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Sequence Diversity and Molecular Evolution of the Heat-Modifiable Outer Membrane Protein Gene (*ompA*) of *Mannheimia* (*Pasteurella*) *haemolytica*, *Mannheimia glucosida*, and *Pasteurella trehalosi*

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The OmpA (or heat-modifiable) protein is a major structural component of the outer membranes of gram-negative bacteria. The protein contains eight membrane-traversing β-strands and four surface-exposed loops. The genetic diversity and molecular evolution of OmpA were investigated in 31 *Mannheimia* (*Pasteurella*) *haemolytica*, 6 *Mannheimia glucosida*, and 4 *Pasteurella trehalosi* strains by comparative nucleotide sequence analysis. The OmpA proteins of *M. haemolytica* and *M. glucosida* contain four hypervariable domains located at the distal ends of the surface-exposed loops. The hypervariable domains of OmpA proteins from bovine and ovine *M. haemolytica* isolates are very different but are highly conserved among strains from each of these two host species. Fourteen different alleles representing four distinct phylogenetic classes, classes I to IV, were identified in *M. haemolytica* and *M. glucosida*. Class I, II, and IV alleles were associated with bovine *M. haemolytica*, ovine *M. haemolytica*, and *M. glucosida* strains, respectively, whereas class III alleles were present in certain *M. haemolytica* and *M. glucosida* isolates. Class I and II alleles were associated with divergent lineages of bovine and ovine *M. haemolytica* strains, respectively, indicating a history of horizontal DNA transfer and assortative (entire gene) recombination. Class III alleles have mosaic structures and were derived by horizontal DNA transfer and intragenic recombination. Our findings suggest that OmpA is under strong selective pressure from the host species and that it plays an important role in host adaptation. It is proposed that the OmpA protein of *M. haemolytica* acts as a ligand and is involved in binding to specific host cell receptor molecules in cattle and sheep. *P. trehalosi* expresses two OmpA homologs that are encoded by different tandemly arranged *ompA* genes. The *P. trehalosi ompA* genes are highly diverged from those of *M. haemolytica* and *M. glucosida*, and evidence is presented to suggest that at least one of these genes was acquired by horizontal DNA transfer.

Mannheimia (*Pasteurella*) *haemolytica* is a commensal of cattle, sheep, and other ruminants, but it also causes bovine and ovine pneumonic pasteurellosis (infecting the respiratory tract), which is responsible for considerable economic losses to the cattle and sheep industries (6, 20, 21). The organism consists of genetically distinct subpopulations that are differentially adapted to and elicit disease in either cattle or sheep (11, 14). *M. haemolytica* possesses various putative virulence determinants (23), including a transferrin receptor (38, 59) and a leukotoxin (26, 49) which are specific for ruminant transferrin (38, 59) and lymphoid cells (4, 7, 27, 49), respectively, and are thought to contribute to the organism's host specificity. However, the molecular basis of host adaptation in the bovine and ovine lineages of *M. haemolytica* remains largely unclear. *Mannheimia glucosida* was previously recognized as the A11 serotype of *M. haemolytica* and represents a heterogeneous group of organisms that are mainly opportunistic sheep pathogens with low virulence (1, 11). *Pasteurella trehalosi* was previously recognized as the T biotype of *M. haemolytica* (51) and is associated exclusively with sheep, in which it causes a systemic disease that is pathologically distinct from pneumonic pasteurellosis (21).

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The OmpA protein is an integral component of the outer membranes of gram-negative bacteria and is highly conserved (3). The protein has characteristic heat-modifiable properties (3), is present at a high copy number ($>10^5$/cell) (22), and is immunogenic (31, 45, 53, 60). Functions that have been attributed to OmpA include the maintenance of outer membrane integrity and cell shape (52), the action of a bacteriophage receptor (10, 34, 35), a role in conjugation (48), and resistance to the bactericidal effect of serum (58). However, OmpA is also involved in adherence to host tissues in *Chlamydia* spp. (36), *Escherichia coli* (44, 55), *Haemophilus influenzae* (24), and *Pasteurella multocida* (9). For *E. coli* and *H. influenzae*, the host cell receptor molecules have been identified (24, 42, 43). The 35-kDa OmpA protein of *E. coli* consists of an N-terminal transmembrane domain (19 kDa) and a C-terminal globular periplasmic domain (16 kDa) (2). The three-dimensional structure of the transmembrane domain has been determined by X-ray crystallography and nuclear magnetic resonance spectroscopy, and it consists of eight membrane-traversing antiparallel β-strands and four relatively long, mobile, hydrophilic surface-exposed loops (2, 39, 40). The periplasmic domain interacts with the underlying peptidoglycan and confers upon OmpA its role in maintaining the structural integrity of the outer membrane (18). The *ompA* gene of *M. haemolytica* has been cloned and sequenced and the immunological properties of OmpA have been investigated (31, 60). However, very little

TABLE 1. Properties of 31 *M. haemolytica*, 6 *M. glucosida*, and 4 *P. trehalosi* isolates

Isolate	ET[a]	Capsular serotype	Host species	*ompA* allele	No. of nucleotides	No. of amino acids	Molecular mass of protein (Da)	GenBank accession no.
M. haemolytica								
PH2	1	A1	Bovine	*ompA1.1*	1,137	379	40,460	AY244653
PH30	1	A1	Bovine	*ompA1.1*	1,137	379	40,460	
PH376	1	A6	Bovine	*ompA1.1*	1,137	379	40,460	
PH346	1	A12	Ovine	*ompA2.1*	1,119	373	39,778	AY244658
PH540	2	A1	Bovine	*ompA1.2*	1,137	379	40,472	AY244654
PH338	3	A9	Ovine	*ompA2.1*	1,119	373	39,778	
PH388	4	A7	Ovine	*ompA2.1*	1,119	373	39,778	
PH50	5	A5	Ovine	*ompA2.1*	1,119	373	39,778	
PH56	5	A8	Ovine	*ompA2.2*	1,119	373	39,794	AY244659
PH238	5	A9	Ovine	*ompA2.1*	1,119	373	39,778	
PH8	6	A1	Ovine	*ompA2.1*	1,119	373	39,778	
PH398	7	A1	Ovine	*ompA2.1*	1,119	373	39,778	
PH284	8	A6	Ovine	*ompA2.1*	1,119	373	39,778	
PH232	9	A6	Ovine	*ompA2.1*	1,119	373	39,778	
PH66	10	A14	Ovine	*ompA2.1*	1,119	373	32,919	
PH706	11	A16	Ovine	*ompA2.1*	1,119	373	32,919	
PH296	12	A7	Ovine	*ompA4.1*	1,104	368	39,151	AY244662
PH396	13	A7	Ovine	*ompA4.1*	1,104	368	39,151	
PH484	14	A7	Ovine	*ompA4.1*	1,104	368	39,151	
PH588	15	A13	Ovine	*ompA4.2*	1,104	368	39,179	AY244663
PH494	16	A2	Bovine-like	*ompA1.4*	1,137	379	40,528	AY244656
PH550	17	A2	Bovine	*ompA1.5*	1,125	375	40,062	AY244657
PH196	18	A2	Bovine	*ompA3.1*	1,122	374	39,911	AY244661
PH526	19	A2	Ovine	*ompA2.3*	1,119	373	39,798	
PH598	20	A2	Ovine	*ompA2.3*	1,119	373	39,798	
PH202	21	A2	Bovine	*ompA1.3*	1,137	379	40,460	AY244655
PH470	21	A2	Bovine	*ompA1.3*	1,137	379	40,460	
PH278	21	A2	Ovine	*ompA2.3*	1,119	373	39,798	AY244660
PH372	21	A2	Ovine	*ompA2.3*	1,119	373	39,798	
PH292	22	A2	Ovine	*ompA2.3*	1,119	373	39,798	
PH392	22	A2	Ovine	*ompA2.3*	1,119	373	39,798	
M. glucosida								
PH344	1	A11	Ovine	*ompA5.1*	1,104	368	39,135	AY244664
PH498	3	A11	Ovine	*ompA5.1*	1,104	368	39,135	
PH240	5	A11	Ovine	*ompA7.1*	1,104	368	39,233	AY244666
PH496	7	UG3	Ovine	*ompA6.1*	1,104	368	39,233	AY244665
PH574	10	UG3	Ovine	*ompA7.1*	1,104	368	39,233	
PH290	16	UG3	Ovine	*ompA6.1*	1,104	368	39,233	
P. trehalosi								
PH68 (NCTC 11550)	19	T3	Ovine	*ompA8.1*	1,095	365	38,633	AY582755
				ompA10.1	1,083	361	37,978	
PH246 (NCTC 10626)	2	T4	Ovine	*ompA8.2*	1,095	365	38,633	AY582756
				ompA10.2	1,083	361	37,978	
PH252 (NCTC 10641)	4	T10	Ovine	*ompA8.3*	1,095	365	38,662	AY582757
				ompA10.3	1,083	361	37,978	
PH254 (NCTC 10370^T)	15	T15	Ovine	*ompA9.1*	1,095	365	38,844	AY582758
				ompA10.4	1,083	361	37,978	

[a] See references 11 and 12.

is known about the role of OmpA in the pathogenesis of bovine and ovine pneumonic pasteurellosis.

The OmpA proteins of bovine and ovine *M. haemolytica* isolates have previously been shown to exhibit interstrain molecular mass heterogeneity that correlates with the host of origin (14). The inference is that OmpA is somehow involved in the adaptation of bovine and ovine *M. haemolytica* strains to cattle and sheep, respectively (i.e., in host specificity). Horizontal DNA transfer and intragenic recombination have played important roles in the evolution of the leukotoxin structural gene (*lktA*) (17) and associated activation (*lktC*) and transport (*lktB* and *lktD*) genes (13) of *M. haemolytica*. In particular, certain *M. haemolytica* strains contain large segments of leukotoxin DNA that have been derived from *M. glucosida* and *P. trehalosi* (17). Similar evolutionary processes may have been involved in the diversification of OmpA, and the objective of the present study was to investigate nucleotide sequence variations of the *ompA* genes of bovine and ovine strains of *M. haemolytica* and of *M. glucosida* and *P. trehalosi*. In particular, we wanted to determine how these variations relate to the observed molecular mass heterogeneity and host association of OmpA in *M. haemolytica* (14) and to ascertain the evolutionary influence, if any, of *M. glucosida* and *P. trehalosi*. The nucleotide sequences were used to infer the evolutionary history of the *ompA* gene in these three species, to detect past recombination events involving the *ompA* gene, and to assess the action of natural selection on amino acid diversity in the different OmpA domains. To accomplish these goals,

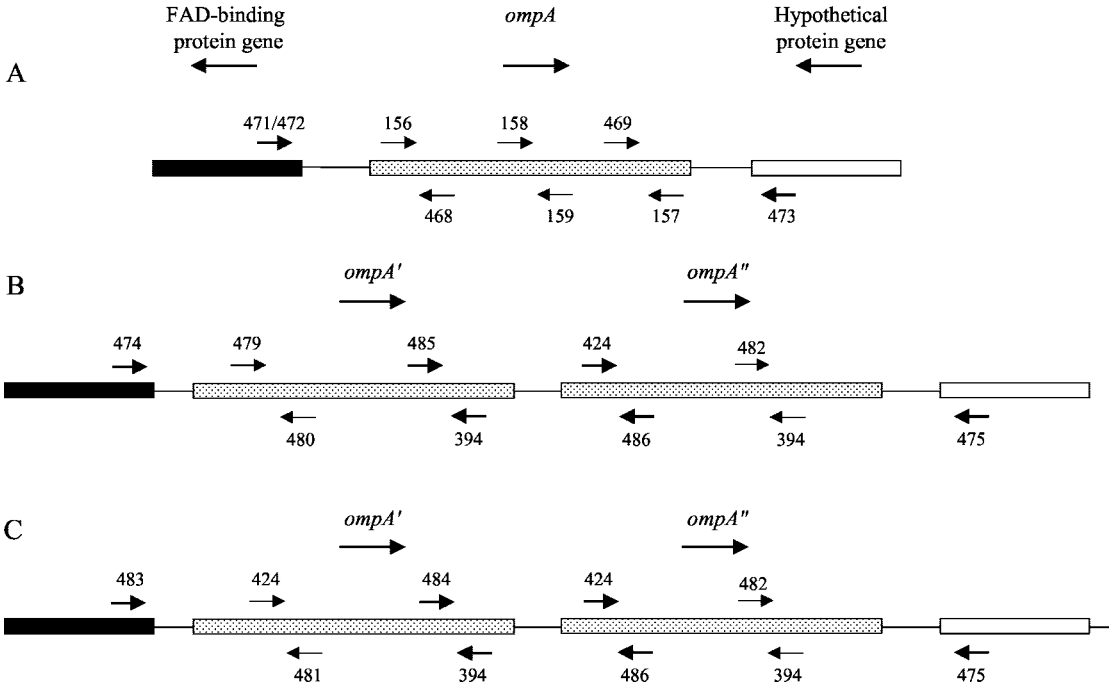

FIG. 1. Locations and numerical designations of PCR amplification and DNA sequencing primers for *M. haemolytica* and *M. glucosida* strains (A), *P. trehalosi* strains PH68, PH246, and PH252 (B), and *P. trehalosi* strain PH254 (C). The relative positions of the primers are represented by arrows; the primers used for PCR amplification are indicated by bold arrows. Arrows beneath gene names indicate the direction of transcription.

we sequenced the *ompA* genes of representative strains of *M. haemolytica*, *M. glucosida*, and *P. trehalosi* and used statistical tools to analyze these data from an evolutionary perspective.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *ompA* genes from 31 *M. haemolytica*, 6 *M. glucosida*, and 4 *P. trehalosi* isolates were sequenced. The 41 strains were well characterized in previous studies (11–17) and were selected to represent specific evolutionary lineages, capsular serotypes, and hosts of origin. Some properties of these isolates are presented in Table 1. The strains were stored at −85°C in 50% (vol/vol) glycerol in brain heart infusion broth and were grown on blood agar (brain heart infusion agar containing 5% [vol/vol] sheep's blood) by overnight incubation at 37°C. Liquid cultures were prepared by inoculating a few colonies into 15 ml of brain heart infusion broth and incubating them overnight at 37°C with shaking at 120 rpm.

SDS-PAGE analysis of *P. trehalosi* OmpA proteins. The preparation of *P. trehalosi* outer membrane proteins (OMPs) and their analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have been described previously (16). The OmpA proteins of *P. trehalosi* were identified by heating the OMP samples at 80, 90, and 100°C for 5 min prior to SDS-PAGE. The molecular masses of individual proteins were calculated with Labworks image acquisition and analysis computer software.

Preparation of chromosomal DNAs. Cells from 1.0-ml overnight liquid cultures were harvested by centrifugation for 1 min at 13,000 × *g* and were washed once in sterile distilled H_2O. DNAs were prepared by use of an InstaGene Matrix kit (Bio-Rad) according to the manufacturer's instructions and were stored at −20°C.

PCR amplification and DNA sequence analysis. The *ompA* gene was found to be located at contigs 87 and 125 of the *M. haemolytica* genome sequence (http://www.hgsc.bcm.tmc.edu/microbial/Mhaemolytica/) by a BLAST analysis with the GenBank *M. haemolytica ompA* sequence (accession no. AF133259). The flanking open reading frames were identified by use of the Lasergene Editseq (DNAstar, Inc.) software application, and the identities of these genes were determined by a BLAST analysis against the GenBank database. The homologous genes were identified in *Actinobacillus actinomycetemcomitans*, *H. influenzae*, and *P. multocida*, and the four sequences were aligned with the Lasergene Megalign (DNAstar, Inc.) software application. Two forward and two reverse universal primers were designed within conserved regions of the four aligned sequences corresponding to each of the two flanking genes for preliminary PCR testing with representative strains of *M. haemolytica*, *M. glucosida*, and *P. trehalosi*. Successfully amplified bands were partially sequenced by use of the same primers, and a second set of internal primers specific for each individual strain was designed. In this way, the primer pairs 471-473 and 472-473 were designed for the amplification of *ompA* from *M. haemolytica* and *M. glucosida* strains, respectively (Fig. 1A). During the course of these preliminary experiments, difficulties were encountered in sequencing the *ompA* gene of *P. trehalosi*, despite the fact that definite PCR products were obtained. The results led us to suspect that two tandem *ompA* genes were present. Consequently, it became necessary to adopt a more complex sequencing strategy in which the *ompA* genes were amplified and sequenced as three separate overlapping fragments (Fig. 1B and C). The primer pairs 474-394, 485-486, and 424-475 were used for *P. trehalosi* strains PH68, PH246, and PH252 (Fig. 1B), and the primer pairs 483-394, 484-486, and 424-475 were used for strain PH254 (Fig. 1C). Both strands of *ompA* were sequenced, and internal sequencing primers were designed as sequence data became available (Fig. 1). The primers were designed with the computer program Primer Designer (version 2.0) and were synthesized by Sigma-GenoSys (Cambridge, United Kingdom). Full details of the PCR and sequencing primers are provided in Table 2.

PCR fragments containing the complete or partial *ompA* gene were amplified from chromosomal DNAs by use of a *Taq* DNA polymerase kit (Boehringer Mannheim) according to the manufacturer's instructions. PCRs were carried out in a Perkin-Elmer 480 DNA thermal cycler using the following amplification parameters: denaturation at 94°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 2 min. Thirty cycles were performed, and a final extension step of 72°C for 10 min was used. The production of PCR amplicons of the expected sizes was confirmed by agarose gel electrophoresis, and the DNAs were purified with a Qiaquick PCR purification kit (Qiagen, Chatsworth, Calif.). DNAs were finally eluted in 30 μl of sterile distilled H_2O and stored at −20°C. Sequence reactions were performed with an ABI Prism Big Dye Terminator cycle sequencing kit (Applied Biosystems) in a GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler. Sequence analysis was performed with an Applied Biosystems 377 DNA sequencer (University of Glasgow Sequencing Service).

Analysis of nucleotide and protein sequence data. Nucleotide sequence data were analyzed and edited with Seqed (Applied Biosystems) and Lasergene (DNAstar, Inc.) sequence analysis software. Phylogenetic and molecular evolu-

TABLE 2. Details of oligonucleotide primers used for PCR and sequencing

Target species or primer no.[a]	Direction	Sequence (5′-3′)	Corresponding nucleotide positions[b]
M. haemolytica and *M. glucosida*			
471	Forward	CCAGTTGCGGTACTTCAG	
472	Forward	CCAGTTGAGGTACTTCAG	
156	Forward	CTCAAGCAGCTCCACAAG	50–67
158	Forward	TAGGTGCTGGTCTTGAGT	515–532
469	Forward	TTAGATGCAGCACACGCT	820–837
473	Reverse	GCTGGTTAAGGCTCTCTA	
157	Reverse	GTTTGCTTCACCGTAACC	1020–1003
159	Reverse	GAGCAGCTAATTCAGGAG	559–542
468	Reverse	GTAACCACCGAATACACC	225–208
P. trehalosi			
474	Forward	GTATCAGTGGCAAGCGAA	
479	Forward	GTGATGGTCCAACTGCTT	494–511
485	Forward	TCGACTTTGGTAAAGCA	1061–1077
424	Forward	GGTGCTAAAGCTGGTTGG	1710–1727
482	Forward	CCAGTTGCTGAGCCAGA	2286–2302
475	Reverse	TATGCAAGCTGGCTAAGG	
394	Reverse	AGCGTGTGCTGCATCTAA	2405–2388
486	Reverse	CCGTATTTACCACCGTT	1783–1767
394	Reverse	AGCGTGTGCTGCATCTAA	1122–1105
480	Reverse	TGCCACGAACACGACCAA	619–602
483	Forward	CTCGGCATAACTATCAGC	
424	Forward	GGTGCTAAAGCTGGTTGG	415–432
484	Forward	TCCAGTAGCAGCTCCTGA	1002–1019
481	Reverse	GAACGCGACCGAAGTAGT	613–596

[a] The positions of the primers are shown in Fig. 1.
[b] Primer positions of internal primers for *M. haemolytica* and *M. glucosida* are based on the strain PH2 sequence (accession no. AY244653) and those for *P. trehalosi* are based on the sequences of strains PH68 (accession no. AY582755) and PH254 (accession no. AY582758).

tionary analyses were conducted with Mega, version 2.1 (30), in conjunction with alignment programs written by T. S. Whittam (Michigan State University). Statistical analyses for clustering of polymorphic sites were performed by the maximum chi-square method (50) with the computer program Maxchi (32, 47). Secondary structure predictions were performed with the Psipred secondary structure prediction method (25; http://bioinf.cs.ucl.ac.uk/psipred/) and the SAM-T99 sequence alignment and modeling system (28; http://www.cse.ucsc.edu/research/compbio/HMM-apps). The *M. haemolytica* and *M. glucosida* OmpA sequences were also aligned and compared with *E. coli* OmpA three-dimensional structural models (MMDB 16249 and PDB IG90 [2]; MMDB 9208 and PDB 1BXW [40]) by use of the computer program Cn3D (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml).

Nucleotide sequence accession numbers. The GenBank accession numbers for the *ompA* sequences obtained in this study are provided in Table 1.

RESULTS

Nucleotide and amino acid variation. The *ompA* genes from 31 *M. haemolytica* isolates representing 12 capsular serotypes and 22 electrophoretic types (ETs) previously defined by multilocus enzyme electrophoresis (11) were sequenced. The *ompA* genes from six isolates representing different ETs of *M. glucosida* (11) and from four isolates representing each of the capsular serotypes T3, T4, T10, and T15 of *P. trehalosi* (12) were also sequenced. PCR errors were shown to be insignificant by duplicate amplification and sequencing of *ompA* for strains PH2, PH376, and PH344. *M. haemolytica* and *M. glucosida* isolates contain a single *ompA* gene, but the *P. trehalosi* strains were shown to possess two tandemly arranged *ompA* genes, *ompA′* and *ompA″* (Fig. 1). The phylogenetic relationships of the *ompA* sequences are shown in Fig. 2. The *ompA* genes of the *M. haemolytica* and *M. glucosida* strains represent two distinct but closely related lineages, A and B. However, the *ompA′* and *ompA″* genes of *P. trehalosi* represent two lineages, C and D, that are as divergent from each other as they are from the *ompA* genes of *M. haemolytica* and *M. glucosida* (Fig. 2). In addition, the *ompA′* gene of strain PH254 shows considerable divergence from that of strains PH68, PH246, and PH252.

The *ompA* genes of the *M. haemolytica* and *M. glucosida* isolates varied from 1,104 to 1,137 nucleotides in length, and the predicted proteins varied from 368 to 379 amino acids in length and from 39,135 to 40,528 Da in molecular mass (Table 1). Since these proteins contain a putative signal sequence of 19 amino acids (1,855 Da) (60), the predicted molecular masses of the putative mature proteins varied from 37,280 to 38,673 Da. Fourteen unique *ompA* sequences, each representing a distinct allele, were identified among the *M. haemolytica* and *M. glucosida* isolates (Fig. 3). The alleles were assigned to seven subclasses, *ompA1* to *ompA7*, based on their overall sequence similarities, and individual alleles within each subclass were designated *ompA1.1*, *ompA1.2*, etc. The *ompA1*- to *ompA4*-type alleles were associated exclusively with *M. haemolytica*, whereas the *ompA5*- to *ompA7*-type alleles were associated only with *M. glucosida*. The subclasses were grouped into four major classes, I to IV, which represent distinct phylogenetic lineages (discussed below). Class I consists of *ompA1*-type alleles, class II consists of *ompA2*- and *ompA3*-type alleles, class III consists of *ompA4*- and *ompA5*-type alleles, and class IV consists of *ompA6*- and *ompA7*-type alleles (Fig. 3). There were 82 (7.2%) polymorphic nucleotide sites and 33 (8.7%) variable inferred amino acid positions among the 14 alleles. Pairwise differences in nucleotide and inferred amino acid sequences between representative pairs of alleles ranged from 1 to 60 nucleotide sites and 1 to 23 amino acid positions.

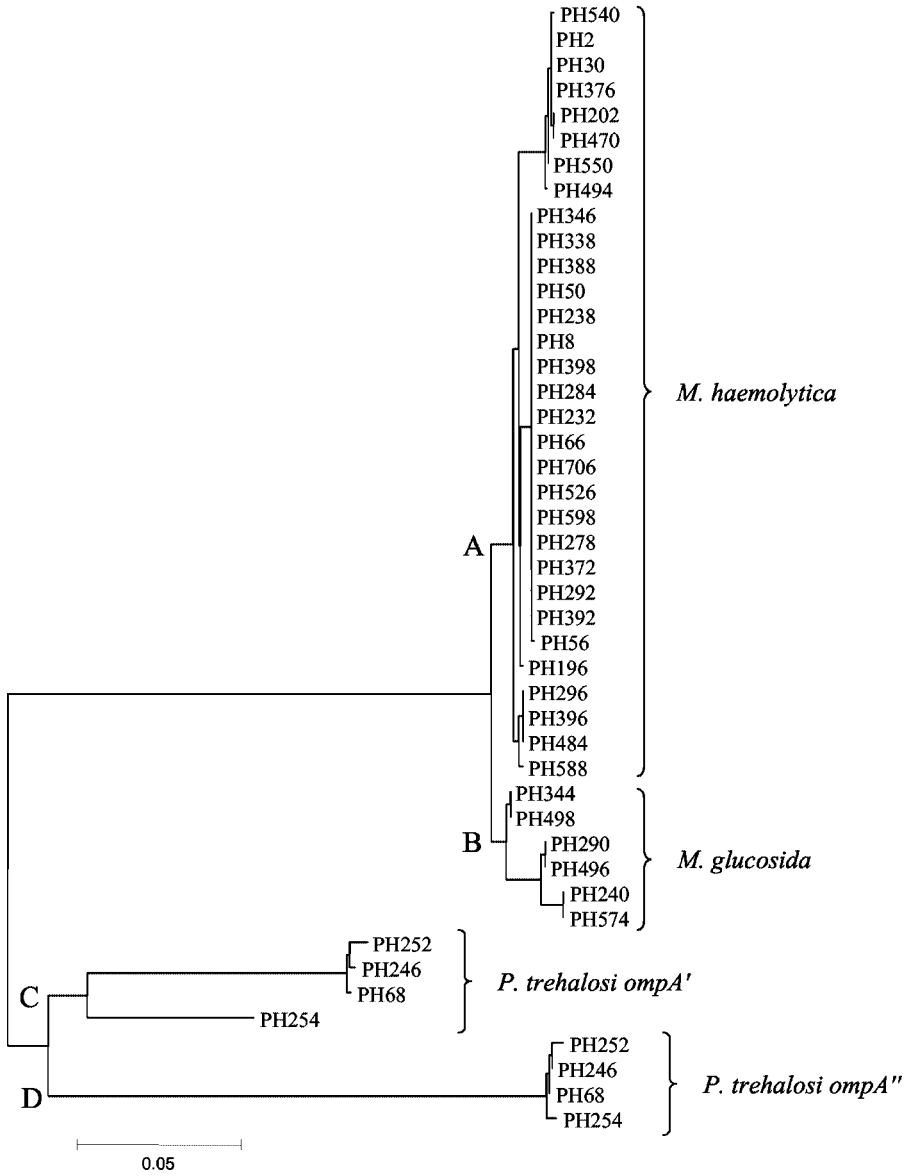

FIG. 2. Neighbor-joining tree representing the phylogenetic relationships of the *ompA* genes of 31 *M. haemolytica*, 6 *M. glucosida*, and 4 *P. trehalosi* strains constructed with the Jukes-Cantor correction for synonymous changes.

The *ompA′* and *ompA″* genes of the *P. trehalosi* isolates each consisted of four unique sequences that represented distinct alleles (Fig. 2 and Table 1). The *ompA′* alleles were assigned to two subclasses, *ompA8* (alleles *ompA8.1* to *ompA8.3*) and *ompA9* (allele *ompA9.1*), whereas the *ompA″* alleles consisted of a single subclass, *ompA10* (alleles *ompA10.1* to *ompA10.4*). The *ompA′* alleles were 1,095 nucleotides long and the predicted proteins were 365 amino acids long, with molecular masses of 38,633 to 38,844 Da (Table 1). Since the putative signal sequence is 20 amino acids (1,918 Da), the molecular masses of the predicted mature proteins varied from 36,714 to 36,926 Da. The *ompA″* alleles were 1,083 nucleotides long and the predicted proteins were 361 amino acids long, with a molecular mass of 37,978 Da; the mature proteins were predicted to have a molecular mass of 36,060 Da. There were 10 (0.9%) polymorphic nucleotide sites and a single variable amino acid position among the three *ompA8*-type alleles and 9 (0.8%) polymorphic nucleotide sites (no amino acid changes) among the four *ompA10*-type alleles. The *ompA9.1* allele differed from the *ompA8.1* to *ompA8.3* alleles at 129 to 136 nucleotide sites and 39 to 40 amino acid positions. Pairwise differences between the *P. trehalosi ompA′* and *ompA″* alleles and those of *M. haemolytica* and *M. glucosida* ranged from 216 to 265 (*ompA′*) and 295 to 307 (*ompA″*) nucleotides and from 84 to 102 (*ompA′*) and 118 to 121 (*ompA″*) amino acids, respectively. Pairwise differences between *ompA′* and *ompA″* alleles ranged from 213 to 231 nucleotides and 83 to 84 amino acids.

The majority of polymorphic nucleotide and inferred amino acid sites in the *ompA* genes of *M. haemolytica* and *M. glucosida* occur in four hypervariable domains located within the surface-exposed loops. The locations of the polymorphic nucleo-

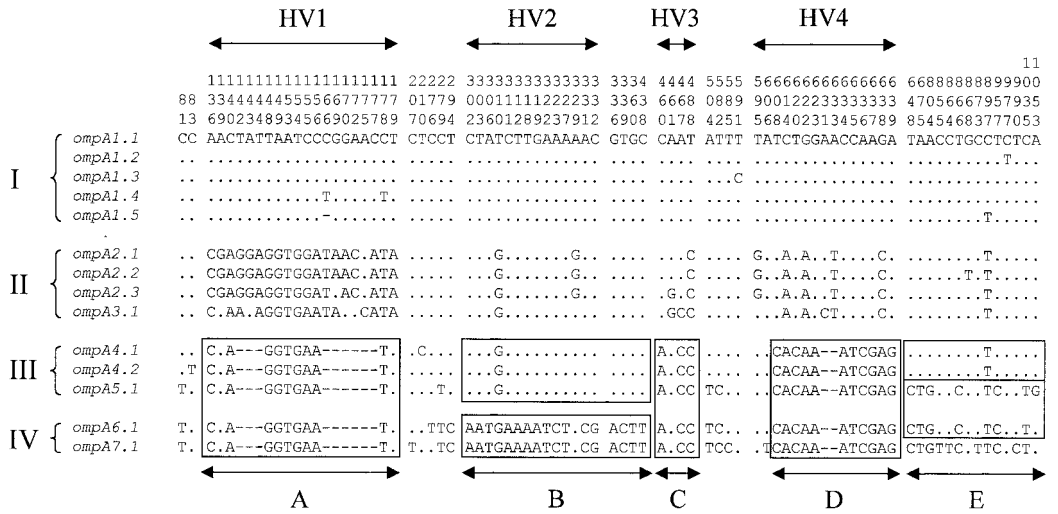

FIG. 3. Distribution of polymorphic nucleotide sites among the 14 *ompA* alleles of *M. haemolytica* and *M. glucosida*. Allele designations are shown to the left of each sequence. Roman numerals I to IV represent the major allele classes. The numbers above the sequences (read vertically) represent the positions of polymorphic nucleotide sites. The dots represent sites where the nucleotides match those of the first (topmost) sequence. Gaps are indicated by dashes. Boxes highlight identical, or nearly identical, segments of DNA (A to E) in class III and IV alleles. HV1 to HV4 represent the hypervariable domains.

tide sites and variable inferred amino acid positions within the *ompA* genes of *M. haemolytica* and *M. glucosida* were strikingly nonrandom in their distribution (Fig. 3 and 4). The majority of polymorphic sites occurred within four hypervariable regions, HV1 to HV4, located in the transmembrane domain. The hypervariable domains together consisted of 53 of 153 (35%) polymorphic nucleotide sites and 29 of 51 (57%) variable amino acid positions. In contrast, the remainder of the *ompA* gene was highly conserved and contained only 29 of 985 (3%) polymorphic nucleotide sites and 4 of 328 (1%) variable inferred amino acid positions. Domains HV1, HV2, and HV4 were characterized by amino acid deletions and/or insertions that accounted for the molecular mass variation of OmpA described above (Fig. 4).

The locations of the four hypervariable regions within the *M. haemolytica* and *M. glucosida* OmpA proteins, in relation to the β-strands and surface-exposed loops of the transmembrane domain, were identified by secondary structure prediction (25, 28), alignment with the three-dimensional structural models of the *E. coli* OmpA protein (2, 40), and comparison with the proposed secondary structure model of the P5 (OmpA) protein of *H. influenzae* (57; also results not shown). In this way, the four hypervariable domains of the *M. haemolytica* and *M. glucosida* OmpA proteins were shown to be located at the distal ends of the surface-exposed loop regions of the eight-stranded β-barrel structure (Fig. 4 and 5).

Evidence for assortative (entire gene) recombination of *ompA* among divergent lineages of *M. haemolytica* and *M. glucosida*. Assortative (entire gene) recombination leads to the presence of identical, or almost identical, alleles in strains that are genetically divergent. The evolutionary relationships of the *M. haemolytica* and *M. glucosida ompA* alleles with respect to the genetic relationships of the strains of origin based on multilocus enzyme electrophoresis data (11) are shown in Fig. 6. The *ompA* alleles were represented by four distinct lineages, I to IV, which correspond to the four classes described above. The association of identical, or almost identical, alleles with divergent lineages of *M. haemolytica* provides strong evidence that these alleles have undergone horizontal transfer and assortative (entire gene) recombination.

In lineage I, alleles *ompA1.1* to *ompA1.5*, which differ from each other at only one to three nucleotide sites (Fig. 3), were associated exclusively with bovine isolates (serotypes A1, A2, and A6) of ETs 1 and 2 (lineage A), 16 and 17 (lineage B), and 21 (lineage C) (Fig. 6 and Table 1). In lineage II, alleles *ompA2.1* to *ompA2.3*, which also differ from each other at only one to three nucleotide sites (Fig. 3), were associated exclusively with ovine isolates (serotypes A1, A2, A5 to A8, A9, A12, A14, and A16) of ETs 3 to 11 (lineage A) and 19 to 22 (lineage C) (Fig. 6 and Table 1). Interestingly, the majority of ovine isolates representing lineage A (ETs 3 to 11) possessed *ompA2.1*, whereas all of the isolates representing lineage C (ETs 19 to 22) possessed *ompA2.3*; these two alleles differ at two nucleotide sites (Fig. 3). In lineage III, alleles *ompA4.1* and *ompA4.2* differ from each other at only two nucleotide sites. However, *ompA4.1* alleles were associated exclusively with ovine serotype A7 isolates of ETs 12 to 14 (lineage B), whereas *ompA4.2* was associated with an ovine serotype A13 isolate of ET 15 (lineage B).

There was also some evidence of assortative recombination in *M. glucosida* since the *ompA5.1* alleles of lineage III were associated with isolates of ETs 1 and 3, and the *ompA6.1* and *ompA7.1* alleles of lineage IV were present in strains of ETs 7 and 16 and ETs 5 and 10, respectively (Fig. 6 and Table 1).

Evidence for intragenic recombination among *ompA* alleles of *M. haemolytica* and *M. glucosida*. Intragenic recombination leads to the formation of linked runs of nucleotides within a sequence whose ancestry is different from other nucleotides in the same sequence (50), i.e., the sequence has a mosaic structure. A visual inspection of the distribution of polymorphic nucleotide sites among the class I and II alleles revealed no evidence of intragenic recombination involving these two

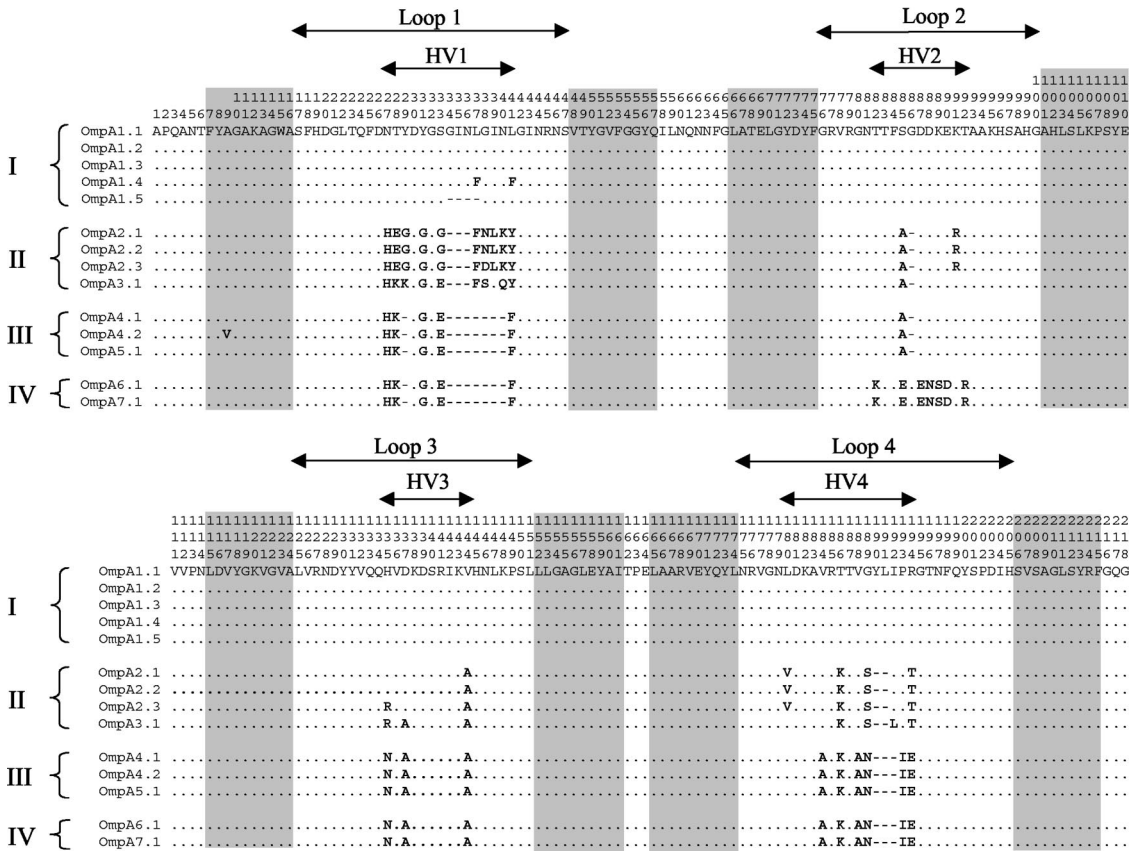

FIG. 4. Distribution of variable inferred amino acid sites in the N-terminal transmembrane domains of the 14 OmpA proteins of *M. haemolytica* and *M. glucosida*. Protein designations are shown to the left of each sequence. Roman numerals I to IV represent the major allele classes. The numbers above the sequences (read vertically) represent amino acid positions. The dots represent sites where the amino acids match those of the first (topmost) sequence. Gaps are indicated by dashes. HV1 to HV4 represent the hypervariable domains within surface-exposed loops 1 to 4. Shaded regions represent predicted membrane-spanning β-strand structures.

groups (Fig. 3). In contrast, there was clear visible evidence of mosaicism within the class III and IV alleles because runs of nucleotides representing recombinant segments were present. For example, segments A, C, and D were identical in all five class III and IV alleles, but the sequence of segment B was very different in *ompA4.1*, *ompA4.2*, and *ompA5.1* compared to that in *ompA6.1* and *ompA7.1* (Fig. 3). In addition, the sequences of segment E were very different in *ompA4.1* and *ompA4.2*, in *ompA5.1* and *ompA6.1*, and in *ompA7.1*.

A pairwise comparison of the *ompA* sequences by the maximum chi-square method identified the locations of significant breakpoints (k_{max}) that represent the end points of recombinant segments in class II, III, and IV alleles. A comparison of alleles *ompA5.1* and *ompA6.1* identified a region from nucleotides 276 to 438 that differs at 11% of the nucleotide sites and is embedded in a region that is almost identical in the two alleles (Fig. 3). A comparison of alleles *ompA2.1* and *ompA4.1* identified a region from nucleotides 640 to 1119 that is identical in both alleles, whereas the region from nucleotides 1 to 639 differs at 3% of the nucleotide sites.

Synonymous and nonsynonymous substitution rates among *ompA* alleles of *M. haemolytica* and *M. glucosida*. To determine how the level of selective constraint varies along the *ompA* genes of *M. haemolytica* and *M. glucosida*, we estimated the numbers of synonymous substitutions per synonymous site (d_S) and nonsynonymous substitutions per nonsynonymous site (d_N) (37) and calculated the d_S/d_N ratios for each of the hypervariable domains and for the combined conserved regions (Table 3). A high d_S/d_N ratio indicates that natural selection at the molecular level is purifying (conservative), acting against mutations resulting in amino acid replacement. Conversely, a d_S/d_N ratio of <1 indicates that selection is diversifying and favors amino acid replacement. The d_S values were four to seven times higher for the hypervariable domains HV1, HV2, and HV4 than for the conserved regions, whereas the d_N values were 2 to 3 orders of magnitude higher for the same domains than for the conserved regions (Table 3). The d_S/d_N ratio for the conserved regions of *ompA* was relatively high, at 35.17, whereas the corresponding values for the hypervariable domains HV1, HV2, and HV4 ranged from 0.52 to 0.77. These data provide strong evidence of a selective constraint against amino acid replacement in the conserved parts of the gene and of diversifying selection in the hypervariable regions.

Evidence for independent transcription and expression of two OmpA proteins in *P. trehalosi*. The upstream intragenic regions of *ompA′* and *ompA″* were 213 and 200 nucleotides long, respectively, in contrast to the downstream intragenic region of *ompA″*, which was only 56 nucleotides long. Putative

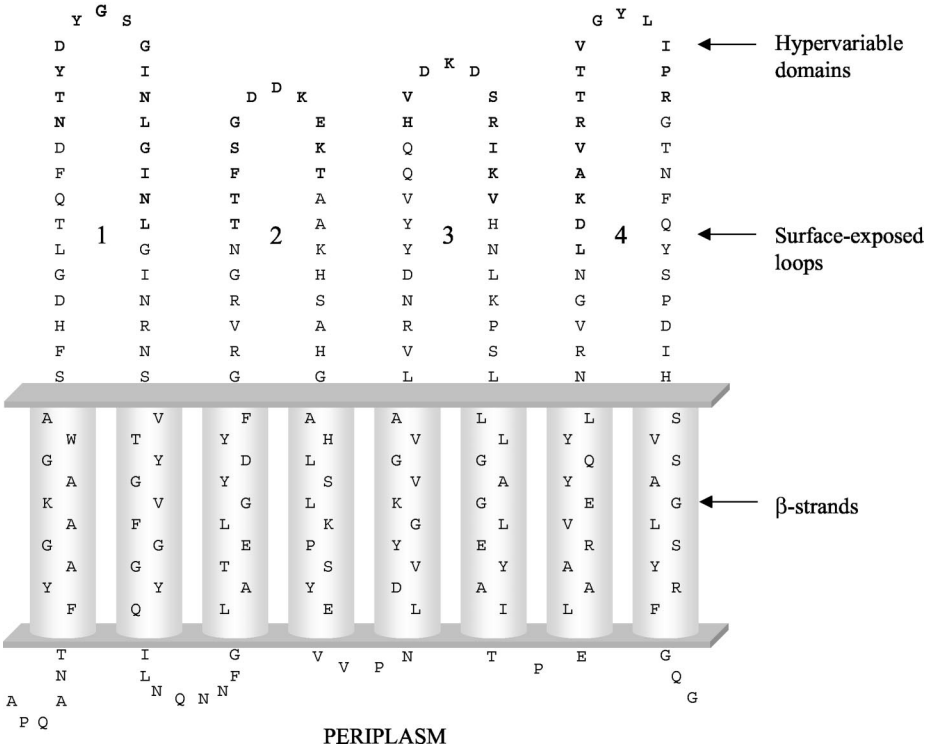

FIG. 5. Proposed secondary structure of N-terminal transmembrane domain of the OmpA proteins of *M. haemolytica* and *M. glucosida*. The sequence is based on OmpA1.1 of strain PH2 (see Fig. 4). The hypervariable domains are shown in bold.

ribosome-binding sequences and promoter sites were identified upstream of the start codons of *ompA′* and *ompA″*, and potential inverted repeat terminator sequences were present downstream of the stop codons of *ompA′* and *ompA″* (results not shown). The existence of putative ribosome-binding, promoter, and terminator sequences associated with *ompA′* and *ompA″* suggests that both genes are independently transcribed.

The expression of two OmpA proteins, OmpA′ and OmpA″, by *P. trehalosi* was confirmed by SDS-PAGE analysis of OMPs after heating the samples at 80, 90, and 100°C (Fig. 7). The OmpA protein undergoes a characteristic shift from low- to high-molecular-mass forms after heating at 100°C. Two low-molecular-mass proteins (29 to 30 kDa) were clearly visible for strains PH68, PH246, and PH252 at 80 and 90°C, whereas these were transformed to two high-molecular-mass proteins (37 to 39 kDa) at 100°C (Fig. 7, lanes 1 to 9, arrows). However, only one low-molecular-mass band was present in strain PH254 at 80 and 90°C (Fig. 7, lanes 10 and 11, arrow), although two bands, of 29 and 39 kDa, were present at 100°C (Fig. 7, lane 12, arrows). The most probable explanation for this difference is that the single 29-kDa band present at 80 and 90°C consists of both proteins (OmpA′ and OmpA″), which in this strain differ in their heat modification properties. Thus, at 100°C the 29-kDa band presumably corresponds to the OmpA′ protein OmpA9.1 (since this band and protein were not present in any of the other isolates), whereas the 39-kDa band represents the OmpA″ protein OmpA10.4.

DISCUSSION

OmpA structure and function in *M. haemolytica*. Three-dimensional structural analyses of the *E. coli* OmpA protein have revealed that the transmembrane domain consists of eight highly conserved membrane-spanning regions and four relatively long, mobile, hydrophilic surface-exposed loops (2, 39, 40). The OmpA proteins of *M. haemolytica* and *M. glucosida* contain four discrete hypervariable domains that are located at the distal ends of the surface-exposed loops (Fig. 5). Similar hypervariable regions correspond to the surface-exposed loops of the OmpA (P5) protein of *H. influenzae* (19, 57). Different selection pressures and evolutionary constraints operate on different parts of the molecule, since the patterns of synonymous and nonsynonymous nucleotide substitution rates vary throughout the *ompA* gene. Amino acid replacement is highly constrained within the conserved regions of OmpA (d_S/d_N = 35.17) because these parts of the molecule correspond to the membrane-spanning and periplasmic domains and cannot tolerate excessive amino acid change. In contrast, diversifying selection operates on the hypervariable domains of the surface-exposed loops because the number of nonsynonymous nucleotide substitutions greatly exceeds the number of synonymous substitutions (d_S/d_N = 0.52 to 0.77 for domains HV1, HV2, and HV4). Similar high rates of nonsynonymous nucleotide substitutions also occur in the four surface-exposed loops of the OmpA (P5) protein of *H. influenzae* (19). Amino acid diversity within the surface-exposed loops is clearly advanta-

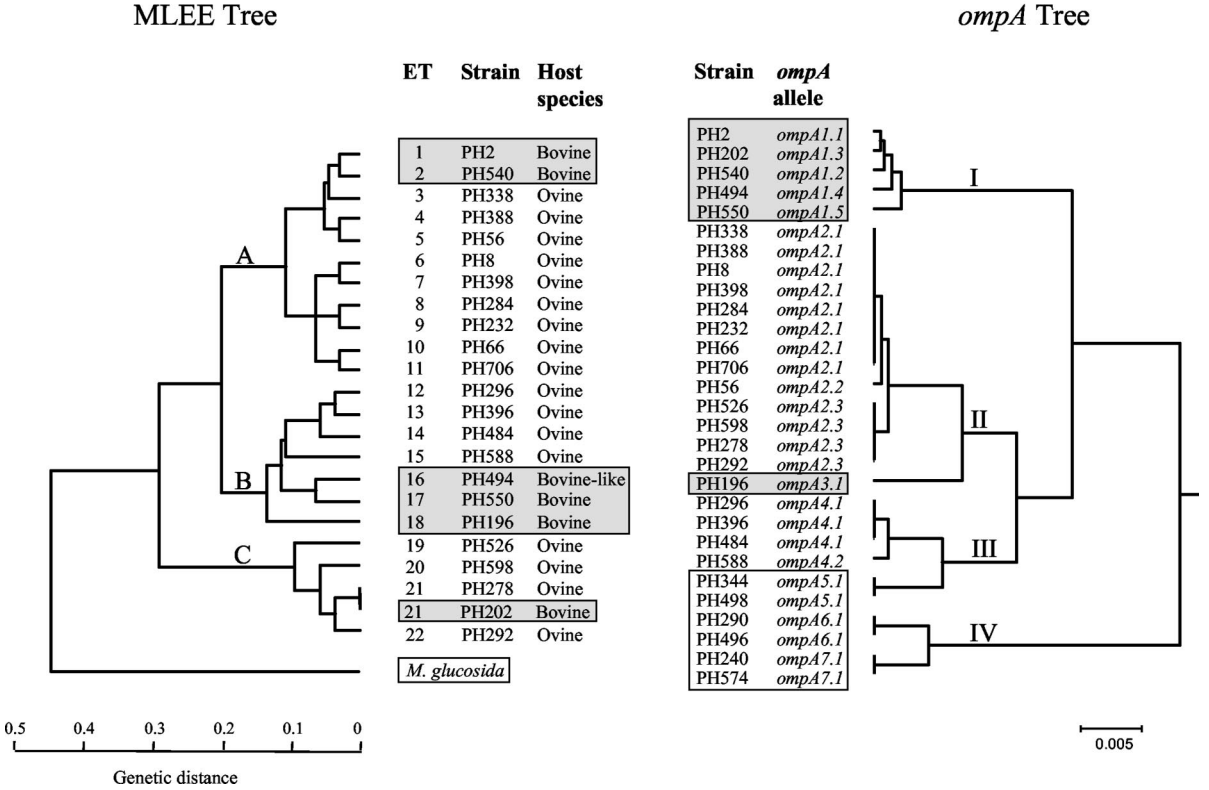

FIG. 6. (Left) Phylogenetic relationships of 178 *M. haemolytica* and 16 *M. glucosida* strains based on electrophoretically demonstrable variation of 18 housekeeping enzymes (11). (Right) Phylogenetic relationships of *ompA* genes from 31 *M. haemolytica* and 6 *M. glucosida* strains. Both trees were constructed by the unweighted pair group method using arithmetic averages. Shaded boxes represent the bovine *M. haemolytica* isolates and *ompA* alleles. White boxes represent the *M. glucosida* isolates and *ompA* alleles.

geous to *M. haemolytica* and suggests that these parts of the molecule play an important role in some aspect of this pathogen's biology.

An increasing body of evidence from other pathogens indicates that OmpA functions as a ligand, is involved in binding to specific host cell receptor molecules, and plays a role in adherence and colonization (9, 24, 33, 42, 44, 46, 55). The exclusive association of the OmpA1- and OmpA2-type proteins with bovine and ovine strains of *M. haemolytica*, respectively, together with evidence that the *ompA1*- and *ompA2*-type genes have undergone horizontal transfer between strains of divergent phylogenetic lineages, indicates that OmpA is under strong selective pressure from the host and plays an important role in host-pathogen relationships. The evidence from other pathogens suggests that the OmpA protein of *M. haemolytica* acts as a ligand and participates in binding to specific host cell receptor molecules in the upper respiratory tracts of cattle and sheep. We propose that the OmpA1 and OmpA2 proteins are involved in binding to bovine and ovine receptors, respectively, and that they play important roles in host specificity. In a similar way, receptor binding of the variable loop regions of the related Opa protein determines tissue tropism in *Neisseria* (5, 41, 56). Although there is no direct evidence to support this hypothesis, a host-specific ligand-like function would account for the variation in the surface-exposed loop regions between the bovine class I and ovine class II proteins and also for the amino acid conservation within each class. Clearly, the surface-exposed loops of OmpA need to be different in bovine and

TABLE 3. Sequence diversity and substitution rates for hypervariable domains HV1 to HV4 and for conserved regions of the *ompA* genes of 31 *M. haemolytica*, 6 *M. glucosida*, and 4 *P. trehalosi* isolates

Domain	Sequence diversity (%)		d_S[a]	d_N[a]	d_S/d_N
	Nucleotide	Amino acid			
HV1	44	67	0.1396 ± 0.1015	0.4064 ± 0.1433	0.71
HV2	42	72	0.0831 ± 0.0601	0.1586 ± 0.0605	0.52
HV3	13	30	0.0000 ± 0.0000	0.0691 ± 0.0404	
HV4	33	53	0.1518 ± 0.1244	0.1954 ± 0.0814	0.77
Conserved regions	3	1	0.0211 ± 0.0046	0.0006 ± 0.0004	35.17

[a] d_S is the number of synonymous substitutions per 100 synonymous sites; d_N is the number of nonsynonymous substitutions per 100 nonsynonymous sites. Values are means ± standard deviations.

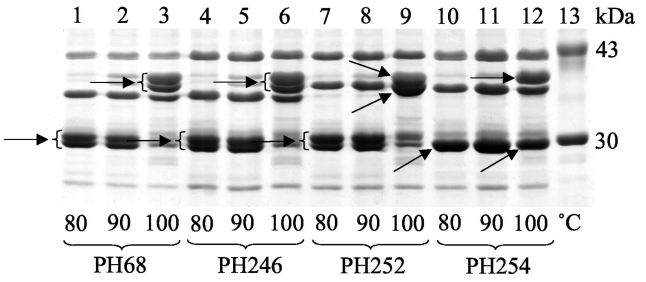

FIG. 7. Coomassie blue-stained SDS-PAGE OMP profiles of *P. trehalosi* strains PH68 (lanes 1 to 3), PH246 (lanes 4 to 6), PH252 (lanes 7 to 9), and PH254 (lanes 10 to 12) after heating at 80°C (lanes 1, 4, 7, and 10), 90°C (lanes 2, 5, 8, and 11), and 100°C (lanes 3, 6, 9, and 12). Molecular mass standards (ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa) are shown in lane 13. Only the relevant part of the gel is shown. Arrows indicate the low- and high-molecular-mass forms of the two OmpA proteins in each strain. The transition is clearly seen between 90 and 100°C. Only one band is present in strain PH254 at 80 and 90°C, whereas two bands occur at 100°C (see the text for further explanation).

ovine strains if cattle and sheep have different receptor molecules, but they would also need to be conserved within each class if they are involved in binding to specific bovine (class I) or ovine (class II) receptor molecules. It is thought that the long, mobile, surface-exposed loops of OmpA are required for interactions with other structures, such as cell receptors and bacteriophages, and that otherwise there is no evolutionary advantage to maintaining them (39, 40). The locations of the four hypervariable domains, HV1 to HV4, at the distal ends of the corresponding loops (Fig. 5) provide further evidence to support the hypothesis that these regions are involved in receptor recognition and binding. Confirmation of this hypothesis will require the production of genetically modified strains and the development of appropriate in vitro adherence assays.

Evolution of OmpA in *M. haemolytica* and *M. glucosida*. The class I, II, and IV *ompA* alleles are associated exclusively with bovine *M. haemolytica*, ovine *M. haemolytica*, and *M. glucosida* strains, respectively. These three groups of alleles have very different nucleotide sequences (Fig. 3) and have evolved independently, by point mutations and the accumulation of insertions and/or deletions, since their divergence from a common ancestor. There is no evidence that intragenic recombination has occurred among these three groups of alleles. In contrast, the class III alleles are associated with strains of both *M. haemolytica* and *M. glucosida* and have mosaic structures that have been derived, by horizontal DNA transfer and intragenic recombination, from class II and IV alleles. A comparison of the *M. glucosida ompA5.1* allele with the *M. glucosida ompA6.1* and *ompA7.1* alleles suggested that *ompA5.1* was derived from an *ompA6.1*-like allele by the acquisition of segment B from an *M. haemolytica* class II allele (Fig. 3). The similarity of the *M. haemolytica ompA4.1* and *ompA4.2* alleles to the *M. glucosida ompA5.1* allele (Fig. 3) also suggests that *ompA4.1* and *ompA4.2* were acquired by horizontal transfer from *M. glucosida*. The occurrence of *ompA4.1* and *ompA4.2* within phylogenetically related serotype A7 and A13 strains of ETs 12 to 14 and 15 (Fig. 6), respectively, indicates that they have a common evolutionary origin. Serotype A7 and A13 *M. haemolytica* strains appear to represent a distinct clonal complex that has undergone frequent recombination with *M. glucosida* because serotype A7 and A13 *M. haemolytica* strains share features of their OMP and lipopolysaccharide profiles with *M. glucosida* isolates (14), and the *lktA* alleles of serotype A7 and A13 *M. haemolytica* strains contain recombinant segments that have been derived from *M. glucosida* isolates (17).

The association of identical, or nearly identical, class I and II *ompA* alleles with divergent phylogenetic lineages of *M. haemolytica* (Fig. 6) suggests that horizontal transfer and assortative (entire gene) recombination have been important factors in the evolution of *ompA*. The data also provide further evidence to support the view that host switching of strains from cattle to sheep and vice versa has contributed to these evolutionary events and to the emergence of new strains (13, 17). The horizontal transfer of class I *ompA1*-type alleles has occurred independently on different occasions within lineages A, B, and C. Bovine A1 and A6 strains of ETs 1 and 2 may have evolved from ovine A1 and A6 strains after transmission of the latter from sheep to cattle and the subsequent acquisition of *ompA1*-type alleles from bovine A2 isolates of ET 16, 17, or 21. Bovine A1 and A6 strains are more closely related to ovine isolates of the same serotypes than they are to bovine A2 strains (11), and they also share very similar *lktA* alleles (17). The horizontal transfer of class II *ompA2*-type alleles has occurred on numerous occasions within lineages A and C. The presence of identical *ompA2.1* alleles in ovine A1, A5 to A7, A9, A12, A14, and A16 strains of ETs 3 to 11 (lineage A) suggests that *ompA2.1* has undergone multiple and recent horizontal gene transfer and recombination events between divergent strains (11). The horizontal transfer of *ompA2.1* has taken place so recently that there has been insufficient time for point mutations to accumulate. In contrast, the capsular polysaccharide antigens within these lineages are extremely diverse (there are at least eight capsular serotypes) and are clearly subject to strong diversifying selection, presumably due to the host immune response. Therefore, the capsular polysaccharide antigens and OmpA proteins of these strains are subject to very different selection pressures that are presumably related to their different functions.

The presence of divergent *ompA* alleles in closely related bovine (*ompA1.3*) and ovine (*ompA2.3*) serotype A2 isolates representing ET 21 provides clues about the evolutionary histories of these strains. One possibility is that the ovine strains evolved from ancestral bovine isolates after transmission of the latter from cattle to sheep. Subsequently, these bovine-derived strains acquired, by horizontal DNA transfer, *ompA2*-type alleles from ovine isolates, and after other evolutionary changes, evolved into the present-day ovine-adapted strains represented in lineage ET 21. Additional evidence to support this hypothesis comes from a comparative sequence analysis of the *lktA* gene (17). Thus, evidence for both the *lktA* and *ompA* genes suggests that strains of a common evolutionary origin have diverged and become adapted to different host species. The trigger for this divergence appears to have been the transmission of isolates from cattle to sheep and vice versa, which is probably linked to the domestication of these species (17).

***P. trehalosi* produces two OmpA homologs.** The discovery that *P. trehalosi* produces two OmpA homologs encoded by different tandemly arranged *ompA* genes was not entirely un-

expected because similar findings have been described for *Aeromonas salmonicida* and *Haemophilus ducreyi* (8, 29). The presence of putative ribosome-binding, promoter, and termination sequences associated with each of the *ompA* genes also provides indirect evidence that they are independently transcribed (8, 54). A retrospective examination of the OMP profiles of a wide range of *P. trehalosi* isolates (16) indicated that the expression of two OmpA homologs is common in this species. The identification of tandem *ompA* genes in another bacterial species suggests that this phenomenon is more widespread in gram-negative bacteria than was previously thought. It also seems likely that a common underlying mechanism is responsible for generating tandem *ompA* genes in certain bacterial species and that the expression of two OmpA homologs provides a selective advantage to these organisms.

It has been suggested that the two *ompA* genes of *A. salmonicida* and *H. ducreyi* have arisen by gene duplication (8, 29). Based on the relatively low level of homology between the two OmpA proteins of *A. salmonicida*, Costello et al. (8) concluded that such a gene duplication event occurred in the distant evolutionary past. However, our data suggest an alternative possibility. The presence of very different *ompA′*-type alleles in strains PH68, PH246, and PH252 (*ompA8.1* to *-8.3*) compared to that in strain PH254 (*ompA9.1*) and the occurrence of very similar *ompA″*-type alleles in the same strains (*ompA10.1* to *-10.4*) indicate that one or the other (or both) of the *ompA8*- or *ompA9*-type alleles has been acquired by horizontal transfer. The low frequency of occurrence of the *ompA9.1* allele suggests that this allele has replaced an *ompA8*-type allele, not vice versa, by horizontal gene transfer. In addition, the low level of similarity between the *ompA′* and *ompA″* alleles, in contrast to the high degree of similarity among the *ompA8*- and *ompA10*-type alleles, is consistent with acquisition by horizontal transfer rather than by gene duplication. If one of the *ompA* genes had arisen by duplication in the distant past, we would also expect to see more divergence among alleles representing each of the *ompA* types, but this is not the case. Therefore, horizontal DNA transfer, rather than gene duplication, might account for the second *ompA* gene in this and other bacterial species.

Finally, it has previously been shown that large segments of DNA from the *lktA* genes of *M. glucosida* and *P. trehalosi* have become incorporated by intragenic recombination into the *lktA* gene of certain *M. haemolytica* serotypes (17). In the case of *ompA*, there was evidence for recombinational exchange involving *M. glucosida* and *M. haemolytica* but not for *P. trehalosi* and *M. haemolytica*. Therefore, we conclude that *M. glucosida* has been involved in frequent recombinational exchanges with certain *M. haemolytica* strains but that recombination between *P. trehalosi* and *M. haemolytica* is much less common.

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