved in replication, transcription, translation and
300 energy metabolism, some encode surface-associated proteins and therefore could be
301 considered potential targets for vaccine development.

302 TraDIS analysis of VP161 and M1404 mutants recovered from BALB/c mice following
303 systemic infection identified 134 genes associated with *in vivo* fitness in at least one tissue
304 and one strain. To validate these results, we generated directed *alsT_1*, *crp* and *cyaA* mutants
305 in strain M1404. Separate infection studies with these mutants showed reduced virulence in
306 mice, confirming each is important for *in vivo* fitness. Additionally, several genes and pathways
307 identified in the TraDIS analysis performed in this study have previously been identified as
308 important for *P. multocida* virulence. Infection studies conducted using mice or poultry have
309 shown that inactivation of *aroA* in *P. multocida* strains X-73, P-1059, and 85020 [37, 38], *tonB*,
310 *exbB*, and *exbD* in strain PM25 [39], *plpB* in strain VP161 [40], and *nanP* and *nanU* in strain
311 P-1059 [41] all resulted in reduced virulence. Furthermore, several *P. multocida aroA* mutants
312 are commercially available for use as live-attenuated vaccines [37, 38]. Signature-tagged
313 mutagenesis studies using strain UC6731 and strain VP161 identified *purN*, *purF, guaB*, *nanE*
314 and *nanU* as important for virulence in mice and/or chickens [24, 42]. Transcriptomics analysis
315 of strain X-73 cells recovered from systemic infections in chickens showed there was
316 increased expression *in vivo* of *purH*, *aspC* and *asnA* [43], and transcriptomic analysis of
317 Pm70 grown in media with limited iron showed increased expression of *hutZ_1* [44]. These
318 previous studies strongly support that our TraDIS analysis has comprehensively identified
319 genes required for *P. multocida* survival *in vivo*. However, surface structures that have
320 previously been proposed to play a role in *P. multocida in vivo* fitness and/or virulence,
321 including filamentous haemagglutinin, fimbriae, and Flp-pili, were not identified as important
322 for *in vivo* fitness in our TraDIS analysis [24, 42, 45, 46]. These surface structures are likely

323 required for initial colonisation of the upper respiratory tract but not when *P. multocida* is
324 delivered via intramuscular, intraperitoneal or intravenous injection. Indeed, *P. multocida*
325 filamentous haemagglutinin mutants have a higher infectious dose (ID_{50}) compared to wild-
326 type parent strains in birds but only when introduced intranasally and not via intramuscular or
327 intravenous route [45, 46]. The TraDIS data presented in this study identified several
328 processes that have not previously been shown to be important for *P. multocida* disease,
329 including ubiquinone biosynthesis, aminodeoxychorismate synthesis, the Mla phospholipid
330 transport system and putative sodium proton antiporters. Importantly, we showed that there
331 were clear differences between pathways and processes required for the two different
332 *P. multocida* strains to survive *in vivo*. The ubiquinone biosynthesis pathway and the putative
333 sodium proton antiporter (PmVP161_0183) were only important for *in vivo* fitness of strain
334 VP161. Conversely, components of the sialic uptake and utilisation pathway, the Mla ABC-
335 transport system, the adenylate cyclase CyaA, the SLP protein PmSLP-3 and its cognate Slam
336 exporter were only important for *in vivo* fitness of strain M1404. This crucially shows that
337 although these *P. multocida* strains share these systems, their importance for survival *in vivo*
338 is strain dependent.

339 Different *P. multocida* strains typically encode two or more SLPs that are exported to the cell
340 surface by a Slam protein [28, 47]. Subunit vaccines formulated using *P. multocida* SLPs
341 (PlpE, PmSLP-3 and PmSLP-1) have been shown to elicit a protective immune response
342 against *P. multocida* strains that produce the cognate SLP [28, 48-50]. Despite both VP161
343 and M1404 encoding two SLPs, our TraDIS analysis only identified PmSLP-3 in M1404 as
344 important for survival in BALB/c mice. PmSLP-1, an SLP that is encoded by a subclade of
345 capsule type A strains isolated from cattle, has recently been shown to bind to complement
346 factor I and block complement-mediated killing [51]. Previous studies have shown that capsule
347 was important for blocking complement-mediated killing in strain VP161 but not for strain
348 M1404 [25, 26]. As such, it is possible that PmSLP-3 is important for blocking complement-
349 mediated killing in M1404. Several SLPs from *Neisseria*, *Actinobacillus*, *Hemophilus* and

350 *Moraxella* spp. have a similar predicted protein fold to *P. multocida* SLPs but have diverse
351 functions, including binding to human iron-binding proteins, lactoferrin and factor H [47]. Thus,
352 despite SLPs having similar structure, each protein can have a different biological function
353 and role in pathogenesis. Given *P. multocida* can persist in several different hosts, it is
354 possible that different SLAM-dependent SLPs are required for survival in different hosts.

355 Most capsule biosynthesis genes and known regulators of capsule biosynthesis were
356 identified as important for *in vivo* fitness, supporting many previous studies demonstrating that
357 capsule is important for *P. multocida* virulence in both type A and B strains [25, 26, 42, 52-54].
358 Additionally, several other genes previously identified in strain VP161 as important for capsule
359 production were also identified by TraDIS as important for *in vivo* fitness, including *prmC*,
360 *epmA*, *epmB* and *ubiX*. However, our data indicated that capsule production is regulated
361 differently in the two strains. The genes encoding the global regulator Fis and the RNA
362 chaperone protein Hfq, previously shown to be important for capsule production in strain
363 VP161 [53, 54], were also identified in this study as important for *in vivo* fitness of VP161 but
364 not M1404. Furthermore, the TraDIS analysis and capsule absorbance assays together
365 showed that CRP-cAMP controls capsule production in these *P. multocida* strains, but in
366 different ways. Disrupting CRP-cAMP by inactivating either *cyaA* or *crp*, abrogated capsule
367 production in strain M1404, showing that CRP-cAMP positively regulates capsule production
368 in this strain. Conversely, disrupting CRP-cAMP by inactivating *cyaA* increased capsule
369 production in strain VP161, indicating that CRP-cAMP represses capsule production in strain
370 VP161. Together, these data show that capsule expression responds differently to CRP-cAMP
371 in these two strains. The TraDIS data also suggested that activation of the catabolite response
372 in strain M1404 may depend on the level of sorbitol. Adenylate cyclase activity is commonly
373 controlled by phosphoenolpyruvate(PEP):carbohydrate phosphotransferase system (PTS)
374 proteins. The PTS system couples carbohydrate import and phosphorylation, with the energy
375 required for import and the donated phosphate provided by PEP [55, 56]. The phosphate is
376 transferred to the carbohydrate via a protein phosphate transfer chain consisting of the general

377 PTS proteins EI and HPr, and the sugar specific EII, with EII generally comprised of three
378 proteins/domains (EIIA, EIIB, EIIC) [57]. Different proteins in the PTS system can allosterically
379 regulate different protein targets depending on whether they are phosphorylated. In *E. coli*,
380 phosphorylated $EIIA^{Glc}$ is known to be involved in inducing adenylate cyclase activity [58, 59],
381 thus allowing cAMP production and activation of the catabolite repressor system when the
382 levels of glucose are low. $EIIA^{Glc}$, encoded by *crr*, was not important for *in vivo* fitness of
383 M1404. However, the sorbitol-specific EIIA protein, encoded by *srlB*, was identified as
384 important for *in vivo* fitness of M1404. This suggests that the absence of sorbitol increases the
385 amount of phosphorylated sorbitol-specific EIIA, which in turn induces adenylate cyclase
386 activity and activates the catabolite response.

387 Several LPS biosynthesis genes were important for fitness in both strains. The LPS of
388 *P. multocida* lacks an O-antigen, making the core-polysaccharide the most distal component
389 of the LPS [60]. The core-polysaccharide is comprised of a conserved inner-core and a
390 variable outer core, and is synthesised by stepwise addition of sugars by different
391 glycosyltransferases [60]. As the function of LPS transferases have been determined for LPS
392 type L1 and L2 strains [61-64], the putative structures required for survival in BALB/c mice can
393 be predicted based on which transferases had reduced fitness in the TraDIS analysis. All
394 *P. multocida* strains examined to date simultaneously produce two inner-core structures
395 (namely glycoforms A and B), with the relative levels of each glycoform determined by the
396 competing activity of KdkA and KdtA. KdkA adds a phosphate to the first 3-deoxy-D-manno-
397 oct-2-ulosonic acid (Kdo) leading to glycoform A production, while KdtA adds a Kdo II to Kdo
398 I leading to glycoform B production [61]. The nascent inner-core is then extended by the
399 heptosyltransferases HptA (specific for glycoform A) and HptB (specific for glycoform B) [61].
400 The TraDIS analysis identified *hptA*, but not *hptB*, as important for fitness in both strain VP161
401 and M1404. Inactivation of *hptA*, but not *hptB*, has previously been shown to attenuate
402 virulence of strain VP161 in chickens [61]. The VP161 *hptA* mutants have severely truncated
403 LPS as the Lipid A – phosphorylated Kdo I cannot be extended in this mutant [61]. The

404 presence of a large amount of severely truncated LPS on the surface make the bacterium
405 vulnerable to the innate immune system [61]. No other LPS transferases were identified as
406 important for fitness of VP161, supporting previous data that showed a full length LPS is not
407 important for fitness of this strain in mice [24]. In strain M1404, the inner-core transferases
408 *hptC*, *hptD* and *gctA* were all important for fitness, indicating that a glycoform A structure of at
409 least Kdo I - Hep I [Hep II – Hep III] – Glucose (Glc) I is required for *in vivo* survival. Additionally,
410 inactivation of the outer-core galactosyltransferase gene *gatD* significantly reduced fitness of
411 M1404 in BALB/c mice. The LPS outer-core structure produced by M1404 is Hep IV – Hep V
412 – N-acetyl glucosamine (GlcNAc) I – Galactose (Gal) I [64]. Identification of *gatD* but not other
413 outer-core transferases as important for fitness suggests that removal of the terminal
414 galactose therefore leaving the GlcNAc as the terminal sugar reduces the viability of M1404
415 in BALB/c mice, but truncations terminating the outer-core at either Hep IV, Hep V or GlcNAc
416 I do not impact fitness. Previous analysis of several *P. multocida* type L3 strains has shown
417 that inactivating mutations in transferase genes that result in different LPS outer-core
418 structures affect virulence in chickens at different levels [65].

419 In this study, we have determined the 100% core-genome for *P. multocida* and identified a set
420 of core genes likely essential for all *P. multocida* strains. Furthermore, we have
421 comprehensively identified genes important for *in vivo* fitness of the type A strain VP161 and
422 the type B strain M1404 during systemic infections in BALB/c mice, with these strains having
423 shared and unique pathways that are important for fitness. Genes identified as important for
424 both strains to survive *in vivo* and conserved across the entire *P. multocida* species represent
425 ideal vaccine targets for generating a vaccine that provides protection against all *P. multocida*
426 strains. Finally, we have shown that capsule production is regulated differently between these
427 two strains, and that a full length LPS is more important in M1404 than VP161 for *in vivo*
428 fitness in mice.

429 **Materials and methods**

430 **Bacterial strains and growth conditions**

431 The bacterial strains and plasmids used in this study are listed in S6 Table (in S1_Text). *E. coli*
432 strains were cultured in lysogeny broth (LB), and *P. multocida* strains were cultured in heart
433 infusion (HI) broth (Oxoid). Solid media was generated by adding 1.5% agar (w/v) to liquid
434 media. When required, antibiotics were added to the media at the following concentrations:
435 kanamycin 50 µg/mL, spectinomycin 50 µg/mL, tetracycline 1.25 µg/mL, ampicillin 100 µg/mL.
436 When required, diaminopimelic acid (DAP) was added to media at a concentration of 100 µM.

437 **Oligonucleotides, DNA extraction, DNA manipulation and DNA sequencing**

438 All oligonucleotides used in this study were synthesised by Sigma, and the sequences of all
439 oligonucleotides are given in S7 Table (in S1_Text). Genomic DNA was extracted using the
440 HiYield Genomic DNA Mini Kit (Real Biotech Corporation) as per the manufacturer's
441 instructions for extraction from Gram-negative bacteria or extracted using Genomic Tip 100/G
442 (QIAGEN) as per manufacturer's instructions. Plasmids were extracted using the NucleoSpin
443 Plasmid Mini Kit (Machery-Nagel) as per the manufacturer's instructions. Polymerase chain
444 reactions (PCR) were performed using either *Taq* DNA polymerase (Roche) or Phusion High-
445 Fidelity DNA polymerase (New England Biolabs), as per the manufacturer's instructions for
446 each polymerase. DNA digests were performed using restriction enzymes sourced from New
447 England Biolabs as per manufacturer's instructions. Ligations were performed using T4 DNA
448 ligase (Roche) as per manufacturer's instructions. DNA was purified using the NucleoSpin Gel
449 and PCR Clean-up Mini Kit (Machery-Nagel). Sanger sequencing reactions were performed
450 as previously described [65]. For whole-genome sequencing of *P. multocida* strain M1404,
451 genomic DNA was submitted to The Ramaciotti Centre for Genomics (University of New South
452 Wales) and sequenced using both an Illumina MiSeq v3, and a Nanopore GridION X5 Mk1
453 using a GridION flow cell (FLO-MIN112) with DNA prepared for sequencing using a Native
454 Barcoding Expansion Kit (SQK-NBD112.24). Additionally, M1404 genomic DNA was also
455 submitted to Plasmidsaurus and sequenced using a Nanopore PromethION using a

456 PromethION flow cell (FLO-PRO114M) with DNA prepared for sequencing using a Rapid
457 Barcoding Kit (SQK-RBK114.96). The M1404 genome was assembled as previously
458 described [7]; see supplementary materials for additional information.

459 **Transformation, site-directed mutagenesis using ClosTron and construction of**
460 **complementation plasmids**

461 Electrocompetent *P. multocida* cells were prepared and transformed as previously described
462 [25]. *P. multocida* site-directed mutants were generated by a modified version of the ClosTron
463 system (also called TargeTron) in the pAL953 plasmid as previously described [65, 66]. Intron
464 insertions into each of the genes of interest were confirmed by PCR-amplification using
465 primers that flanked the gene of interest, and by Sanger sequencing directly from genomic
466 DNA extracted from putative mutants with oligonucleotide BAP6544, which binds within the
467 intron and directs sequencing into the adjacent region of the genome. For complementation,
468 the wild-type gene was amplified using Phusion polymerase and cloned into pAL99S under
469 the control of the *tpiA* promoter. The sequence of the cloned gene and promoter were
470 confirmed by Sanger sequencing. Complementation plasmids or pAL99S empty vector were
471 used to transform the corresponding mutant strains to produce the complemented mutant and
472 empty vector strains, respectively.

473 **Conjugation conditions and generation of the *P. multocida* strain M1404 *Himar1***
474 **transposon mutant library**

475 Conjugation between an *E. coli* donor and *P. multocida* recipient was performed by filter
476 mating as previously described [27], with minor modifications. The donor used in this study
477 was *E. coli* strain JKE201 [67], a diaminopimelic acid (DAP) auxotrophic strain that cannot
478 grow without the addition of DAP in the media. Filters containing the conjugation mix were
479 incubated on HI agar plates containing DAP for one hour. The conjugation mix was recovered
480 from the filter by vortexing the filter in 1 mL of HI broth. The conjugation mix was plated onto
481 LB agar with ampicillin and DAP to recover donor *E. coli*, HI agar to recover all *P. multocida*
482 recipients, and HI agar with spectinomycin to recover *P. multocida Himar1* mutants.

483 *P. multocida Himar1* transconjugants were recovered from initial selection plates after
484 overnight growth and replated on fresh HI agar containing spectinomycin to ensure selection
485 against mutants with insertions in essential genomic regions, and to increase culture density
486 for library storage. Following the second overnight growth, the *P. multocida Himar1* mutants
487 were recovered in glycerol broth (30% glycerol in HI broth) and stored at -80°C. A total of
488 ~1.12 x 10^6 M1404 *Himar1* mutants were pooled to generate the final M1404 mutant library.

489 **Mouse systemic infections**

490 Animal experiments were performed according to Victorian State Government regulations,
491 approved by the Monash University Animal Ethics Committee (Monash University AEC no.
492 MARP/2023/36738). Each *P. multocida* systemic infection was performed with an equal
493 number of male and female 6–10-week-old BALB/c mice (housed separately) supplied by
494 Monash Animal Research Platform (Monash University), with mice housed in the Animal
495 Research Laboratory (Monash University). *P. multocida* strains used for systemic infections
496 were grown in HI medium to early-exponential phase (OD_{600} ~0.25). Mice were each injected
497 intraperitoneally using a 27-gauge needle with a 200 µL total volume containing one of the
498 following: ~1 x 10^7 CFU of the *P. multocida* VP161 transposon mutant library, ~1 x 10^7 CFU
499 of the *P. multocida* strain M1404 transposon mutant library, ~5 x 10^4 CFU of wild-type strain
500 M1404, or ~1 x 10^5 CFU of one of the directed M1404 mutants (S1 Table in S1_Text). Mice
501 were monitored using a clinical score monitoring system for systemic infections in mice [68];
502 mice were monitored hourly and reached humane endpoints when they had a clinical score of
503 4 in a single category, or a cumulative clinical score of 10 or more. Mice were humanely
504 euthanised using CO_2 asphyxiation followed by cervical dislocation. Blood samples were
505 collected via cardiac puncture, and liver and spleen samples were recovered and
506 homogenized. Tissue and blood samples were plated at the appropriate dilutions onto HI agar
507 to obtain single colonies and determine the bacterial burden in the bloodstream or tissue. For
508 TraDIS analysis, to ensure there were enough cells for genomic DNA extraction, blood and
509 homogenized tissue samples were plated neat onto heart infusion agar and grown overnight

510 before bacteria were recovered. Samples of the VP161 and M1404 *Himar1* mutant libraries
511 used to inoculate the mice were also plated neat onto HI agar in the same way as the bacteria
512 recovered from mice. For DNA isolation, bacterial samples recovered from solid media using
513 1 x PBS were centrifuged at 13,000 x *g* then the cell pellet was resuspended 1 x PBS to wash
514 cells before being centrifuged again as above. The cell pellets generated were then used to
515 extract genomic DNA.

516 **Transposon-directed insertion site sequencing library production, Illumina sequencing**
517 **and bioinformatic analysis of TraDIS libraries**

518 TraDIS libraries were prepared for Illumina sequencing as previously described (29). TraDIS
519 libraries were sequenced by The Ramaciotti Centre for Genomics at the University of New
520 South Wales using an Illumina NextSeq1000 P1 with 100 bp-single end reads. TraDIS data
521 was analysed using the Bio-TraDIS toolkit and modified scripts, and replicates compared by
522 principal component analysis, as previously described [27]. Briefly, genes essential for growth
523 in rich media were identified by comparing normalized UIS per gene plotted on a histogram.
524 Genes important for fitness were identified by comparison of read count per gene between
525 each mouse TraDIS library and the input rich medium TraDIS library. Genes with $\log_2$ fold-
526 change < -2 and a *q*-value < 0.01 were called as having a fitness cost, and genes with a $\log_2$
527 fold-change > 2 and a *q*-value < 0.01 as having a fitness benefit. Full data sheets are provided
528 as Supplemental Material S1_Data, S2_Data, S3_Data and S4_Data.

529 To identify protein homologs between strains, all coding sequences from *P. multocida* strains
530 VP161 and M1404 were analysed using eggNOG mapper 5.0 [69] and then matched by seed
531 ortholog value; proteins with the same seed ortholog value were taken as homologs. Proteins
532 without a match in eggNOG and non-coding sequences were compared using the Basic Local
533 Alignment Search Tool (BLAST) [70], with homologs called when there was >90% amino
534 acid/nucleotide identity over >90% of the query. Venn diagrams were generated using
535 https://eulerr.co/. Heatmaps were generated in R using the ggplot2 package [71]. String was
536 used to identify genes involved in the same pathway or processes, and to identify pathways

537 and processes enriched in each dataset [72]. Roary analysis was performed using the VP161
538 and M1404 genomes, and an additional 167 complete *P. multocida* genomes obtained from
539 the BV-BRC database (in April 2024). Pan-genome analysis and maximum-likelihood core-
540 genome phylogenies were generated using Roary and IQ-TREE as previously described [7].

541 **Capsule absorbance assays**

542 Capsule absorbance assays were performed as previously described [7]. For assessing
543 capsule production in strain VP161 and derivative strains, cells were cultured in liquid broth
544 and samples taken to assess capsule production at early stationary phase (7 h, OD_{600} ~2.2).
545 For strain VP161, the amount of hyaluronic acid was determined against a standard curve of
546 absorbance values obtained using different amounts of purified hyaluronic acid (Sigma
547 catalogue number H5388). For strain M1404 and derivative strains, cells were cultured
548 overnight on heart infusion agar and then recovered by adding 1 x PBS to the plate and
549 scraping. As purified polysaccharide equivalent to the *P. multocida* type B capsule is not
550 commercially available to use as a standard, the absorbance values were normalised against
551 the optical density values of the culture used for each capsule extraction. Statistical
552 comparisons between strains were performed using a Mann-Whitney U-test.

553 **Data availability**

554 The M1404 genome was submitted to Genbank with the biosample accession number
555 SAMN49532731. The TraDIS data analysis output files have been uploaded to the NCBI Gene
556 Expression Omnibus and are available under accession number GSE300598.

557 **Supporting information**

558 S1_Text: Additional materials and methods and supporting tables, figures and figure legends.
559 S2_Text: Newick format maximum-likelihood core-genome phylogeny of 169 *P. multocida*
560 strains.
561 S3_Text: Annotated assembly of *P. multocida* strain M1404 used for TraDIS analysis in this
562 study.
563 S1Fig: S1 Figure
564 S2Fig: S2 Figure

565 S3Fig: S3 Figure

566 S4Fig: S4 Figure

567 S5Fig: S5 Figure

568 S6Fig: S6 Figure

569 S7Fig: S7 Figure

570 S8Fig: S8 Figure

571 S9Fig: S9Figure

572 S1_Data: All TraDIS data from this study.

573 S2_Data: TraDIS data for genes identified as essential for *P. multocida* strains VP161 and
574 M1404 growth in rich media.

575 S3_Data: TraDIS data for genes identified as having an *in vivo* fitness-cost.

576 S4_Data: TraDIS data for genes identified as having an *in vivo* fitness-benefit.

577

578 **Acknowledgements**

579 We would like to acknowledge the Monash Bioinformatics Platform for assistance with
580 bioinformatic analysis. We would like to acknowledge Abbey Allgood and Irene Alevizos for
581 assistance provided while plating bacteria during the mouse infections.

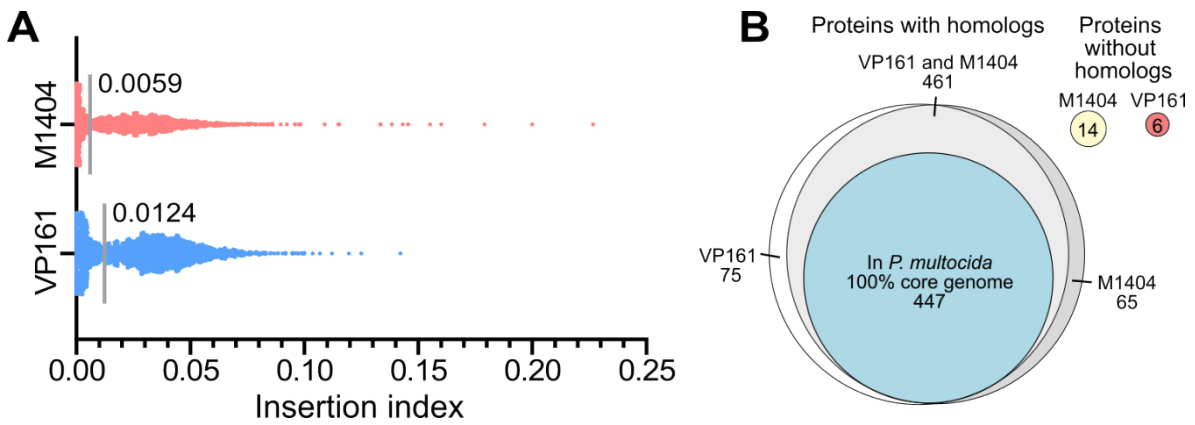
582 **Tables**

583 **Table 1.** Number of genes identified in *P. multocida* strain VP161 or M1404 that when
584 disrupted by a *Himar1* transposon insertion resulted in decreased or increased *in vivo* fitness.

	VP161				M1404			
	Blood	Liver	Spleen	Total	Blood	Liver	Spleen	Total
Decreased fitness	59	57	50	63	85	75	74	94
Increased fitness	4	6	6	7	6	5	4	7

585

586 **Figures**



587

588 **Fig 1.** Identification and comparison of genes essential for *P. multocida* strains VP161 and
589 M1404 to grow in rich media. Both the VP161 and M1404 *Himar1* mutant libraries were grown
590 to mid-exponential phase in heart infusion (HI) broth, before being plated on HI agar. Viable
591 mutants were recovered, used to generate TraDIS libraries that were sequenced by Illumina
592 sequencing, and the data analysed using Bio-TraDIS and related scripts. **A.** Insertion indexes
593 (number of unique *Himar1* transposon insertion sites divided by gene length) for all genes
594 following growth in HI broth. The cut-off for essentiality is marked by a grey line for each
595 dataset. **B.** Comparison of essential protein encoding genes present in both strain VP161 and
596 M1404, or only one of the strains. Protein homologs between strains were identified using
597 eggNOG matches, or by having > 90% amino acid identity. A 100% *P. multocida* core-genome
598 was identified in strains VP161 and M1404 along with 167 complete, publicly available
599 *P. multocida* genomes using Roary, and compared using a > 90% amino acid identity cut-off
600 to the essential proteins identified in both strains VP161 and M1404.

601

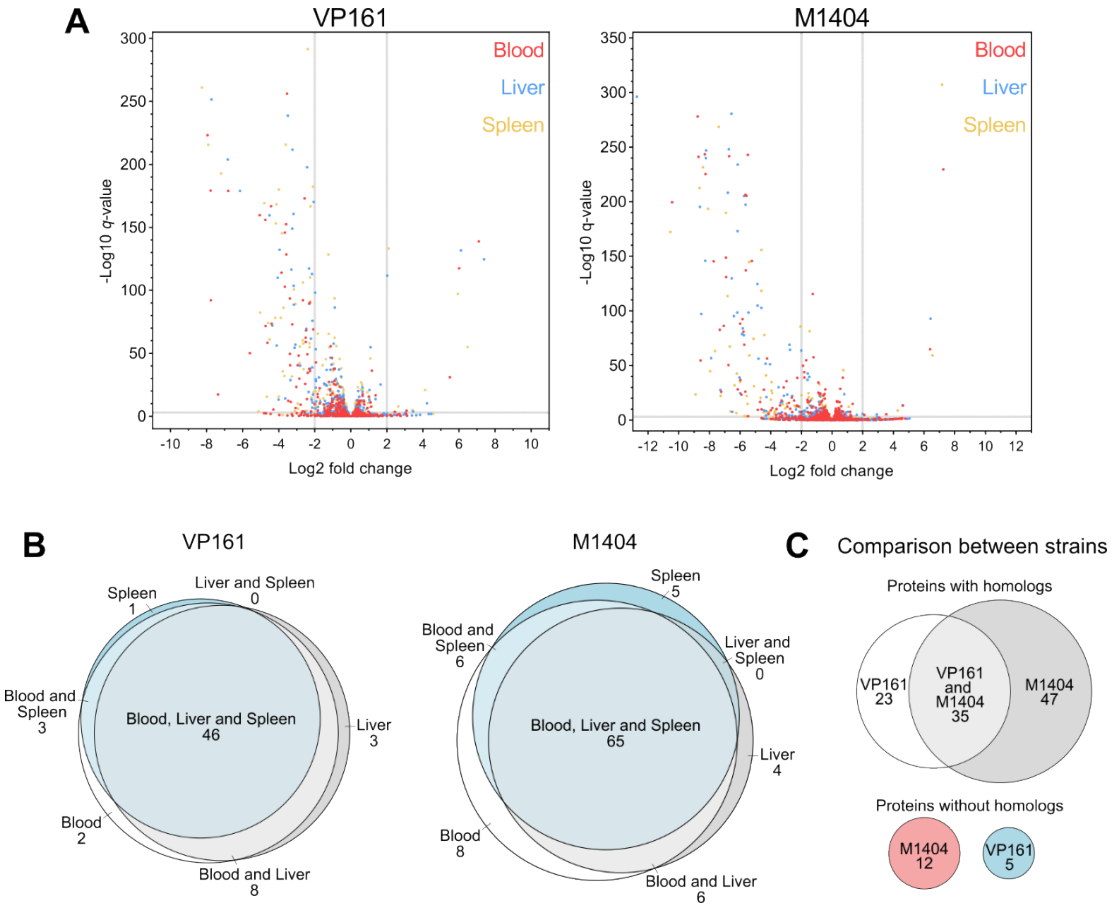


Fig 2. Identification and comparison of genes associated with *in vivo* fitness for *P. multocida* strain VP161 and M1404 during systemic infections in mice. The *P. multocida* strain VP161 and M1404 *Himar1* libraries were individually used in systemic infections in BALB/c mice. Surviving mutants were recovered from the bloodstream, liver and spleen, and then plated on heart infusion agar. Genomic DNA was extracted from surviving mutants and used to generate TraDIS libraries. TraDIS libraries were sequenced via Illumina sequencing and the data analysed using Bio-TraDIS and related scripts. **A.** Volcano plots showing the $\log_2$ fold-change in read count per gene and $-\log_{10}$ *q*-value for all genes in the bloodstream (red), liver (blue), and spleen (gold) TraDIS libraries compared to the rich media TraDIS library. Genes were identified as *in vivo* fitness-associated if they had a four-fold increase or decrease in read count, with a *q*-value of < 0.01 (cut-offs shown as grey lines). **B.** Venn diagrams showing the number of genes identified as important for *in vivo* fitness that were shared between the bloodstream, liver, and spleen TraDIS libraries. **C.** Comparison of *in vivo* fitness genes between strain VP161 and M1404. Protein homologs between strains were identified using matching eggNOG IDs, or by having > 90% amino acid identity and > 90% coverage.

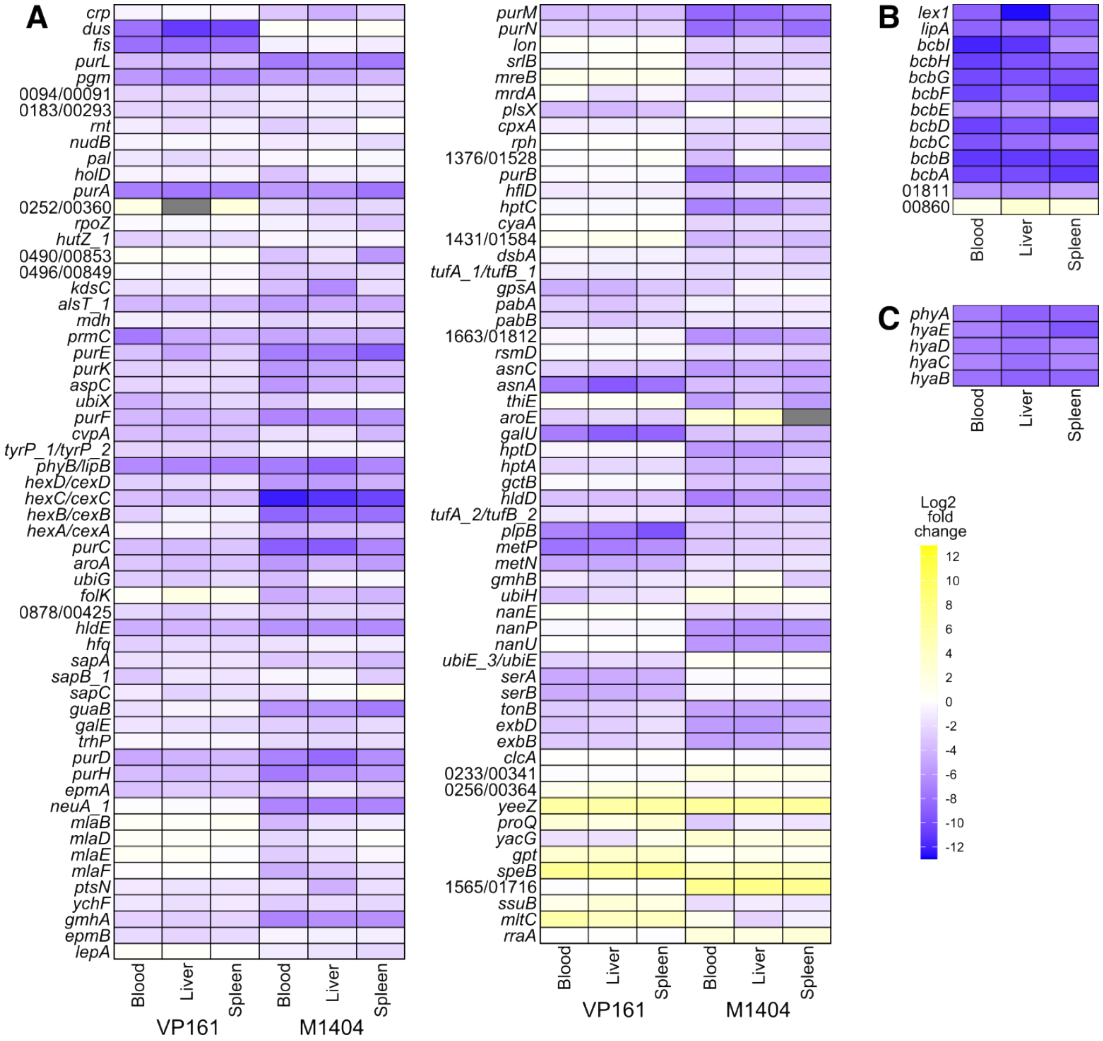


Fig 3. Heat maps showing the $\log_2$ fold-change in reads for *in vivo* fitness-associated genes. The $\log_2$ fold-change in reads per gene was identified using the fitness.comparison R-script, using the rich media TraDIS data as the control, and each of the bloodstream, liver, and spleen TraDIS libraries as the condition. Genes were included in the heatmap if they were identified as *in vivo* fitness-associated in any one of the TraDIS libraries. The gene *clcA*, which is known not to impact *in vivo* fitness [61], was included as a control. **A.** *In vivo* fitness-associated genes that are present in both strains. For genes that are annotated with different names or are identified only by different locus tags, both names or locus tags are given, with the VP161 name or locus tag first followed by the M1404 name or locus tag. For the locus tags, the prefix (PmVP161_ or M1404_) has been omitted. **B.** *In vivo* fitness-associated genes in M1404 with no homolog in VP161. **C.** *In vivo* fitness-associated genes in VP161 with no homolog in M1404.

632

633 **Fig 4.** Kaplan-Meier survival curves for mice infected with *P. multocida* wild-type M1404 or an

634 M1404 directed mutant. Groups of six BALB/c mice (three male and three female) were

635 inoculated via intraperitoneal injection with wild-type strain M1404, the M1404 *alsT_1* mutant,

636 the M1404 *crp* mutant, or the M1404 *cyaA* mutant (colony forming units ranged between 2 x

637 10^4 and 2 x 10^5). Statistical differences in survival between strains was determined using a

638 log-rank Mantel-Cox test; *** $P < 0.001$.

639

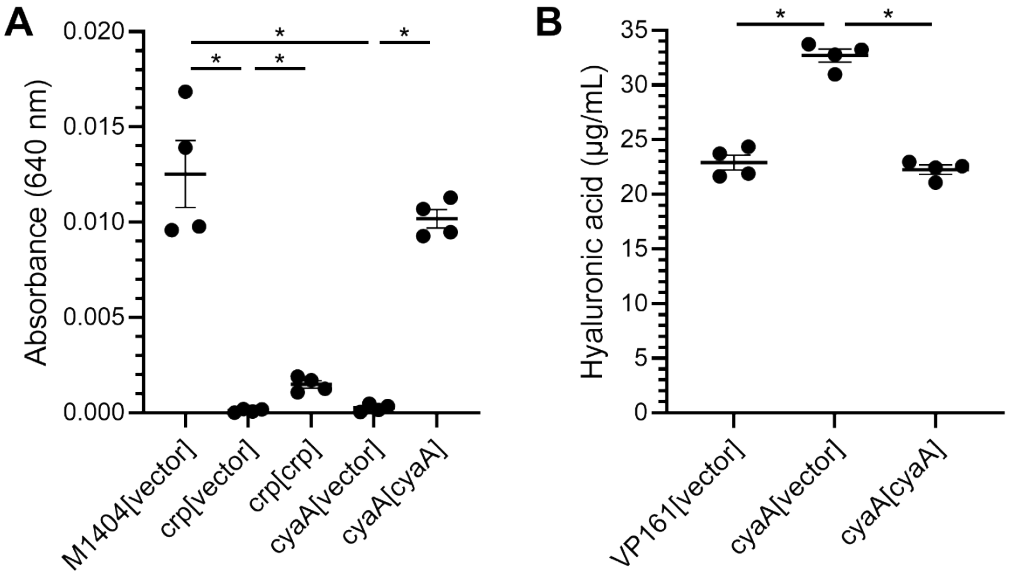


Fig 5. Capsule produced by VP161 and M1404 wild-type and mutant strains. Capsule absorbance assays were performed by mixing capsule extracted from cells with stains-all solution and measuring the absorbance at 640 nm. **A.** Capsule measured from wild-type M1404 harbouring empty vector, the M1404 *crp* mutant and M1404 *cyaA* mutant harbouring either empty vector or the appropriate complementation plasmid. Capsule was extracted from cells recovered from overnight growth on heart infusion agar. **B.** Capsule measured from wild-type VP161 harbouring empty vector, and the VP161 *cyaA* mutant harbouring empty vector or an expression plasmid containing an intact copy of the VP161 *cyaA* gene. Capsule was extracted from cells grown in heart infusion broth to early stationary phase (7 h). Error bars represent mean ± standard error of the mean, and statistical differences in capsule production between samples were determined using Mann-Whitney U-tests; * $P < 0.05$.

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