Signature-Tagged Mutagenesis of Pasteurella multocida Identifies Mutants Displaying Differential Virulence Characteristics in Mice and Chickens

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Received 14 April 2003/Returned for modification 30 May 2003/Accepted 16 June 2003

Pasteurella multocida is the causative agent of fowl cholera in birds. Signature-tagged mutagenesis (STM) was used to identify potential virulence factors in a mouse septicemia disease model and a chicken fowl cholera model. A library of P. multocida mutants was constructed with a modified Tn916 and screened for attenuation in both animal models. Mutants identified by the STM screening were confirmed as attenuated by competitive growth assays in both chickens and mice. Of the 15 mutants identified in the chicken model, only 5 were also attenuated in mice, showing for the first time the presence of host-specific virulence factors and indicating the importance of screening for attenuation in the natural host.

Pasteurella multocida is a capsule, gram-negative cocco-bacillus and is the causative agent of avian fowl cholera, bovine hemorrhagic septicemia, atrophic rhinitis in pigs, and snuffles in rabbits (30). To date, few virulence factors of P. multocida have been identified; they include the capsule in serogroups A and B (7, 10), PMT toxin in strains causing atrophic rhinitis (16), putative filamentous hemagglutinins PhfB1 and PhfB2 (17), and several iron acquisition proteins, such as TonB, ExbD, and ExbB (6, 17, 31).

Recently, studies have been undertaken to identify the genes involved in the pathogenesis of pasteurellosis, including in vivo expression technology (22), signature-tagged mutagenesis (STM) (17), and whole-genome expression profiling (8). These genomic-scale methods have identified some true virulence factors and virulence-associated genes, including those involved in iron transport and metabolism as well as nucleotide and amino acid biosynthesis. However, many genes identified by these analyses have no known function, and neither in vivo expression technology nor whole-genome expression profiling gives direct information about the importance of genes in virulence.

STM allows the large-scale screening of mutants for reduced survival in vivo, colonization and adhesion defects, and decreased invasive ability (3, 9, 20). The method has been applied to many bacterial species, including a previous study on a bovine isolate of P. multocida using a tagged mini-Tn10 (17). This previous study used a septicemic mouse model of infection and identified 25 P. multocida genes that were important for survival and growth in vivo. However, one-third of the 62 mutants identified in that study contained multiple transposon insertions, and in these instances, it was not possible to determine the gene responsible for the attenuated phenotype (17).

In this study, we report the screening of an STM bank of P. multocida mutants in two animal models to identify, for the first time, potential host-specific virulence factors. We screened the mutant library in mice, the most widely used P. multocida animal model, and in chickens, the natural host.

Modification of Tn916 for use in STM of P. multocida. The transposon Tn916 has been shown to transpose in a quasirandom fashion into the P. multocida genome (14). In addition, tetracycline resistance was stable in the absence of selection over many generations, and the transposon could be introduced into P. multocida by electroporation rather than conjugation, avoiding the need for a recipient strain carrying a characterselectable marker (14). However, in our study, the inherent instability of the transposon in the base plasmid pAM120 (18) resulted in an inadequate number of tagged transposons being generated for library construction. To overcome this problem, we constructed a smaller version of the transposon, designated Tn916EΔC, that was stable for manipulation in Escherichia coli and, when required, could be restored to an active transposon by removal of the emr(B) cassette from the int gene (Fig. 1).

Briefly, two PCR products were generated from pAM120. The first was generated with primers BAP1127 (AGCAGTTCTAGATGATGATACTGTCCTC) and BAP1126 (TCGCTGCTCGAATATCCTCGCCAG) and included the right and left junctions of the transposon, the 3′ end of the int gene, and the entire base plasmid (Fig. 1). An XhoI site and XbaI site were introduced near the right junction of the transposon and the 3′ end of the int gene, respectively. The second fragment, amplified with BAP1128 (TCATCATCTAGAACACTGTTCTTGTG) and BAP1129 (TTTGGTACTCGAAGAACGGGAG), included the 5′ end of the int gene (and an XbaI site) and the entire tetracycline resistance gene, tet(M). An XhoI site was also generated at the end of this fragment upstream of the tet(M) gene. A third fragment of DNA containing an erm(B) gene conferring erythromycin resistance was purified after digestion of the plasmid pJIR599 (4) with XhoI and inserted into the XbaI site engineered within the int gene. All three fragments were then ligated, and the subsequent E. coli transformants were screened for the correct plasmid profile. The

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erm(B) insertion resulted in the inactivation of transposon excision during DNA manipulations, and the XbaI site located at either end of the erm(B) gene allowed the removal of the cassette when transposition was required. The modified transposon was designated Tn916ΔC, as it conferred erythromycin resistance (E) and no longer contained the conjugation genes (ΔC). Tn916ΔC in pAM120 was designated pPBA1638, which is a suicide vector in *P. multocida*. Prior to transposition into *P. multocida*, the erm(B)cassette was removed by enzyme digestion and religation. Tn916ΔC was shown to transpose in a quasirandom fashion in *P. multocida*, and most mutants had only a single transposon insertion (data not shown).

**Construction and cloning of DNA tags.** The variable oligonucleotide used for construction of the tagged Tn916ΔC was essentially as described previously (20). To ensure tag fidelity, we constructed the tag from three oligonucleotides (Fig. 2). The first, designated BAP1298 [GTACAACCTCAAGCTT(NK)20GAATTCGGTTAGAATG], was a 72-bp oligonucleotide, which included the 40-bp variable region. The remaining bases required for cloning into Tn916ΔC were synthesized on oligonucleotides BAP1299 (ATAAACCCGGGTACAACTCAAGCT) and BAP1300 (GCATCCCGGGCATTC TACCCGGAATTC), and each included an XhoI site for insertion into the BspEI site of Tn916. Double-stranded tags were generated by annealing BAP1298 and BAP1300 and allowing T4 polymerase to fill the 5′ single-stranded extensions. The resulting double-stranded molecule was further extended by PCR amplification using oligonucleotides BAP1299 and BAP1300 (Fig. 2).

**Generation of a *P. multocida* signature-tagged Tn916ΔC library.** For construction of the tagged transposon library, the plasmid pPBA1638 was digested with BspEI (Fig. 1) and ligated to XhoI-digested DNA tags, generating a plasmid which could no longer be digested by either enzyme. Prior to electroporation into competent *E. coli* DH5α cells, the ligated products were digested with BspEI to digest any plasmid not containing a DNA tag.

A library of 42 uniquely tagged Tn916ΔC transposons in the plasmid pPBA1638 was constructed by selecting tagged transposons that would act as efficient templates for PCR and hybridize specifically in DNA dot blot hybridizations. Prior to transposition into *P. multocida*, the erm(B) cassette was removed from the plasmids by digestion with XbaI and self-ligation. Each plasmid harboring a uniquely tagged transposon was then introduced separately into *P. multocida* strain VP161 by electroporation. Mutant pools, each containing 42 mutants,
were assembled by selecting a single *P. multocida* colony from each transformation plate, and the procedure was repeated until 10 pools were assembled.

Other studies have indicated that the animal model chosen for STM studies can influence the genes identified, as some virulence genes will be host specific or involved in host adaptation (47). We therefore chose to use two animal models, namely, the mouse, which has been used regularly as a model for fowl cholera, and the chicken, a natural host for *P. multocida*.

**Screening of the *P. multocida* mutant library in vivo.** For each input pool, the mutants were grown separately overnight, and an aliquot from each culture was transferred to a fresh tube. A sample of the mix was then spread onto brain heart infusion (BHI) plates with tetracycline and grown overnight. Colonies were washed from the plate and used to prepare genomic DNA representing the input pool.

For screening in mice, female, inbred BALB/c mice, aged 8 to 10 weeks, were injected intraperitoneally with approximately 10^5 CFU (approximately 2 × 10^5 CFU/mutant) from the input pool to ensure that all mutants were well represented. This dose gave reproducible disease progression, and all mice showed terminal signs of disease within 6 h, when blood was recovered and the animals were euthanized in accordance with animal ethics requirements. After infection, blood was diluted fivefold in BHI and spread onto BHI plates containing tetracycline. At least 5,000 colonies were washed from these plates and used to prepare genomic DNA representing the output pool.

Each pool of mutants was tested in two mice, and the relative abundance of each tag in the input and output pools was determined by dot blot hybridization. Genomic DNA from bacteria isolated from both animals was pooled prior to generation of the output probe and therefore represented the average output profile from two animals.

For preparation of the labeled probe, genomic DNA isolated from each pool was used as a template for PCR amplification with the primers BAPI129 and BAPI1414 (GTGCAT GAAATAATATACGAGT), generating an 800-bp fragment that contained the unique tag region. The amplified products were digested with *Hind*III and *Eco*RI (located at the end of the variable tag region) followed by *Klenow* end filling. The digested DNA was then separated on a 20% polyacrylamide gel, eluted overnight (2), and then labeled with the digoxigenin oligonucleotide 3′ end labeling kit (Roche Diagnostics). For preparation of the target DNA for high-stringency dot blot hybridizations, each tag was amplified separately by PCR with the primers BAPI1414 and BAPI1129, and 1 ng of amplified product was spotted onto nylon filters.

Mutants were identified as displayed reduced growth in vivo if the hybridization with the corresponding target DNA was stronger with the input probe than with the output probe. Mutants identified in the first screening were collected into a new pool, injected into a mouse, and used in a second STM screen. After the second STM screening, 15 mutants were identified that were potentially attenuated. Each mutant was then tested in a competitive growth assay together with wild-type strain VP161.

For screening of each STM pool in chickens, duplicate chickens (commercially obtained Leghorn cross) were infected by injection of approximately 10^5 CFU of the pool into the breast muscle. Infections were allowed to proceed until the onset of terminal signs of infection (12 to 16 h), whereupon blood was recovered and the birds were euthanized in accordance with animal ethics requirements. An output probe was generated from the bacteria recovered from each bird and used separately in dot blot hybridizations to determine the relative abundance of each tagged mutant.

Thirty-five mutants were identified as having reduced output hybridization signals and subsequently tested individually for reduced growth in vivo with competitive growth assays.

**Identification of attenuated strains in both animal hosts.** Competitive growth assays were used to quantify the relative growth rates of putative attenuated mutants. Log-phase cultures of mutant and wild-type were mixed at a ratio of 1:1, and serial 10-fold dilutions were prepared. To determine the input ratio of wild-type to mutant organisms, 100 μl of the appropriately diluted culture was plated immediately onto NB agar in order to obtain single colonies representing the input pool. For the in vivo growth assays, animals were infected with approximately 10^5 CFU, and blood samples were obtained at the terminal stages of the disease (6 h for mice or 12 to 18 h for chickens). Blood was diluted twofold in BHI containing heparin and plated onto NB agar. For the in vitro growth assay, a 100-fold dilution of the mixed bacteria was grown in BHI at 37°C with shaking for the same length of time as the animal infections, diluted appropriately, and spread onto NB agar.

After growth on nonselective plates, a minimum of 100 individual colonies was patched onto NB agar with or without tetracycline. Mutants were identified as attenuated if the proportion of mutants obtained after in vivo growth was significantly less than the proportion obtained after in vitro growth. Significance was determined by calculating the *P* value by an approximate *z* test for the difference in two proportions (Table 1). The relative competitive index (rCI) was determined by dividing the percentage of tetracycline-resistant colonies (transposon mutants) obtained in vivo by the percentage of tetracycline-resistant colonies obtained in vitro (Table 1).

Of the 15 mutants originally identified in mice, only 5 were significantly attenuated, as determined by the competitive growth assay in mice (Table 1). Subsequent competitive growth assays in chickens showed that all five mutants were also attenuated in chickens, although the level of attenuation varied between the two hosts.

Fifteen of the 35 mutants identified in the STM screening in chickens showed significantly reduced growth in competitive growth assays in chickens. Each of these was also tested in competitive growth assays in mice to assess whether there was a difference in the level of attenuation between the two animal models (Table 1). Only 5 of the 15 mutants were also attenuated in mice; these were the same mutants identified in the mouse STM screening.

Southern hybridization confirmed single transposon insertions in all mutants. To identify the exact site of transposon insertion, inverse PCR was performed using Sau3AI-digested and ligated genomic DNA as the template and oligonucleotides BAPI1128 and BAP2252 (ACATAGAATAGGCTTTTACGAGC) (36). The amplified products were sequenced directly by using BAP2252, and *P. multocida* DNA sequence
Adjoining the Tn916 sequence was searched against the GenBank database with the BLASTX algorithm (1).

**Attenuated strains with insertions in metabolic genes.** We identified a number of mutants that had transposon insertions affecting genes encoding biosynthetic enzymes, including purN, pyrF, and deoC. Previous global screenings in bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Yersinia enterocolitica* also identified many biosynthetic genes which were important for growth in vivo (11, 13, 26). The mutant AL252 had an insertion in the purN gene and was attenuated.

<table>
<thead>
<tr>
<th>Group and mutant</th>
<th>Disrupted gene or region</th>
<th>Predicted function or homologue</th>
<th>Competitive growth assay results in:</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Mouse</td>
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<td></td>
<td></td>
<td></td>
<td>rCI</td>
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<td>Attenuated in chickens and mice</td>
<td>dcaA</td>
<td>Phosphoethanolamine transferase</td>
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<td>AL249</td>
<td>pm0855 (flp1)</td>
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<td>deoC</td>
<td>Deoxyribose synthesis</td>
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<td>AL389</td>
<td>ponC</td>
<td>Penicillin binding, murein synthesis</td>
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<td>Homology with HI0902; probable integral membrane protein</td>
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<td>AL407</td>
<td>295 bp upstream of tfoX</td>
<td>Between tfoX and a 16S RNA gene</td>
<td>3.15</td>
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*Values represent the rCI calculated for individual animals, determined by dividing the in vivo competitive index (CI) by the in vitro CI. Each CI value was determined by dividing the output mutant/wild-type ratio by the input mutant/wild-type ratio.

*P* values were calculated by using an approximate *z* test for the difference in two proportions. The test was performed to determine if the proportion of mutants in vivo was significantly less than the proportion obtained in vitro.
in both mice and chickens (Table 1). This gene encodes the enzyme 5’-phosphoribosylglycinamidyltransferase, which catalyzes the fourth step of de novo purine biosynthesis (48). The ability to synthesize purine nucleotides from simple precursors has been identified as being important for bacterial growth in vivo, and it has been reported recently that several of the genes involved in purine biosynthesis in P. multocida are up-regulated in bacteria harvested from the blood of infected chickens (8). In addition, in a previous STM study on a bovine isolate of P. multocida in mice, a purF mutant was identified, the product of which is also involved in the de novo purine biosynthesis pathway (17). The mutant AL388 (Table 1) was attenuated only in chickens and had an insertion in the pyrF gene, which encodes an orotidine 5’-monophosphate decarboxylase that in E. coli is involved in pyrimidine biosynthesis (34).

The mutant AL387 had an insertion upstream of deoC and deoR, and this strain was attenuated in chickens but not in mice. The E. coli homologs of these genes encode a deoxyribose phosphate aldolase and a repressor protein, respectively, and are predicted to be part of the nucleoside catabolism pathway. In E. coli, purine and pyrimidine nucleosides and deoxynucleosides can be utilized in the nucleoside catabolic pathway to extract pentose or deoxypentose for use as a carbon and energy source. DeoC converts a deoxyribose intermediate to glyceraldehyde-3-P and acetaldehyde, which can be used in glycolysis and the TCA cycle, respectively. In E. coli, four genes encoding the deoxynucleoside enzymes are located in two adjacent operons, and the DeoR repressor protein, encoded elsewhere, binds to two DNA binding sites upstream of the deoC, -A, -B, and -D genes, thereby generating a loop in the DNA and preventing initiation of transcription (32). In P. multocida, the gene arrangement is different, with only deoC and deoR being adjacent to each other. Without a functional deoxynucleoside aldolase enzyme, the mutant will be unable to convert the deoxyribose intermediates to the final products in the pathway. In a rich in vitro medium, this pathway may not be essential for the mutant to grow normally, but in chickens, the bacteria may need to utilize nucleosides as an alternative source of carbon and energy. It is also possible that in the mutant, the transposon insertion upstream of deoC and deoR affects the transcriptional regulation of the deo genes, as the DeoR repressor protein may no longer be synthesized.

Mutants with altered cell envelopes. Three genes predicted to be involved in cell envelope biosynthesis were identified: ponC, pm1294, and dcaA. The mutant AL389 had a transposon insertion in ponC, a homologue of the E. coli gene pbpC, which encodes the protein PBP1C that has a penicillin-binding domain and functions as a transglycosylase for murein polymerization (45). Murein is required for the biosynthesis of the murein sacculus, the scaffolding structure of the bacterial cell wall. Mutants in the pbpC gene in E. coli are viable in vitro, but the cross-linkage of the murein structure is different from that of the wild type, with mainly nascent murein being used for the biosynthesis of the sacculus instead of a combination of pre-existing murein strands with newly synthesized ones (37, 45). The P. multocida ponC mutant was also viable in vitro but showed significant attenuation in chickens, perhaps indicating that the changed structure of the murein sacculus affects the integrity of the cell wall, making the mutant more susceptible to osmotic stress in vivo.

Three mutants, namely, AL251, AL390, and AL391, were identified with insertions in the gene pm1294. This gene is predicted to encode a heptosyl transferase, as the amino acid sequence displayed 58% identity to the heptosyltransferases II and III from Haemophilus ducreyi. These H. ducreyi proteins are glycosyltransferases responsible for the addition of heptose to lipooligosaccharide (15). The three P. multocida mutants had the transposon inserted at the same site, but in one, AL390, the transposon was in the opposite orientation. Interestingly, each mutant showed different levels of attenuation in mice and chickens. Strain AL251 was significantly attenuated in mice, whereas AL390 and AL391 were not. In chickens, all three strains were attenuated, but AL251 and AL391 were more so than AL390. Although the mutant AL251 was significantly attenuated in mice, with an average rCI value of 0.62, it was more highly attenuated in chickens, with an average rCI of less than 0.01. Interestingly, the strain AL391 had the same insertion site and transposon orientation as AL251 and was also highly attenuated in chickens, but in contrast, it showed no attenuation in mice. Preliminary data on the virulence of strains AL251 and AL391 indicate that they are both unable to cause disease in chickens.

The mutant AL249 had an insertion in the gene annotated as dcaA, whose protein’s amino acid sequence has a high degree of identity with LptA and Lpt3 from Neisseria meningitidis (12, 28, 31). In N. meningitidis, lpt-3 and lptA encode phosphoethanoloamine transferases which are required for the addition of phosphoethanolamine (PEtn) to specific sites within the inner core of lipopolysaccharide (LPS) (12, 28). The inactivation of lpt-3 in N. meningitidis resulted in bacteria that were lacking PEtn on the LPS structure, and the mutants were less resistant to bactericidal killing and in vitro opsonophagocytosis (28). The dcaA gene has also been identified as being significantly up-regulated in P. multocida during infections in chickens (8). Preliminary structural information on the P. multocida LPS purified from the wild-type VP161 indicates that a PEtn molecule is attached to the LPS (data not shown). Moreover, based on the positions of PEtn in N. meningitidis (28) and Haemophilus influenzae (38, 39), it is unlikely that the lack of the PEtn in the mutant would have any significant effect on the LPS structure that would result in major structural changes in the outer membrane. Therefore, the attenuation observed in the P. multocida mutant AL249 is likely to be a direct effect of an altered LPS. Taken together, these data are consistent with a more important role for LPS in the progression of fowl cholera in chickens than in the progression of pasteurellosis in mice. Currently, studies are being undertaken to analyze the LPS composition of the mutant AL251 compared to wild-type LPS.

Notably, in the previous STM study of a bovine pneumonia isolate of P. multocida in a septicemic mouse model, another LPS mutant was identified with an insertion in lgtC, encoding a galactosyltransferase, the homologue of which in H. influenzae is responsible for the addition of galactose to the LPS oligosaccharide (17, 21).

Mutants with insertions in genes encoding outer membrane proteins and surface structures. Fimbriae have previously been identified on the surface of P. multocida cells and genes
encoding putative fimbriae, and fibrils are present in the *P. multocida* genome (23, 31, 42, 44). However, it has not been determined which of the fimbrial proteins are present within the observed structures and what role, if any, they play in the pathogenesis of disease. In this study, we identified one attenuated mutant, namely, AL250, with the transposon inserted between the start codon and the putative promoter of the gene *pm0855*. The deduced amino acid sequence of *pm0855* has 43% identity with Flp1 from *Actinobacillus actinomycetemcomitans*, and the gene is located at the start of a potential operon containing 14 genes. This putative *P. multocida* operon also contains other genes with similarities to those from the *flp* operon of *A. actinomycetemcomitans*, including genes encoding tight adherence proteins (Tad) that are thought to be involved in the synthesis of long filamentous fibrils (24). A second *flp* homologue, *flp2*, was identified adjacent to *flp1*, but this putative gene has not been annotated in the published *P. multocida* genome sequence (31). These two *P. multocida* genes contain an Flp motif that has been predicted to be characteristic of a new pilin gene subfamily (25).

In a previous STM study on *P. multocida*, a tadD mutant was shown to be attenuated. The *tadD* gene is positioned downstream of the *flp* genes but potentially within the same operon (17). To determine if the *tadD* gene was affected in the *flp* mutant AL250, we measured the levels of RNA expressed from selected genes within the putative operon. Quantitative analysis of mRNA levels indicated that only the *flp1* and, to a lesser extent, *flp2* mRNAs had reduced levels in the mutant, while the mRNA expression levels of genes downstream of *flp2* were identical to those of the wild type (data not shown). This finding indicates that there may be a promoter located downstream of *flp2*. Given that the transposon was located between the putative promoter and the start codon, it was surprising that there was any mRNA expression from the *flp1* gene in the mutant.

In competitive growth assays, the *flp* mutant was consistently attenuated in mice. However, in chickens, there was variation in the level of attenuation between individual birds, with the *flp* mutant being able to grow at levels similar to that of the wild type in one of the four chickens tested. This may indicate real variability in the growth of the strain in different birds or may indicate that there is some instability of the mutant.

Finally, a mutant was identified with an insertion in *pm1069*, the amino acid sequence of which has significant similarity to the *E. coli* protein FadL and the P1 outer membrane protein from *H. influenzae*. In *E. coli*, FadL has been identified as an outer membrane porin required for the uptake of long-chain fatty acids (5, 29). Mutants lacking FadL are unable to grow on medium containing long-chain fatty acids as a sole carbon source (35). A search of the PM70 genome sequence showed that homologues of two other proteins required for the uptake of long-chain fatty acids, namely, FadR and FadD, are also present. The *H. influenzae* homologue P1 has been considered a potential vaccine candidate for both type b and nontypeable strains of *H. influenzae*, as both polyclonal and monoclonal antibodies raised against P1 protect against bacteremia in the infant rat model (19, 27). In *H. influenzae* type b, a P1 mutant was shown to grow at the same rate as wild-type *H. influenzae* in vitro and could still induce bacteremia in the infant rat model (33). However, analysis of a P1 mutant in *H. influenzae* biogroup aegyptius demonstrated that although both the wild type and mutant grew at the same rate under anaerobic conditions, the expression of P1 was up-regulated in the wild-type strain (46). The *P. multocida* mutant AL386 was attenuated only in chickens, suggesting that the uptake of long-chain fatty acids as an energy source is required only during infections in chickens and not during in vitro growth or during infections in mice.

**Concluding remarks.** We have screened 420 *P. multocida* STM mutants in both mice and chickens and identified 15 attenuated mutants and 13 disrupted genes or regions. Ten of the insertions caused attenuation in chickens but not in mice, indicating for the first time *P. multocida* virulence genes that may be host specific. Five mutants were attenuated in both hosts, and no mutants were identified that were attenuated in mice but not in chickens.

Significantly, in this study, we did not identify any of the genes identified in a previous STM study that used a bovine pneumonia isolate of *P. multocida* in a mouse infection model. However, several genes were identified that encoded proteins involved in similar pathways (17). These differences are unsurprising, as the studies utilized different transposons, the *P. multocida* strains were isolated from two very different diseases (fowl cholera and bovine pneumonia), and each strain exhibited different infection kinetics in the mouse model. Furthermore, it is likely that neither study approached saturation coverage of the genome.

Mutant pools were introduced into the intraperitoneal cavity of mice and via the breast muscle of chickens. While these inoculation routes reliably cause disease in both animal models, these infection routes would not allow the detection of bacteria with mutations in genes involved in the initial attachment and colonization of mucosal surfaces. Previous studies have indicated that birds can be infected via the respiratory tract, and it has been demonstrated that *P. multocida* can adhere to turkey air sac macrophages (40, 41, 43). Future studies will aim to detect mutants unable to attach and colonize by using intratracheal inoculation of the STM pools into chickens.

This work was funded in part by grants from the Australian Research Council, the Australian Centre for Agricultural Research, and the Rural Industries Research and Development Corporation. We thank the staff at Veterinary Pathology, University of Queensland, for their valuable assistance with the chicken experiments and Vicki Vallance and Ian McPherson for their excellent technical assistance. We also thank Aidan Sudbury for valuable statistical advice and Trudi Bannam for critical reading of the manuscript.

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