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Research Article

A preliminary survey of *M. hyopneumoniae* virulence factors based on comparative genomic analysis

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Abstract

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia (PEP), a major problem for the pig industry. The mechanisms of *M. hyopneumoniae* pathogenicity allow to predict the existence of several classes of virulence factors, whose study has been essentially restricted to the characterization of adhesion-related and major antigenic proteins. The now available complete sequences of the genomes of two pathogenic and one non-pathogenic strain of *M. hyopneumoniae* allowed to use a comparative genomics approach to putatively identify virulence genes. In this preliminary survey, we were able to identify 118 CDSs encoding putative virulence factors, based on specific criteria ranging from predicted cell surface location or variation between strains to previous functional studies showing antigenicity or involvement in host-pathogen interaction. This survey is expected to serve as a first step towards the functional characterization of new virulence genes/proteins that will be important not only for a better comprehension of *M. hyopneumoniae* biology, but also for the development of new and improved protocols for PEP vaccination, diagnosis and treatment.

Key words: porcine enzootic pneumoniae, virulence, host-pathogen interaction, antigen, adhesin.

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Introduction

Virulence is one of the possible outcomes of host-pathogen interaction and may involve a plethora of components from both partners in this mutual relationship. Pathogen-centered views of virulence consider that pathogens are distinguished from nonpathogens by their expression of virulence factors (Casadevall and Pirofski, 2001), which are encoded by the so called virulence genes. The concept of virulence genes is also rather ample and may vary according to the definition of virulence that is being used (Wassenaar and Gaastra, 2001). Essentially, they can be divided in true virulence genes, virulence-associated genes, and virulence life-style genes. True virulence genes are only those that encode factors directly involved in causing disease. However, true virulence factors may be regulated, activated or may require the activity of virulence-associated genes, and virulence life-style genes may be necessary to enable the pathogen to colonize its host.

The identification of virulence genes is based on different experimental criteria that range from mere antigenicity analysis of protein products to genetic studies involving inactivation or complementation of specific genes (Wassenaar and Gaastra, 2001). The advent of whole-genome sequencing triggered a new revolution in infectious disease research, as it allowed, in association with the improvement of bioinformatic methods, the extensive identification of candidate virulence genes in several pathogenic organisms (Weinstock, 2000; Allan and Wren, 2003; Chen *et al*., 2005).

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia (PEP), a major problem for the pig industry. This bacterium is an extracellular pathogen, which colonizes the pig respiratory epithelium and induces PEP by first damaging the ciliated epithelial cells of the trachea, bronchi, and bronchioles (DeBey *et al*., 1992). However, the mechanisms underlying *M. hyopneumoniae*-induced ciliary damage or loss of cilia are not well understood (Park *et al*., 2002). Despite of the relatively low exposure of the bacterium to the host immune system, *M. hyopneumoniae* causes a general immunosupression (Adegboye, 1978) and also induces a localized autoimmune response in infected animals, which is responsible for additional damage to the respiratory epithelium (Suter *et al*., 1985). The lesions associated with *M. hyopneumoniae* infection appear to be primarily the result of an induced

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host immune reaction and inflammatory response, rather than due to direct toxic effects of molecules produced by the bacterial cells (Razin *et al*., 1998).

The study of virulence factors in *M. hyopneumoniae* has been centered in the characterization of adhesion mediating molecules, especially the P97 adhesin (see, for example, Zhang *et al*., 1994; Chen *et al*., 1998; Hsu and Minion, 1998a 1998b; Djordjevic *et al*., 2004). However, the mechanisms of *M. hyopneumoniae* pathogenicity allow to predict the existence of several other classes of not yet identified virulence factors, including genes/proteins involved with secretion and/or traffic between host and pathogen cells, or with evasion and/or modulation of the host immune system. The now available complete sequences of the genomes of two pathogenic and one non-pathogenic strain of *M. hyopneumoniae* (Minion *et al*., 2004; Vasconcelos *et al*., 2005) allowed to use a comparative genomics approach to putatively identify new virulence genes. For that, we made a preliminary survey of CDSs that can be assigned as virulence genes. Although this survey is not intended, by any means, to be definitive, we expect that it may contribute to the identification of candidate virulence genes for future functional studies. The study of these genes and their corresponding products will be important not only for a better comprehension of host-pathogen interactions, but also for the improvement of PEP vaccines and diagnostic methods, as well as for the development of therapeutic drugs.

Material and Methods

Finished and annotated genome sequences of *M. hyopneumoniae* strains 232, J and 7448 have been recently published (Minion *et al*., 2004; Vasconcelos *et al*., 2005). Strains 232, and 7448 were characterized as pathogenic, while strain J is a non-pathogenic one, with reduced capacity to adhere to porcine cilia.

These genomic sequences were used in a systematic search for protein encoding sequences (CDSs) corresponding to candidate virulence factors. A CDS was considered as a putative virulence gene when fulfilling one or, preferentially, more of the following criteria: (i) previous description as a *M. hyopneumoniae* virulence gene based on experimental studies; (ii) orthology to previously described virulence genes in related bacteria; (iii) presence in the pathogenic *M. hyopneumoniae* strains and absence from the non-pathogenic one; (iv) demonstration of antigenicity of its product; (v) probable localization of its product in the bacterial surface (vi) sequence variability between strains; (vii) probable association of its product to one of the known *M. hyopneumoniae* pathogenicity mechanisms; (viii) possibility that its product is a component of a bacterial pathogenicity-related mechanism.

The systematic search for CDS coding for candidate virulence factors was performed in public databases and using the System for Automated Bacterial Integrated Annotation (SABIA) (Vasconcelos *et al.*, 2005). Comparative sequence analyses were performed using the programs Clustal X (Thompson *et al*., 1997), GeneDoc (Nicholas *et al.*, 1997) and Blast (Zhang and Maden, 1997). The definition of orthologous and paralogous sequences was based on a tblastx cutoff E value of $1e^{-5}$, with at least 60% query coverage and 50% identity; in special cases, these criteria were relaxed as indicated, according to peculiarities of specific gene families. The subcellular localization prediction was made using the PSORT program (Rey *et al*., 2005).

Sequence variability between some corresponding CDSs of *M. hyopneumoniae* strains other than J, 7448 and J was determined by PCR amplification of selected regions and DNA sequencing. DNA sequencing reactions were performed using the DYEnamic ET dye terminator cycle sequencing (MegaBACE) kit and run on MegaBACE 1000 capillary sequencers (Amersham Biosciences). Sequences were assembled using Phred and Staden package (Staden *et al*., 2003).

Results and Discussion

Table 1 summarizes the CDSs encoding putative virulence factors found in the genome sequences from *M. hyopneumoniae* strains J, 7448, and 232. The listed CDS-encoded products were assigned as virulence factors based on previously described results with *M. hyopneumoniae*, other mycoplasma species or other related bacterial species. They were divided in adhesins, variable antigens, other antigenic proteins, proteases and chaperones, strain-specific gene clusters, and other possible virulence factors. Each of these classes of putative *M. hyopneumoniae* virulence factors is briefly described and discussed below.

Adhesins

The adherence of *M. hyopneumoniae* to ciliated epithelium is necessary to induce colonization of the swine respiratory tract, and therefore, the adherence of the bacteria to host cells is an important initial step in the pathogenesis. The adherence process is mainly mediated by receptor-ligand interactions and the *M. hyopneumoniae* proteins (adhesins) possibly involved in these interactions are obvious candidates to be virulence factors. As previously described (Vasconcelos *et al*., 2005) and shown on Table 1, CDSs corresponding to several previously described mycoplasma adhesins (MgPa, LppS, LppT, P69, P76, P97, P102, P110, P146, and P216) were identified in the sequenced *M. hyopneumoniae* genomes. The presence of the same adhesin CDSs in all three sequenced *M. hyopneumoniae* genomes and the high degree of overall identity (> 94%) between the deduced amino acid sequences of the J, 7448 and 232 ortholog adhesins does not allow any obvious correlation to explain the differential virulence simply based on different adhesin repertoires.

Table 1 - List of CDSs encoding virulence factors in *M. hyopneumoniae* strains J, 7448 and 232, and identities between the corresponding orthologous deduced amino acid sequences of the different strains. The CDSs were classified according to the text and include CDSs encoding both previously described and putative virulence factors. Any additional criterium(a) used to assign a given CDS product as a virulence factor is(are) indicated (superscripted numbers), as well as any additional criterium(a) to establish orthology (superscripted letters). CDS numbers are according to GenBank and SABIA.

CDS product	CDS			Identity (%)		
	J	7448	232	J x 7448	J x 232	7448 x 232
Adhesins						
P29[11]	MHJ_0357	MHP7448_0361	mhp372	100	100	100
P69[1, 11]	MHJ_0358	MHP7448_0362	mhp373	99.30	99.30	99.65
P76[1, 3, 4, 6]	MHJ_0494	MHP7448_0497	mhp494	99.34	95.66	95.78
P97[1, 2, 3, 6, 8, 9]	MHJ_0194 (operon I)	MHP7448_0198	mhp183	92.72	94.45	95.49
	MHJ_0105 (operon II)	MHP7448_0108	mhp271	95.91	96.01	96.67
	MHJ_0264 (operon III)	MHP7448_0272	mhp107	98.34	99.51	98.44
P102 [1, 9]	MHJ_0195 (operon I)	MHP7448_0199	mhp182	98.67	97.90	98.23
	MHJ_0104 (operon II)	MHP7448_0107	mhp272	96.42	96.94	97.25
	MHJ_0263 (operon III)	MHP7448_0271	mhp108	97.95	98.99	98.59
	MHJ_0102[a]	MHP7448_0105[a]	-	99.00		
P146 [1, 3, 6]	MHJ_0663	MHP7448_0663	mhp684	92.46	97.12	94.19
P216[1, 3, 6]	MHJ_0493	MHP7448_0496	mhp493	97.88	97.13	97.35
LppS [1, 7, 11]	MHJ_0369	MHP7448_0373	mhp385	97.05	99.19	96.86
LppT [1, 3, 7, 11]	MHJ_0368	MHP7448_0372	mhp384	98.22	96.55	97.28
MgPa [9, 11]	MHJ_0006	MHP7448_0006	mhp006	99.35	99.38	98.75
	MHJ_0005	MHP7448_0005	mhp005	99.39	100	99.39
Variable antigens						
P95[1, 6, 11]	MHJ_0096	MHP7448_0099	mhp280	97.12	97.99	96.34
Hypothetical protein H1[1, 6]	MHJ_0441	MHP7448_0443	mhp445	96.31	98.22	96.25
Hypothetical protein H2[6]	MHJ_0444	MHP7448_0445	mhp447	84.04	88.11	87.14
Hypothetical protein H3 [6]	MHJ_0350	MHP7448_0355	mhp366	95.68	96.40	95.53
Hypothetical protein H4 [6]	MHJ_0032	MHP7448_0036	mhp037	94.44	97.91	96.10
Hypothetical protein H5 [6]	MHJ_0662	MHP7448_0662	mhp683	91.85	94.41	94.49
Hypothetical protein H6 [6]	MHJ_0442	MHP7448_0444	mhp446	88.54	85.87	86.36
Conserved hypothetical protein CH1 [6]	MHJ_0089	MHP7448_0092	mhp287	97.80	98.51	98.27
Other antigenic proteins						
Previously described antigens						
P36 (L-lactate dehydrogenase, ldh) [8, 11]	MHJ_0133	MHP7448_0137	mhp245	100	99.68	99.68
P46 [1, 8, 11]	MHJ_0511	MHP7448_0513	mhp511	99.52	100	99.52
P115 (ABC transporter ATP-binding protein P115-like) [1, 11]	MHJ_0657	MHP7448_0657	mhp678	99.59	99.28	99.28
P60 (p60-like lipoprotein) [11]	MHJ_0348	MHP7448_0353	mhp364	98.31	99.25	99.06
P65 (prolipoprotein p65) [8, 11]	MHJ_0656	MHP7448_0656	mhp677	99.04	99.56	98.88
P80 [1, 11]	MHJ_0347	MHP7448_0352	mhp363	99.40	99.71	99.43
NnrdF (ribonucleoside-diphosphate reductase beta chain) [1, 7, 8]	MHJ_0217	MHP7448_0223	mhp156	100	99.71	99.71
Pyruvate dehydrogenase (beta chain)[1, 3, 4]	MHJ_0112	MHP7448_0116	mhp264	100	100	100
Elongation factor EF-tu[4]	MHJ_0524	MHP7448_0523	mhp540	99.75	99.75	100
Membrane lipoproteins						
Lipoprotein Lp1[1, 11]	MHJ_0213	MHP7448_0217	mhp164	95.92	99.44	99.44
Lipoprotein Lp2[1, 11]	MHJ_0364	MHP7448_0368	mhp379	98.99	99.66	99.33
Lipoprotein Lp3[1, 11]	MHJ_0324	MHP7448_0333	mhp345	99.05	99.05	98.10
Lipoprotein Lp4[1, 11]	MHJ_0362	MHP7448_0366	mhp377	99.59	98.91	99.04
Lipoprotein Lp5[1, 11]	MHJ_0363	MHP7448_0367	mhp378	100	99.85	99.85
Lipoprotein Lp6[1, 11]	MHJ_0364	MHP7448_0368	mhp379	98.99	99.66	99.33
Lipoprotein Lp7[11]	MHJ_0374	MHP7448_0378	mhp390	98.68	98.68	99.34
Lipoprotein Lp8[11]	MHJ_0622	MHP7448_0621	mhp640	99.38	99.12	98.99
Proteases and chaperones						
Proteases						
Signal peptidase I[1, 7, 10]	MHJ_0022	MHP7448_0026	mhp028	98.63	98.63	100
Lipoprotein signal peptidase [1, 7, 10]	MHJ_0027	MHP7448_0031	mhp032	98.10	97.47	98.5
ATP-dependent protease binding protein[7]	MHJ_0098	MHP7448_0101	mhp278	99.27	99.57	99.71
Heat shock ATP-dependent protease (Lon)[7, 10]	MHJ_0525	MHP7448_0524	mhp541	99.89	100	99.89
Aminopeptidase [3, 7, 10]	MHJ_0125	MHP7448_0129	mhp252	99.44	100	99.44

Table 1 (cont.)

CDS product	CDS			Identity (%)		
	J	7448	232	J x 7448	J x 232	7448 x 232
Subtilisin-like serine protease[1, 7]	MHJ_0085	MHP7448_0332	mhp292	93.12	92.67	94.83
Methionine aminopeptidase (map) [1, 7]	MHJ_0169	MHP7448_0173	mhp209	99.20	100	99.2
Leucyl peptidase[7]	MHJ_0461	MHP7448_0464	mhp462	98.68	99.56	98.68
Proline dipeptidase[7]	MHJ_0378	MHP7448_0382	mhp394	100	99.40	99.40
Oligoendopeptidase F (pepF) [7, 10]	MHJ_0522	MHP7448_0521	mhp520	99.19	99.18	98.68
xaa-proaminopeptidase[7]	MHJ_0659	MHP7448_0659	mhp680	97.97	98.84	98.55
o-syaloglicoprotein endopeptidase[1, 7]	MHJ_0636	MHP7448_0635	mhp656	100	100	100
Chaperones						
Hsp70 - Chaperone (dnaK)[1, 4, 5, 7, 10]	MHJ_0063	MHP7448_0067	mhp072	99.83	99.50	99.67
Trigger factor (prolyl isomerase) Tig[7, 10]	MHJ_0145	MHP7448_0149	mhp233	99.31	99.35	99.54
Heat-shock protein (dnaJ) [5, 7, 10]	MHJ_0064	MHP7448_0068	mhp073	99.73	98.94	98.48
Heat-shock protein (grpE) [5, 7, 10]	MHJ_0011	MHP7448_0011	mhp011	98.80	99.60	99.19
ftsY - cell division protein[7]	MHJ_0008	MHP7448_0008	mhp008	99.70	99.70	100
ftsH - cell division protein[1, 7]	MHJ_0202	MHP7448_0206	mhp175	100	99.86	99.86
ftsZ - cell division protein[1, 7]	MHJ_0406	MHP7448_0393	mhp407	99.38	99.38	99.38
Member of the HSP33 family of disulfide bond chaperones[1, 7]	MHJ_0144	MHP7448_0148	mhp234	100	100	100
Metal-dependent proteases and putative molecular chaperones[7]	MHJ_0652	MHP7448_0652	mhp673	96.40	94.80	95.20
Metal-dependent proteases and putative molecular chaperones[7]	MHJ_0538	MHP7448_0537	mhp554	98.30	98.30	98.86
Strain-specific gene clusters						
ICEH-related						
Hypothetical protein	-	-	mhp131[d, e]			
Translation elongation factor	-	-	mhp430[d, e]			
Putative ICEF product	-	-	mhp521[d, e]			
Hypothetical protein	-	-	mhp525[d, e]			
Hypothetical protein	-	-	mhp528[d, e]			
Hypothetical protein	-	-	mhp529[d, e]			
Hypothetical protein	-	-	mhp536[d, e]			
Elongation factor P	-	-	mhp431[d, e]			
TRSE-like[1, 7, 11]	MHJ_0239[c]	MHP7448_0247[d, 12]	mhp133[d, 12]	100	99.33	99.33
TRSE-like[1, 7, 11]		MHP7448_0416	-			
TRSE-like[1, 7, 11]	-	-	mhp531[d, e]			
TRSE-like[1, 7, 11]	-	-	mhp532[d, e]			
DNA processing protein SMF	MHJ_0241[c]	MHP7448_0249[12]	mhp130[12]	99.78	99.78	98.68
Hypothetical protein	-	MHP7448_0290	mhp535			89.42
Hypothetical protein		MHP7448_0418				
Hypothetical protein	-	MHP7448_0412				
Hypothetical protein		MHP7448_0413	mhp538			73.23
Putative ICEF II[11]	-	MHP7448_0414	mhp534[b]			43.56
Putative ICEF II[11]	-	MHP7448_0415	mhp533[b]			35.75
Hypothetical protein	-	MHP7448_0417	mhp530[b]			49.35
Hypothetical protein	-	MHP7448_0419	mhp527[b]			44.16
SSB (single binding protein)[11]	-	MHP7448_0420	mhp522[b]			34.23
Hypothetical protein	-	MHP7448_0422	mhp523[b]			39.17
TraK putative ICEF-IIA[1, 7, 11]	-	MHP7448_0423	mhp526			50.45
Putative ICEF product[1, 11]	-	MHP7448_0424	mhp089[b]			32.04
Restriction-modification enzymes						
Type I specific subunit[7, 10]	MHJ_0283	-	-			
Type I specificity subunit [7, 10]	-	MHP7448_0292	-			
Type I specificity subunit [7, 10]	-	-	mhp303			
Conserved hypothetical (Type II methyltransferase subtype: gamma)[7, 10]	MHJ_0319	MHP7448_0327	mhp340	98.96	99.58	98.15
Cytosine specific DNA methylase (type II methyltransferase)[7]	MHJ_0615	MHP7448_0614	mhp633	97.86	98.35	97.86
DNA adenine methylase (DamP) type II restriction enzyme and methyltransferase subtype: alpha[7, 10]	MHJ_0623	MHP7448_0622	mhp 641	98.93	98.86	99.75

Table 1 (cont.)

CDS product	CDS			Identity (%)		
	J	7448	232	J x 7448	J x 232	7448 x 232
Type II restriction enzyme and methyltransferase subtypes: G, gamma[1, 7, 10]	-	MHP7448_0291	-			
Type III methyltransferase subtype: beta[7, 10]	MHJ_0423	MHP7448_0388	mhp400	53.17	97.75	97.75
Type III methyltransferase subtype: beta[7, 10]	MHJ_0383 MHJ_0308 MHJ_0382 MHJ_0399	-	-			
Type III methyltransferase subtype: beta[7, 10]	-	MHP7448_0410 MHP7448_0316	-			
Type III methyltransferase[7, 10]	-	-	mhp330 mhp427			
Other possible virulence factors						
Hemolysin-related proteins						
afuC (Hemolysin secretion ATP-binding protein, plasmid - *Escherichia coli* - TCP family)[1, 7, 10]	MHJ_0379	MHP7448_0383	mhp395	99.87	99.61	99.48
HlyA (TlyA)[1, 7, 10]	MHJ_0159	MHP7448_0163	mhp218	99.16	98.73	98.73
HlyC[1, 7, 10]	MHJ_0643	MHP7448_0643	mhp663	100	100	100
Putative hemolysin ABC transporter[1, 7]	MHJ_0156	MHP7448_0160	mhp222	99.71	99.85	99.56
Nucleases						
Excinuclease ABC subunit C[1, 7]	MHJ_0062	MHP7448_0066	mhp070	100	98.81	98.81
Excinuclease ABC subunit B[1, 7]	MHJ_0648	MHP7448_0648	mhp669	99.09	98.48	98.18
Putative 5'-3' exonuclease [1, 7]	MHJ_0582	MHP7448_0581	mhp548	99.32	100	99.87
Nuclease lipoprotein[7]	MHJ_0262	MHP7448_0270	mhp109	98.98	98.21	100
VACB-like ribonuclease II (vacB)[7, 10]	MHJ_0033	MHP7448_0037	mhp038	99.42	99.71	99.41
Membrane nuclease lipoprotein (mnuA) [1, 7]	MHJ_0581	MHP7448_0580	mhp597	98.35	98.35	98.90
Oxidative stress-related proteins						
Thiol peroxidase[5, 7, 11]	MHJ_0093	MHP7448_0096	mhp283	100	100	100
Neutrophil activating factor (napA)[7, 10]	MHJ_0454	MHP7448_0457	mhp456	96.53	100	97.22

Additional criteria to define a CDS product as a virulence factor: [1]Probable membrane or secreted protein (according to PSORT). [2]Experimental evidence of proteolytic processing (Djordjevic *et al.*, 2004). [3]Experimental evidence of post-translational modification (phosphorylation) (Pinto and Ferreira, in press). [4]Experimental evidence of antigenicity (our unpublished results). [5]Environmental stress related. [6]Variable length of tandem aminoacid repeats between strains. [7]Orthology to previously described virulence factors in related bacteria. [8]Previous description as a *M. hyopneumoniae* virulence factor based on experimental studies. [9]Probably associated to one of the known *M. hyopneumoniae* pathogenicity mechanisms.
[10]Component of a possible bacterial pathogenicity-related mechanism. [11]Previous description as a virulence factor in others mycoplasmas. [12]Not strain specific, but potentialy related to conjugative DNA transfer.
Additional orthology criteria: [a]Frameshifted p102-related CDSs; orthology based on nucleotide homology (deduced aminoacid sequences with homology below the cutoff value established to define CDS orthologs). [b]Identity below the established cutoff value (see Material and Methods); orthology based also on CDS position (between ICEH-related inverted repeats), and tblastx, tblasn, and psiblast with an e value of at least $1e^{-10}$. [c]Orthologous to 7448 and 232 ICEH CDSs (although strain J does not contains an ICEH). [d]Assigned as an ICEH-related CDS based on homology to CDSs from integrative and conjugative elements of *Mycoplasma fermentas* or *Spiroplasma* spp. [e]Assigned as an ICEH-related CDS based on position between ICEH-related inverted repeats. - Not found.

The hypothesis of differences in the expression patterns of these adhesins in distinct strains, however, can not be discarded and remains to be investigated. Regarding this aspect, it is interesting to consider the cases of cytadherence-related operons, from which adhesin CDSs may be coordinately expressed under the control of common regulatory sequences. Cythaderence-related operons were described for *M. genitalium* (Musatovova *et al*., 2003) and, for *M. hyopneumoniae*, there are at least seven examples of this kind of organization, listed in Table 2. Experimental evidence of polycistronic transcription from P97/P102 operons has been recently provided by Adams *et al*. (2005).

MgPa has been described as one of the protein components of the so-called tip organelle, a multiproteic attachment structure considered to be critical for surface parasitism and successful colonization of mammalian cells in some pathogenic mycoplasmas (*e.g. M. pneumoniae* and *M. genitalium*) (Layh-Schmitt and Harkenthal, 1999; Musatovova *et al*., 2003). However, in *M. hyopneumoniae*, the other proteins necessary to form a tip organelle are missing (Vasconcelos *et al*., 2005), indicating that MgPa, as well as its counterpart (MgPa-like) encoded by the same operon, might mediate adhesion in another molecular context.

The P97 adhesin is regarded as an important virulence determinant, being considered responsible, at least in part, for *M. hyopneumoniae* adherence to the swine respiratory cilia. The P97 mediated-adhesion depends on a cilium binding site located in the R1 amino acid repeat sequence of

Table 2 -Putative cythaderence-related operons in the *M. hyopneumoniae* genome.

Operon name	CDS[1] products
MgPa operon	MgPa and MgPa-like proteins
LppS/T operon[2]	LppS and LppT lipoproteins
p97/p102 operon I	p97 and p102 adhesins
p97/p102 operon II[3]	p97-like and p102-like proteins
p97/p102 operon III	p97-like and p102-like proteins
p146 operon	p146 adhesin and hypothetical lipoprotein
p216/p76 operon[2]	p216 and p76 adhesins

[1]The corresponding CDS numbers in the *M. hyopneumoniae* J, 7448 and 232 genomes are shown on Table 1.

the protein (Hsu and Minion, 1998b), and it was demonstrated that a minimum number of eight R1 repeat units are required for cilium binding (Minion *et al*., 2000). The P97 CDS of P97/P102 operon I, corresponding to the previously described *M. hyopneumoniae* P97 gene (Hsu and Minion, 1998a), codes for P97 proteins with 10, 9, and 15 R1 repeat units in strains J, 7448 and 232, respectively, all above the above cited minimum number and, therefore, potentially able to mediate adhesion. The fact that the non-pathogenic J strain, with reduced adhesion capacity to porcine cilia (Zhang *et al*., 1995), has an apparently functional P97 adhesin reinforces the idea of the multifactorial nature of the adhesion process.

The presence of P97-like proteins in P97/P102 operons II and III, and the recently described P97 post-translational proteolytic processing Djordjevic *et al*., 2004) add further complexity to the system. It has been recently demonstrated that all P97 paralog CDSs are transcribed during experimental infection with the 232 strain (Adams *et al*., 2005) and this simultaneous expression, associated with the possible proteolytic processing of the corresponding proteins, would generate a plethora of surface antigenic variants (see below), while keeping the capacity to mediate cell adhesion. It has been recognized, given the importance of the P97 adhesin to virulence, that its posttranslational cleavage may play an important role in the disease process (Djordjevic *et al*., 2004).

Similarly to their P97 counterparts, the P102 adhesin paralogs encoded by P97/P102 operons I, II or III are only 21-43% identical in each of the sequenced *M. hyopneumoniae* genomes. Unlike P97, however, P102 does not present variable amino acid tandem repeats between strains and, according to Djordjevic *et al*. (2004), no evidence for the above discussed posttranslational proteolytic cleavage was found for this adhesin. Regarding its expression, Adams *et al.* (2005) have demonstrated that, in the 232 strain, P102 paralog CDSs are transcribed during experimental infection, and the protein can be immunologically detected in *M. hyopneumoniae* adhered to epithelium cilia. The 7448 and J strains, but not the 232 one, present an additional frameshifted P102 CDS (see footnotes on Table 2), and it is interesting to note that this frameshift is not in the same position in the two strains, suggesting independent origins.

We have been also able to experimentally demonstrate post-translational modifications that generate alternative forms with different pI for at least 11 *M. hyopneumoniae* proteins, five of which are adhesins (Pinto and Ferreira, in press; Table 1). These modifications, which include but are not necessarily restricted to phosphorylation, further increase the repertoire of *M. hyopneumoniae* protein variants. Noteworthy, some of them, as in the cases of P76 and P97, occur concomitantly to the proteolytic processing discussed above. It will be important to investigate whether different post-translational processing patterns occur among different *M. hyopneumoniae* strains and are important for virulence, an issue that is being addressed by our group.

Variable antigens

Bacterial virulence factors, particularly surface-located molecules, are frequently antigenic and their antigenic properties are often associated, directly or indirectly, with pathogenesis. For instance, the generation of antigenic variants may be an important way to evade the host immune response, or the elicited immune response may be itself part of the disease process, as in the case of pathogen-induced autoimmunity.

For *M. hyopneumoniae*, there is evidence pointing to the occurrence of antigenic variation with possible correlation with virulence. Besides P97 (see above), several other *M. hyopneumoniae* CDSs encode proteins with variably reiterated amino acid repeat regions amongst different strains, including those coding for the P76, P146, and P216 adhesins. Variations in the number of amino acid repeats between J, 7448, and 232 orthologs were also identified for at least seven other CDS products annotated as membrane proteins (Vasconcelos *et al*., 2005), and this has been also demonstrated for additional CDSs and other *M. hyopneumoniae* strains by PCR amplification and DNA sequencing of repeat regions (Castro *et al.*, 2006).

The variably reiterated amino acid repeat regions found in *M. hyopneumoniae* encoded adhesins and other putative surface proteins result from variation in the number of tandem nucleotide repeat units within the corresponding CDSs. The occurrence of these repeated nucleotide motifs, however, is not restricted to CDSs, being found also in intergenic regions. In both cases, the variability in the length of these repetitive sequences is probably a consequence of slippage within them during replication (Henderson *et al*., 1999). Such slippage has also the potential to generate events of phase variation, but interestingly, this was not observed for any of the deduced protein variants identified so far in different *M. hyopneumoniae* strains, since the variable number of corresponding nucleotide repeats did not result in any alteration in translational frame.

This suggests a strong pressure for the maintenance of integrity of these proteins, while allowing the occurrence of variation in the number of amino acid repeats within them.

Besides leading to the generation of proteins with functional or antigenic variations when occurring within the corresponding CDSs, the presence of tandem nucleotide repeats within regulatory regions may lead also to on-off switching of the associated genes. The presence of variably repeated nucleotide sequences was identified in intergenic upstream flanking regions of at least 41 *M. hyopneumoniae* CDSs of strains J, 7448 and 232 (Vasconcelos *et al*., 2005). Among them are the CDS coding for the P97 like and LppS adhesins and the P65 antigen (see below). The apparent switch off of P97, at least at the protein level, has been demonstrated for some *M. hyopneumoniae* field strains (Assunção *et al.*, 2005), but the possible corresponding transcriptional inactivation was not yet assessed.

It has been also recently shown (Djordjevic *et al*., 2004) that P97 and some other high-molecular-weight proteins undergo proteolytic processing and that their cleavage products are translocated to and remain associated with the *M. hyopneumoniae* cell surface during growth, at least *in vitro*. Such processing was regarded as important for the establishment of the *M. hyopneumoniae* cell surface phenotype and this consideration gains in importance when we consider that, for different strains, fragments of several proteins, some of them with different numbers of amino acid repeats, are being presented to the host. This may have roles both to determine different adhesion properties for each *M. hyopneumoniae* strain and to evade or immunomodulate the host immune system.

The specific proteolytic processing of surface proteins must involve an equally specific set of proteases, that remain to be identified among the putative proteases already annotated for *M. hyopneumoniae* J, 7448 and 232 (Minion *et al*., 2004; Vasconcelos *et al.*, 2005). These surface protein processing proteases could themselves be regarded as virulence factors, assuming that they are responsible for the generation of cell surface phenotypes that are determinants of pathogenicity. *M. hyopneumoniae* proteases with putative roles in pathogenicity are discussed below (see *Proteases and nucleases* section).

Other antigenic proteins

Not all antigens are virulence factors, but virulence gene products are often immunogenic and responsible for acquired immunity that protects against disease (Wassenaar and Gaastra, 2001). Genes for nine previously described mycoplasma proteins not related to adhesins or other virulence factors but with a well established antigenic character and relevance for diagnosis and/or vaccination were also found in the genomes of *M. hyopneumoniae* J, 7448 and 232 strains. These antigens, namely P36, P46, P60, P65, P80, P95, P115, nrdF and napA, are very similar (96-100% identity at the amino acid level) in the three strains, which does not allow any inference of a more obvious role in pathogenesis for any of them. However, the P36 antigen, despite corresponding to a lactate dehydrogenase enzyme, is apparently not expressed in some field strains (Assunção *et al*., 2005), which suggests that it can not be merely regarded as a constitutive antigenic protein, as could be initially expected. P65 may be also differentially expressed between strains, since, as discussed above, the putative regulatory region of the corresponding CDS presents tandem nucleotide repeats associated with transcriptional on-off switching. Since P65 is a major antigenic surface lipoprotein (Schmidt *et al*., 2004), the occasional switching off of the P65 gene in individuals of a colonizing population of *M. hyopneumoniae* would be of immunological relevance, and would also be one of the factors contributing to the infection outcome.

Among the prominent surface antigens detected on the cellular membrane of mycoplasmas there are a large number of lipoproteins. In *M. hyopneumoniae* and other mycoplasmas, these membrane lipoproteins are likely to play a role in pathogenicity mechanisms, since they are known to induce pro-inflammatory cytokines (Brenner *et al.*, 1997; Herbelin *et al.*, 1994). Furthermore, membrane lipoproteins are among the most dominant antigens in mollicutes, and many of them are known to be variable amongst different strains, undergoing size and antigenic variations (Razin *et al*., 1998).

Proteases and chaperones

Proteases are often involved in the virulence of gram-positive pathogens (Minion *et al*., 2004). In the *M. hyopneumoniae* genome, at least twelve putative protease CDSs have been identified, including those coding for five aminopeptidases and two serine proteases (Minion *et al*., 2004; Vasconcelos *et al*., 2005). Five of these proteases have characteristics of membrane or secreted proteins (see Table 1), and, as such, they could be virulence life style factors, involved in the acquisition of metabolic precursors from the host.

One or more members of the *M. hyopneumoniae* protease repertoire are thought to be responsible for the above discussed (see *Variable antigens* section) posttranslational processing of P97 and other surface proteins (Djordjevic *et al*., 2004), with potential implication in cell adhesion properties and virulence. CDSs coding for signal peptidases I and II were also found in the *M. hyopneumoniae* genome (Vasconcelos *et al*., 2005) and their products may be related to pathogenicity. Similar signal peptidases have been described as bacterial virulence factors (Mei *et al*., 1997; Reglier-Poupet *et al*., 2003), and, in *M. hyopneumoniae*, they could be also important for proteolytic processing and translocation of adhesion related and/or antigenic proteins to the cell surface.

Chaperones can be often considered as virulence-associated proteins or even true virulence factors. For in-

stance, chaperone-mediated protein refolding may be relevant for the posttranslational processing of proteins that are virulence factors, or some chaperones may be strongly immunogenic, eliciting an immune response that is responsible for part of the pathological damage (Scherm *et al*., 2002; Minion *et al*., 2004). In the *M. hyopneumoniae* genome, at least ten chaperone-related CDSs have been annotated (Minion *et al*., 2004; Vasconcelos *et al*., 2005), including those encoding a ClpB homolog, possibly implicated in the P97 posttranslational processing (Minion *et al*., 2004), and an hsp70, which is strongly antigenic (our unpublished results).

Strain-specific gene clusters

Strain-specific genomic regions may include genes that are determinants of phenotypes important for virulence, like in the case of pathogenicity islands, already described in several bacterial genomes (Oelschlaeger and Hacker, 2004). In the alignment of the genome sequences of the *M. hyopneumoniae strains* J, 7448, and 232, two relatively large regions came to attention due to their presence exclusively in one or two of these strains (Vasconcelos *et al*., 2005). One of these regions, similar to a *Mycoplasma fermentans* integrative conjugal element of (ICEF) (Calcutt *et al*., 2002), was initially described as a 7448 strain-specific genomic segment, with a length of 22.3 kb and containing 14 CDSs (Vasconcelos *et al.*, 2005). It was considered a probable *M. hyopneumoniae* integrative conjugal element, named ICEH, and its presence was also verified in the pathogenic strain 232 and in another unrelated, pathogenic Brazilian field isolate, but not in the J strain. A more refined *in silico* analysis has extended the 7448 strain ICEH boundaries, so that it now includes around 30 kb and at least 23 CDSs (Pinto *et al*., unpublished results), some of which, however, are also shared with the ICEH-less strain J. Among the ICEH CDSs, there are at least seven related to conjugative and integrative genes, whose presence is suggestive of the element' s mobility. Additional evidence for the ICEH's mobile character and activity are the presence of flanking direct repeat sequences, possibly generated by target site duplication, and PCR detection of its circular extrachromosomal forms (Vasconcelos *et al.*, 2005). In the strain 232 genome, there are at least four ICEH-related regions, with 10 kb, 0.4 kb, 6.7 kb and 26.4 kb, including at least 22 CDSs, at least 12 of them orthologous to CDSs of the strain 7448 ICEH (see Table 1).

The verified presence of a potentially mobile element like the ICEH in three *M. hyopneumoniae* pathogenic strains, but not in a non-pathogenic one is suggestive of its involvement with virulence. It has recently been shown that in some pathogenic bacteria components of the conjugation machinery are virulence-associated factors, being responsible for intercellular delivery of effector molecules that modify host cell functions in favour of the pathogen (Seubert *et al.*, 2003; Schroder and Dehio, 2005). Whether the putative ICEH-mediated transfer mechanisms are used for the delivery of secretion substrates to host cells and whether this is a determinant of the *M. hyopneumoniae* ability to colonize and persist in the swine respiratory epithelium, however, are questions that demand future experimental work.

Another *M. hyopneumoniae* strain-specific region is exclusive of the non-pathogenic strain J, and includes some CDSs coding for type III restriction-modification (R-M) system components and putative transposases (Vasconcelos *et al*., 2005; Brochi *et al*., unpublished results). Bacterial DNA restriction-modification (R-M) enzymatic systems have been related to protection of bacteria against invasion by phage and foreign DNA or as a cause of genome rearrangements in cases in which the corresponding genes behave like selfish mobile elements (Kobayashi, 2001). These functions are not directly related to virulence, but it has been demonstrated for different bacteria, including *M. pulmonis* (Gumulak-Smith *et al*., 2001), *Helicobacter pylori* (Salaun *et al*., 2005), and *Bacteroides fragilis* (Cerdeno-Tarraga *et al*., 2005), that R-M genes belong to a group of phase-variable genes that may play a role in determining initial fitness for colonization of the host and subsequent niche adaptation, acting, therefore, as virulence life-style genes.

Besides the J strain-exclusive type III R-M CDS set, the genomes of *M. hyopneumoniae* strains J, 7448, and 232 present also additional CDSs related to type I, II or III R-M systems, some of them common to the three strains and others exclusive of one strain or shared by only two of them (Brochi *et al*., unpublished results; Table 1). It is interesting to note that several of these putative R-M genes present unusual sequence features, such as the presence of nucleotide repeats within their coding or regulatory regions, translational frameshifts, or flanking insertion sequences (Vasconcelos *et al*., 2005). This suggests that these *M. hyopneumoniae* R-M genes may be targets of genomic rearrangements and nucleotide repeat-related on-off switching or phase variation, which could be associated to virulence. The effective contribution of the observed *M. hyopneumoniae* inter-strain variation in R-M systems to virulence remains to be confirmed. However, it is possible that, as proposed for *M. pulmonis* (Gumulak-Smith *et al*., 2001), the status of R-M systems is a determinant of differential cell growth rates, and its variation could generate cell subpopulations better adapted to colonize the host.

Other possible virulence factors

Hemolysin-mediated hemolysis is another common potential virulence factor among mollicutes (Chambaud *et al*., 2001) and membrane-associated hemolysin activity has been described for *M. hyopneumoniae* J (Minion and Jarvill-Taylor, 1994). This activity has been commonly associated with the HlyA, HlyC and ACP proteins (Trent *et al.*, 1999), whose genes are simultaneously present in *M.*

pulmonis, *U. urealyticum*, and *M. penetrans* (Glass *et al*., 2000; Chambaud *et al*., 2001, Sasaki *et al*., 2002). In *M. hyopneumoniae* J, 7448, and 232 strains, however, CDSs for HlyA and HlyC were found, but no typical ACP or ACP-synthase (the enzyme that catalyzes ACP acylation) CDSs were identified. A CDS for a putative hemolysin ABC exporter (Koronakis *et al*., 1995) was also found in M. *hyopneumoniae*, as well as in all other mollicute genomes so far characterized.

Membrane or secreted nucleases, as their protease counterparts (see section *Proteases and chaperones*, above) may be important to define the colonizing capacity of mycoplasmas, helping these microorganisms to acquire from the host the metabolic precursors they are unable to synthesize (Bendjenatt *et al*., 1999; Chambaud *et al*., 2001). Eleven nuclease CDSs have been annotated in the *M. hyopneumoniae* genome, from which four are predicted to be membrane or secreted proteins. Among the *M. hyopneumoniae* nucleases, there is a VACB-like ribonuclease II, whose orthologs have been described as virulence factors in *Shigella flexneri* and *E. coli* (Cheng *et al*., 1998).

For *M. pulmonis* infections, the production of hydrogen peroxide has been suggested to be a virulence factor (Brennan and Feinstein, 1969; Chochola *et al*., 1995). In this context, a thiol peroxidase, a thiol dependent antioxidant enzyme, could also be considered a virulence factor, assuming it would help the pathogen to survive in a peroxide-rich environment. Among the characterized mollicute genomes, thiol peroxidase encoding CDSs were found only in *M. hyopneumoniae*, *M. synoviae*, and *M. pulmonis* (Chambaud *et al*., 2001;Vasconcelos *et al*., 2005), suggesting that this activity is not essential or is supplied by other related enzymes in other mycoplasma species.

Another possible virulence factor which may be related to oxidative stress is the neutrophil-activating protein (napA), encoded by an ortholog of a previously described *Helicobacter pylori* virulence gene. *H. pylori* napA induces neutrophils to produce reactive oxygen species and promotes neutrophil adhesion to endothelial cells (Yoshida *et al*., 1993; Evans *et al*., 1995). Moreover, by activating neutrophils and inducing a moderate inflammatory reaction leading to alteration of the epithelial tight junctions and basal membranes, *H. pylori* napA possibly promotes the release of nutrients from the mucosa to support bacterial growth (Blaser, 1993; Montecucco and Pappuoli, 2000). Whether *M. hyopneumoniae* napA would be capable of causing one or more of theses effects on the swine epithelium remains to be experimentally investigated.

Concluding Remarks

Genomic comparisons among pathogenic and non-pathogenic strains of a pathogen can reveal genes whose products are potentially related to virulence, like those described for *M. hyopneumoniae* in this article. Functional studies of these genes and their products are expected to help elucidating molecular mechanisms important for *M. hyopneumoniae* infection of and survival in its host environment. Once elucidated, components of such mechanisms will be natural targets for the development of effective and specific therapeutical agents against PEP.

Furthermore, bacterial virulence factors, particularly surface-located molecules, are often immunogenic and make good vaccine antigens (Allan and Wren, 2003). Considering that virulence factors are required for the pathogen to infect and survive in its host, their use as vaccinal antigens has the potential to generate a vaccine-stimulated immune response that neutralizes infection and offers protection against the disease. Therefore, several of the identified putative *M. hyopneumoniae* virulence factors are natural candidates for cloning and production of recombinant molecules (either DNA or proteins) to be evaluated as components of a possible recombinant vaccine against PEP.

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Internet Resource

Phred software, www.phrap.org/phredphrapconsed.html.

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