Differences between predicted outer membrane proteins of *Pasteurella multocida*, *Histophilus somni*, and genotype 1 and 2 *Mannheimia haemolytica* strains isolated from cattle

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Abstract: Common bacterial causes of bovine respiratory disease (BRD) include *Histophilus somni*, *Mannheimia haemolytica*, and *Pasteurella multocida*. Within *M. haemolytica*, two major genotypes are commonly found in cattle (1 and 2); however, genotype 2 strains are isolated from diseased lungs much more frequently than genotype 1 strains. Outer membrane proteins (OMPs) of *H. somni*, *P. multocida*, and genotype 2 *M. haemolytica* may be important factors for acquired host immunity. The predicted OMP differences between genotypes 1 and 2 *M. haemolytica* have been previously identified. In this study, we expanded the focus to include bovine-isolated strain genomes representing all three species and the two *M. haemolytica* genotypes. Reported here are the core genomes unique to each of them, core genomes shared between some or all combinations of the three species and two *M. haemolytica* genotypes, and predicted OMPs within these core genomes. The OMPs identified in this study are potential candidates for further studies and the development of interventions against BRD.

Key words: *Histophilus somni*, *Mannheimia haemolytica*, *Pasteurella multocida*, outer membrane proteins, bovine respiratory disease.

Résumé : Les bactéries le plus souvent en cause dans les maladies respiratoires des bovins (MRB) incluent *Histophilus somni*, *Mannheimia haemolytica* et *Pasteurella multocida*. Au sein du *M. haemolytica*, deux génotypes majeurs sont communément rencontrés chez les bovins (génotypes 1 et 2), mais les souches du génotype 2 sont isolées de poumons atteints beaucoup plus souvent que les souches du génotype 1. Les protéines de la membrane externe (PME) de *H. somni*, *P. multocida* et du génotype 2 de *M. haemolytica* pourraient constituer des facteurs importants pour l'immunité acquise chez l'hôte. Des différences prédites au niveau des PME entre les génotypes 1 et 2 de *M. haemolytica* ont été identifiées précédemment. Dans ce travail, les auteurs élargissent la portée de ces recherches en étudiant les génomes de souches isolées de bovins pour les trois espèces et les deux génotypes de *M. haemolytica*. Les auteurs rapportent ici les génomes partagés qui sont uniques à chacune d'elles, les portions de ces génomes qui sont communes à toutes ou à certaines combinaisons de ces trois espèces et deux génotypes de *M. haemolytica*, ainsi que les PME prédites au sein de ces génomes partagés. Les PME identifiés dans ce travail constituent des candidats potentiels pour des études plus approfondies et pour la mise au point de méthodes d'intervention pour contrer les MRB. [Traduit par la Rédaction]

Mots-clés : *Histophilus somni*, *Mannheimia haemolytica*, *Pasteurella multocida*, protéines de la membrane externe, maladies respiratoires des bovins.

Introduction

Bovine respiratory disease (BRD) is a multifactorial disease that affects cattle worldwide, with severe animal health, well-being, and economic ramifications (Rice et al. 2007). BRD can result from combinations of stress, host genetics, and viral and bacterial infections (Taylor

et al. 2010). *Histophilus somni*, *Mannheimia haemolytica*, and *Pasteurella multocida* are all members of the Pasteurellaceae family and are common bacterial causes of BRD (Klima et al. 2019; Klima et al. 2020). These gram-negative bacteria are opportunistic pathogens that colonize the nasopharynx of healthy cattle and can invade their lungs and cause BRD during times of general poor health, stress, and viral

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infections (Amat et al. 2019; Caswell 2014; Dabo et al. 2007; Singh et al. 2011).

Outer membrane proteins (OMPs) belonging to pathogenic members of the *Pasteurellaceae* family may be important antigens for acquired bovine immunity directed against them (Ackermann and Brogden 2000; Confer and Ayalew 2018). Previously, several differences in the predicted outer membrane proteomes of genotype 1 and genotype 2 *M. haemolytica* have been identified, including a porin, ligand-gated channel, protease S6, and three adhesins that were intact in genotype 2 and not genotype 1 strains (Clawson et al. 2020). The two genotypes were originally identified by whole genome sequencing of 1133 *M. haemolytica* strains (Clawson et al. 2016). Genotype 1 is represented by commensal-like serotype A2 strains, whereas genotype 2 is represented by more BRD pathogenic serotype A1 and A6 strains (Clawson et al. 2020). Thus, OMPs specific to genotype 2 *M. haemolytica* represent interesting targets for the study of genotype 2 specific pathogenesis and immunogenicity.

Here, we expanded the analyses conducted on genotypes 1 and 2 *M. haemolytica* predicted OMPs. We included additional available whole genomes of *M. haemolytica*, and those of *H. somni* and *P. multocida* that all originated from bovine-isolated strains to identify OMPs of the core genome of each species, as well as the shared core genome of each combination of species. By identifying OMPs that are shared by the core genomes of all combinations of these three species of *Pasteurellaceae*, it may be possible to identify epitopes that provide a common immune response to all three of them, or epitopes that provide an immune response specific to two or one species, as well as genotypes of *M. haemolytica*.

Materials and methods

Whole genomes of *M. haemolytica* ($n = 84$), *H. somni* ($n = 21$), and *P. multocida* ($n = 29$) isolated from cattle were downloaded from GenBank on 17 April 2020 (File S1¹). To ensure consistent annotation, all genomes were annotated using DEFAST (v1.2.6) (Tanizawa et al. 2018). To identify homologous coding sequences across species, a pan-genome analysis was performed with EDGAR (v2.3) (Blom et al. 2016) using *H. somni* genome CP000436.1 as a reference. The pan-genome analysis output was sorted to extract the core genome of each individual species and the core genome shared between species; a 95% cutoff was applied to be considered core. Amino acid sequences of the core genomes were analyzed by BOMP (Berven et al. 2004) to identify beta-barrel motifs and predict OMPs. Coding sequences that were predicted to be localized to the outer membrane were then analyzed by Boctopus2 (Hayat et al. 2016) to confirm the outer membrane prediction as well as to identify the specific amino acids likely to be found in the extracellular loop (EL) domains of the OMP.

Python scripts were written and used to extract amino acid sequences from the sorted EDGAR output (<https://github.com/EmilyWynn/EDGAR-protein-grabber>). To measure the similarity of the predicted OMPs across species, alignments were made using a BLOSUM62 scoring matrix for both the full-length proteins and their predicted EL domains.

Ethics approval

This research project did not involve experimental work with humans or animals.

Results

A pan-genome of 7577 genes was identified from the 134 genomes of the three *Pasteurellaceae* species analyzed in this study (File S2¹). A total of 206 genes were unique to *H. somni*, 581 were unique to *M. haemolytica* (found in both genotypes), 143 were unique to genotype 1 *M. haemolytica*, 104 were unique to genotype 2 *M. haemolytica*, and 346 were unique to *P. multocida* (Fig. 1A; File S2¹).

Three genes encoding predicted OMPs were identified in the shared core genome of all three species: a TonB-dependent receptor protein TBDR, an OMP assembly factor BamA, and an LPS assembly protein LptD (Fig. 1B; Files S3 and S4¹). The consensus sequence of all LptD protein isoforms was 793 amino acids long, with 224 amino acids predicted to localize to the EL domains (File S5¹). The full-length LptD protein had a minimum similarity of 83.5% among all three species, and the predicted EL domains had a minimum similarity of 81.9% (Table 1; File S6¹). The consensus sequence of all BamA protein isoforms was 814 amino acids long, with 185 amino acids predicted in the EL domains (File S5¹). The full-length BamA protein had a minimum similarity of 84% among all three species, and the predicted EL domains had a minimum similarity of 71.7% (Table 1; File S6¹). The consensus sequence of all TBDR protein isoforms was 1019 amino acids long, with 481 amino acids predicted in the EL domains. The full-length TBDR protein had a minimum similarity of 63.4% among all three species, and the predicted EL domains had a minimum similarity of 57.3% (Table 1; File S6¹). Another TBDR-encoding gene was shared between *M. haemolytica* and *P. multocida* strains, in addition to the one observed across all three species (Fig. 1; File S5¹).

Three genes encoding predicted OMPs were identified in the shared core genome of *M. haemolytica* and *P. multocida*, but not in *H. somni*: an exo-alpha-sialidase, a TonB-dependent receptor protein, and a TPR repeat-containing protein (Files S3 and S4¹). The exo-alpha-sialidase had a consensus sequence of 806 amino acids long, with 7 amino acids predicted in an EL domain (File S5¹). This EL domain had 100% similarity between *M. haemolytica* and *P. multocida* (Table 1; File S6¹). The TonB-dependent receptor protein had a consensus sequence 811 amino acids long, with 384 amino acids predicted in

¹Supplementary data are available with the article at <https://doi.org/10.1139/gen-2021-0038>.

Fig. 1. (A) The number of genes in the shared and unique core genomes of *Histophilus somni*, the two major genotypes of *Mannheimia haemolytica*, and *Pasteurella multocida*. Core genome analysis was done by EDGAR. (B) Predicted outer membrane proteins (OMPs) in the shared and unique core genomes of *H. somni*, the two major genotypes of *M. haemolytica*, and *P. multocida*. OMP beta-barrel predictions were analyzed by BOMP and Boctopus2.

the EL domains (File S5¹). The TPR repeat-containing protein has a consensus sequence of 605 amino acids long, with 185 amino acids predicted in the EL domains (File S5¹).

One gene encoding a predicted OMP was identified in the shared core genome of *M. haemolytica* and *H. somni*, but not in *P. multocida*, an enterobactin receptor FetA. The consensus sequence of the FetA protein was 709 amino acids long, with 304 amino acids predicted in the EL domains. This FetA protein had a minimum of 83% overall similarity between *M. haemolytica* and *H. somni*, and 70% similarity in the EL domains (Table 1; File S6¹).

At the single-species level of specificity, *P. multocida* strains had nine genes encoding predicted OMPs that were not detected in *M. haemolytica* and *H. somni*: a phospholipase A, two autotransporter outer membrane beta-barrel domain-containing proteins, an OmpV family protein, an exo-alpha-sialidase, an uncharacterized OMP, an OMP P2, and two TonB-dependent receptor proteins (Files S3 and S4¹). Regarding *H. somni*, four genes encoding predicted OMPs were identified that were not detected in *M. haemolytica* or *P. multocida*: a porin family protein, OmpP1, a ligand-gated channel protein, and an uncharacterized

Table 1. Predicted outer membrane proteins (OMPs) and minimum similarity (BLOSUM62) of full-length protein and extracellular loop regions.

Gene	<i>Histophilus somni</i>	<i>Mannheimia haemolytica</i> genotype 1	<i>Mannheimia haemolytica</i> genotype 2	<i>Pasteurella multocida</i>	Full length OMP minimum similarity (%)	OMP extracellular loop minimum similarity (%)
Hemolysin activator	X			X	85.9	80.7
Beta 1,4-xylosidase		X	X		100	100
DUF560		X	X		82.5	89.6
FhuA		X	X		99.8	99.2
FhuE		X	X		99.6	98.9
HemeC		X	X		93.8	79.5
HuxB		X	X		99.6	98.6
Maltoporin		X	X		99.2	99.4
OmpP5		X	X		93.3	87.8
Porin		X	X		100	100
S8 serine protease		X	X		94.3	94
Serine protease		X	X		100	100
TBDR 1		X	X		99.4	99.7
TBDR 2		X	X		98.9	99.1
TBDR 3		X	X		100	100
S6 IgA peptidase			X		61.5	81.6
YadA			X		72.4	100
Exo-alpha-sialidase		X	X	X	83.4	100
TBDR		X	X	X	66.8	63
TPR		X	X	X	70.6	61
OmpW	X		X	X	68.4	56.6
FetA	X	X	X		83	70
Hypothetical protein	X				81.2	67.8
Ligand-gated channel	X				94.4	86.9
OmpP1	X				99.5	97.8
Porin	X				93.3	94
Autotransporter 1				X	94.5	92.7
Autotransporter 2				X	98.8	96.4
Exo-alpha-sialidase				X	90.6	55.9
Outer membrane protein				X	76.6	59.7
OmpP2				X	68	46.4
OmpV				X	99.2	96.7
Phospholipase A				X	100	100
TBDR 1				X	99.4	98.2
TBDR 2				X	99.3	98.1
BamA	X	X	X	X	84	71.7
LptD	X	X	X	X	83.5	81.9
TBDR	X	X	X	X	63.4	57.3

hypothetical protein (Files S3 and S4¹). For *M. haemolytica*, 14 genes were identified that were predicted to encode OMPs that were not detected in *P. multocida* and *H. somni*: three TonB-dependent receptor proteins, a heme/hemopexin utilization protein C, a heme/hemopexin transporter protein HuxB, a ferrichrome porin FhuA, a ferricrhodotorulic acid/ferriccoprogen receptor FhuE, a Beta-1,4-xylosidase, an S8 serine peptidase, a porin family protein, a DUF560 containing protein, a Maltoporin-1, a serine protease, and OmpP5 (Files S3 and S4¹). For a full list of these proteins, as well as their minimum similarity within a species, see Table 1.

Discussion

The bovine nasopharynx supports a diverse microbial community that can include commensal bacteria as well

as pathogens (Amat et al. 2019). Identifying OMPs that are specific to particular pathogenic species or that are shared between multiple pathogens is an important step towards developing treatment strategies that can target pathogenic species without disrupting the commensal microbiome. Here, we identified the shared and unique core genomes of *H. somni*, *P. multocida*, and both major genotypes of *M. haemolytica* and identified predicted OMPs that are unique or shared between all combinations of these species.

Previously, a peptidase S6, porin, ligand-gated channel protein, and three trimeric autotransporter adhesins (B, D, and G) were found to be specific to genotype 2 *M. haemolytica* versus genotype 1 (Clawson et al. 2020). The peptidase S6, ligand-gated channel, and adhesin G

OMPs identified in that study were annotated as S6-IgA-peptidase, HemeC, and YadA family protein, respectively, by DFAST in this study. Both the S6-IgA peptidase and YadA family protein were genotype 2 *M. haemolytica* specific (Fig. 1B).

DFAST annotation of the HemeC gene reported previously as a ligand-gated channel pseudogene in genotype 1 *M. haemolytica* assigned an alternate start codon in genotype 1 strains slightly downstream of the original NCBI annotated start codon. Thus, the gene was not annotated as a pseudogene by DFAST, and further analysis is needed to determine whether a functional protein is expressed. The porin previously described as genotype 2 *M. haemolytica* specific did not meet the 95% cutoff to be included in the core genome; as noted previously, not all genotype 2 strains carry this gene (Clawson et al. 2020).

Adhesin B of genotype 2 *M. haemolytica* is split into two genes (B1 and B2) in genotype 1 strains (Clawson et al. 2020). In this study, genotype 1 *M. haemolytica* B2 adhesin was matched to the full-length adhesin B of genotype 2 strains, as the two regions share high homology (Clawson et al. 2020). However, the genotype 1 B2 adhesin isoform is only 67% of the amino acid size of the predominant adhesin B isoform of genotype 2 strains. DFAST annotated the genotype 1 *M. haemolytica* version of adhesin D with an alternate start codon that is downstream of the original start codon and multiple stop codons annotated for the gene by NCBI. However, this genotype 1 *M. haemolytica* version of adhesin D is only 62% of the amino acid length of the predominant adhesin D isoform of genotype 2 strains. Thus, genotype 1 *M. haemolytica* adhesin B and D isoforms remained markedly different from those of genotype 2 strains in the analyses performed in this study. None of the six OMPs previously found to be specific to genotype 2 *M. haemolytica* were detected in the genomes of *H. somni* or *P. multocida*.

The bovine lung is an iron-poor environment; thus, the acquisition and utilization of iron is very important. Many siderophores canonically associated with iron uptake in pathogenic bacteria are apparently absent in *M. haemolytica* (Reissbrodt et al. 1994), but several proteins involved in iron uptake have been identified (Roehrig et al. 2007). Several of the proteins identified in the outer membrane core of the *Pasteurellaceae* species in this study, such as the TonB-dependent receptors, are likely involved in iron binding and uptake (Kingsley et al. 1996; Reissbrodt et al. 1994). The pervasiveness and diversity of these proteins, both across and within species, indicates the importance of iron uptake as a limiting factor within the nasopharyngeal niche and makes these proteins potential targets for host immune responses.

LptD is a protein involved in the assembly of lipopolysaccharides (LPS) in the outer membrane. LPS is an endotoxin and a general virulence attribute of gram-negative bacteria, including *Pasteurellaceae* species (Harper and Boyce 2017). In other gram-negative pathogens, LptD has been

shown to be an immunogenic target (Zha et al. 2016). Thus, its presence in the shared core genome of the three species examined in this study and the high similarities of its predicted EL domains make it a promising candidate for the treatment of BRD and warrants further study. BamA is a part of the outer membrane protein assembly complex (Albrecht et al. 2014). In other gram-negative pathogens, BamA has been shown to be an immunogenic target (Guan et al. 2016; Luther et al. 2019).

The *OmpW* gene identified in this study is present in the core genomes of *H. somni*, *P. multocida*, and genotype 2 *M. haemolytica*, but absent in genotype 1 *M. haemolytica*. Thus, *OmpW* could be targeted as an intervention strategy against the three *Pasteurellaceae* pathogens without disturbing the more commensal *M. haemolytica* genotype 1 strains and disrupting the microbial community (Harper and Boyce 2017). The *OmpW* protein of *P. multocida* and other gram-negative pathogens has been shown to be immunogenic (Huang et al. 2015; Mohanty et al. 2019).

The OMPs identified in this study may be available for antibody recognition. Within the shared outer membrane core genome of the three *Pasteurellaceae* species, there are regions of the predicted EL domains that are highly conserved among all three species. This conservation indicates that a host immune response to one or more of the species-wide, conserved OMPs identified in this study could potentially provide cross-immunity to homologous proteins in the other *Pasteurellaceae* species. This is an area of future investigation. While intra-species cross-protection at the strain or serotype level has been observed for *M. haemolytica* (Confer and Ayalew 2018) and *P. multocida* (Rimler 1996), evidence of cross-reactivity between the species is more limited. However, it has been observed between *P. multocida* and *M. haemolytica* antigens on outer membrane vesicles in mice (Roier et al. 2013). Additionally, potassium thiocyanate extracts of *Pasteurella haemolytica* have protected mice from challenge with *P. multocida* (Mukkur 1977), and *Haemophilus somni* rabbit immune sera have been shown to agglutinate both *P. multocida* and *P. haemolytica* (Canto et al. 1983). Of note, *H. somni* and *P. haemolytica* have been reclassified as *H. somni* and *M. haemolytica*, respectively (Angen et al. 1999, 2003).

It would be beneficial for the approach used in this study to be applied to deeper populations of *H. somni* and *P. multocida*, as was previously done for *M. haemolytica*. We used bovine-isolated strain genomes available in GenBank for *P. multocida* and *H. somni*, which probably did not adequately represent the strains populations present in cattle either in the United States or elsewhere throughout the world. It would also be beneficial for future studies to include *H. somni* and *P. multocida* strains from cattle that did not have or go on to develop BRD. This could potentially provide a level of resolution required to identify OMPs and other genetic determinants that are shared between pathogenic strains and those that have evolved

into a more constrained commensal biological state. It could also lead to the identification of OMPs that are conserved across some or all three of the *Pasteurellaceae* species and that associate specifically with BRD pathogenesis.

Declarations

Competing interests

The authors declare that there are no competing interests.

Author contributions

E.L.W. contributed to project conceptualization, methodology, formal analyses, and writing of the manuscript. M.L.C. contributed to project conceptualization, supervision, reviewing, and editing of the manuscript.

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Data availability

The authors confirm that all supporting data, code, and protocols have been provided within the article or through supplementary data files and a GitHub link. All sequences used in this study are publicly available in GenBank.

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The use of product and company names is necessary to accurately report the methods and results; however, the United States Department of Agriculture (USDA) neither guarantees nor warrants the standard of the products, and the use of names by the USDA implies no approval of the product to the exclusion of others that may also be suitable. The USDA is an equal opportunity provider and employer.

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