Genomic differentiation of *Mannheimia haemolytica* genotypes

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Abstract

Mannheimia haemolytica is an opportunistic bacterial pathogen associated with the economically costly bovine respiratory disease. Two genotypes have been described, of which genotype 2 is more strongly associated with disease. Several previous studies have investigated the genomic differences between the genotypes and/or the major serotypes (1, 2 and 6) of *M. haemolytica*, however we still lack a clear basis for the greater disease association of genotype 2 (serotypes 1 and 6) and demonstrations of phenotypic differences are scarce. This work builds upon previous investigations to identify genes that differentiate the two genotypes with a particular focus on genes that may play a role in virulence and fitness in the respiratory tract microbiome. We identified 422 genotype differentiating genes in a collection of 208 *M. haemolytica* genomes (62 genotype 1, 146 genotype 2). Genotype differentiating genes including genotype-associated variants of a TonB-dependent siderophore receptor homolog, transferrin binding protein B, leukotoxin A, and IgA1 proteases. We also identified a genotype 1 associated lytic transglycosylase, and a genotype 2 specific highly immunogenic outer membrane lipoprotein. These results expand our knowledge of what differentiates the genotypes of *M. haemolytica* and provides information that can be utilized to inform diagnostic strategies. The results also highlight the relative lack of information regarding *M. haemolytica* from sheep and goats and geographic regions other than Canada and the United States.

Introduction

Mannheimia haemolytica is a gram-negative opportunist bacterial pathogen associated with bovine respiratory disease (BRD) [1,2]. The beef industry is estimated to lose more than $3

billion per year globally due to BRD [3,4]. Two genotypes of *M. haemolytica* have been described based on whole genome sequencing and single-nucleotide polymorphism (SNP) analysis and multiple serotypes have also been defined [5,6]. The three most common serotypes (1, 2 &, 6) have been found to correspond to genotypes with serotypes 1 & 6 corresponding to genotype 2 (G2) and serotype 2 corresponding to genotype 1 (G1) [1,5,7,8]. Genotype 2 has been more frequently associated with clinical cases of BRD, however the genetic and phenotypic basis for this apparent association is unclear [1,9–14].

Differences in disease association between strains of the same species of bacteria is relatively common. For example, commensal and pathogenic strains of *E. coli* differ in their possession of virulence factors such as adhesins, toxins such as the Shiga toxin, and other effectors such as the cytotoxic necrotizing factors [15]. Other species known to exhibit differences in disease outcome based on strain diversity include *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Salmonella enterica* [16]. In addition to virulence factors, adaptations that confer increased fitness, such as greater ability to acquire iron and other nutrients, can contribute to disease association by allowing a pathogenic strain to outcompete non-pathogenic strain(s) present in the same environment [17].

Previous research into the virulence factors of *M. haemolytica* has focused on leukotoxin, lipopolysaccharide, adhesins and various proteases [11,18–20]. The response of *M. haemolytica* to iron limitation has also been studied, however most previous studies have only examined the virulence factors and metabolism of serotype 1 isolates [21]. Serotype associated differences in leukotoxin have been demonstrated, including differing degrees of cytoxic activity [1,22,23]. Serotype 1 has also been demonstrated to invade differentiated bovine bronchial epithelial cells

in vitro while serotype 2 was unable to, suggesting that serotype 1 (included in G2) likely possesses virulence factors facilitating this invasion that serotype 2 (included in G1) lacks [24]. Expanding on these previous observations and examining them through a genotype rather than serotype lens will help elucidate the clinical relevance of genotype in *M. haemolytica*. Also, as diagnostic laboratories continue to move toward molecular methods and away from techniques like serology for many pathogens, understanding the association of genotype with pathogenicity is increasingly important.

Several previous studies have investigated the phenotypic and genetic differences between the two genotypes of *M. haemolytica*. Phenotypic differences described to date are distinct matrix-assisted laser desorption/ionization - time of flight mass spectrometry (MALDI-TOF) profiles and colony morphology [25,26]. A pangenome analysis of 69 *M. haemolytica* genomes identified 112 G1 specific, and 179 G2 specific genes including seven G2 specific predicted outer membrane protein encoding genes that were hypothesized to play a role in the greater disease association of genotype 2 [27]. Another study examined a collection of 11 *M. haemolytica* genomes for potential differences in virulence factors between the serotypes 1, 2 and 6 [28]. The authors reported more prophages in serotypes 1 and 6 than in serotype 2, which could influence virulence due to the presence of virulence factors in these prophages. Self-targeting CRISPR spacer sequences were detected in serotypes 1 and 6, which may enhance immune evasion or adhesion during infection by modulating sialic acid expression on the cells surface. ARG-carrying ICEs were more prevalent in serotypes 1 and 6 than serotype 2, which could enhance survival of antibiotic exposure [28]. In our own previous investigation of macrolide resistance in *M. haemolytica*, we found that genotype was not associated with differences in phenotypic resistance after accounting for the prevalence of ICEs, however we did

identify a resistance-associated single nucleotide polymorphism in the L22 ribosomal protein that was exclusive to G2 [29].

In addition to the demonstration of genotype differentiation by MALDI-TOF [25], a loop-mediated amplification (LAMP) assay has also been developed to differentiate the *M. haemolytica* genotypes [30]. The rationale for application of such genotype specific diagnostics beyond research studies and the potential value of their adoption in routine diagnostic investigations depends on a better understanding of the roles of *M. haemolytica* genotypes in bovine respiratory disease. In the current study, we take advantage of the greater number of *M. haemolytica* whole genome sequences now available to identify genetic differences between the two genotypes. In our examination of >200 *M. haemolytica* genomes, particular attention was given to genotype differentiating genes that are predicted to encode virulence factors and those implicated in processes that affect fitness in the respiratory tract such as iron acquisition and carbohydrate utilization.

Materials & Methods

Genome sequences

M. haemolytica whole genome sequences defined as complete by NCBI were downloaded from the NCBI Genome database on March 25, 2024, using the NCBI Datasets command line tools [31]. To avoid duplication all .gca files that had a matching .gcf file were removed. The remaining genome sequences were genotyped using an *in silico* PCR assay in Unipro UGENe v45.1 and primers from a genotype specific LAMP assay [30,32]. Primers from the LAMP assay included a pair targeting G1 specific adhesin pseudogene B1, G2 adhesin G

(CP017538.1 locus tag: BG586_06285; Forward: 5'-GCA CAT TAA AAT ATA GCA GCT TTG-3'; Reverse: 5'-CTA AGC CAG AGT GAT CCG-3'), and leukotoxin D (*lktD*) (CP005972.1 locus tag: F382_07400; Forward: 5'-ACT GAA AA TTC ACA CTA TAG GTG-3'; Reverse: 5'-GCT AAT ATG TTT AAT TCG ACC AGT T-3'). The *lktD* primers were used to confirm identity of genomes as *M. haemolytica*. In initial testing G1 specific primers failed to produce any predicted products in our G1 reference strain and so were discarded. New G1 specific primers targeting the G1 specific adhesin pseudogene B1 were developed using Primer 3 v 4.1.0 (CP017495.1 locus tag BG548_01640; Forward: 5'-ACT AAT CTG AAG AGC GGC GT-3'; Reverse: 5'-TTG GTG GTT GCT GCT TGT AC-3') [33–35]. Successfully genotyped NCBI genomes were combined with a set of 99 genome sequences previously generated by our lab (BioProject: PRJNA1088094)(30) and reference genomes (G1 = CP017518; G2 = CP017519) for both genotypes to generate the final collection of 209 genomes (62 G1, 147 G2) (S1 Table).

Pangenome construction, annotation, and classification

The pangenome of 209 genomes was constructed using PanTools v 4.3.2 with FASTA files, gff3 annotation files, and genotype designation as inputs [36–38]. Genomes were assigned a new unique identifier based on their input order during pangenome construction (S1 Table).

Pantools groups protein sequences from the input genomes into homology groups based on their similarity. Homology groups also contain the nucleotide sequences encoding the constituent protein sequence(s). Homology groups were generated using the group command with the relaxation parameter set to 2. The relaxation parameter is a combination of four sub-parameters (intersection rate, similarity threshold, mcl inflation, and contrast) where a lower number represents a stricter set of thresholds. Given the large number of genomes and

anticipated sequence variation, we set the relaxation parameter to 2 to reduce the chances of minor differences preventing similar proteins from being grouped together.

Genome metrics such as genome size, GC content, number of genes, etc. were determined using the metrics command in PanTools [36]. One genome (Genome 130/38241 S-19) was approximately twice as large as all others and appeared to represent a chimeric assembly. This genome was excluded from all subsequent analyses reducing the genome collection to a total of 208 (62 G1, 146 G2).

Homology groups were classified as core (present in ≥ 90%, 187/208 genomes), unique (present in only one single genome), or accessory (present in 2-186 genomes). Homology groups were additionally classified as either shared (present in ≥ 90% genomes of one genotype), exclusive (solely present in genomes of one genotype), or specific (present in ≥ 90% genomes of one genotype, and no genomes of the other genotype) [36]. To allow for instances in which a homology group was strongly associated with one genotype yet still present in small numbers of the other genotype, all homology groups not initially classified as core or unique were further manually examined. Homology groups that were present in ≥ 95% (60/62 G1, 138/146 G2) of one genotype while also being present in ≤ 5% (3/62 G1, 7/146 G2) of the other genotype were classified as being genotype associated.

Genomes were functionally annotated using InterProScan v 5.64-96.0 with SignalP v 4.1 database integrated [39–41]. Biosynthetic gene cluster annotations were generated using antiSMASH 7.1.0 with default parameters [42]. InterPro and antiSMASH annotations were added to the pangenome, with InterProScan annotations including InterPro, Pfam, GO, and SignalP annotations [36].

Multiple sequence alignments of constituent nucleotide sequences were generated for all homology groups in the pangenome with MAFFT and consensus sequences from the alignments were generated to represent the genotype specific and associated homology groups [36,43]. Consensus sequences were used as queries for BLASTx searches using the bacterial/archaeal translation code against the NCBI refseq_select database [44–48]. All other parameters were kept as default. Searches were performed March 20-25, 2025. The top hit for each search was manually recorded to provide further evidence of the predicted function, facilitate human interpretation, and identify functions that may not have been annotated by any of the other approaches. The entire pangenome database is available in S1 Dataset [43].

The nucleotide sequences of several previously characterized genes upregulated in response to iron limitation, and previously identified proteases were used as BLAST queries against the pangenome using the blast command in PanTools to determine if these previously characterized genes were present in our dataset [18,21,36]. Both alignment threshold and minimum identity were set to 90. Query nucleotide sequences are available S2 Table.

Tet(H) gene abundance

Tet(H) genes were used as a proxy for the presence of integrative and conjugative elements (ICE) due to the strong correlation between presence of tet(H) and presence of ICE [12,49]. The reference tet(H) nucleotide sequence (Accession Number: Y15510.1 (nucleotides 2099-3301)) was downloaded from the comprehensive antibiotic resistance database (CARD) on July 26, 2024, and used as nucleotide BLAST query against the pangenome database using the inbuilt PanTools blast command. Minimum identity and alignment threshold were both set to 90%.

Leukotoxin operons

Reference nucleotide sequences for each of the component genes of the *M. haemolytica* leukotoxin operon (*lktCABD*) were downloaded from GenBank (AF314508.2) and used as queries against the pangenome BLAST database with percent identity and alignment threshold both set to 90%. Consensus nucleotide sequences of G1 and G2 versions of all leukotoxin operon genes were extracted and translated in Geneious 2024.0.5. Predicted protein sequences for the G1 and G2 versions of all leukotoxin operon genes were aligned against one another in Geneious 2024.0.5.

Annotation of carbohydrate active enzymes

Carbohydrate active enzymes (CAZymes) were annotated using a local installation of dbCAN v 4.1.1 software with integrated SignalP v 4.1 database and DIAMOND, HMMER, and HMMER dbcan_sub prediction algorithms [50]. Annotation was performed on each genome sequence individually using a custom BASH script [43]. dbCAN annotations that had supporting annotations from at least three tools, and contained a predicted signal peptide were selected for further analysis.

Protein structure prediction and visualization

Protein sequences were submitted to the AlphaFold Server for structural prediction [51]. Models with the highest-ranking score were downloaded and visualized in PyMol version 2.5.2 (Schrödinger, LLC).

Statistics

A Fisher's exact test and odds ratio comparison was performed to determine if the prevalence of tet(H) was different between genotypes. Both tests were performed in Microsoft Excel for Microsoft 365 using Real Statistics Resource Pack. A p-value ≤ 0.05 was considered significant.

A one-way ANOVA (Kruskal-Wallis), and post-hoc pairwise Mann-Whitney t-tests were performed in GraphPad PRISM v 10.4.1 to determine if there was a difference in genome size between G1 and G2 with or without ICE. A p-value ≤ 0.05 was considered significant.

Results & Discussion

Description of genome collection

M. haemolytica genomes designated as complete were retrieved from NCBI and after removal of duplicates, 121 genomes were available for further analysis. *In silico* PCR with modified primers from a previously published genotyping assay resulted in the expected amplicons for 108/121 genomes. No *in silico* genotyping PCR products were obtained for 13 genomes and so they were removed from further analyses leaving 108 genomes.

Three of the 13 isolates that could not be genotyped also failed to produce any predicted product for the *lktD in silico* PCR. Two of these genomes have since been removed by the RefSeq curation team for either containing too many frameshift mutations (GCF_900474405) or failing to pass a completeness check (GCF_023516295). All three genome sequences that failed to produce an *lktD* product were from sheep isolates (NCTC 10643, NCTC 10609) or from

culture collections from the 1960s (ATCC 29701). Of the other ten genomes that were not genotyped by *in silico* PCR, four were from a German study examining macrolide resistance in *M. haemolytica* [49], and three were from sheep isolates collected in 1951 or 1968 and sequenced as part of the United States Department of Agriculture (USDA) *M. haemolytica* sequencing project (BioProject: PRJNA172854). The last three isolates were all generated from non-nasopharyngeal cattle samples as part of either the USDA sequencing project (GCF_000819525, GCF_023516255) or a German study (GCF_020971705) and had a CheckM completeness <92.5%.

Given the variety of the sources of the isolates we were unable to genotype it is difficult to determine a general cause, however the assay developed may struggle with genomes derived from ovine isolates, non-North American isolates, and non-nasopharyngeal isolates. Whether the failure of the *in silico* assay to genotype isolates from these origins represents true genotypic differences is unclear and requires further investigation. Difficulties in genotyping these isolates likely reflects the lack of diversity in available *M. haemolytica* genomes. For example, of the 158 annotated complete *M. haemolytica* genome sequences available in the NCBI genome database as of April 4, 2025, only 22 were from sources other than collections from the USDA or the University of Saskatchewan.

The successfully genotyped genomes were combined with 99 genomes from our previous work (BioProject Accession PRJNA1088094) and reference genomes for G1 and G2 as described in the Methods. One additional genome was removed following discovery that it was likely a chimera, leaving a final dataset 208 *M. haemolytica* whole genomes with 62 G1 and 146 G2 representatives (S1 Table). Most genomes were from isolates originating from feedlot cattle in either Canada or the United States between 2003 and 2021. The lack of geographic diversity

and absence of genomes from ovine isolates means that the results presented in this work may not represent the characteristics of *M. haemolytica* genomes outside of the Canadian and American feedlot industries.

Genome properties & Tet(H) abundance

The two genotypes were compared to one another on a broad set of genome metrics, such as genome size, GC content, etc., to determine if there were any high-level differences in the genomic structure and content of the two genotypes. G2 genomes were larger, had more predicted protein-coding sequences, and had slightly lower GC content than G1 genomes. No differences were identified in the number of tRNA or rRNA genes or the proportion of the genome covered by genes (Table 1).

Table 1. Comparison of genome features of G1 and G2. Values are the average ± standard deviation. P-value ≤ 0.05 was considered significant.

Metric	**Genotype 1 (n = 62)**	**Genotype 2 (n = 146)**	**t-test *P*-value**
Genome Size (Mb)	2.592 ± 0.045	2.719 ± 0.048	<2e-16
GC Content (%)	41.14 ± 0.026	41.04 ± 0.031	<2e-16
Gene Count	2,561.7 ± 100.9	2,798.9 ± 116.4	<2e-16
Genome Covered by Genes (%)	88.17 ± 1.5	88.73 ± 1.2	0.0097
tRNA Count	64.31 ± 1.70	64.17 ± 2.04	0.61
rRNA Count	18.98 ± 4.3	19.68 ± 2.4	0.24

The larger size of G2 genomes may be attributable to a greater prevalence of integrative and conjugative elements (ICEs), large (~20 kb to >500 kb) mobile genetic elements that are known to be associated with antimicrobial resistance and are more frequently identified in G2 genomes [5,29]. To investigate this possibility, we used the proxy measure of the presence of

tetracycline resistance gene tet(H) due to its strong association with ICEs [12,49]. Tet(H) was identified significantly more frequently in G2 (67/146, 45.9%) than G1 (1/62, 1.6%) (Fisher's *p*-value = 4.39×10^{-12} Odds Ratio: 0.02 ± 1.02), however, G2 genomes were larger than G1 regardless of the presence of ICE (Figure 1). The larger size of G2 genomes regardless of presence of ICE suggests that G2 genomes carry a wider repertoire of genes that may allow them to be more adaptable to a wider variety of environments than the smaller G1 genomes. Considering average bacterial gene sizes, the difference in the median genome size of G2 without ICE and G1 (~100 kb) could represent dozens of G2-specific genes. Additionally, the greater prevalence of ICEs in G2 suggests that this more disease associated genotype is also more likely to possess antimicrobial resistance genes.

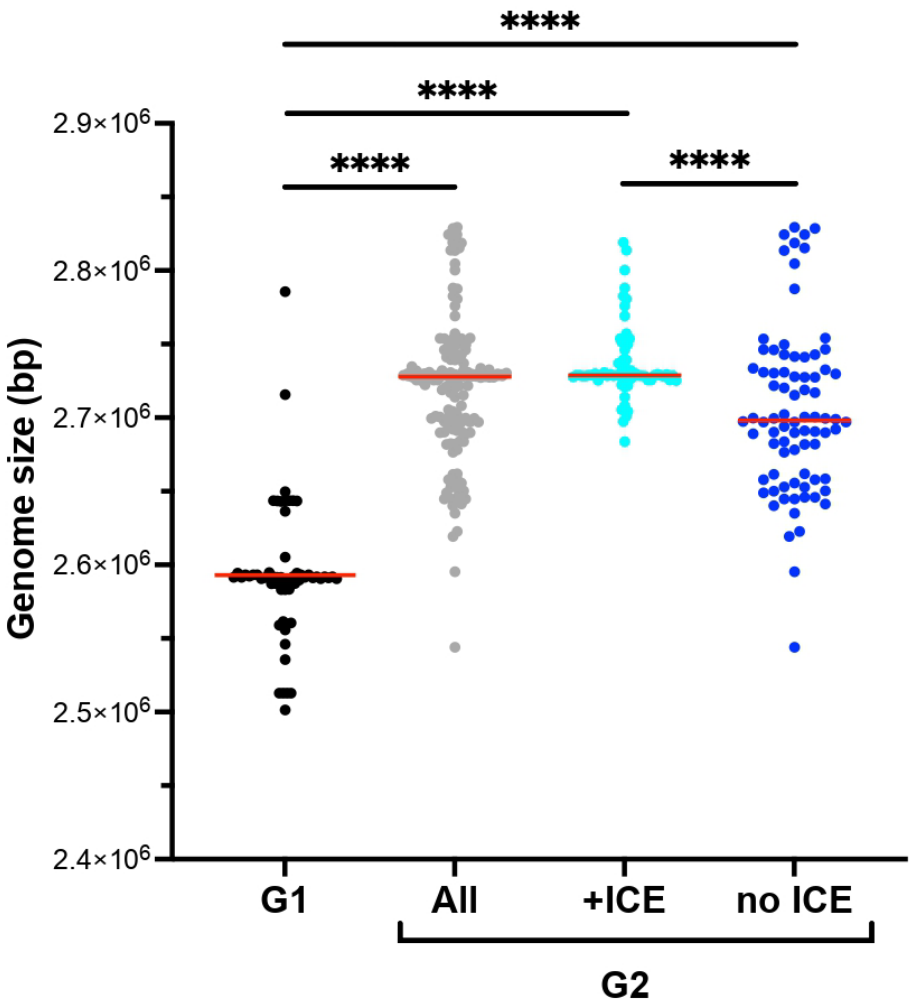

Figure 1. Genome sizes of Genotype 1 (G1), Genotype 2 (G2), G2 possessing integrative and conjugative elements (ICEs), and G2 without ICEs (no ICE). Red bars indicate mean. Significant differences ($P < 0.0001$) are indicated (one way ANOVA (Kruskal-Wallis), post-hoc pairwise Mann-Whitney t-tests).

Pangenome analysis

The pangenome calculation resulted in the identification of "homology groups": clusters of sequences from input genomes that exceed a given similarity threshold. We then examined the distribution of homology groups across all *M. haemolytica* genomes to identify those that are core, unique, accessory, shared, genotype specific or genotype associated as defined in the methods. A total of 4,038 homology groups were identified of which 2,007 (49.7%) were core,

1,473 (36.48%) were accessory, and 558 (13.82%) were unique. Subsequent analyses focused on accessory homology groups as these groups were most likely to differ in prevalence between the two genotypes. We identified 280 G1 exclusive, 2,169 G1 shared, and 135 G1 specific homology groups and 790 G2 exclusive, 2,342 G2 shared, and 233 G2 specific homology groups. To focus our attention on the homology groups most likely to define and differentiate genotypes, the shared or exclusive categories were further examined to identify those homology groups that were present in ≥ 95% of one genotype and ≤ 5% of the other genotype, resulting in 21 G1 associated, and 33 G2 associated homology groups (S3-S4 Tables). Identification of genotype specific and associated genes within this relatively large genome collection further reinforces previous findings that the two genotypes carry distinct sets of genes [27]. A substantial proportion of the proteins encoded by the specific and associated homology groups were mobile genetic element (MGE) associated (transposases, toxin-antitoxin systems, prophage associated proteins, etc.) or hypothetical or unknown function proteins (Figure 2).

The larger number of G2 specific or associated homology groups compared to G1 specific or associated groups is consistent with the larger size of G2 genomes (Figure 1) and the larger number of specific and associated homology groups encoding MGE-associated proteins in G2 suggests that these elements are more common in G2 than G1, consistent with previous observations [9,12,27,28,49]. The number of hypothetical/unknown proteins is also unsurprising as ~22% of all domain families in Pfam v32.0 are defined as “domains of unknown function” and a recent examination of >30,000 bacterial genomes and metagenomes showed that 40-60% of genes are of unknown function [52,53]. Homology groups predicted to encode unknown/hypothetical proteins, and MGE-associated proteins were excluded from further analysis in this study but certainly represent an opportunity for future investigation.

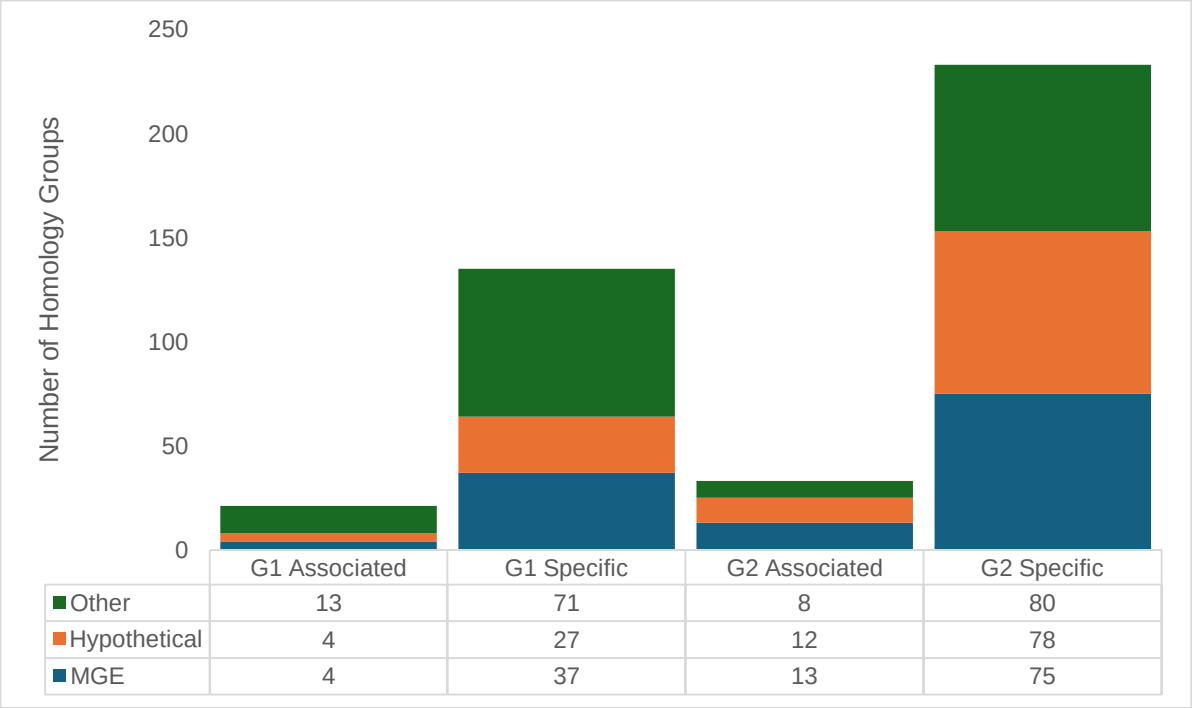

Figure 2. Genotype specific/associated homology groups. Mobile Genetic Element (MGE) associated proteins included transposases, plasmid toxin-antitoxin systems, prophage associated proteins, etc. Hypothetical proteins included those annotated as hypothetical proteins as well as proteins annotated with a domain of unknown function (DUF) which did not appear to be MGE-associated and did not have any putative functions. Proteins that did not fall into either of the two preceding categories were placed into the Other category. G1 = Genotype 1 G2 = Genotype 2.

Based on inspection of the complete inventory of pangenome homology groups (S3-4 Tables), we identified a subset of specific/associated homology groups that may be associated with reported differences between disease association of G1 and G2 (Table 2).

Table 2. Genotype association and predicted functions of selected homology groups. Annotation notes summarize the functional annotations from InterProScan for each homology group. BLASTX search used consensus nucleotide sequence of the homology group as query against the refseq_select database. Reported top BLASTx hits were from *M. haemolytica* unless otherwise stated.

Homolog group	Genotype association	InterProScan Annotation	BLASTx hit (top)	Predicted Function
2042480	G2 specific	TonB-dependent receptor.	TonB-dependent siderophore receptor	Iron Acquisition
2042481	G1 associated	TonB dependent receptor protein.	TonB-dependent siderophore receptor	Iron Acqusition
2041941	G2 specific	TonB Dependent Receptor	TonB-dependent receptor plug-domain containing protein	Iron Acquisition
2944545	G2 specific	Transferrin Binding	Transferrin-binding protein-like solute	Iron Acquisition

		Protein (TpbB)	binding protein	
2040773	G1 specific	Transferrin Binding Protein	Transferrin-binding protein-like solute binding protein. *Mannheimia pernigra*	Iron acquisition
2042189	G2 specific	Peptidase S6/Autotransporter/IgA1 protease	S6 Family Peptidase	Host immune evasion
2040817	G1 associated	Peptidase/Pectate Lyase/IgA1 Protease	S6 family peptidase	Host immune evasion
2044609	G2 specific	N/A	Outer membrane lipoprotein PlpE.	Highly immunogenic outer membrane protein
2042729	G1 associated	MltB-like Transglycosylase	Lytic murein transglycosylase. *Mannheimia glucosida*	Cell wall remodeling/recycling

Homology groups 2042480 (G2 specific) and 2042481 (G1 associated) were both annotated as encoding a TonB-dependent siderophore receptor. The consensus amino acid sequences of these homology groups were 84.4% identical, and both contained consensus TonB-dependent transporter (TBDT) motifs. In addition, the predicted 3D structures demonstrated the canonical 22 β-strand barrel structure with an N-terminal plug domain characteristic of TBDTs (Figure 3A). The amino acid sequence differences between the G1 and G2 sequences were concentrated in the extracellular loops responsible for substrate binding, suggesting that these two proteins may differ in their ability to bind their substrate [54,55] or they may bind different substrates (Figure 3B). TonB dependent receptors (TBDTs) are transporters often involved in iron acquisition, specifically in the uptake of small, secreted iron scavenging molecules known as siderophores [54,55]. Homology groups 2042480 and 2042481 also produced BLAST hits to a previously characterized siderophore (ferric enterobactin) receptor (*frpB*) homolog that was upregulated in *M. haemolytica* in response to iron limitation [21]. Identification of a putative siderophore receptor in *M. haemolytica* is unexpected as *M. haemolytica* is not known to produce or utilize siderophores and has been demonstrated to be unable to utilize ferric enterobactin so it is likely that the identified TBDTs have some other function besides siderophore uptake [11,21].

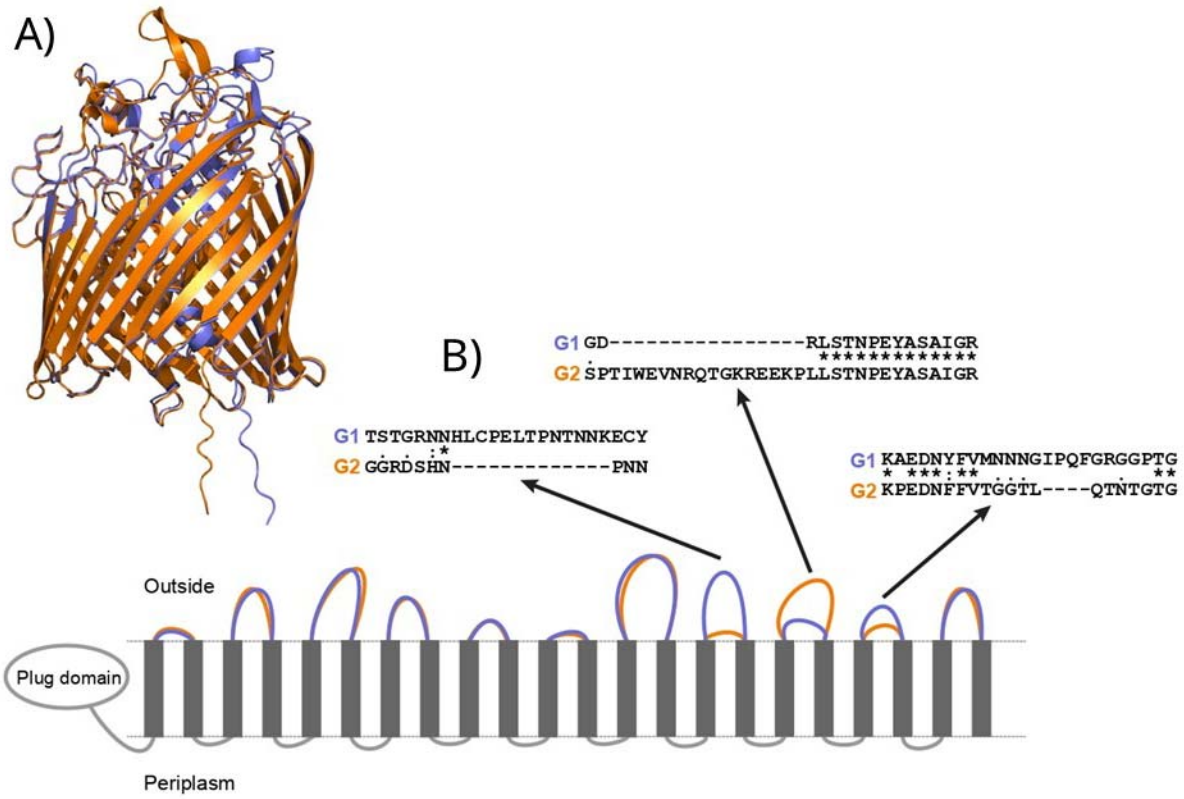

Figure 3. A) Predicted 3D structures of Genotype 1 (G1, Purple) & Genotype 2 (G2, Orange) ferric enterobactin receptor (frpB) homolog proteins. 3D structure conforms to canonical TonB-dependent receptor structures. B) 2D representation of tertiary structure of G1 and G2 frpB homologs. Each grey rectangle represents one of the 22 β-strand sheets, and the size of the extracellular/periplasmic loops are shown. Differences in the amino acid sequence for select extracellular loops are shown in further detail. Difference in extracellular loops may contribute to differences in substrate binding efficiency and/or result in interactions with completely different substrates.

G2 specific homology group 2041941 was also predicted to encode a TBDT, and no similar G1 specific/associated protein was identified and the consensus sequence of 2041941 did not correspond to previously identified genes upregulated in response to iron limitation [21]. The identification of another G2 specific TBDT suggests that G2 may have a greater ability to uptake iron and/or other substrates. TBDT have been implicated in transport of a variety of substrates in

Gram-negative bacteria including siderophores as well as vitamin B_{12} and carbohydrates [55]. Further examination is required to determine the function of this TBDT and whether it provides a competitive advantage to G2.

Other iron acquisition proteins include transferrin binding proteins A and B (*tpbAB*), both of which are found in *M. haemolytica* [11]. Transferrin is an iron-carrying glycoprotein found in mammalian circulatory systems that transports iron throughout the body and serves as a sink for any excess free iron released into the circulatory system [56]. TpbB is a surface lipoprotein that extends outwards from the outer membrane to the extracellular space where it binds to transferrin and brings the transferrin closer to TbpA, a TBDT in the outer membrane that transports the transferrin into the periplasm [56]. To investigate any genotype associations of transferrin binding proteins, previously characterized *tbpA* and *tbpB* nucleotide sequences from *M. haemolytica* were used as BLAST queries against the pangenome database. Core homology group 2044546 contained hits for *tbpA* while *tbpB* hits were solely identified in G2 specific homology group 2044545 with no hits in any G1 genomes. These results suggest that *tbpA* is highly conserved within *M. haemolytica* and likely plays a key role in iron metabolism. In contrast while no BLAST hits were produced to the query *tbpB* nucleotide sequence in G1 genomes, G1 specific homology group 2040773 was predicted to encode for a transferrin binding protein. The amino acid sequences of G1 and G2 variants of the putative tbpB (homology groups 2040773 and 2044545) were compared and found to be 56.5% identical and 81.2% similar. Despite the relatively low similarity of these two proteins, the predicted 3D structures were very similar to one another as well as the crystal structure for the TbpB from *Actinobacillus pneumoniae* (S1 Figure). A difference in the TbpB structure between the genotypes was observed in the N lobe with G2 appearing to have a longer alpha-helix structure in the N-lobe, which is the

region of the protein involved in transferrin binding [56]. Genotype variation of a transferrin binding protein further suggests they may differ in their ability to acquire iron, which may partially explain observed differences in disease association between the two genotypes. The genotype variants of TbpB identified here may also reflect host preferences of the genotypes as TbpB is selective for host transferrin and differences between TbpB proteins has been partially attributed to this host selectivity [56].

IgA proteases are metalloproteases that cleave immunoglobulin A (IgA), a critical component of the mucosal immune system. Two IgA proteases (IgA1 and IgA2) have been previously identified and characterized in a G2 (serotype 1) *M. haemolytica* isolate [18]. We identified a G2 specific IgA protease (homology group 2042189) and a G1 associated IgA protease (2040817) (Table 2). To determine the relationship of these sequences to the previously reported IgA proteases, a BLAST search was performed using previously reported nucleotide sequences for IgA1 and IgA2 as queries against the pangenome database (IgA1:NZ_DS264709 Locus Tag: MHA_RS00165IgA2: NZ_DS264612.1 Locus Tag: MHA_RS09765). At least one BLAST hit was returned for IgA2 in all but five of the included genomes with hits grouped into five homology groups (2041035, 2041036, 2041399, 2041400, and 2043723). Homology groups 2041305 and 2041306 were accessory, while 2041399 and 2041400 were unique suggesting some minor variations are likely present within the pangenome for this IgA2 protease. Homology group 2043723, however, was a core group suggesting that this IgA2 protease is a core gene of *M. haemolytica* and is not genotype differentiating. In contrast, a BLAST hit was returned for IgA1 in 146/209 of the genomes with hits grouped into one of three homology groups (2040817, 2041298, and 2042189). Homology group 2041298 was found in only one genome so was not

examined any further. Group 2040817 is a G1 associated group while 2042189 is a G2 specific group and thus these groups appear to represent genotype variants of an IgA protease.

Comparison of the consensus sequences of the two IgA1-like groups revealed that the nucleotide sequence for 2040817 is 2,281 bp while the sequence for 2042189 is 4,512 bp (94% identical across 2,281 shared positions). The G1 associated consensus sequence is much smaller than the previously reported 1,503 amino acids (4,509 bp) for IgA1 and 1,398 amino acids (4,194 bp) for IgA2 while the G2 specific consensus sequence is slightly larger than both previously reported IgA protease sequences. The smaller size of the G1 sequence suggests that this sequence may represent a truncated protease or difficulties in the gene finding process due to the large size of these genes [18]. The slightly larger size of the G2 specific sequence may similarly be due to gene finding issues or in generating the consensus sequence. While the identified G1 specific sequence may not represent a complete protease sequence, the observation of a G2 specific sequence and possibility of genotype variation remains of interest and warrants further investigation. Should both genotypes have differing IgA protease sequences the potential difference(s) in their ability to evade the host immune system may contribute to differences in disease association.

Pasteurella lipoproteins (Plp) are a group of several immunogenic outer membrane lipoproteins identified in *M. haemolytica* [57]. G2 specific homology group 2044609 was predicted to encode PlpE, which has been investigated as a potential vaccine target for *M. haemolytica* because of its immunogenicity [58–60]. Aside from the immunogenic role of PlpE relatively little is known regarding its function although it is important in complement-mediated killing of *M. haemolytica* cells in bovine hosts. Previous investigations of PlpE sequence diversity across serotypes showed that the serotype 1 and 6 (G2) sequences were highly

conserved whereas serotype 2 (G1) PlpE sequences were extremely heterogenous, however, antibodies raised against serotype 1 PlpE cross reacted with serotype 2 bacteria [61]. No G1 associated or specific *plpE* gene was identified in this study which may be due to the previously observed heterogeneity of this sequence in G1 genomes or potentially due to the relative lack of available G1 genomes compared to G2. The identification of G2 specific PlpE suggests that G2 isolates may induce stronger immune reactions than G1 isolates, however further research is needed to investigate the prevalence of PlpE in G1and the immunogenicity of G1 PlpE compared to G2-derived PlpE.

Homology group 2040817 (G1 specific) was predicted to encode a lytic transglycosylase and is explored in more depth below in the discussion of carbohydrate active enzymes (CAZymes).

Leukotoxin operon

Leukotoxin (Lkt) is a critical virulence factor in *M. haemolytica* and is produced by both genotypes (all serotypes) [62]. Lkt is encoded in an operon consisting of four genes *(lktCABD*). *lktA* encodes the protoxin, *lktC* encodes the acylating enzyme essential for post-translational modification and activation of the protoxin, and the products of *lktB* and *lktD* are involved in secretion of the toxin [23,63]. *lktCABD* genes were identified and the predicted protein sequences for the Lkt operon genes from G1 and G2 were aligned (Figure 4A). Within the Lkt operon the predicted proteins of the *lktC, lktB* and *lktD* genes were 95.2%, 99.6% and 100% identical, respectively, suggesting these genes are under conservation pressure due to their roles in regulating the secretion and function of Lkt [63,64]. In contrast, LktA protein sequences were only 89% identical. Differences in the predicted LktA proteins of G1 and G2 were concentrated

in the N-terminal and C-terminal regions of the protein (Figure 4B). In leukotoxins the N-terminus is involved in pore-forming activities while the C-terminus is involved in export and host immune system recognition [63]. As such any differences in the N-terminal and/or C-terminal regions may potentially influence the activities of the Lkt and thus may result in virulence differences between the two genotypes.

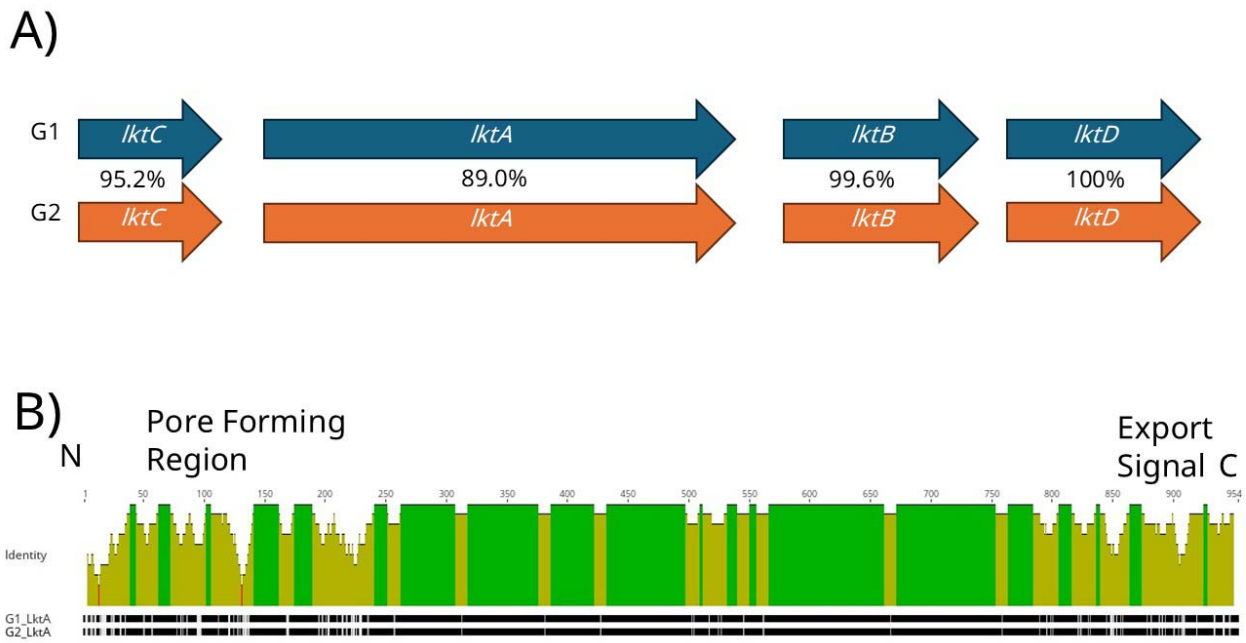

Figure 4. A) Percent identity between the predicted protein sequence of each of the four genes in the leukotoxin operon in G1 and G2. B) Sequence alignment of G1 and G2 LktA amino acid sequence with a sliding window size of 10. A larger peak indicates a greater number of matching amino acids within the window for that position.

In a previous study, Davies *et al*. examined the sequence diversity of leukotoxin in 31 *M. haemolytica*, 6 *Mannheimia glucosida*, and 4 *Pasteurella* [*Bibersteinia*] *trehalosi* isolates identified eight *M. haemolytica* allelic variants of *lktA* [23]. Five of the alleles (A6-A10) were identified solely in ovine isolates, allele A3 was only identified in bovine isolates, while A1 and A2 alleles were identified in both ovine and bovine isolates. The A1 allele was subdivided into 5

groups (A1.1-A1.5) with A1.1 solely occurring in bovine isolates from serotype 1 (n = 3) and 6 (n = 1) isolates (corresponding to G2). In bovine isolates from serotype 2 (corresponding to G1), the authors reported detection of alleles A2, A2.1, A2.2, and A3. LktA sequences of study genomes were compared with representative amino acid sequences for each allele and subgroup downloaded from NCBI Genbank. Within G2, 141/146 LktA sequences were 100% identical to the A1.1 allele and another 4/146 were >99.5% identical to A1.1, consistent with the observations of Davies *et al*. (S5-7 Tables). The only exception was the LktA from Genome 2, that shared 100% amino acid identity with A1.3 and A1.5 (S7 Table). Interestingly, all but one of the G1 LktA sequences were 99.5-100% identical to the A8.1 allele (Genome 157 was identical to A2) (S5 and S8-9 Tables). The A8.1 allele was found in serotype 2 (G1) isolates by Davies *et al*., however, all the isolates in that study were ovine (n = 3), and A8.1 was also detected in ovine serotype 7 isolates (n = 3). The identification of A8.1 across many bovine origin genomes and nearly complete absence of A2 alleles suggests that G1 from Canada and the U.S.A has diverged from the G1 found in the United Kingdom where most of the isolates used by Davies *et al*. were collected and further demonstrates that A8.1 is not restricted to ovine isolates [23]. Whether there is a relationship between specific LktA alleles and respiratory disease severity in cattle warrants further investigation.

CAZymes

Carbohydrate active enzymes (CAZymes) are enzymes whose function has been biochemically verified to be involved in the metabolism of carbohydrates. We chose to investigate any differences in CAZymes between the two genotypes since these may indicate differences in ability to metabolize certain carbohydrates, and thus in the nutritional niches of the genotypes [65]. We were particularly interested in enzymes predicted to be secreted into either

the periplasmic or extracellular space (i.e. contain a signal peptide) and are thus most likely to be involved in interactions with the external environment. A total of six CAZymes containing signal peptides were identified in the pangenome (S10 Table) (50). At least one CAZyme was predicted in all genomes except for Genome 86/GCF_022404935, which was removed from this analysis. CAZymes identified were members of families GH23 (lytic transglycosylase), GH33 (sialidase), GH13_19 (amylose glucosidase), GT9 (glycosyltransferase), GH102 (lytic transglycosylase) and GH103 (lytic transglycosylase). Only GH103 (homology group 2042729) was genotype associated, present in 60/61 (98.4%) G1 genomes and 2/146 (1.37%) G2 genomes. The GH103 family consists of a group of lytic transglycosylases that cleave the β-1,4 linkage between *N*-acetylmuramoyl and *N*-acetylglucosaminyl residues in peptidoglycan and belong to family 3 of the transglycosylases with structures similar to lysozyme [66,67]. Numerous biological functions have been attributed lytic transglycosylases, however the only clear role is that they are involved in cell wall remodelling [66–69]. Whether this genotype difference in lytic transglycosylases is associated with phenotypic differences in colony morphology of the two genotypes [26] or other differences in cell wall maintenance will require further investigation.

Conclusions

We identified a total of 422 *Mannheimia haemolytica* genotype differentiating genes (135 G1 specific, 21 G1 associated, 233 G2 specific, and 33 G2 associated), including 125 with unknown functions and 132 that are associated with mobile genetic elements. Genotype associated genes and variants that may contribute to differences in disease association include TonB-dependent transporters, IgA proteases, a highly immunogenic outer membrane protein (PlpE), a lytic transglycosylase, and genotype variants of the leukotoxin gene. The list of

genotype differentiating genes provides a shortlist of targets that warrant further investigation to provide a mechanistic understanding of phenotypic differences between the *M. haemolytica* genotypes and inform rational strategies for *M. haemolytica* and bovine respiratory disease diagnostics.

Acknowledgements

The authors are grateful to Dr. David Palmer (Department of Chemistry, University of Saskatchewan) for assistance with the protein structure alignments, and to Dr. Maarten Voordouw (Department of Veterinary Microbiology, University of Saskatchewan) for assistance with statistical analysis and approaches.

Supporting Information

S1 Table. Summary of Genomes Included in Pangenome Analysis

S2 Table. Accession numbers of sequences used as BLAST queries against pangenome database.

S3 Table. Overview of pangenome classification of all homology groups. Genes matching is the number of genes that were found to correspond to the given homology group, as this referred to genes the number may be greater than or less than the number of genomes examined. For pangenome classification: core = group was present in ≥90% of all genomes (187/209); unique = Group was present in exactly one genome; Accessory = Group was present in somewhere between 2-186 genomes. For phenotype (Genotype) classification: Shared = Group was present in ≥90% of the genomes of the given genotype; exclusive = Group was present solely in

genomes of the given genotype; Specific = Group was exclusive to one genotype and present in ≥ 90% of that genotype; Associated = Group was present in ≥90% of the genomes of one genotype and ≤5% of the other genotype.

S4 Table. Genotype Specific and Associated Homology Groups. Annotation notes column provides summary of the interpro, pfam and GO functional annotations. Genes matching is the number of genes that were found to correspond to the given homology group, as this referred to genes the number may be greater than or less than the number of genomes examined. For pangenome classification: core = group was present in ≥90% of all genomes (187/209); unique = Group was present in exactly one genome; Accessory = Group was present in somewhere between 2-186 genomes. For phenotype (Genotype) classification: Shared = Group was present in ≥90% of the genomes of the given genotype; exclusive = Group was present solely in genomes of the given genotype; Specific = Group was exclusive to one genotype and present in ≥ 90% of that genotype; Associated = Group was present in ≥90% of the genomes of one genotype and ≤5% of the other genotype.

S5 Table. Pairwise comparisons of amino acid sequences of genotype 1 (G1) and genotype 2 (G2) Leukotoxin A consensus sequences against representatives of LktA alleles from Davies *et al*. 2001

S6 Table. Pairwise comparison of all genotype 1 LktA nucleotide sequences.

S7 Table. Pairwise comparison of genotype 1 LktA amino acid sequences that had a divergent LktA nucleotide sequence to representative sequences of LktA alleles identified in Davies *et al*. 2001.

S8 Table. Pairwise comparison of all genotype 2 LktA nucleotide sequences.

S9 Table. Pairwise comparison of genotype 1 LktA amino acid sequences that had a divergent LktA nucleotide sequence to representative sequences of LktA alleles identified in Davies *et al*. 2001.

S10 Table. Summary of CAZyme Analysis Results. Only CAZymes that were predicted to contain a signal peptide and were annotated by 3 of the utilized annotation tools (dbCAN_sub, DIAMOND, & HMMER) were included. No CAZymes were predicted in genome 86/GCF_022404935 and so was excluded from this analysis.

S1 Figure. Predicted structures of G1 and G2 variants of a predicted transferrin binding protein B (TbpB). (A) The crystal structure of TbpB from *Actinobacillus pleuropneumoniae* (PDB accession 3HOL) was used as a reference. Major domains include the N-lobe and C-lobe, each containing a beta-barrel and a “handle”. The reference structure is “rainbow” coloured from N terminus (blue) to C terminus (red). (B) Alignment of G1 and G2 variants. (C) G2 aligned to reference. (D) G1 aligned to reference. The G1 variant has an elongated alpha-helix in the N-lobe handle domain relative to G2 and the reference. The N-lobe has been demonstrated to interact with transferrin. Structures and alignments were visualized in PyMol.

References

1. Rice JA, Carrasco-Medina L, Hodgins DC, Shewen PE. *Mannheimia haemolytica* and bovine respiratory disease. Animal Health Research Reviews. 2007;8: 117–128. doi:10.1017/S1466252307001375

2. Highlander SK. Molecular genetic analysis of virulence in *Mannheimia (Pasteurella) haemolytica*. FBL. 2001;6: 1128–1150. doi:10.2741/highland

3. DeDonder KD, Apley MD. A literature review of antimicrobial resistance in pathogens associated with bovine respiratory disease. Animal Health Research Reviews. 2015;16: 125–134. doi:10.1017/S146625231500016X

4. Blakebrough-Hall C, McMeniman JP, González LA. An evaluation of the economic effects of bovine respiratory disease on animal performance, carcass traits, and economic outcomes in feedlot cattle defined using four BRD diagnosis methods. Journal of Animal Science. 2020;98: skaa005. doi:10.1093/jas/skaa005

5. Clawson ML, Murray RW, Sweeney MT, Apley MD, DeDonder KD, Capik SF, et al. Genomic signatures of *Mannheimia haemolytica* that associate with the lungs of cattle with respiratory disease, an integrative conjugative element, and antibiotic resistance genes. BMC Genomics. 2016;17: 982. doi:10.1186/s12864-016-3316-8

6. Biberstein EL, Gills M, Knight H. Serological types of Pasteurella hemolytica. Cornell Vet. 1960;50: 283–300.

7. Al-Ghamdi GM, Ames TR, Baker JC, Walker R, Chase CCL, Frank GH, et al. Serotyping of *Mannheimia (Pasteurella) haemolytica* Isolates from the Upper Midwest United States. J VET Diagn Invest. 2000;12: 576–578. doi:10.1177/104063870001200617

8. Mason C, Errington J, Foster G, Thacker J, Grace O, Baxter-Smith K. *Mannheimia haemolytica* serovars associated with respiratory disease in cattle in Great Britain. BMC Veterinary Research. 2022;18: 5. doi:10.1186/s12917-021-03121-3

9. Klima CL, Alexander TW, Hendrick S, McAllister TA. Characterization of *Mannheimia haemolytica* isolated from feedlot cattle that were healthy or treated for bovine respiratory disease. Can J Vet Res. 2014;78: 38–45.

10. Arnal JL, Fernández A, Vela AI, Sanz C, Fernández-Garyzábal JF, Cid D. Capsular type diversity of *Mannheimia haemolytica* determined by multiplex real-time PCR and indirect hemagglutination in clinical isolates from cattle, sheep, and goats in Spain. Veterinary Microbiology. 2021;258: 109121. doi:10.1016/j.vetmic.2021.109121

11. Confer AW, Martino P. *Mannheimia haemolytica*. CABI Compendium. 2017;CABI Compendium: 70912. doi:10.1079/cabicompendium.70912

12. Klima CL, Zaheer R, Cook SR, Booker CW, Hendrick S, Alexander TW, et al. Pathogens of Bovine Respiratory Disease in North American Feedlots Conferring Multidrug Resistance via Integrative Conjugative Elements. Journal of Clinical Microbiology. 2014;52: 438–448. doi:10.1128/jcm.02485-13

13. Holman DB, McAllister TA, Topp E, Wright A-DG, Alexander TW. The nasopharyngeal microbiota of feedlot cattle that develop bovine respiratory disease. Vet Microbiol. 2015;180: 90–95. doi:10.1016/j.vetmic.2015.07.031

14. Noyes NR, Benedict KM, Gow SP, Booker CW, Hannon SJ, McAllister TA, et al. *Mannheimia haemolytica* in feedlot cattle: prevalence of recovery and associations with antimicrobial use, resistance, and health outcomes. J Vet Intern Med. 2015;29: 705–713. doi:10.1111/jvim.12547

15. Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2: 123–140. doi:10.1038/nrmicro818

16. Nysten J, Sofras D, Dijck PV. One species, many faces: The underappreciated importance of strain diversity. PLOS Pathogens. 2024;20: e1011931. doi:10.1371/journal.ppat.1011931

17. Hacker J, Carniel E. Ecological fitness, genomic islands and bacterial pathogenicity. A Darwinian view of the evolution of microbes. EMBO Rep. 2001;2: 376–381. doi:10.1093/embo-reports/kve097

18. Ayalew S, Murdock BK, Snider TA, Confer AW. *Mannheimia haemolytica* IgA-specific proteases. Vet Microbiol. 2019;239: 108487. doi:10.1016/j.vetmic.2019.108487

19. Singh K, Ritchey JW, Confer AW. *Mannheimia haemolytica*: Bacterial–Host Interactions in Bovine Pneumonia. Vet Pathol. 2011;48: 338–348. doi:10.1177/0300985810377182

20. Kisiela DI, Czuprynski CJ. Identification of *Mannheimia haemolytica* Adhesins Involved in Binding to Bovine Bronchial Epithelial Cells. Infection and Immunity. 2009;77: 446–455. doi:10.1128/iai.00312-08

21. Roehrig SC, Tran HQ, Spehr V, Gunkel N, Selzer PM, Ullrich HJ. The response of *Mannheimia haemolytica* to iron limitation: Implications for the acquisition of iron in the bovine lung. Veterinary Microbiology. 2007;121: 316–329. doi:10.1016/j.vetmic.2006.12.013

22. Davies RL, Baillie S. Cytotoxic activity of *Mannheimia haemolytica* strains in relation to diversity of the leukotoxin structural gene lktA. Vet Microbiol. 2003;92: 263–279. doi:10.1016/s0378-1135(02)00408-x

23. Davies RL, Whittam TS, Selander RK. Sequence Diversity and Molecular Evolution of the Leukotoxin (lktA) Gene in Bovine and Ovine Strains of *Mannheimia (Pasteurella) haemolytica*. J Bacteriol. 2001;183: 1394–1404. doi:10.1128/jb.183.4.1394-1404.2001

24. Cozens D, Sutherland E, Lauder M, Taylor G, Berry CC, Davies RL. Pathogenic *Mannheimia haemolytica* Invades Differentiated Bovine Airway Epithelial Cells. Infect Immun. 2019;87: 10.1128/iai.00078-19. doi:10.1128/iai.00078-19

25. Loy JD, Clawson ML. Rapid typing of *Mannheimia haemolytica* major genotypes 1 and 2 using MALDI-TOF mass spectrometry. J Microbiol Methods. 2017;136: 30–33. doi:10.1016/j.mimet.2017.03.002

26. Wynn EL, Schuller G, Loy JD, Workman AM, McDaneld TG, Clawson ML. Differentiation of *Mannheimia haemolytica* genotype 1 and 2 strains by visible phenotypic characteristics

on solid media. Journal of Microbiological Methods. 2020;171: 105877. doi:10.1016/j.mimet.2020.105877

27. Clawson ML, Schuller G, Dickey AM, Bono JL, Murray RW, Sweeney MT, et al. Differences between predicted outer membrane proteins of genotype 1 and 2 *Mannheimia haemolytica*. BMC Microbiology. 2020;20: 250. doi:10.1186/s12866-020-01932-2

28. Klima CL, Cook SR, Zaheer R, Laing C, Gannon VP, Xu Y, et al. Comparative Genomic Analysis of *Mannheimia haemolytica* from Bovine Sources. PLOS ONE. 2016;11: e0149520. doi:10.1371/journal.pone.0149520

29. Deschner D, Voordouw MJ, Fernando C, Campbell J, Waldner CL, Hill JE. Identification of genetic markers of resistance to macrolide class antibiotics in *Mannheimia haemolytica* isolates from a Saskatchewan feedlot. Appl Environ Microbiol. 2024;90: e00502-24. doi:10.1128/aem.00502-24

30. Dassanayake RP, Clawson ML, Tatum FM, Briggs RE, Kaplan BS, Casas E. Differential identification of *Mannheimia haemolytica* genotypes 1 and 2 using colorimetric loop-mediated isothermal amplification. BMC Research Notes. 2023;16: 4. doi:10.1186/s13104-023-06272-8

31. O'Leary NA, Cox E, Holmes JB, Anderson WR, Falk R, Hem V, et al. Exploring and retrieving sequence and metadata for species across the tree of life with NCBI Datasets. Sci Data. 2024;11: 732. doi:10.1038/s41597-024-03571-y

32. Okonechnikov K, Golosova O, Fursov M, UGENE team. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics. 2012;28: 1166–1167. doi:10.1093/bioinformatics/bts091

33. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3--new capabilities and interfaces. Nucleic Acids Res. 2012;40: e115. doi:10.1093/nar/gks596

34. Kõressaar T, Lepamets M, Kaplinski L, Raime K, Andreson R, Remm M. Primer3_masker: integrating masking of template sequence with primer design software. Bioinformatics. 2018;34: 1937–1938. doi:10.1093/bioinformatics/bty036

35. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007;23: 1289–1291. doi:10.1093/bioinformatics/btm091

36. Jonkheer EM, van Workum D-JM, Sheikhizadeh Anari S, Brankovics B, de Haan JR, Berke L, et al. PanTools v3: functional annotation, classification and phylogenomics. Bioinformatics. 2022;38: 4403–4405. doi:10.1093/bioinformatics/btac506

37. Sheikhizadeh S, Schranz ME, Akdel M, de Ridder D, Smit S. PanTools: representation, storage and exploration of pan-genomic data. Bioinformatics. 2016;32: i487–i493. doi:10.1093/bioinformatics/btw455

38. Anari SS, Ridder D de, Schranz ME, Smit S. Pangenomic read mapping. bioRxiv; 2019. p. 813634. doi:10.1101/813634

39. Jones P, Binns D, Chang H-Y, Fraser M, Li W, McAnulla C, et al. InterProScan 5: genome-scale protein function classification. Bioinformatics. 2014;30: 1236–1240. doi:10.1093/bioinformatics/btu031

40. Blum M, Chang H-Y, Chuguransky S, Grego T, Kandasaamy S, Mitchell A, et al. The InterPro protein families and domains database: 20 years on. Nucleic Acids Res. 2021;49: D344–D354. doi:10.1093/nar/gkaa977

41. Nielsen H. Predicting Secretory Proteins with SignalP. Methods Mol Biol. 2017;1611: 59–73. doi:10.1007/978-1-4939-7015-5_6

42. Blin K, Shaw S, Augustijn HE, Reitz ZL, Biermann F, Alanjary M, et al. antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation. Nucleic Acids Res. 2023;51: W46–W50. doi:10.1093/nar/gkad344

43. Deschner D, Hill JE. Genomic Differentiation of *Mannheimia haemolytica* genotypes. FigShare; 2025. doi:10.6084/m9.figshare.28872536

44. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. BMC Bioinformatics. 2009;10: 421. doi:10.1186/1471-2105-10-421

45. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25: 3389–402.

46. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215: 403–10.

47. Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. J Comput Biol. 2000;7: 203–214. doi:10.1089/10665270050081478

48. Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA. Database indexing for production MegaBLAST searches. Bioinformatics. 2008;24: 1757–1764. doi:10.1093/bioinformatics/btn322

49. Kostova V, Hanke D, Kaspar H, Fiedler S, Schwarz S, Krüger-Haker H. Macrolide resistance in *Mannheimia haemolytica* isolates associated with bovine respiratory disease from the German national resistance monitoring program GERM-Vet 2009 to 2020. Front Microbiol. 2024;15. doi:10.3389/fmicb.2024.1356208

50. Zheng J, Ge Q, Yan Y, Zhang X, Huang L, Yin Y. dbCAN3: automated carbohydrate-active enzyme and substrate annotation. Nucleic Acids Research. 2023;51: W115–W121. doi:10.1093/nar/gkad328

51. Abramson J, Adler J, Dunger J, Evans R, Green T, Pritzel A, et al. Accurate structure prediction of biomolecular interactions with AlphaFold 3. Nature. 2024;630: 493–500. doi:10.1038/s41586-024-07487-w

52. Vanni C, Schechter MS, Acinas SG, Barberán A, Buttigieg PL, Casamayor EO, et al. Unifying the known and unknown microbial coding sequence space. Brown CT, Storz G, Brown CT, Smith B, editors. eLife. 2022;11: e67667. doi:10.7554/eLife.67667

53. Lobb B, Tremblay BJ-M, Moreno-Hagelsieb G, Doxey AC. PathFams: statistical detection of pathogen-associated protein domains. BMC Genomics. 2021;22: 663. doi:10.1186/s12864-021-07982-8

54. Ferguson AD, Deisenhofer J. TonB-dependent receptors-structural perspectives. Biochim Biophys Acta. 2002;1565: 318–332. doi:10.1016/s0005-2736(02)00578-3

55. Silale A, Van Den Berg B. TonB-Dependent Transport Across the Bacterial Outer Membrane. Annu Rev Microbiol. 2023;77: 67–88. doi:10.1146/annurev-micro-032421-111116

56. Pogoutse AK, Moraes TF. Iron acquisition through the bacterial transferrin receptor. Crit Rev Biochem Mol Biol. 2017;52: 314–326. doi:10.1080/10409238.2017.1293606

57. Ayalew S, Shrestha B, Montelongo M, Wilson AE, Confer AW. Identification and immunogenicity of *Mannheimia haemolytica* S1 outer membrane lipoprotein PlpF. Vaccine. 2011;29: 8712–8718. doi:10.1016/j.vaccine.2011.08.074

58. Ayalew S, Confer AW, Blackwood ER. Characterization of immunodominant and potentially protective epitopes of *Mannheimia haemolytica* serotype 1 outer membrane lipoprotein PlpE. Infect Immun. 2004;72: 7265–7274. doi:10.1128/IAI.72.12.7265-7274.2004

59. Confer AW, Ayalew S, Panciera RJ, Montelongo M, Wray JH. Recombinant *Mannheimia haemolytica* serotype 1 outer membrane protein PlpE enhances commercial *M. haemolytica* vaccine-induced resistance against serotype 6 challenge. Vaccine. 2006;24: 2248–2255. doi:10.1016/j.vaccine.2005.11.036

60. Pandher K, Confer AW, Murphy GL. Genetic and immunologic analyses of PlpE, a lipoprotein important in complement-mediated killing of Pasteurella haemolytica serotype 1. Infect Immun. 1998;66: 5613–5619. doi:10.1128/IAI.66.12.5613-5619.1998

61. Ayalew S, Blackwood ER, Confer AW. Sequence diversity of the immunogenic outer membrane lipoprotein PlpE from *Mannheimia haemolytica* serotypes 1, 2, and 6. Vet Microbiol. 2006;114: 260–268. doi:10.1016/j.vetmic.2005.11.067

62. Oppermann T, Busse N, Czermak P. *Mannheimia haemolytica* growth and leukotoxin production for vaccine manufacturing — A bioprocess review. Electronic Journal of Biotechnology. 2017;28: 95–100. doi:10.1016/j.ejbt.2017.06.001

63. Narayanan SK, Nagaraja TG, Chengappa MM, Stewart GC. Leukotoxins of gram-negative bacteria. Vet Microbiol. 2002;84: 337–356. doi:10.1016/s0378-1135(01)00467-9

64. Larsen J, Pedersen AG, Davies RL, Kuhnert P, Frey J, Christensen H, et al. Evolution of the leukotoxin promoter in genus *Mannheimia*. BMC Evolutionary Biology. 2009;9: 121. doi:10.1186/1471-2148-9-121

65. Drula E, Garron M-L, Dogan S, Lombard V, Henrissat B, Terrapon N. The carbohydrate-active enzyme database: functions and literature. Nucleic Acids Res. 2022;50: D571–D577. doi:10.1093/nar/gkab1045

66. Dik DA, Marous DR, Fisher JF, Mobashery S. Lytic transglycosylases: concinnity in concision of the bacterial cell wall. Crit Rev Biochem Mol Biol. 2017;52: 503–542. doi:10.1080/10409238.2017.1337705

67. Martinez-Bond EA, Soriano BM, Williams AH. The mechanistic landscape of Lytic transglycosylase as targets for antibacterial therapy. Curr Opin Struct Biol. 2022;77: 102480. doi:10.1016/j.sbi.2022.102480

68. Suvorov M, Lee M, Hesek D, Boggess B, Mobashery S. Lytic Transglycosylase MltB of *Escherichia coli* and Its Role in Recycling of Peptidoglycan Strands of Bacterial Cell Wall. J Am Chem Soc. 2008;130: 11878–11879. doi:10.1021/ja805482b

69. Scheurwater E, Reid CW, Clarke AJ. Lytic transglycosylases: bacterial space-making autolysins. Int J Biochem Cell Biol. 2008;40: 586–591. doi:10.1016/j.biocel.2007.03.018