1   Rationally designed *mariner* vectors to allow functional genomic analysis of

2   *Actinobacillus pleuropneumoniae* and other bacteria by transposon-directed insertion-

3   site sequencing (TraDIS)

4

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23

24   Running head: *mariner* mutagenesis of *A. pleuropneumoniae* and *P. multocida*

25

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29   **Abstract**

30

31   Transposon Directed Insertion Sequencing (TraDIS) is a high-throughput

32   method for mapping insertion sites in large libraries of transposon mutants. The

33   *Himar1* (*mariner*) transposon is ideal for generating near-saturating mutant

34   libraries, especially in AT-rich chromosomes, as the requirement for integration is

35   a TA dinucleotide. In this study, we generated two novel *mariner* vectors,

36   pTsodCPC9 and pTlacPC9 (differing only in the promoter driving expression of the

37   transposase gene), in order to facilitate TraDIS identification of conditionally

38   essential genes in *Actinobacillus pleuropneumoniae* and other bacteria. Using the

39   pTlacPC9 vector, we have generated, for the first time, saturating *mariner* mutant

40   libraries in both *A. pleuropneumoniae* and *Pasteurella multocida* that showed a

41   near random distribution of insertions around the respective chromosomes. A

42   preliminary screen of 5000 mutants each identified 8 and 15 genes, respectively,

43   that are required for growth under anaerobic conditions.

44

45   **Importance**

46

47   Comprehensive identification of conditionally essential genes requires

48   efficient tools for generating high-density transposon libraries that, ideally, can be

49   analysed using next-generation sequencing methods. The *mariner* transposon

50   has been used for mutagenesis of a wide variety of bacteria, however plasmids for

51   delivery of this transposon do not necessarily work well in all bacteria. In

52   particular, there are limited tools for functional genomic analysis of

53   *Pasteurellaceae* species of major veterinary importance, such as swine and cattle

54   pathogens, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida*,

55   respectively. Here, we have developed plasmids that allow delivery of *mariner* and

56   the production of genome saturated mutant libraries for both of these pathogens,

57   but which should also be applicable to a wider range of bacteria. High-throughput

58   screening of the generated libraries will identify mutants required for growth under

59   different conditions, including *in vivo*, highlighting key virulence factors and

60   pathways that can be exploited for development of novel therapeutics and

61   vaccines.

62

63   **Introduction**

64

65   *Actinobacillus pleuropneumoniae*, a member of the *Pasteurellaceae*, is the

66   causative agent of porcine pleuropneumonia, a highly contagious, often fatal,

67   respiratory disease that causes considerable economic losses to the swine

68   industry worldwide (1). Certain virulence factors have been shown to have specific

69   roles in the pathogenesis of *A. pleuropneumoniae* infection including RTX toxins,

70   capsule, lipopolysaccharide and various outer membrane proteins (2, 3). In

71   addition, two signature-tagged mutagenesis (STM) studies have identified a large

72   number of genes that contribute to the ability of *A. pleuropneumoniae* to survive

73   and cause disease in pigs, though neither screen was saturating (4, 5).

74   For more than two decades, Tn*10* has been the transposon of choice for

75   generating libraries of random mutants of *A. pleuropneumoniae* for STM and other

76   studies (4-9). However, Tn*10* has an insertion site preference for GCTNAGC (10),

77   and different insertional hotspots were reported in both *A. pleuropneumoniae* STM

78   studies (4, 5, 11), limiting the usefulness of this transposon for creating a fully

79   saturating library. Clearly, a more random transposon mutagenesis system in *A.*

80   *pleuropneumonia*e is required in order to allow genome-wide analysis of fitness

81   using high-throughput sequencing methods such as Transposon Directed

82   Insertion-site Sequencing (TraDIS) that not only precisely map, but also

83   quantitatively measure the relative abundance of each transposon insertion in a

84   pool of mutants (12-14).

85   The *Himar1* (*mariner*) transposon, originally isolated from the horn fly,

86   *Haematobia irritans,* has been shown to insert randomly into the chromosomes of

87   a wide range of bacteria with a dinucleotide target of "TA" (15). In particular,

88   *mariner* has been used for mutagenesis of *Haemophilus influenza* (12, 16)*,*

89   another member of the *Pasteurellaceae,* with a comparatively AT-rich genome, like

90   *A. pleuropneumoniae*.

91   The aim of this study was to develop a *Himar1 mariner* mutagenesis system

92   for use in *A. pleuropneumoniae*, and other bacteria such as *Pasteurella multocida*,

93   that would be amenable to TraDIS (14).

94

95   **Results**

96

97   **Construction and evaluation of *mariner* vectors**

98

99   Two novel mobilizable *mariner* delivery vectors, pTsodCPC9 and pTlacPC9

100 (Figure 1), differing only in the promoter driving expression of the C9 transposase

101 gene, were successfully generated using pGEM-T as the vector backbone.

102 Conjugal transfer into *A. pleuropneumoniae* MIDG2331 from the DAP-dependent

103 MFD*pir*, achieved for both vectors, confirmed that the *oriT* and *traJ* sequences

104 were sufficient to facilitate mobilization. Comparison of results for *A.*

105 *pleuropneumoniae* indicated that although the *sodC* promoter is constitutively

106 expressed (17), IPTG induction of expression of the C9 transposase from the *lac*

107 promoter resulted in higher frequencies of transposition (10^{-6}-10^{-8} compared to 10^{-7}-10^{-10}).

108 ^{7}-10^{-10}). Furthermore, similar frequencies of transposition were found in the

109 genome of *P. multocida* MIDG3277 (10^{-6}-10^{-8}) using pTlacPC9, whereas no

110 transposants were recovered using pTsodCPC9 in this species. Therefore, the

111 pTlacPC9 vector was used for generation of libraries in both *A. pleuropneumoniae*

112 and *P. multocida*.

113   Colony PCR revealed that initial transconjugants retained

114 extrachromosomal plasmid, as indicated by amplification of both the

115 chloramphenicol (Cm) cassette and the *oriT*/*traJ* sequence (data not shown).

116 Following subculture on selective agar, only the Cm cassette could be amplified

117 from selected mutants, indicating loss of the plasmid and integration of the

118 transposon into the chromosome. Southern Blot (not shown) and linker-PCR

119 (Figure 2) confirmed single insertions in different locations in 12 randomly selected

120 mutants. Insertions were stable in the absence of selection for 20 generations.

121

122 **TraDIS analysis of the *A. pleuropneumoniae* and *P. multocida mariner***

123 **libraries**

124

125   Analysis of linker-PCR products generated from DNA (+/- ISceI digestion)

126 from the pooled *A. pleuropneumoniae mariner* library containing $\geq$ 43,000

127 transconjugants showed a dominant plasmid-specific band only in the sample that

128 was not treated with ISceI (Figure 3). Both samples showed a strongly stained

129 smear of DNA ranging from 100 to 500 bp in size, indicating a good distribution of

130 insertions in the library. Subsequent TraDIS analysis of the ISceI-digested *A.*

131 *pleuropneumoniae* library DNA generated 16,565,883 sequence reads from the 5'

132 end of the *mariner* transposon. Alignment of the reads against the closed

133 MIDG2331 genome (18) identified 56,664 unique insertion sites in total, with near

134 random distribution around the entire genome (Figure 4A).

135   For analysis of the *P. multocida mariner* library, it was first necessary to

136 generate a draft genome sequence for the isolate used, MIDG3277. Sequencing

137 on an Illumina HiSeq 2000 yielded 2285355 pairs of 100 bp sequence reads, which

138 were assembled into 160 contigs, which were ordered based on a comparison with

139 the genome of *P. multocida* PM70 (GenBank accession AE004439; Figure 5). The

140 assembly had a total size of 2,562,694 bp, with an n50 of 77,141 bp. Annotation of

141 the contigs identified 2490 coding sequences, together with 35 tRNA genes and 4

142 rRNA genes.

143   TraDIS analysis of the ISceI-digested *P. multocida* library DNA generated

144 46,809,272 sequence reads, from the 5' end of the *mariner* transposon. Alignment

145 of the reads with the draft genome sequence of MIDG3277 identified 147,613

146 unique insertion sites, with distribution around the entire genome (Figure 4B).

147

148 **Preliminary screen for anaerobic mutants**

149

150   Of approximately 5000 mutants each of *A. pleuropneumoniae* and *P.*

151 *multocida* (from 4 different matings in both cases) screened for the presence of

152 insertions in genes required for anaerobic growth, 19 and 15 mutants, respectively,

153 were identified that failed to grow. Linker-PCR produced amplicons of different

154 sizes that, when sequenced, mapped to unique TA dinucleotides in 8 different

155 genes in the *A. pleuropneumoniae* MIDG2331 genome (Table 2), and 15 different

156 sites (some intergenic) in the *P. multocida* MIDG3277 genome (Table 3).

157

158 **Discussion**

159

160   The *mariner* transposon does not require host-specific factors for

161 transposition and has a minimal requirement for TA dinucleotides as its target site,

162 allowing for greater distribution in genomes compared to transposons such as Tn*5*

163 and Tn*10*, which have "hot spots" for insertion. Tn*10* insertion at hotspots was a

164 severe limitation in our previous *A. pleuropneumoniae* signature tagged

165 mutagenesis study (4, 11). Given the relatively AT rich genomes of

166 *Pasteurellaceae* species, *mariner* should allow for creation of saturating mutant

167 libraries in *A. pleuropneumoniae* and *P. multocida*.

168   Preliminary (unpublished) investigations in our laboratory using the vector

169 pMinihimarRB1 (19), a kind gift from Dr D. Saffarini, revealed that *mariner* is

170 functional in *A. pleuropneumoniae*, however disadvantages including high

171 background with kanamycin selection and retention of plasmid in initial

172 transconjugants limited the usefulness of this vector for genome-wide analysis of

173 fitness using TraDIS, a next generation sequencing method for mapping insertion

174 sites (14). We therefore decided to construct a *mariner* delivery vector

175 incorporating the following desired components: a stringent selection gene carried

176 by the mini-transposon; presence of DNA uptake sequences flanking the selection

177 gene to allow for easy transfer of mutations between different strains via natural

178 transformation; the presence of paired ISceI restriction sites just outside of the

179 mini-transposon element to allow for elimination of plasmid reads during TraDIS;

180 sequences to allow mobilization of the vector from a conjugal donor strain; and the

181 C9 hyperactive mutant of the *Himar1 mariner* transposase gene (20), under control

182 of either a constitutive or inducible promoter, encoded adjacent to the mini-

183 transposon element to ensure stability of insertions.

184   Two novel mobilizable *mariner* vectors, pTsodCPC9 and pTlacPC9, were

185 developed in this study. Both contain *mariner* mini-transposon elements carry a

186 Cm resistance gene, known to provide stringent selection of mutants in *A.*

187 *pleuropneumoniae* (21), flanked by paired DNA uptake sequences for each of *A.*

188 *pleuropneumoniae*, *H. influenzae* and *Neisseria* spp. (22, 23). Although initially

189 designed for mutagenesis of *A. pleuropneumoniae*, all features included were

190 designed to facilitate use in other Gram-negative bacteria. As it is often desirable

191 to transfer mutations into wild-type strains to confirm the effect(s) of gene mutation,

192 DNA uptake sequences were designed into the transposon in order to facilitate

193 transfer of mutations by natural transformation in the species which selectively take

194 up DNA containing these elements (22, 23).

195   Restriction barriers can prevent or decrease the efficiency of plasmid

196 delivery by electroporation into various bacteria (24, 25), we therefore added

197 the *oriT* transfer origin and the *traJ* gene from the broad host-range conjugative

198 plasmid RP4 (26) to facilitate delivery of the vectors by conjugation. Successful

199 conjugal transfer of both vectors into MIDG2331 confirmed that these sequences

200 were sufficient to allow mobilization from the DAP-dependent *Escherichia coli*

201 strain MFD*pir* (27) which carries loci coding for the complete RP4 conjugation

202 machinery. It is likely that the presence of the *oriT* alone would have been sufficient

203 for mobilization, however, as binding of TraJ to the *oriT* initiates formation of the

204   transfer machinery (26), a copy of this gene was included in the vectors. Use of

205   MFD*pir* facilitates conjugation into wild-type recipient bacteria, instead of antibiotic

206   resistant derivatives, as selection of transconjugants is achieved on agar that does

207   not contain DAP which is required for growth of the donor strain (27).

208   The hyperactive C9 mutant transposase gene, previously shown to

209   enhance the efficiency of *mariner* transposition (20, 28) was placed under

210   transcriptional control of either the *A. pleuropneumoniae sodC* promoter, which we

211   have shown to be active under all conditions investigated so far, both in *A.*

212   *pleuropneumoniae* as well as in other *Pasteurellaceae* species (17), or the *E. coli*

213   *lac* promoter which allows induction by IPTG, and which is functional in a variety

214   of bacteria including *A. pleuropneumoniae* (4). Although both resulting plasmids,

215   pTsodCPC9 and pTlacPC9, were functional in *A. pleuropneumoniae*, the former

216   generated a lower frequency of transposants in this species, and only the latter

217   generated transposants in *P. multocida*. Furthermore, IPTG induction of

218   expression of the C9 transposase gene from the *lac* promoter resulted similar

219   frequencies of transposition in both *A. pleuropneumoniae* and *P. multocida*,

220   indicating it will likely be a useful genetic tool for other bacteria.

221   Using the pTlacPC9 vector, saturating libraries were generated for both *A.*

222   *pleuropneumoniae* (insertions on average every 41 bp) and *P. multocida*

223   (insertions on average every 17 bp), with insertions randomly distributed around

224   the respective chromosomes. In both cases, pooled mutant libraries were prepared

225   from initial selective plate cultures of the transconjugants (from multiple mating

226   experiments) in order to limit selective expansion of clones. Under these

227   conditions, extrachromosomal plasmid retained in the library can lead to high

228   numbers of sequencing reads, interfering with TraDIS mapping. By engineering

229   paired ISceI restriction sites (18 bp sequences not usually found in bacterial

230   genomes) flanking the mini-transposon element in our vectors, we were able to

231   effectively eliminate retained plasmid from the libraries prior to sequencing.

232   Both *A. pleuropneumoniae* and *P. multocida* are members of the

233   *Pasteurellaceae* and are facultative anaerobes that infect the respiratory tracts of

234   animals. Anaerobic growth is known to contribute to virulence of *A.*

235   *pleuropneumoniae* (29). However, little is known about the importance of genes

236   contributing to anaerobic growth in *P. multocida*. In this study, we have screened

237   a limited number of *mariner* mutants of both pathogens to identify genes important

238   for anaerobic growth in order to further validate the randomness of insertions in

239   these libraries and usefulness of our approach. Of the 19 *A. pleuropneumoniae*

240   mutants sequenced, 15 unique insertions in 8 different genes were identified

241   (Table 2). Of 15 *P. multocida* mutants sequenced, all were in unique sites in the

242   genome, with some insertions mapped to intergenic sites (Table 3). In both cases,

243   mutants were selected from only four separate mating experiments, so it is

244   possible that the four duplicate insertions mapped for the *A. pleuropneumoniae*

245   mutants were due to clonal expansion following insertion, even though mutants

246   were randomly selected from the primary counter selection plate. As clonal

247   expansion can skew libraries for over representation of more rapidly growing

248   mutants, steps should be taken to limit this prior to TraDIS analysis.

249   For both organisms, genes of the *atp* operon encoding the F_0F_1 ATP

250   synthase appear to be essential for anaerobic growth. In *A. pleuropneumoniae*, 13

251   mutants had disruptions in four genes of the *atp* operon (nine unique sites). In *P.*

252   *multocida*, two of the anaerobic mutants had insertions that mapped to sites

253   upstream of genes in the *atp* operon (56 bp upstream of *atpI*, the first gene in the

254   operon; and 24 bp upstream of *atpG*). These intergenic insertions likely disrupt

255   transcription of all, or part, of the *atp* operon, and therefore production of functional

256   F_0F_1 ATP synthase. STM studies have previously identified genes of this operon

257   as important for *in vivo* survival of both *A. pleuropneumoniae* (4, 5), and of *P.*

258   *multocida* (30), but the F_0F_1 ATP synthase has not previously been reported as

259   essential for anaerobic growth of these bacteria. The number of unique *mariner*

260   insertions mapping to the *atp* operons of these species in our current study

261   provides strong evidence for the requirement of a functional F_0F_1 ATP synthase for

262   their anaerobic growth. In the absence of oxidative phosphorylation, the F_0F_1

263   enzyme complex extrudes protons at the expense of ATP hydrolysis, to generate

264   the driving force for solute transport and to maintain an acceptable intracellular pH

265   value, and is required for function of certain enzymes involved in anaerobic

266   respiration, such as formate hydrogenlyase (FHL) (31). Furthermore, activity of

267   both FHL and F_0F_1-ATPase was found to facilitate the fermentative metabolism of

268   glycerol in *E. coli* (32).

269   An insertion in *nhaB*, encoding a Na^+/H^+ antiporter, was also found to be

270   important for anaerobic growth of *A. pleuropneumoniae* in our current screen. As

271   with the *atp* operon, *nhaB* has been shown to be important for *in vivo* growth of *A.*

272   *pleuropneumoniae* (33). The importance of interactions between proton and

273   sodium cycles during both aerobic and anaerobic growth has been reported for

274   certain pathogenic bacteria (34). The relative contributions of proton and sodium

275   cycles to anaerobic growth of *A. pleuropneumoniae* and *P. multocida* warrant

276   further investigation.

277   The importance of menaquinone biosynthesis for anaerobic growth of *P.*

278   *multocida* was indicated by single insertions in each of *menA*, *menB,* and *menC*,

279   as well as a single insertion three bp upstream of *aroG* (likely disrupting

280   transcription), and two in *aroA*. Menaquinone is involved in anaerobic electron

281   transport, and is derived from chorismate (pathway includes *menA*, *menB*, and

282   *menC*), which in turn is derived from shikamate (pathway includes *aroA* and *aroG*).

283   Two *A. pleuropneumoniae* (*pflB* and *fumC*) genes identified encode proteins

284   with known roles in anaerobic pathways. There were three separate insertions in

285   *pflB* encoding pyruvate formate lyase which catalyses generation of formate via

286   decarboxylation of puruvate. As mentioned above, the FHL complex is important

287   in anaerobic respiration where it catalyses the conversion of formate into CO_2 and

288   H_2 (35). A single insertion was mapped to *fumC* encoding fumarate hydratase, the

289   enzyme responsible for catalysing the conversion of malate to fumarate. Fumarate

290   has previously been shown to be an essential terminal electron acceptor during

291   anaerobic respiration in *A. pleuropneumoniae* (29, 36).

292   Two of the *P. multocida* (*ccmD* and *sucC*) genes identified also have known

293   anaerobic functions. Genes of the *ccm* operon are required for maturation of c-

294   type cytochromes, including those involved in the electron transfer to terminal

295   reductases of the anaerobic respiratory chain with nitrate, nitrite or TMAO

296   (trimethylamine-N-oxide) as electron acceptors (37). The *sucC* gene encodes the

297   beta subunit of succinate-CoA ligase, an enzyme in the aerobic citrate (TCA) cycle,

298   where it catalyses hydrolysis of succinyl-CoA to succinate (coupled to the

299   synthesis of either GTP or ATP). This enzyme also mediates the reverse reaction

300   when required for anabolic metabolism, which can be particularly important under

301   anaerobic conditions where the generation of succinyl-CoA via the oxidative

302   pathway from 2-oxoglutarate is repressed (38, 39).

303   The remaining mutants in both *A. pleuropneumoniae* and *P. multocida* have

304   insertions in (or upstream of) genes not directly linked with anaerobic growth,

305   however further investigation is warranted to determine their possible

306   contributions.

307   It is clear from our results that we have successfully constructed a *mariner*

308   mini-transposon delivery vector capable of generating extremely large numbers of

309   random mutants in *A. pleuropneumoniae*, *P. multocida*, and likely in other Gram-

310   negative bacteria, which is amenable to genome-wide analysis of fitness using

311   TraDIS. In preliminary experiments we have established that the pTlacPC9 vector

312   can be used in *Aggregatibacter actinomycetemcomitans* and *Mannheimia*

313   *haemolytica*, though it remains to be determined if the generated libraries are

314   saturating.

315   The limited number of anaerobic-essential genes, identified individually via

316   phenotypic analysis in the current study, mapped to different insertion sites,

317   including some which had not previously been associated with anaerobic growth

318   of *A. pleuropneumoniae* and *P. multocida.* This suggests that TraDIS analysis of

319   the pooled *mariner* libraries subjected to the same screen will identify many more

320   genes with functions contributing to anaerobic fitness. In future work, we will screen

321   our *mariner* libraries under different *in vitro* and *in vivo* growth conditions,

322   broadening our understanding of conditionally essential genes in the

323   *Pasteurellaceae*.

324   **Materials and Methods**

325

326   **Bacterial strains and culture**

327

328   For generation of mariner transposon libraries, we chose two different

329 *Pasteurellaceae* species, *A. pleuropneumoniae* and *P. multocida*. The *A.*

330 *pleuropneumoniae* clinical serovar 8 isolate used, MIDG2331, was previously

331 shown to be genetically tractable and has been fully sequenced (18). The *P.*

332 *multocida* isolate was recovered from the respiratory tract of a calf in Scotland in

333 2008, and was shown to be sequence type 13, and part of clonal complex 13, in a

334 multi-species multilocus sequence typing (MLST) study (40). This isolate, which

335 has been labeled MIDG3277 in our collection, can be found in the pubMLST

336 database (https://pubmlst.org/bigsdb?db=pubmlst_pmultocida_isolates) under the

337 isolate name 22/4. The *Pasteurellaceae* isolates were routinely propagated at

338 37°C with 5% CO_2 on Brain Heart Infusion (Difco) plates supplemented with 5%

339 horse serum and 0.01% NAD (BHI-S-NAD). The *E. coli* strains used were: Stellar

340 [*F⁻, ara,Δ(lac-proAB) [Φ 80d lacZΔM15], rpsL(str), thi, Δ(mrr-hsdRMS-mcrBC),*

341 *ΔmcrA, dam, dcm*]; and MFD*pir* [MG1655 RP4-2-Tc::[*ΔMu1::aac*(3)*IV-ΔaphA-*

342 *Δnic35-ΔMu2::zeo] ΔdapA::(erm-pir) ΔrecA*] (27). *E. coli* strains were maintained

343 in Luria-Bertani (LB). Where appropriate, ampicillin (Amp; 100 µg/ml),

344 chloramphenicol (Cm; 20 and 1 µg/ml for *E. coli* and *A. pleuropneumoniae*,

345 respectively), and 0.3 mM diaminopimelic acid (DAP: required for growth of the

346 MFD*pir* strain), were added to media.

347

348

349 **Genome sequencing, assembly and annotation**

350

351   To obtain a draft genome sequence suitable for TraDIS analysis, genomic

352 DNA was extracted from the *P. multocida* MIDG3277 strain using the FastDNA

353 Spin kit (MP Biomedicals), and sequenced using an Illumina HiSeq 2000 at the

354 Wellcome Sanger Institute. Illumina adapter sequences were trimmed from the

355 reads using Cutadapt (41), and the trimmed reads were assembled using SPAdes

356 (42). The assembled contigs were aligned to the complete genome sequence of

357 *P. multocida* Pm70 (GenBank accession AE004439) using nucmer version 4.0.0

358 (43). Using the alignment, the contigs were reordered and reoriented to match the

359 Pm70 reference genome, with one contig manually split where it overlapped the

360 Pm70 origin. The reordered contigs were annotated using Prokka version 1.11

361 (44).

362

363 **Construction of the *mariner* mini-transposon vectors**

364

365   All primers are listed in Table 1. CloneAmp HiFi PCR Premix (Takara) was

366 used to amplify sequences for cloning, and the QIAGEN Fast Cycling PCR Kit

367 (Qiagen) was used for verification of clones, using the respective manufacturer's

368 protocols. When required, blunt PCR products were A-Tailed using 5 U Taq

369 polymerase and 0.2mM dATP prior to TA cloning into pGEM-T (Promega),

370 according to the manufacturer's protocol. Also, when required, DpnI digestion was

371 used to remove plasmid DNA template from PCR products prior to cloning. All

372 ligation products and In Fusion cloning products were transformed into *E. coli*

373 Stellar cells (Clontech) by heat shock, according to the manufacturer's protocol.

374   A *mariner* mini-transposon encoding Cm resistance was constructed in

375 stages using pGEM-T as the vector backbone. Initially, the Cm cassette flanked

376 by *A. pleuropneumoniae* uptake signal sequences (USS) was amplified from

377 pUSScat (21) using primers CmDUSUSS_for and CmDUSUSS_rev, which further

378   added DNA uptake sequences for *Neisseria* spp*.* and *H. influenzae* on both sides

379   of the cassette. The resulting 956 bp amplicon was A-tailed and cloned into pGEM-

380   T yielding the plasmid pTCmDUSUSS. The *mariner* inverted repeat (IR) sequence

381   (TAACAGGTTGGCTGATAAGTCCCCGGTCT) was then added to either side of

382   the CmDUSUSS cassette in two subsequent rounds of PCR amplification and

383   cloning into pGEM-T. In the first round, primers CmDUSUSS_IRleft and

384   CmDUSUSS_IRright added the last 19 bases of the *mariner* inverted repeat to

385   either side of the cassette. The full IR sequence was then used as a primer

386   (Himar_IR) to amplify the transposon cassette prior to cloning into pGEM-T to yield

387   pTHimarCm. Finally, paired I-SceI restriction sites were added to either side of the

388   transposon by PCR using primers ISceI_left and ISceI_right, and the product was

389   cloned into pGEM-T to yield pTISceHimarCm. The full insert was sequenced using

390   M13_for and M13_rev primers prior to further modifications of the plasmid.

391   All further cloning steps were performed using the In Fusion HD cloning kit

392   (Clontech), according to the manufacturer's protocol. A sequence containing the

393   *oriT* and *traJ* gene was amplified from pBMK1 (45), a generous gift from Gerald

394   Gerlach, using primers oriTtraJ_left and oriTtraJ_right, and cloned into NsiI/SalI

395   cut pTISceHimarCm to yield pTISceHimarCmoriT. The C9 hyperactive *Himar1*

396   transposase gene (20), amplified from pCAM45 (46) using primers C9_right and

397   either C9sodC_left or C9lacP_left, was fused by overlap-extension (OE-PCR) PCR

398   to either the *A. pleuropneumoniae sodC* promoter amplified from pMK-Express

399   (17) using primers sodCPC9_left and sodCPC9_right, or to the *lac* promoter

400   amplified from pBluescript II KS (Agilent Technologies) using primers lacPC9_left

401   and lacPC9_right. The *sodCP*-C9 and *lacP*-C9 OE-PCR products were cloned into

402   ZraI cut pTISceHimarCmoriT to yield the *Himar1 mariner* mini-transposon delivery

403 vectors pTsodCPC9 and pTlacPC9, respectively. All inserts were confirmed by

404 sequencing.

405   Purified pTsodCPC9 and pTlacPC9 plasmids were electroporated into the

406 *E. coli* conjugal donor strain MFD*pir* (27), a generous gift from Jean-Marc Ghigo,

407 with transformants recovered on LB containing 20 µg/ml Cm and 0.3 mM DAP.

408

409 **Bacterial mating and generation of *mariner* mutant libraries in *A.***

410 ***pleuropneumoniae* and *P. multocida***

411

412   Initially, the two different *mariner* mini-transposon delivery vectors,

413 pTsodCPC9 and pTlacPC9, were evaluated for their ability to produce Cm-

414 resistant mutants in *A. pleuropneumoniae* serovar 8 strain MIDG2331 following

415 conjugal transfer from the DAP-dependent *E. coli* MFD*pir* donor strain. For mating

416 experiments, donor and recipient bacteria were grown separately in broth culture

417 to an optical density at 600 nm (OD_{600}) of approximately 1.0 (cultures were

418 adjusted to equivalent OD_{600}). Two hundred microliters of recipient strain were

419 mixed with 0 to 200 µl of donor strain (to give ratios of 1:1, 1:2, 1:4 and 1:8, as well

420 as a control of recipient only). The bacteria were pelleted and re-suspended in 200

421 µl of 10 mM $MgSO_4$, and 20 µl aliquots were spotted onto 0.45-µm nitrocellulose

422 filters (Millipore) placed onto BHI-S-NAD agar supplemented with DAP and, when

423 required for induction of the *lac* promoter, 1 mM IPTG (isopropyl-ß-D-

424 galactopyranoside). Plates were incubated overnight at 37°C with 5% CO_2, after

425 which bacteria were recovered in 1 ml of sterile phosphate-buffered saline and

426 aliquots were plated onto BHI-S-NAD agar supplemented with 1 µg/ml Cm.

427 Selected transconjugants were tested, both before and after subculture on

428   selective agar, by colony PCR for the presence of the Cm cassette using primers

429   CmDUSUSS_for and CmDUSUSS_rev, and for presence of the plasmid backbone

430   using primers oriTtraJ_left and oriTtraJ_right.

431   For construction of the *A. pleuropneumoniae mariner* library, a total of 14

432   matings were performed using the MFD*pir* + pTlacPC9 donor, with between 1400

433   and 6200 MIDG2331 transconjugants per mating resuspended in 3 ml BHI broth

434   containing 50% (v/v) glycerol and stored in 1 ml aliquots at -80°C. A combined

435   pooled library of transconjugants (56,664 insertion sites) was generated by mixing

436   equal aliquots of each of the individual mutant pools. Southern blot and linker-PCR

437   using AluI-digested DNA were performed as previously described (24, 47) in order

438   to confirm single random insertion of the transposon in selected transconjugants.

439   The pTlacPC9 vector was further assessed for the ability to generate Cm-

440   resistant mutants in the related bacterium, *P. multocida.* In total, 9 separate

441   matings were performed using the MFD*pir* + pTlacPC9 donor and *P. multocida*

442   MIDG3277 recipient, with between 2388 and 20,640 transconjugants selected per

443   mating. Mutants were stored at -80°C as separate pools, and a combined pool

444   (147,613 insertion sites), in 1 ml aliquots in BHI broth containing 50% (v/v) glycerol,

445   as above.

446

447   **TraDIS analysis of the *A. pleuropneumoniae* and *P. multocida mariner***

448   **libraries**

449

450   Genomic DNA was extracted from the pooled libraries of mutants using the

451   FastDNA Spin kit (MP Biomedicals), according to the manufacturer's protocol for

452   bacterial cells. To assess the distribution of insertions in the pools prior to TraDIS,

453 linker-PCR was performed as previously described (24) with the exception that, for

454 PCR amplification, primer L-PCR-C was paired with either IR_left_out or

455 IR_right_out (see Table 1) in place of L-PCR-L or L-PCR-R, respectively. For

456 comparison, 2.5 µg aliquots of genomic DNA that were either untreated, or

457 digested with ISceI to remove reads from residual plasmid, were used for linker

458 PCR amplification of the left flank sequences, and the products were separated on

459 2% Nusieve agarose. Subsequently, 2 µg of ISceI-digested DNA was used to

460 prepare Illumina libraries for TraDIS analysis, as previously described (25).

461 The TraDIS libraries were sequenced on an Illumina HiSeq 2500 at the

462 Wellcome Sanger Institute. For the *A. pleuropneumoniae* library, TraDIS reads

463 were mapped to the closed whole genome sequence of MIDG2331 (accession

464 number LN908249). The *P. multocida* TraDIS reads were mapped to the draft

465 genome of MIDG3277, constructed as described above. Reads were mapped

466 using BWA mem (48), with an increased penalty for 5' clipping (-L 100,5). To

467 reduce background noise, aligned reads which did not match a TA site at the 5'

468 end of the alignment were excluded from further analysis.

469

470

471 **Preliminary screen for anaerobic mutants**

472

473 Approximately 5000 mutants each for *A. pleuropneumoniae* and *P.*

474 *multocida* (from 4 separate mutant pools) were screened for insertions in genes

475 required for survival during anaerobic growth. The mutant pools were plated on

476 BHI-S-NAD supplemented with 1 µg/ml Cm at a density of 75 to 150 colonies per

477 plate. Following overnight incubation at 37°C with 5% CO_2, colonies were

478   transferred by replica plating onto 2 fresh selective plates. One plate was incubated

479   with 5% CO_2, and the other was placed in an anaerobic jar, at 37°C overnight.

480   Mutants that failed to grow anaerobically were re-tested to confirm the growth

481   defect, and the site of transposon insertion was determined for each by direct

482   sequencing of linker-PCR products.

483

484   **Nucleotide sequence accession numbers**

485

486   The complete sequences of the two *mariner* delivery vectors, pTsodCPC9

487   and pTlacPC9, have been deposited in Genbank under accession numbers

488   MH644834 and MH644835, respectively. The draft genome sequence of the

489   porcine clinical respiratory isolate of *P. multocida*, MIDG3277, has been deposited

490   in the European Nucleotide Archive (ENA) under the accession number

491   ERZ681052, with the raw reads available under the accession number

492   ERR200085. The raw TraDIS reads for *A. pleuropneumoniae* are available in ENA

493   under the accession number ERR271132. The raw TraDIS reads for *P. multocida*

494   are available in ENA under the accession numbers ERR744003, ERR744016,

495   ERR752316, ERR752329, ERR755725 and ERR755738.

496   **Acknowledgements**

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521   **References**

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693

694   Table 1. Primers used in this study.

Name	Sequence
CmDUSUSS_for	CGCGG<u>ATGCCGTCTG</u>*AAGTGCGGT***ACAAGCGGT**CGGCAATAGTTACC
CmDUSUSS_rev	CGCG*AAGTGCGGT*<u>ATGCCGTCTGA</u>**ACAAGCGGT**TTCAACTAACGGG
CmDUSUSS_IRleft	CTGATAAGTCCCCGGTCTGCAGGCGGCCGCACTAGTGATTC
CmDUSUSS_IRright	CTGATAAGTCCCCGGTCTCGAAGTGCGGTATGCCGTCTG
Himar_IR	TAACAGGTTGGCTGATAAGTCCCCGGTCT
ISceI_left	***TAGGGATAACAGGGTAAT***CATGGCCGCGGGATTAACAGGTTG
ISceI_right	***ATTACCCTGTTATCCCTA***CGGCCGCACTAGTGATTAACAGG
M13_for	GTAAAACGACGGCCAGTG
M13_rev	GGAAACAGCTATGACCATG
oriTtraJ_left	CCGCCTGCAGGTCGACAAAACAGCAGGGAAGCAGCGCTTTTC
oriTtraJ_right	ACTCAAGCTATGCATGCATGGGGACGTGCTTGGCAATC
sodCPC9_left	CGAATTGGGCCCGACCGCCAACCGATAAAACCTACATTTTGC
sodCPC9_right	CCTCCTTTTCTAGTCGCGGTACCGTCGACTGCAGAATTC
lacPC9_left	CGAATTGGGCCCGACGTGAGCGCAACGCAATTAATGTGAGTTAG
lacPC9_right	CCTCCTTTTCTAGTCGGCGTAATCATGGTCATAGCTGTTTCC
C9sodC_left	AGTCGACGGTACCGCGACTAGAAAAGGAGGATTCCTCATATGG
C9lacP_left	GACCATGATTACGCCGACTAGAAAAGGAGGATTCCTCATATGG
C9_right	CCGGGAGCATGCGACCCAGTGTGCTGGAATTCGCCCTTAGC
L-PCR-C	GATAAGCAGGGATCGGAACC
IR-Left_out	CACTTCAGACGGCATCCGCGAATC
IR-Right_out	GTTGAAACCGCTTGTTCAGACGGC

695   Specific DNA uptake sequences for *Neisseria* spp. (underlined), *H. influenza* (italics), *and A. pleuropneumoniae* (bold) are indicated in the CmDUSUSS_for and
696   CmDUSUSS_rev sequences
697   The ISceI restriction site is indicated in bold italic text
698   The 15 base overhangs for In Fusion cloning are shown in smaller font on the 5' end of primers oriTtraJ_left to C9_right
699

700 Table 2. Location of *mariner* insertions in anaerobic mutants of *A. pleuropneumoniae*

701

Gene	Product of disrupted gene	Gene location[a]	Location of insert[a]
nhaB	Na+/H+ antiporter protein	363852..365393C[b]	364160
pflB	Formate acetyltransferase	1178587..1180899C	1179054
			1179780
			1180383
atpC	ATP synthase epsilon chain	1922342..1922761C	1922665
atpG	ATP synthase gamma chain	1924188..1925054C	1924771
			1924970[c]
atpA	ATP synthase alpha chain	1925077..192661C	1925488
			1925791
			1925826[c]
			1926363
atpB	ATP synthase A chain	1928081..1928869C	1928204[c]
			1928575[c]
fumC	Fumarate hydratase	2051573..2052967C	2052243
MIDG2331_02098	D-alanyl-D-alanine carboxypeptidase	2163589..2164257C	2163863

702 [a]Location in the complete genome of MIDG2331 (accession number LN908249)

703 [b]Start and end positions of genes are indicated; those on the complementary strand are shown with the suffix C

704 [c]Insertions at these locations were mapped in 2 mutants each

705 Table 3. Location of *mariner* insertions in anaerobic mutants of *P. multocida* MIDG3277

706

Gene	Product of disrupted gene	Gene location[a]	Location of insert[a]
MIDG3277_1258	predicted glycosyl transferase	contig26:104818..105990[b]	contig26:105340
aroG	deoxy-7-phosphoheptulonate synthase	contig17:68162..69247	contig17:68149[c]
intergenic			contig37:10847[d]
MIDG3277_1016	Hypothetical protein[e]	contig21:76828..77169C	contig21:77129
aroA	3-phosphoshikimate 1-carboxyvinyltransferase	contig21:79640..80962	contig21:80029
			contig21:80287
sucC	Succinate--CoA ligase subunit beta	contig3:86080..87246	contig3:86908
ccmD	Heme exporter protein CcmD	contig1:16084..16287	contig1:16136
atpI	F0F1 ATP synthase subunit I	contig38:36319..36726	contig38:36262[f]
atpG	F0F1ATP synthase subunit gamma	contig38:40520..41389	contig38:40497[g]
MIDG3277_0379	Hypothetical protein	contig6:168..512[h]	contig6:434
pfeA	Ferric enterobactin receptor	contig32:62820..64826C	contig32:64243
menA	1,4-dihydroxy-2-naphthoate octaprenyltransferase	contig28:97896..98801C	contig28:98875
menB	1,4-dihydroxy-2-naphthoyl-CoA synthase	contig28:34911..35768C	contig28:35383
menC	O-succinylbenzoate synthase	contig28:33645..34658C	contig28:34159

707 [a]Location in the draft genome of MIDG3277 (accession number ERZ681052)
708 [b]Start and end positions of genes are indicated; those on the complementary strand are shown with the suffix C
709 [c]Insertion located 13 bp upstream of the start of *aroG*; insertion likely disrupts transcription of *aroG*
710 [d]Insertion located 75 bp upstream of the start of *degQ* and 147 bp upstream of the start of MIDG3277_0890 encoding a putative NAD(P)H nitroreductase; insertion likely
711 disrupts transcription of *degQ*
712 [e]This protein shares 100% identity with PM0836 (Genbank accession AAK02920) which was reported to be in vivo expressed (49)
713 [f]Insertion located 57 bp upstream of the start of *atpI* (MIDG3277_1814); insertion likely disrupts transcription of *atpI* and remaining genes in the *atp* operon
714 [g]Insertion located 23 bp upstream of *atpG*; insertion likely disrupts transcription of *atpG* and remaining genes in the *atp* operon
715 [h]This predicted CDS is on a small contig and may be part of a larger gene (100% identity with the last 104 AAs of a 818 AA hypothetical protein
716 P1062_0208970; Genbank accession ESQ7176

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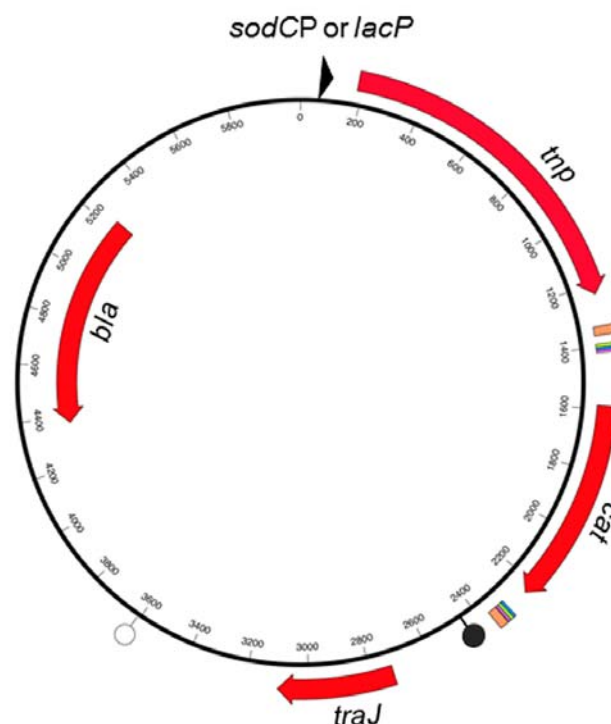722

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728   Figure 1. General map of the *mariner* vectors, pTsodCPC9 and pTlacPC9,

729   differing only in the promoter for the *tnp* gene. Genes *tnp* (*mariner* transposase

730   C9 mutant), *cat* (chloramphenicol resistance), *traJ* (plasmid transfer gene), and

731   *bla* (ampicillin resistance) are indicated by the appropriately labelled solid red

732   arrows. The origin of plasmid transfer (*oriT*) is shown as a filled lollipop (♦), and

733   the origin of plasmid replication (*colE1*) as a hollow lollipop (♀). Coloured blocks

734   flanking the *cat* gene indicate the locations of the *Himar1* inverted repeats (thick

735   orange), and paired copies of DNA uptake sequences for *Neisseria* spp. (thin

736   green), *H. influenzae* (thin pink) and *A. pleuropneumoniae* (thin blue).

737   Arrowhead upstream of C9 *tnp* gene indicates the promoter sequences for

738   either the *A. pleuropneumoniae sodC* or the *E. coli lac* gene, depending on the

739   vector (pTsodCPC9 and pTlacPC9, respectively).
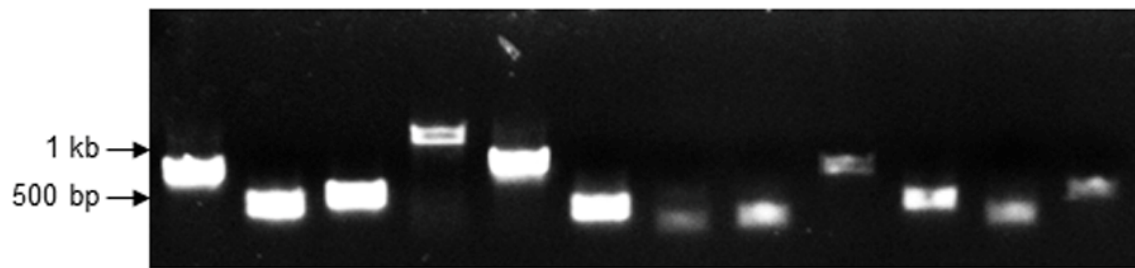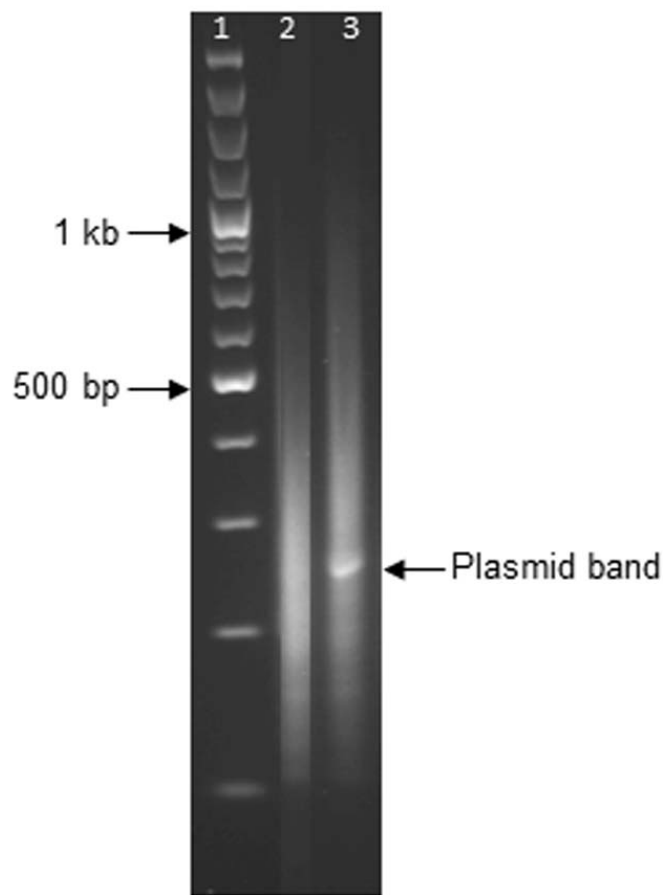
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744 Figure 2. Linker PCR products for 12 randomly selected *A. pleuropneumoniae*

745 mutants. Sequences flanking the *mariner* insertions were amplified from AluI-

746 digested linker-ligated DNA fragments using primers L-PCR-C and IR-Left_out

747 (for amplification of the left flank).

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Figure 3. Comparison of linker PCR-products generated from pooled *A. pleuropneumoniae* library genomic DNA +/- ISceI digestion. Lanes: 1) 100 bp ladder; 2) ISceI-treated sample; 3) untreated sample. Amplification products were generated for the left flanking sequences, as in Figure 2. The dominant plasmid band in the untreated sample is indicated.
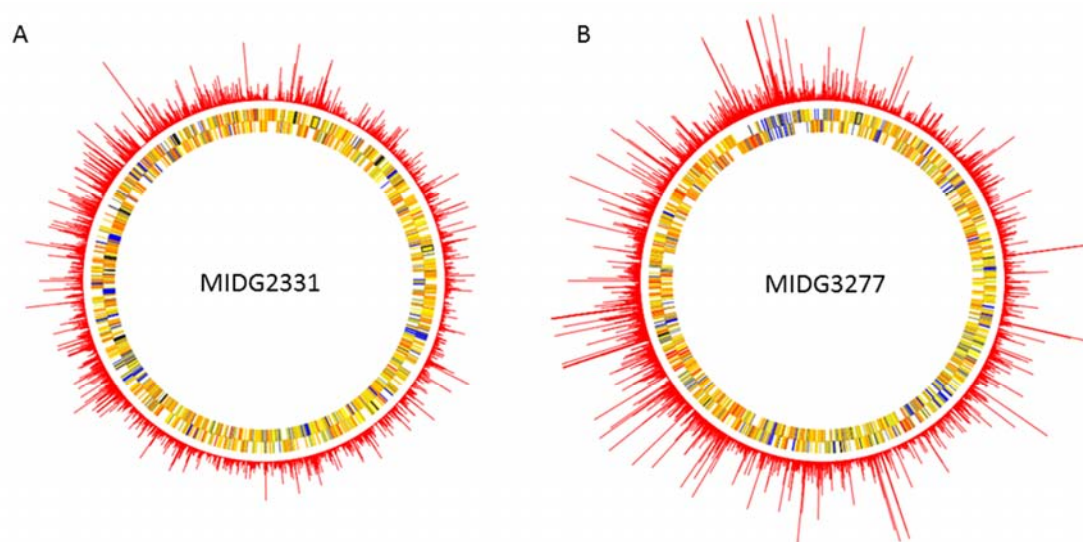
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Figure 4. Distribution of *mariner* insertions identified in the *A. pleuropneumoniae* and *P. multocida* genomes. TraDIS reads for the respective pooled libraries were mapped to A) the complete genome of *A. pleuropneumoniae* MIDG2331 (accession number LN908249); and B) the draft genome of *P. multocida* MIDG3277 (accession number ERZ681052). Each spike plotted from the chromosome represents a single insertion site, with the length of each spike proportional to the number of mapped sequence reads from that insertion site. 56,664 unique insertion sites were identified in the *A. pleuropneumoniae* library, and 147,613 in *the P. multocida* library. In the *P. multocida* dataset, there were several insertion sites with large numbers of mapped reads. To enable insertions with fewer reads to be seen clearly, read coverage has been capped to a maximum of 50,000 in B (the true maximum coverage at an insertion site was 136,062 reads).

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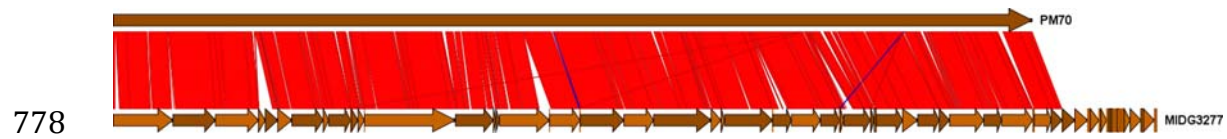
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780 Figure 5. A comparison of the *P. multocida* MIDG3277 draft assembly with the

781 complete genome sequence of *P. multocida* PM70. Arrows indicate the position

782 and orientation of the contigs. Red blocks indicate matches in the same

783 orientation, blue blocks indicate matches in the reverse orientation. The

784 assembled contigs of MIDG3277 were ordered based on this comparison,

785 which was performed using NUCmer 4.0 (43).