UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

ESTUDOS PROTEÔMICOS DO PATÓGENO SUÍNO *Mycoplasma hyopneumoniae*

TESE DE DOUTORADO

PAULO MARCOS PINTO

PORTO ALEGRE, MARÇO DE 2009.

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TESE SUBMETIDA AO PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR DO CENTRO DE BIOTECNOLOGIA DA UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL COMO REQUISITO PARCIAL PARA OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS.

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PORTO ALEGRE, MARÇO DE 2009.

Este trabalho foi desenvolvido no Laboratório de Genômica Estrutural e Funcional, no Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul.

"A razão é o passo, o aumento da ciência, o caminho e o benefício da humanidade o fim."

Thomas Hobbes (5 de abril de 1588 – 4 de dezembro de 1679)

DEDICO

Ao meu sobrinho Gabriel Pinto Machado, o qual me fez entender muitas das singularidades do mundo apenas pelo seu existir.

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SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES XI

LISTA DE FIGURAS XIII

RESUMO XIV

ABSTRACT XV

1. INTRODUÇÃO 1

1.1. A suinocultura 1

1.2. *Mycoplasma hyopneumoniae* e pneumonia enzóotiva suína 5

1.2.1. Taxonomia e morfologia 5

1.2.2. Ocorrência e distribuição 6

1.2.3. Patologia 7

1.2.4. Diagnóstico 9

1.2.5. Profilaxia e tratamentos 10

1.2.6. Vacinas 11

1.3. Estudos genômicos de *Mycoplasma hyopneumoniae* 12

1.4. Estudos transcriptômicos de *M. hyopneumoniae* 14

1.5. Estado da arte da análise proteômica 15

1.5.1. Estudos proteômicos de micoplasmas e *M. hyopneumoniae* 20

2. OBJETIVOS 23

2.1. Objetivo Geral 23

2.2. Objetivos Específicos 23

3. ARTIGO 1 24

4. ARTIGO 2 36

5. DISCUSSÃO GERAL 73

6. CONCLUSÕES 79

7. PERSPECTIVAS 81

8. REFERÊNCIAS BIBLIOGRÁFICAS 82

ANEXOS 105

ANEXO I 106

ANEXO II 125

ANEXO III 136

ANEXO IV 145

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

°C	Grau Celsius
2DE	Eletroforese bidimensional
ABIPECS	Associação Brasileira das Indústrias Produtoras e Exportadoras de Carne Suína
ATCC	*American Type Culture Collection*
BCIP	5-bromo-4-cloro-3'-indolilfosfato (sal de p-toluidino)
CapLC	Cromatografia líquida capilar
CDS	Seqüência codificadora de DNA
CF	Fixação de complemento
CFU	Unidades formadoras de colônia
CHAPS	3-[(3-colamidopropil)-dimetilamonio]-1-propano sulfonato
COG	*Clusters of orthologous groups*
DNA	Ácido desoxirribonucléico
DTT	Ditiotreitol
EDTA	Ácido etilenodiaminotetracético
ELISA	*Enzyme-linked immunosorbent assay*
emPAI	*Exponentially modified protein abundance index*
ESI	Ionisação por *electrospray*
FAO	*Food and Agricultural Organization*
g	Grama
h	Hora
Hz	Hertz
ICEH	Elemento conjugativo integrativo de *M. hyopneumoniae*
IEF	Focalização isoelétrica
IF	Imunofluorescência
IgA	Immunoglobulina A
IgG	Immunoglobulina G
IHA	Hemoaglutinação indireta
IHQ	Imunohistoquímica
IPG	Gradiente de pH imobilizado
kb	Quilobase
kDa	Quilodalton
kg	Quilograma
L	Litro
LC-MS/MS	Cromatografia líquida acoplada a espectrometria de massas em tandem
M	Molar
MDLC	Cromatografia líquida multidimensional
MALDI	Ionização/dessorção por laser assistida por matriz
MAPA	Ministério da Agricultura, Pecuária e Abastecimento
mg	Miligrama
min	Minuto
mL	Mililitro
mM	Milimolar
mRNA	RNA mensageiro
MudPIT	*Multidimensional protein identification technology*

MW	Peso molecular
MS	Espectrometria de massas
NBT	Cloreto de p-nitro azul de tetrazólio
NPS-RP-HPLC	Cromatografia de fase revesa não-porosa
PAGE	Eletroforese em gel de poliacrilamida
PBS	Tampão fosfato-salina
PCR	Reação em cadeia da DNA-polimerase
PES	Pneumonia enzoótica suína
pH	Potencial de hidrogênio iônico
pI	Ponto isoelétrico
PMF	*Peptide mass fingerprint*
PRDC	Complexo de doenças respiratórias de suínos
PRRSV	Vírus da síndrome respiratória e reprodutiva suína
PVDF	Fluoreto de polivinilideno
SDS	Dodecilsulfato de sódio
SIV	Vírus influenza suíno
SECEX	Secretaria de Comércio Exterior
SPF	*Specific Pathogen Free*
TCA	Ácido tri-cloro acético
TNF	Fator de necrose tumoral
TOF	Tempo de vôo
USDA	Departamento de Agricultura dos Estados Unidos
μL	Microlitro

LISTA DE FIGURAS

Figura 1.1. Produção de carnes (aviária, bovina e suína) nos últimos 40 anos.

Figura 1.2. Percentual da produção de carne suína no Brasil no ano de 2005, por estado.

Figura 1.3. Exportação de carne suína brasileira no ano de 2005, participação por estado.

Figura 1.4. Destruição dos cílios do epitélio ciliado do trato respiratório de suíno experimentalmente inoculado com *M. hyopneumoniae*.

RESUMO

Mycoplasma hyopneumoniae é um importante patógeno suíno, sendo o agente da pneumonia enzoótica suína. Recentemente, o genoma de três cepas (J, 7448 e 232) de *M. hyopneumoniae* foi seqüenciado. Inicialmente, realizamos uma análise proteômica baseada em eletroforese bidimensional (2DE), estudos imunológicos e espectrometria de massas para a cepa patogênica 7448 de *M. hyopneumoniae*. Foram produzidos mapas proteômicos em duas faixas de pH (3-10 e 4-7). Um total de 31 produtos gênicos foram experimentalmente verificados. Em uma análise imunológica foi possível identificar pelo menos cinco proteínas antigênicas de *M. hyopneumoniae*. Seguindo este primeiro estudo prospectivo da cepa 7448, foi realizada uma análise proteômica comparativa de três cepas de *M. hyopneumoniae*, uma não-patogênica (J) e duas cepas patogênicas (7448 e 7422). Na comparação por 2DE foi possível identificar diferenças no nível de expressão entre as cepas em pelo menos 67 *spots* protéicos. Para uma análise mais ampla dos perfis protéicos das três cepas foi utilizada uma estratégia baseada em LC-MS/MS. Nas três cepas, 231 proteínas foram identificadas, correspondendo a cerca de 35% da capacidade codificadora do genoma de *M. hyopneumoniae*. A classificação funcional das proteínas identificadas sugere diferenças fisiológicas entres as cepas não-patogênicas e patogênicas. A aplicação de uma proteômica quantitativa *label-free* (o *exponentially modified protein abundance index*) demonstrou diferenças significativas nos níveis de expressão de pelo menos 64 proteínas. Por fim, uma análise imunológica baseada em 2DE utilizando um anticorpo monoclonal contra a repetição R1 da proteína P97, foi capaz de identificar um padrão de clivagem proteolítica diferencial entre as três cepas de *M. hyopneumoniae*.

ABSTRACT

Mycoplasma hyopneumoniae is an important pathogen for pigs, being the causative agent of enzootic pneumonia. Recently, the genome sequences of three strains, J, 7448 and 232 have been reported. Here, we describe the results of a proteomic analysis, based on two-dimensional gel electrophoresis of soluble protein extracts, immunoblot and mass spectrometry from the *M. hyopneumoniae* pathogenic strain 7448. A preliminary *M. hyopneumoniae* proteome map in two pH ranges (3–10 and 4–7) was produced. A total of 31 different coding DNA sequences were experimentally verified. Moreover, at least five highly antigenic proteins of *M. hyopneumoniae* were identified by immunoblots. Following the previous report of a proteomic survey of the pathogenic 7448 strain, we performed comparative protein profiling of three *M. hyopneumoniae* strains, namely the non-pathogenic J strain and the pathogenic strains 7448 and 7422. In 2DE comparisons, we were able to identify differences in expression levels between strains for 67 proteins. For more comprehensive protein profiling, an LC-MS/MS strategy was used. Overall, 231 different proteins were identified, corresponding to 35% of the *M. hyopneumoniae* genome coding capacity. The functional classification of identified proteins was suggestive of physiological differences between the non-pathogenic and the pathogenic strains. Also, application of the exponentially modified protein abundance index demonstrated significant differences in the expression levels of proteins detected in more than one strain, confirming over-expression in one or two of the strains for 64 proteins. 2DE immunoblot analyses allowed the identification of differential proteolytic cleavage patterns of the P97 adhesin in the three strains.

1. INTRODUÇÃO

1.1. A suinocultura

Após a virada do século XX, a carne suína manteve o desempenho já conquistado e consolidado no final dos anos 1900, continuando a ser a fonte de proteína animal mais importante para a alimentação do homem em qualquer canto do mundo. Levando-se em conta dados publicados pela FAO (*The Food and Agriculture Organization of the United Nations* – Organização para a Agricultura e a Alimentação das Nações Unidas), percebe-se que até meados dos anos 80 as carnes de suínos e bovinos (com ligeira vantagem para a carne bovina), eram as principais fontes de suprimento de proteína animal para o consumo humano. A partir de então, a carne bovina começou a perder terreno para a carne de frango. Nos anos 90 a produção mundial de carne de frango chegava à casa dos 20% praticamente igualando-se ao volume produzido de carne bovina. Naquela década, a situação entre carne bovina e carne de frango ficou bastante estável, todavia ainda com ligeira vantagem para a carne bovina. A produção de carne suína apresentou estabilidade no período de 1961 a 2000, situando-se ao redor de 33% do volume das carnes produzidas no mundo. A carne bovina que vinha em queda livre até 1995, estabilizou-se a partir de então e voltou a apresentar juntamente com a carne suína forte crescimento a partir de 2000 (Figura 1.1). Enfim, pode-se afirmar com base nestes dados de que a carne suína é atualmente a principal fonte de proteína animal produzida no mundo desde a década de 1980.

A suinocultura industrial brasileira exibe indicadores de produtividade do Primeiro Mundo. O Brasil continuou na posição de quarto maior produtor mundial de carne suína, com 2,7 milhões de toneladas em 2003, o que representou uma queda de 6% em relação ao

ano anterior. O resultado refletiu, evidentemente, o impacto do sistema de cotas adotado pela Rússia, o maior mercado da carne suína brasileira.

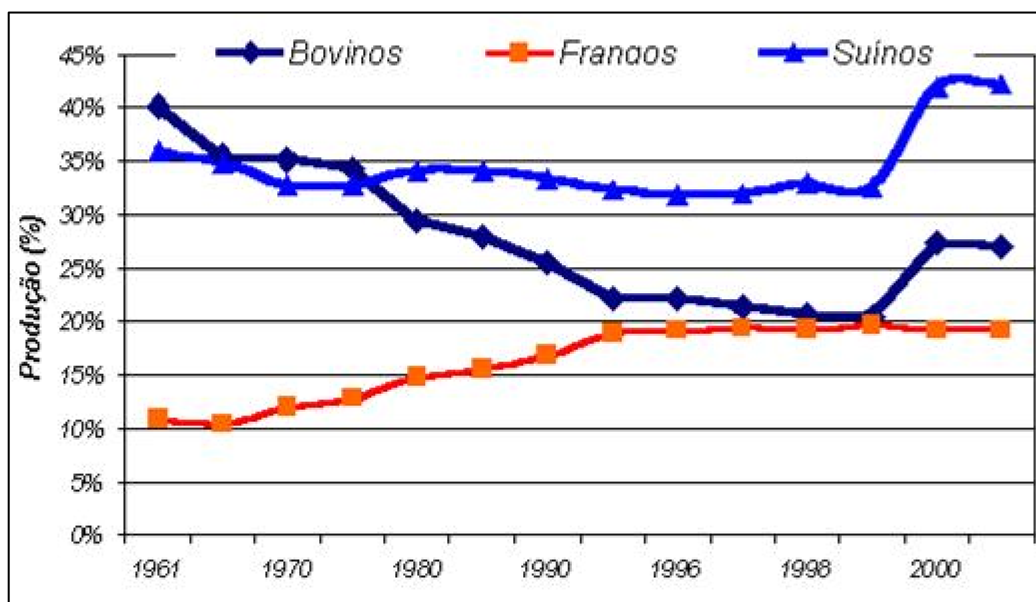

Figura 1.1. Produção de carnes (aviária, bovina e suína) nos últimos 40 anos. Fonte: FAO, 2003.

Mais uma vez o desempenho do Brasil, quarto maior exportador mundial, foi superior à média mundial, já que os embarques de 2003, ao totalizarem 491 mil toneladas, exibiram um incremento de 3,3% em relação a 2002.

A cadeia produtiva da suinocultura brasileira representa 1% do PIB nacional e é responsável pela renda de 2,7 milhões de pessoas, das quais 733 mil dependem diretamente da atividade (ANTUNES, 2001). Presente em quase metade das propriedades rurais do país, a suinocultura contribui para a viabilização de pequenos e médios produtores agrícolas, tradicionalmente utilizando mão-de-obra familiar; o que gera empregos e fixa o trabalhador ao campo (GOMES *et al.*, 1992). Os estados da Região Sul lideram o volume de produção, cuja atividade é desenvolvida, na maioria, por pequenas propriedades que participam de sistemas integrados coordenados por agroindústrias ou cooperativas

(BOHRER, 1993). No Rio Grande do Sul, os suínos estão presentes em 76% dos estabelecimentos rurais, dos quais 16% comercializam os animais; estando a maior parte do rebanho concentrada em propriedades com até 50 hectares (VIOLA & BARTELS, 1993).

As Figuras 1.2 e 1.3 apresentam a participação dos Estados brasileiros na produção de suínos e na exportação da carne suína brasileira, com destaque para a Região Sul. Como podemos observar, a Região Sul detém 62% do rebanho suíno brasileiro e responde por 83% de toda carne suína exportada pelo Brasil. Em 2005, só o Estado do Rio Grande do Sul apresentou um incremento de mais de 34% de carne suína exportada em relação ao ano anterior (SECEX, 2006).

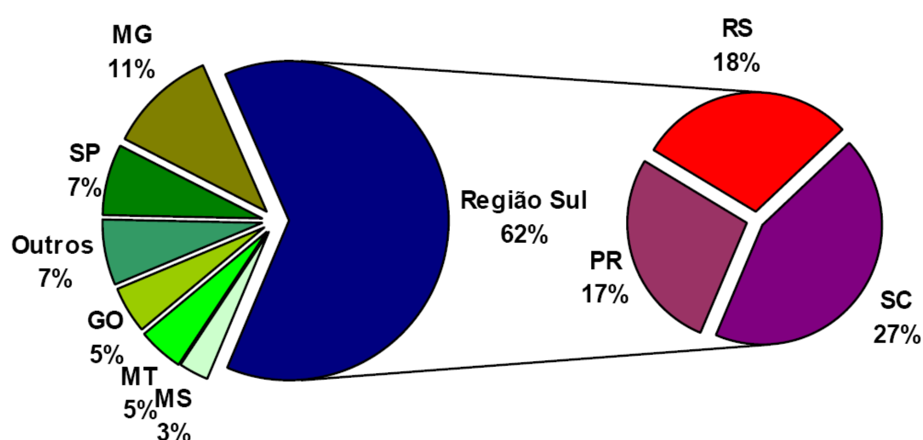

Figura 1.2. Percentual da produção de carne suína no Brasil no ano de 2005, por estado. (Fonte: ABIPECS, 2006).

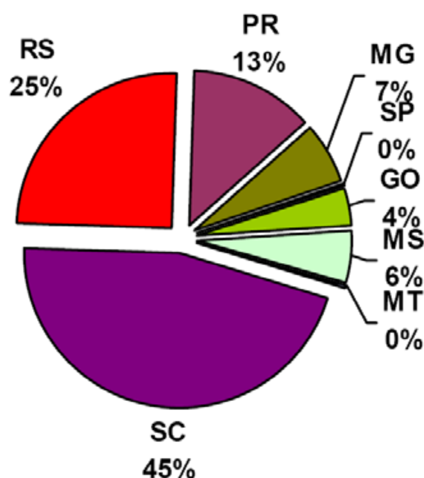

Figura 1.3. Exportação de carne suína brasileira no ano de 2005, participação por estado. (Fonte: ABIPECS, 2006).

Existem vários pontos críticos à expansão e melhoria do setor suinícola brasileiro, destacando-se a ineficiência e a baixa produtividade na produção de terminados e as dificuldades técnicas para o controle de doenças, visando garantir maior produtividade e melhor qualidade de produtos finais, incrementando o consumo e aumentando as exportações (GOMES *et al.*, 1992).

Diversas doenças podem afetar os animais, retardando seu crescimento, reduzindo o ganho de peso e até mesmo causando sua morte. Em relação às doenças de importância econômica, GOMES *et al.* (1992) identificaram as doenças respiratórias e entéricas como as mais relevantes e indicaram como necessidades prioritárias de pesquisa e desenvolvimento o estudo do ecossistema de suínos e sua associação com a ocorrência de doenças multifatoriais; a pesquisa e o desenvolvimento de vacinas mais eficientes; e a melhoria das técnicas de diagnóstico.

As doenças respiratórias são economicamente devastadoras para a suinocultura mundial (CHAE, 2005). *Mycoplasma hyopneumoniae* é uma das mais importantes causas

de doenças em suínos, envolvida na pneumonia enzoótica suína (PES) e no complexo de doença respiratória suína (do inglês, *porcine respiratory disease complex* ou PRDC). Além de *M. hypneumoniae* outras bactérias secundárias como *Pasteurella multocida*, *Streptococcus suis*, *Haemophilus parasuis*, e *Actinobacillus pleuropneumoniae* fazem parte deste complexo (KIM *et al.*, 2003). Recentemente o termo PRDC tem sido adotado para descrever uma doença respiratória severa que se desenvolve pela combinação de patógenos virais e bacterianos. Os mais comuns componentes do PRDC são *M. hyopneumoniae*, o vírus influenza suíno (SIV), o vírus da síndrome respiratória e reprodutiva suína (PRRSV) e bactérias secundárias subseqüentes (KIM *et al.*, 2003).

1.2. *Mycoplasma hyopneumoniae* e pneumonia enzóotiva suína

O *M. hyopneumoniae* é o agente causador da chamada broncopneumonia micoplásmica ou pneumonia enzoótica suínas (PES), sendo o agente mais comumente encontrado em doenças pulmonares infecciosas nos suínos. É uma das doenças mais comuns e economicamente importantes da suinocultura (ROSS, 1999).

1.2.1. Taxonomia e morfologia

Taxonomicamente a bactéria *Mycoplasma hyopneumoniae* está classificada dentro da classe *Mollicutes*, ordem *Mycoplasmatales*, família *Mycoplasmataceae* e gênero *Mycoplasma*. A ausência de parede celular é utilizada para separar as micoplasmas de outras bactérias, incluindo-as na classe Mollicutes (do latim *mollis* = macio, *cutis* = pele). Esta classe é composta pelos gêneros *Acholeplasma*, *Anaeroplasma*, *Asterosplama*, *Mycoplasma*, *Spiroplasma* e *Ureaplasma* (WALKER, 2003). Segundo FODDAI *et al.* (2005) exitem mais de 120 espécies associadas a estes gêneros

A morfologia de *M. hyopneumoniae* é bastante variada, em função da ausência de parede celular, podendo a célula apresentar-se em forma de pêra, esférica, espiral ou filamentosa. É corada insatisfatoriamente pelo Método de Gram, sendo recomendadas as colorações de Giemsa, Castañeda, Dienes e Novo Azul de Metileno. Possuem uma membrana trilaminar simples composta de proteínas, glicoproteínas, glicolipídeos, fosfolipídeos e colesterol, sendo este último responsável pela rigidez e estabilidade osmótica da membrana (WALKER, 2003). Além disso, necessitam que haja um contato íntimo com as células do hospedeiro, para que seja capaz de suprir suas necessidades nutricionais indispensáveis para sobrevivência (CLARK, 2005).

1.2.2. Ocorrência e distribuição

Segundo CLARK (2005), por muitos anos acreditou-se que a dependência em relação ao seu hospedeiro, fizesse com que os micoplasmas apresentassem uma forte especificidade pelo mesmo. No entanto, PITCHER & NICOLAS (2005) salientam que há espécies que são extremamente espécie-específicas enquanto outras têm uma ampla variedade de hospedeiros. Da mesma forma, certas espécies que eram encontradas apenas em animais, foram isoladas em seres humanos, os quais, na maioria das vezes, se encontravam imunocomprometidos. Esses autores ainda acrescentaram que a espécie hospedeira pode ser simplesmente aquela na qual a espécie determinada de micoplasma é isolada com maior freqüência. CLARK (2005) concorda com esse fato e ainda acrescenta que o hospedeiro perfeito é aquele que é portador do micoplasma sem demonstrar nenhuma evidência desta infecção. STAKENBORG *et al.* (2005) também concordam que é possível a transferência de agentes entre hospedeiros de espécies diferentes.

Micoplasmas são encontrados na natureza como parasitas dos mais variados organismos, como por exemplo, mamíferos (inclusive humanos), répteis, peixes, artrópodes e plantas (RAZIN, 1992). *M. hyopneumoniae* tem distribuição mundial com uma incidência próxima de 100% dos rebanhos suínos (FLECK & SNELSON 2004).

1.2.3. Infecção e patologia

A maioria dos membros da classe *Mollicutes* é patogênica e coloniza uma grande variedade de hospedeiros, incluindo animais, plantas e insetos (WALKER, 2003).

A infecção de *M. hyopneumoniae* resulta em taxas de crescimento diminuídas e pobre conversão alimentar, além de ser caracterizada por uma tosse crônica seca não-produtiva. Causa uma doença com atraso no ganho de peso, alta morbidade e baixa mortalidade. Entretanto, a PES e o PRDC são doenças muito severas resultando na diminuição de ganho de peso diário e eficiência de alimentação, anorexia, febre, tosse e dispnéia em suínos de 18-20 semanas (SOBESTIANSKY *et al.*, 1999). POINTON *et al.* (1985) relataram que a taxa de crescimento de suínos com PES, com peso entre 50 e 80 kg, sofre um decréscimo de 12,7% enquanto que em animais entre 8 e 85 kg, a redução atingiu 15,9%.

M. hyopneumoniae coloniza as células do epitélio ciliado do trato respiratório dos suínos, resultando na destruição destes cílios e na perda do sistema de defesa mucociliar (Figura 1.4) (HAESEBROUCK *et al.*, 2004). A função destes cílios é a de expelir partículas do trato respiratório e de servir como uma barreira física inicial. A perda do aparato mucociliar permite o aumento da colonização de bactérias secundárias como *Pasteurella multocida*. O organismo interage também com células imunes no trato respiratório, o que resulta na liberação de citocinas, como o fator de necrose tumoral (TNF)

e interleucinas e na ativação de linfócitos (CHOI *et al.*, 2006). Esta interação geralmente minimiza a capacidade do sistema imune de responder eficientemente à infecção (THANAWONGNUWECH *et al.*, 2004). As lesões observadas nos pulmões de suínos infectados apresentam-se de cor púrpura a cinza (MORRISON *et al.*, 1985). Lesões microscópicas incluem a acumulação de neutrófilos em torno da via aérea e alvéolos. Infecções secundárias por bactérias e fatores de gerenciamento, como superlotação e pobre ventilação, influenciam na severidade das lesões.

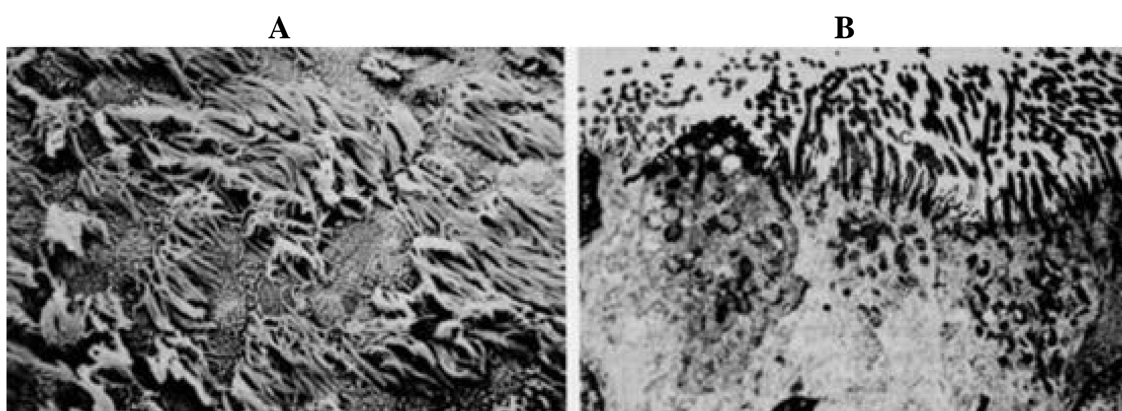

Figura 1.4. Destruição dos cílios do epitélio ciliado do trato respiratório de suíno experimentalmente inoculado com *M. hyopneumoniae*. A) Suíno afetado; B) suíno não afetado. Aumento 2.900 X (modificado de IRIGOYEN *et al.*, 1998).

O contato direto com secreções do trato respiratório parece ser a maneira de transmissão mais comum (SOBESTIANKY *et al.*, 2001). A transmissão aérea também foi proposta (STARK *et al.*, 1992; RAUTIAINEN & WALLGREN 2001). A PES torna-se estabelecida em um rebanho e é mantida através do contato direto de suíno com suíno (FLECK & SNELSON, 2004; MORRIS *et al.*, 1995).

M. hyopneumoniae tem tempo de incubação relativamente longo (10-16 dias ou mais) (HAESEBROUCK *et al.*, 2004) mas, pode ser alterado por condições estressantes,

tais como a densidade populacional aumentada, a ventilação inadequada, e a exposição a outros patógenos. Estes fatores contribuem ao aparecimento de sinais clínicos no período final de crescimento, embora a infecção real ocorra freqüentemente em porcos mais jovens.

1.2.4. Diagnóstico

Devido às características singulares dessa enfermidade, o diagnóstico presuntivo depende da conjunção dos sinais clínicos (tosse crônica não-produtiva, redução do crescimento, baixa mortalidade e desuniformidade dos lotes) e dos aspectos macroscópicos (áreas e consolidação pulmonar de cor púrpura a cinza) e microscópicos das lesões (hiperplasia linfóide peribronquiolar) (AHRENS & FRIIS, 1991; ROSS & STEMKE, 1995; DONE, 1996). Para a confirmação do diagnóstico, faz-se necessária a utilização de recursos laboratoriais complementares (sorologia, PCR) a fim de se evitar diagnóstico inconclusivo e controverso sobre a etiologia do processo (SOBESTIANSKY *et al.*, 1999). Os testes sorológicos mais comuns utilizados para diagnóstico da PES incluem hemoaglutinação indireta (IHA), fixação de complemento (CF) e testes de ELISA. Um teste de ELISA indireto chamado de "Tween 20" e um teste de ELISA bloqueador foram desenvolvidos. Tem sido demonstrado que o Tween 20 detecta infecções inicias, de 5 a 7 semanas após a infecção, até 52 semanas (PIFFER *et al.*, 1998). O teste ELISA bloqueador tem menor reatividade cruzada com outras micoplasmas (ROSS & STEMKE, 1995).

Apesar de o isolamento de *M. hyopneumoniae* ser o "padrão ouro" de diagnóstico, este microrganismo é muito difícil de ser isolado e identificado, pois necessita de meios especiais e pode levar de 4 a 8 semanas para se multiplicar. Além disso, obter culturas puras de *M. hyopneumoniae* pode ser difícil por causa da presença de micoplasmas comensais com crescimento rápido, tais como *M. hyorhinis* e *M. flocculare*, tornando o

diagnóstico via cultivo, às vezes, não exeqüível (THACKER, 2004). Tentativas para diagnosticar a infecção por *M. hyopneumoniae* em rebanhos utilizando apenas dados coletados em abatedouro têm sido propostas (NOYES *et al.*, 1990), no entanto, em muitos casos, as lesões por micoplasma resultantes de infecção precoce estão consolidadas e não são óbvias ao abate. Portanto, o impacto da infecção por *M. hyopneumoniae* pode ser subestimado se recair apenas sobre análises de abatedouro (SITJAR *et al.*, 1996).

Com o desenvolvimento da análise por PCR a eficiência na detecção do *M. hyopneumoniae* aumentou significativamente (ARTIUSHIN *et al.*, 1993). Numerosos estudos avaliaram os vários locais de coleta (no suíno) e os potenciais usos da PCR para detectar com eficiência esse agente (SORENSEN *et al.*, 1997; CALSAMIGLIA *et al.*, 2000; KURTH *et al.*, 2002). O tecido pulmonar é o melhor local de coleta para a detecção deste microrganismo pela PCR, havendo variações quando se coleta material da cavidade nasal (SORENSEN *et al.*, 1997; KURTH *et al.*, 2002). Lavados traqueais analisados pela PCR podem também ser usados para detectar com eficiência este agente (VERDIN *et al.*, 2000; KURTH *et al.*, 2002).

Em adição à PCR, a detecção do *M. hyopneumoniae* no tecido pulmonar pode ser feita usando análise por imunofluorescência (IF), imunohistoquímica (IHQ) e hibridização *in situ* (AMANFU *et al.*, 1984; SORENSEN *et al.*, 1994; KWON & CHAE, 1999). São requeridos tecidos congelados para a detecção de *M. hyopneumoniae* e microscópio de fluorescência, para análise por IF, fazendo com que este procedimento seja difícil em nível de campo (THACKER, 2004).

1.2.5. Profilaxia e tratamentos

Vários antibióticos têm sido avaliados para um efetivo tratamento e/ou controle de infecções de *M. hyopneumoniae* (THOMÁS *et al.*, 2003). Antibióticos derivados de tetraciclina demonstram um impacto variável sobre as infecções geradas por *Mycoplasma* spp. (RAZIN *et al.*, 1998). Embora, as tetraciclinas não previnam a infecção e as lesões tendem as se desenvolver após a parada da terapia (SWITZER & ROSS, 1975), um estudo indica que a administração de oxitetraciclinas durante a fase de lactação e em fases iniciais da doença podem reduzir a pneumonia induzida por *M. hyopneumoniae* em suínos adultos (SCHEIDT *et al.*, 1990). Entretanto, um estudo realizado no Japão demonstrou um aumento da resistência a clorotetraciclinas (INAMOTO *et al.*, 1994). Houve um relato que a tilosina reduz a severidade da PES quando injetada em dose 10mg/Kg diária, iniciando em um dia antes da exposição e continuando por três dias após a infecção (GOODWIN *et al.*, 1972). Devido à falta de parede celular, todos os isolados de micoplasmas são resistentes a ampicilinas β-lactâmicas, penicilina e ceftiofur, pois esses antibióticos atuam na inibição da síntese de parede celular bacteriana (FLECK & SNELSON, 2004).

1.2.6. Vacinas

Vacinas vêm sendo desenvolvidas para minimizar os efeitos clínicos da infecção por *M. hyopneumoniae* e prover alguma proteção contra o desenvolvimento das lesões de pulmões. Entretanto, há, na literatura certa contradição sobre os reais efeitos das vacinas até hoje produzidas.

As vacinas disponíveis no mercado são bacterinas, as quais apresentam elevado custo de produção, principalmente devido ao crescimento fastidioso deste agente. HAESEBROUCK *et al.* (2004) demonstrou que uma bacterina comercial não apresenta uma proteção satisfatória. Entretanto, alguns estudos sobre o efeito da vacinação revelaram

que as vacinas melhoram significativamente, o ganho diário médio em relação aos controles não vacinados (JENSEN *et al.*, 2002) e diminuem a severidade dos sintomas e lesões, diminuindo o tempo para o abate, além de reduzir o gasto com medicamentos e a variabilidade de peso entre os animais (THACKER & THACKER, 2001).

Tem sido demonstrado que as vacinas contra *M. hyopneumoniae* estimulam a produção de anticorpos IgG e IgA contra o mesmo, seguida da mudança e diminuição da produção de citocinas pró-inflamatórias, em particular, TNF. Embora, mesmo que a vacinação não resulte em uma prevenção da colonização ou infecção, parece ocorrer uma minimização dos efeitos inflamatórios seguidos da infecção (THACKER *et al.*, 2000).

Desta forma, estudos vêm sendo desenvolvidos buscando novas alternativas para o diagnóstico e profilaxia da PES, uma vez que a mesma apresenta elevado impacto econômico, principalmente na região Sul do Brasil. As vacinas de terceira geração em fase de testes são baseadas em antígenos nativos da bactéria, em antígenos recombinantes ou em formulações com DNA (CHEN *et al.*, 2003; LIN *et al.*, 2003; OKAMBA *et al.*, 2007). Porém, o número de antígenos caracterizados até o momento é bastante restrito, sendo que destes somente dois foram testados em um ensaio de imunoproteção em suínos e protegeram apenas parcialmente os animais (FAGAN *et al.*, 2000; CHEN *et al.*, 2003). A identificação e caracterização imunológica de um número maior de proteínas antigênicas do agente podem propiciar o desenvolvimento de uma nova vacina recombinante contra PES.

1.3. Estudos genômicos de *Mycoplasma hyopneumoniae*

O recente seqüenciamento de genomas completos de diferentes espécies do gênero *Mycoplasma* (FRASER *et al.*, 1995; HIMMELREICH *et al.*, 1996; CHAMBAUD *et al.*,

2001; SASAKI *et al.*, 2002; PAPAZISI *et al.*, 2003; JAFFE *et al.*, 2004; MINION *et al.*, 2004; WESTBERG *et al.*, 2004; VASCONCELOS *et al.*, 2005; SIRAND-PUGNET *et al.*, 2007; DYBVIG *et al.*, 2008) tem atraído considerável atenção para a biologia destas bactérias, os menores organismos auto-replicativos já conhecidos. Além de identificar, em termos moleculares, o que poderia ser um genoma mínimo (conceito do genoma mínimo, ver HUTCHISON *et al.*, 1999), avanços consideráveis foram alcançados para o entendimento da patogênese das micoplasmas. Os mais importantes achados são os relacionados à interação das bactérias com o sistema imune do hospedeiro, ativação de macrófagos, indução de citocinas, componentes de membrana de micoplasma atuando como superantígenos e manifestações autoimune (LLEWELYN & COHEN, 2002). A evasão do sistema imune do hospedeiro por variação dos antígenos de superfície de micoplasmas, bem como a definição molecular de adesinas de micoplasma, tem ganhado atenção e alguns relatos genômicos, inclusive em *M. hyopneumoniae*.

Micoplasmas são caracterizados pela ausência de parede celular, baixo conteúdo de GC e pequenos genomas. Estas bactérias contêm os menores genomas auto-replicativos conhecidos e têm sido descritas como células mínimas (FRASER *et al.*, 1995; HIMMELREICH *et al.*, 1997). Os genomas das micoplasmas, muitos dos quais estão seqüenciados ou em processo de seqüenciamento, são também especialmente reduzidos, variando entre 580 e 1.350 kb.

A partir da disponibilização das seqüências completas dos genomas de uma cepa patogênica 232 (MINION *et al.*, 2004) e das cepas J (não-patogênica) e 7448 (patogênica) de *M. hyopneumoniae*, por parte da Rede Sul de Análise de Genomas e Biologia Estrutural (PROGENESUL) (VASCONCELOS *et al.*, 2005) permitiu uma abordagem comparativa entre estas três cepas além de outras cepas de *M. hyopneumoniae*. Essa análise comparativa

foi capaz de identificar regiões cepas-específicas que podem ser relacionadas com patogenicidade. Uma seqüência particularmente interessante é o ICEH (*Integrative Conjugal Element of Mycoplasma hyopneumoniae*) um elemento integrativo conjugativo presente em todos os genomas das cepas patogênicas de *M. hyopneumoniae* e ausente na cepa não-patogênica (MINION *et al.*, 2004; VASCONCELOS *et al.*, 2005; PINTO *et al.*, 2007).

1.4. Estudos transcriptômicos de *M. hyopneumoniae*

A obtenção da seqüência de nucleotídeos do genoma de um organismo, por si só, constitui apenas um primeiro passo que, no entanto, abre caminho à realização de muitos outros estudos. De fato, através da inspeção da seqüência do genoma não é possível obter informação sobre o nível de expressão de genes e proteínas, ou sobre características das proteínas expressas, tais como o seu tempo de meia vida, a sua localização subcelular, eventuais modificações pós-traducionais, interações proteína-proteína e proteína-DNA, a estrutura e a função biológica das proteínas.

Visto que, para cada tipo de célula e condição ambiental, se podem verificar diferenças no nível de expressão, modificação e localização das proteínas, bem como na interação de determinadas proteínas com outras proteínas, a exploração do proteoma continua a exigir o desenvolvimento de equipamentos e abordagens experimentais. Um esforço significativo tem sido dirigido às técnicas envolvidas na proteômica quantitativa, de modo a viabilizar a sua exploração na elucidação de aspectos da regulação da expressão genética global. Esta análise complementa a monitorização de expressão gênica global ao nível do mRNA (análise transcriptômica), a qual não permite apreciar aspectos de regulação que ocorrem pós-transcrição e pós-tradução.

Para *M. hyopneumoniae* existem alguns trabalhos que tem como objetivo uma análise de genômica funcional, quase todas focados em estudos transcriptômicos, estudando a diferença em nível de mRNA em diferentes condições, como por exemplo, estresse térmico (MADSEN *et al.*, 2006), estresse oxidativo (SCHAFER *et al.*, 2007) e durante a infecção (MADSEN *et al.*, 2008).

Mesmo com sua importância em análises filogenéticas, seqüências gênicas e tecnologias de DNA recombinante, o genoma completo do organismo gera somente uma visão relativamente estática do potencial funcional de um organismo, sendo limitado, quando é necessário, o conhecimento das proteínas realmente expressas em uma condição fisiológica especifica ou a identificação das modificações pós-traducionais (LOTTSPEICH, 1999; DUTT & LEE, 2000). A população de mRNAs, da mesma forma que o genoma, tem limitações em proporcionar tais informações (LOTTSPEICH, 1999). Desta forma, a análise proteômica torna-se importante para estudos de comparações da expressão gênica em diferentes estados fisiológicos de um organismo.

1.5. Estado da arte da análise proteômica

O termo “proteoma” foi proposto em 1993 por Mark Wilkins e Keith Williams para referir-se à identificação sistemática do complemento protéico total do genoma (BLACKSTOCK & WEIR, 1999; LOPEZ, 1999), ou seja, se refere ao conjunto de proteínas que são expressas pelo genoma de um organismo ou tipo celular (HAYNES & YATES, 2000). Entretanto, este termo pode também ser usado em um sentido menos universal, para referir-se ao conjunto de proteínas que está sendo expresso num dado momento da vida da célula (ABBOTT, 1999). As proteínas são moléculas muito ricas em informação biológica e suas, atividades específicas, estado de modificação, associação com

outras biomoléculas e os níveis de expressão são essenciais para a descrição de sistemas biológicos (AEBERSOLD, 2003).

A Proteômica tem como objetivo estudar as propriedades das proteínas, seus níveis de expressão, suas funções, modificações pós-traducionais, interações entre proteínas e mecanismos regulatórios (BLACKSTOCK & WEIR, 1999). Uma descrição completa do proteoma de um organismo fornece, não apenas um catálogo do conjunto de proteínas que está sendo expresso pelo genoma, mas também dados de expressão celular sob condições definidas e a distribuição dessas proteínas na célula (CASH, 1998). Portanto, o passo do genoma para o proteoma é um passo do nível da informação genética para a função celular. As concentrações de proteínas que são alteradas em determinadas condições fisiológicas mostram quais funções sofrem modificações (JUNGBLUT, 2001).

A identificação rápida e genérica de proteínas requer um método eficiente para relacionar informações no nível de proteínas com informações no nível do genoma (TYERS & MANN, 2003). Em contraste à análise clássica de proteínas, na qual se analisavam proteínas individualmente, a análise proteômica tem uma vantagem única, em que o estado fisiológico pode ser obtido independentemente da atividade biológica da proteína individual, pois é identificado um conjunto de proteínas que podem ser correlacionadas com vias metabólicas (LOTTSPEICH, 1999).

Para a análise de proteoma, dois princípios são utilizados: o de separação e o de identificação. As duas técnicas de separação mais utilizadas são a eletroforese bidimensional e a cromatografia multidimensional. Na eletroforese bidimensional, as proteínas são separadas por uma focalização isoelétrica (IEF) e, posteriormente, por massa molecular (SDS-PAGE). Essa estratégia possibilita alta resolução para separar proteínas de um extrato protéico complexo (JOUBERT *et al.*, 2000). Na cromatografia

multidimensional, as proteínas são separadas pela utilização de cromatografia em várias colunas, com princípios de separação distintos, geralmente empregando-se a cromatografia em colunas de troca iônica e fase reversa (MAYNARD *et al.*, 2004).

Tirando partido da informação que tem sido disponibilizada, em resultado da execução dos projetos genomas, emergiu, recentemente, uma nova abordagem experimental em Biologia, conhecida por análise Proteômica. Esta consiste no estudo das proteínas expressas a partir de um genoma, o denominado proteoma. A evolução desta abordagem experimental tem resultado do desenvolvimento, integração e automatização de uma variedade de técnicas e equipamentos que permitem separar, identificar, quantificar e caracterizar proteínas, bem como relacionar essa informação com a obtida por outras abordagens, através da bioinformática.

Atualmente, a eletroforese bidimensional em gel de poliacrilamida (2DE) é o método mais eficiente de separação simultânea de centenas ou milhares de proteínas e uma das principais técnicas utilizadas em análises proteômica. Em análises 2DE, as proteínas são separadas com base em duas das suas propriedades: numa primeira dimensão, de acordo com o seu ponto isoelétrico (pI) e, numa segunda dimensão, em gel de SDS-PAGE, de acordo com o seu peso molecular (MW). Os fundamentos desta abordagem foram apresentados, pela primeira vez, por O'Farrell e Klose em 1975 (O´FARRELL, 1975; KLOSE, 1975). Desde então, têm-se verificado melhoramentos substanciais na qualidade e reprodutibilidade dos resultados obtidos, em conseqüência do desenvolvimento de equipamentos e reagentes específicos. Hoje em dia, a aplicação da 2DE permite uma alta resolução das várias espécies protéicas presentes numa amostra biológica, de uma forma reprodutível. Além da sua alta resolução e reprodutibilidade, a 2DE permite também separar as várias formas protéicas que tenham sofrido modificações pós-traducionais. A

separação destas formas é possível visto que essas modificações conferem propriedades diferentes à proteína, em particular, um diferente pI ou peso molecular. Por exemplo, consegue-se distinguir as formas fosforilada e não-fosforilada de fosfoproteínas porque este tipo de modificação pós-tradução implica uma alteração do seu pI (YAMAGATA *et al.*, 2002). Modificações do tipo acetilação, glicosilação ou hidrólise específica de determinados peptídeos, alteram o peso molecular da proteína modificada e, eventualmente, o seu pI, tornando assim possível a detecção dessas formas com base nos géis 2DE. Ainda outro tipo de modificação que pode afetar as proteínas, em células expostas a agressão oxidativa, é a carbonilação. A estimativa do nível de carbonilação, que ocorre nos resíduos de histidina, lisina e arginina das proteínas, é encarada como uma boa medida da extensão dos danos causados pelo estresse oxidativo. Este nível de carbonilação pode ser determinado, de forma específica, proteína a proteína, após marcação dos grupos carbonilo com DNPH (2,4-di-nitrofenil-hidrazina). As proteínas carboniladas, após separação por 2DE, podem ser detectadas por imunodetecção com um anti-corpo específico para o DNP (di-nitrofenilo) (KOROLAINEN *et al.*, 2002).

Garbis *et al.* (2005), revisando sobre limitações nas técnicas proteômicas, mostraram que é possível identificar maior número de proteínas utilizando as técnicas de cromatografia líquida multidimensional (MDLC) do que as técnicas de eletroforese bidimensional. O uso de sistemas de MDLC, além de melhorar a resolução da separação cromatográfica, simplifica e prepara a amostra para a análise direta em espectrômetro de massas. Dados obtidos nos bancos genômicos favorecem a análise do proteoma, aliando espectrometria de massa com predições *in silico* do proteoma (CAGNEY *et al.*, 2003).

Em estudos de proteômica quantitativa, a análise comparativa dos proteomas obtidos em duas (ou mais) situações em estudo, tem por objetivo identificar e quantificar

as possíveis alterações ao nível da abundância relativa de cada espécie protéica separada nos géis 2DE. Desta forma, torna-se possível identificar as proteínas envolvidas na resposta celular à alteração imposta. Geralmente, é construído um mapa de referência do proteoma de células cultivadas na condição de referência. Este mapa é construído através da catalogação das várias espécies protéicas separadas em géis 2DE, após a sua identificação. Embora existam já mapas disponíveis para os proteomas de um número significativo de organismos inclusive para algumas espécies de *Mycoplasma* (HOOGLAND *et al.*, 2000; BABNIGGE & GIOMETTI, 2004), as particularidades da técnica utilizada em cada laboratório podem dificultar o recurso aos mapas de referência disponíveis, além de que estes podem não incluir as proteínas que, em um determinado estudo, interessa analisar. Torna-se, assim, freqüentemente necessária a construção de um mapa, baseado nas condições experimentais escolhidas para a execução das experiências de 2DE, que possa, posteriormente, servir de base de análise para todos os trabalhos sobre o mesmo sistema biológico em que o protocolo de 2DE aplicado seja semelhante. No entanto, tendo em consideração os custos inerentes à identificação exaustiva de todas as proteínas separadas por 2DE, muitas vezes opta-se só pela identificação das proteínas cuja abundância varia no caso particular em análise.

Após coloração ou marcação das proteínas, a intensidade de cada spot de proteína detectada nos géis é registrada, digitalmente, sob forma de imagem. Essa informação é posteriormente tratada usando software adequado a quantificação e análise comparativa da intensidade dos vários spots obtidos nos géis 2DE. No mercado, existem várias soluções de softwares, desenvolvidas para a análise dos resultados obtidos em géis 2D (ex. Phoretix 2D, Nonlinear Dynamics; PDQuest, Biorad; Melanie, GeneBio; Decyder, Amersham Biosciences). Estes permitem padronizar as diferenças existentes de gel para gel (através

de métodos de normalização), quantificar e comparar a intensidade (quantidade) relativa de cada proteína presente nos diferentes géis.

A possibilidade de identificar rapidamente um grande número de proteínas através de espectrometria de massa (MS) foi um enorme avanço na área da proteômica. Em 2002, John B. Fenn e Koichi Tanaka receberam o prêmio Nobel em química pelo desenvolvimento das técnicas de ionização brandas utilizadas em ionização por electrospray (ESI) e ionização/dessorção a laser assistida por matriz (MALDI), respectivamente. Estas técnicas são consideradas brandas pela sua capacidade de gerar íons a partir de macromoléculas não voláteis sem ou com muito pouca fragmentação da molécula analisada. A espectrometria de massa determina a massa molecular através da relação carga/massa (m/z) de íons gerados a partir da molécula analisada. Atualmente MALDI e ESI são as tecnologias utilizadas para ionização das proteínas e sua subseqüente identificação (KÖPKE, 2003). A espectrometria de massa permite a identificação de proteínas em pequenas concentrações por seqüenciamento ou por características como modificações póstraducionais, peso molecular e impressão digital de massas (MO & KARGER, 2002).

A tripsina, normalmente utilizada para a digestão das proteínas, faz clivagem específica C-terminal adjacente a resíduos de arginina e lisina, gerando um conjunto de peptídeos únicos cujas massas são determinadas por espectrometria de massa (SHEVCHENKO *et al.*, 1996; CAGNEY *et al.*, 2003) e que podem ser considerados como impressão digital daquela molécula (QUADRONI & JAMES, 1999).

1.5.1. Estudos proteômicos de micoplasmas e *M. hyopneumoniae*

As bactérias respondem a condições de estresse ambiental através de modificações nos padrões de expressão gênica. Muitos investigadores têm verificado este fenômeno através do uso de eletroforese bidimensional. Esta abordagem pode ser utilizada mesmo com pouca informação a respeito da identidade das proteínas, servindo como um sistema de análise para ajudar em investigações futuras. Muitos dos estudos iniciais com esta abordagem foram usados para formar um catálogo das proteínas sintetizadas e de seus padrões de síntese em condições definidas, sem informações a respeito de sua identidade (CASH, 2002).

Projetos de caracterização proteômica em ampla escala já foram desenvolvidos com vários microrganismos, dentre eles *Saccharomyces cerevisiae*, *Escherichia coli*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Ochrobactrum anthropi*, *Salmonella entérica*, *Spiroplasma melliferum*, *Synechocystitis* spp, *Dictyostelium discoideum* e *Rhizobium leguminosarum* (HAYNES & YATES, 2000). No gênero *Mycoplasma* alguns estudos proteômicos também já foram descritos (REGULA *et al.*, 2000; WASINGER *et al.*, 2000; UEBERLE *et al.*, 2002; JAFFE *et al.*, 2004). Dentre os estudos proteômicos, destaca-se a análise comparativa dos "fostoproteoma" entre *M. genitalium* e *M. pneumoniae* (SU *et al.*, 2007). Este trabalho foi a primeira análise comparativa entre os proteomas de duas espécies distintas de micoplasma, além de identificar proteínas com uma modificação pós-traducional (fosforilação).

Com relação a análises proteômicas existem trabalhos que utilizam algumas técnicas (como 2DE e espectrometria de massas) para caracterização de proteínas específicas como a P97 (DJORDJEVIC *et al.*, 2004), P76 (BRUNETT *et al.*, 2006) e a P216 (WILTON *et al.*, 2009), entretanto, nenhuma análise proteômica global foi realizada até agora. Portanto, uma análise funcional comparativa entre cepas de *M. hyopneumoniae*

permitiria a identificação de diferenças tanto qualitativas quanto quantitativas no perfil protéico, através da utilização de ferramentas proteômicas para tal. Poderia também ser identificados a partir desta análise fatores de virulência, que, no futuro, poderão servir como alvos para o desenvolvimento de vacinas e/ou diagnóstico.

2. OBJETIVOS

2.1. Objetivo Geral

Realizar uma análise proteômica comparativa de diferentes cepas (não-patogênicas e patogênicas) de *Mycoplasma hyopneumoniae* na tentativa de identificar proteínas possivelmente envolvidas em patogenicidade e/ou virulência, e também antígenos com potencial para diagnóstico e/ou vacinação.

2.2. Objetivos Específicos

i) Produzir mapas proteômicos de referência em diferentes faixas de pH para *M. hyopneumoniae*, incluindo a identificação de padrões de processamento pós-traducional;

ii) Realizar a análise proteômica comparativa das cepas J, 7448 e 7422 de *M. hyopneumoniae* por eletroforese bidimensional e MALDI-TOF MS/MS e identificar proteínas antigênicas e padrões de processamento pós-traducional;

iii) Realizar a análise proteômica comparativa das cepas J, 7448 e 7422 de *M. hyopneumoniae* por LC-ESI MS/MS.

3. ARTIGO 1

No ARTIGO 1 (Pinto *et al.*, 2007), foi descrito o primeiro estudo proteômico de *M. hyopneumoniae*.

Neste trabalho foi realizada uma análise proteômica baseada em eletroforese bidimensional (2DE), estudos imunológicos e espectrometria de massas, com objetivo de identificar produtos gênicos e proteínas antigênicas para a cepa patogênica 7448 de *M. hyopneumoniae*. Foram produzidos mapas proteômicos em duas faixa de pH (3-10 e 4-7). Um total de 31 produtos gênicos, incluindo três hipotéticos, foram experimentalmente verificados pela identificação dos produtos protéicos por espectrometria de massas. Em uma análise imunológica foi possível identificar pelo menos cinco proteínas antigênicas de *M. hyopneumoniae*, incluindo quatro novos (uma proteína de choque térmico 70, um fator de alongamento Tu, a subunidade E1-beta da piruvato desidrogenase e a proteína de membrana P76).

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Proteomic survey of the pathogenic *Mycoplasma hyopneumoniae* strain 7448 and identification of novel post-translationally modified and antigenic proteins

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Abstract

Mycoplasma hyopneumoniae is an important pathogen for pigs, being the causative agent of enzootic pneumonia. Recently, the genome sequences of three strains, J, 7448 and 232 have been reported. Here, we describe the results of a proteomic analysis, based on two-dimensional gel electrophoresis of soluble protein extracts, immunoblot and mass spectrometry, which was carried out aiming the identification of gene products and antigenic proteins from the *M. hyopneumoniae* pathogenic strain 7448. A preliminary *M. hyopneumoniae* proteome map in two pH ranges (3–10 and 4–7) was produced. A total of 31 different coding DNA sequences (CDSs), including three hypothetical ones, were experimentally verified with the identification of the corresponding protein products by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. According to the Clusters of Orthologous Groups (COG) functional classification, the identified proteins were assigned to the groups of metabolism (13), cellular processes (5) and information and storage processing (4). Nine of the identified proteins were not classifiable by COG, including some related to cytoadherence and possibly involved in pathogenicity. Moreover, at least five highly antigenic proteins of *M. hyopneumoniae* were identified by immunoblots, including four novel ones (a heat shock protein 70, an elongation factor Tu, a pyruvate dehydrogenase E1-beta subunit and the P76 membrane protein). The now available proteome map is expected to serve as a reference for comparative analyses between *M. hyopneumoniae* pathogenic and non-pathogenic strains, and for methabolic studies based on cells cultured under modified conditions.
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Keywords: Proteomics; Antigenic proteins; Adhesins; HSP70; *Mycoplasma hyopneumoniae*

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1. Introduction

Mycoplasma hyopneumoniae is highly infective for swines and is the causative agent of enzootic pneumonia (EP), a disease characterized by sporadic, dry and non-productive cough, retarded growth and inefficient food conversion (Maes et al., 1996). EP is responsible for major economical losses in pig industry. Despite presenting low direct mortality rates, it causes increased susceptibility to secondary respiratory infections, due to the *M. hyopneumoniae*-associated deactivation of mucociliary functions.

There have been several efforts to identify and characterize *M. hyopneumoniae* antigens (see, for example, Subramaniam et al., 2000; Yang et al., 2005; Castro et al., 2006; Chen et al., 2006; Conceição et al., 2006; Meens et al., 2006). The recently published genome sequences of three *M. hyopneumoniae* strains, two pathogenic (232 and 7448) and one non-pathogenic (J) (Minion et al., 2004; Vasconcelos et al., 2005), will contribute to such efforts, accelerating the process of identification of novel antigenic proteins. These antigens, besides being potentially useful for immunodiagnosis and/or vaccination, may be relevant as virulence factors (Wassenaar and Gaastra, 2001; Ferreira and Castro, 2007), and their characterization may help to elucidade mechanisms of pathogenicity. Regarding immunodiagnosis, there is an obvious need for better antigens, in order to improve the serological methods currently used for pig herd surveying, an important step prior to preventive or therapeutical measures (Thacker, 2004; Kim et al., 2006; Lorenzo et al., 2006).

A proteomics approach based on two-dimensional gel electrophoresis (2DE), mass spectrometry (MS) and genomic data is a powerful tool for genome expression profiling. Proteome analysis has proven to be useful for bacteria, as a complementary method to support genome annotation and protein identification (Ueberle et al., 2002; Jaffe et al., 2004), and to characterize antigenic, differentially expressed or post-translationally modified proteins (Lebeau et al., 2005). 2DE mapping of *M. hyopneumoniae* proteins will improve genome annotation and help to characterize the extent of post-translational modifications and to identify potential virulence factors.

In this work, a preliminary 2DE map of *M. hyopneumoniae* is described and the repertoire of proteins identified by tandem MS is discussed. Moreover, we identified novel antigenic proteins from the *M. hyopneumoniae* pathogenic strain 7448 with potential for use in diagnosis.

2. Materials and methods

2.1. Bacterial strain, cultivation and cell extracts

M. hyopneumoniae strain 7448 was isolated from an infected swine in Lindóia do Sul, Santa Catarina, Brazil. When inoculated in specific pathogen free (SPF) pigs, this strain caused disease and consistently produced the characteristic symptoms of EP (Vasconcelos et al., 2005). Isolation and cultivation were performed in standard conditions, as described by Friis (1975), with cells grown in 2 l of medium, until a density corresponding to 10^8 CFU ml^{-1}. Cells were harvested by centrifugation at 18,000 $\times$ g for 10 min and resuspended in 1 ml of 25 mM Tris–HCl, pH 7.2. Cell suspensions were then lysed by sonication (25 Hz in a VC601 Sonics and Materials Inc. sonicator) in an ice bath by five 30 s cycles with 1 min interval between pulses. Proteins were quantified using the Bradford method (Bio-Rad Protein Assay, Bio-Rad).

2.2. Two-dimensional gel electrophoresis

Protein samples were solubilized in isoelectric focusing buffer (IEF buffer) containing 7 M urea, 2 M thio-urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT) and 0.2% (v/v) ampholytes pH 3–10 (Bio-Rad). The 17 cm immobilized pH gradient (IPG) strips (pH 3–10 or 4–7, Bio-Rad) were passively rehydrated for 16 h with 300 μl of cell extract samples containing 1–4 mg of protein. Isoelectric focusing was performed in a Protean IEF cell system (Bio-Rad) with up to 50,000 VH at a maximum voltage of 10,000 V. Strips were equilibrated for 15 min in equilibration buffer I (30%, v/v, glycerol, 6 M urea, 1% DTT, a trace of Bromophenol Blue) and for 15 min in equilibration buffer II (equilibration solution I with DTT replaced by 4% iodoacetamide). In the second dimension, IPG strips were run vertically onto SDS-PAGE 12% gels using PROTEAN® II xi 2D Cell (Bio-Rad). Gels were subsequently stained with Coomassie Brilliant Blue R250 (Bio-Rad) or with silver. The stained gels were

scanned with an Image Scanner (Amersham Biosciences) and analysed with the PDQuest Basic-8.0 software (Bio-Rad), followed by additional visual analysis.

2.3. Anti-M. hyopneumoniae sera

Three SPF (*Mycoplasma* spp.) swines were weekly immunized by two intramuscular injections with an emulsion containing equal parts of *M. hyopneumoniae* total protein extract (1 mg) and incomplete Freund's adjuvant. Nine weeks after the first injection, blood was collected and serum samples were evaluated by ELISA. Specific titles from 1:7000 to 1:10,000 were obtained and the serum with the highest title was used in the presented immunoblot experiments. All procedures and experiments involving animals were performed according to Brazilian laws and were approved by the Ethical Committees of the Universidade Federal do Rio Grande do Sul and of the CNPSA/EMBRAPA.

2.4. Immunoblotting

Proteins resolved by 2DE were electroblotted onto PVDF membranes (Amersham Biosciences). Blotted membranes were blocked with 5% (w/v) skimmed milk powder in PBS (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) and then incubated with the pig anti-*M. hyopneumoniae* serum diluted 1:2000 in blocking solution. Membranes were washed three times in PBS for 10 min, incubated with a secondary antibody (anti-pig IgG alkaline phosphatase conjugate, Sigma) (1:2000), washed and developed with NBT/BCIP (Sigma) according to the manufacturer.

2.5. Protein identification

Protein spots were manually excised from Coomassie stained gels and in-gel digested with trypsin. Gel plugs were treated in three washing steps with 180 μl of 50% acetonitrile and 50 mM ammonium bicarbonate for 15 min, followed by one washing step with 180 μl of acetonitrile. After the washing procedures, gel plugs were dried by vacuum centrifugation and digested for 18–24 h at 37 °C using 12 μl of 10 $\mu g\ ml^{-1}$ modified porcine trypsin (sequencing grade modified trypsin, Promega Corporation) diluted to 25 mM in NH_4HCO_3. After tryptic digestion, peptides were extracted in two washing steps with 50 μl of 50% acetonitrile and trifluoroacetic acid (TFA) for 1 h. Extracted peptides were dried and resuspended in 10 μl of Milli-Q water. A sample of 1 μl of crude digest was mixed with 1.0 μl of α-cyano-4-hydroxycinnamic acid (10 $mg\ ml^{-1}$ in 0.1% TFA in 1:1 acetonitrile/methanol) and an aliquot of 0.5 μl was delivered to the target plate and dried at room temperature. MS was performed on an AB 4700 TOF/TOF Proteomics Analyzer (Applied Biosystems), operated in the reflector mode for MALDI-TOF peptide mass fingerprint (PMF) or collision induced dissociation-MALDI-MS/MS (MS/MS). Each spectrum was produced by accumulating data from 2000 consecutive laser shots. All samples analysed by PMF were additionally analysed by MS/MS. For that, up to five PMF precursor ions per sample were chosen and submitted to the MS/MS analysis. Peptides were identified by matching the measured monoisotopic masses to theoretical monoisotopic masses generated using the MASCOT search engine (http://www.matrixscience.com) via *in silico* digest using *M. hyopneumoniae* J and 7448 strains genome generated protein databases, available from the System for Automated Bacterial Integrated Annotation—SABIA (Almeida et al., 2004). Maximum mass errors of 100 and 200 ppm were allowed for validation of PMF and MS/MS protein identifications, respectively.

3. Results

3.1. Two-dimensional electrophoresis

In order to resolve the prominent proteins of *M. hyopneumoniae* 7448, we performed 2DE from protein extracts from bacteria grown for 21 days in standard culture conditions. As technical controls, all protein preparations and subsequent 2DE were repeated three times, with a very high degree of reproducibility (data not shown).

About 350 prominent proteins spots were resolved in Coomassie stained 2DE gels with pH 3–10 IPG (IPG 3–10) strips (Fig. 1A), with molecular weights ranging from 18 to 216 kDa. Silver staining did not

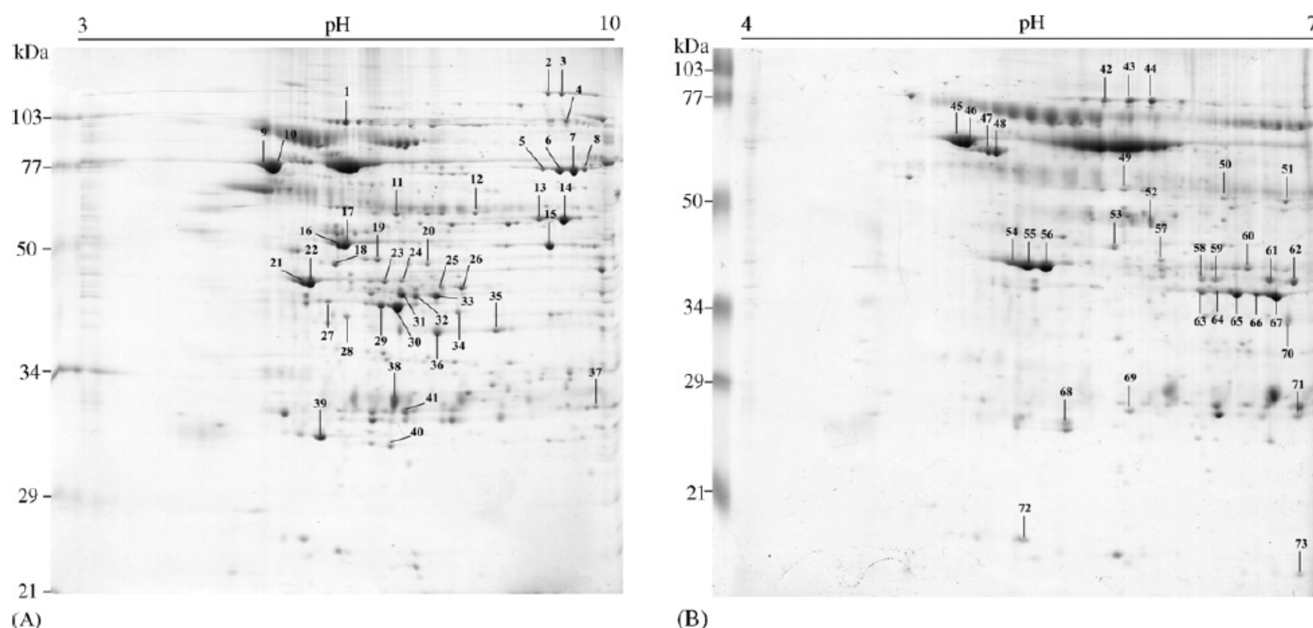

Fig. 1. 2DE gels of *M. hyopneumoniae* strain 7448. Proteins (2 mg) were separated by IEF using 17 cm IPG strips (A) pH 3–10 and (B) pH 4–7, followed by SDS-PAGE on 12% gels, and stained with 0.1% Coomassie Brilliant Blue R250. The approximate molecular weights are shown on the left of the gel and the acid-to-alkaline gradient is from left to right. The spot numbers correspond to those identified by MS and listed in Table 1.

increase this number significantly, allowing the detection of about 400 prominent protein spots, 92% of which also detected by Coomassie staining (data not shown). The number of detected protein spots corresponds to more than 50% of the 671 predicted *M. hyopneumoniae* 7448 protein gene products. Around 280 protein spots were resolved when pH 4–7 IPG (IPG 4–7) strips were used (Fig. 1B), a number corresponding to the expected, since more than 30% of protein spots resolved in IPG 3–10 are in the pH 7–10 range.

3.2. Mass spectrometry and data analysis

For a preliminary 2DE mapping of *M. hyopneumoniae* 7448 proteins, protein spots resolved in IPG 3–10 and IPG 4–7 were excised, in-gel digested with trypsin and analysed by MS techniques (PMF and MS/MS). MS identification was obtained for 73 of the analysed protein spots, 41 from IPG 3–10 and 32 from IPG 4–7 (Table 1).

The 73 identified protein spots corresponded to 31 different proteins. The molecular mass (MW) and the isoeletric point (p*I*) of each protein spot were experimentally determined and compared with gene-deduced MW/p*I* coordinates obtained from SABIA database (http://www.genesul.lncc.br/finalMP). Most (53 out of 73) of the gel-estimated MW/p*I* corresponded very well to the theoretical values. For other 20 spots (indicated in Table 1), on the other hand, experimentally determined MW and/or p*I* values were significantly distinct from the predicted ones, suggesting a relatively high level of post-translational modification. Some proteins, namely P97, P146, P216, LPPT, L-lactate dehydrogenase (P36), an elongation factor Tu (EF-TU), pyruvate dehydrogenase E1-alpha subunit (PDHA), pyruvate dehydrogenase E1-beta subunit (PDHB), an aminopeptidase, phosphate acetyltransferase (PTA), glyceraldehyde 3-phosphate dehydrogenase (GAP) and a conserved hypothetical protein, presented isoforms with charge differences, while others, namely P76, heat shock protein 70 (HSP70) and adenine phosphoribosyltransferase (APT), presented charge and mass variations. In most cases, two or three forms were identified for a given protein, but, in the cases of PTA (spots 58, 59, 61 and 62), PDHB (spots 63–67) and P97 (spots 5–8), a larger number of corresponding spots were found, including spot trains of high-MW protein isoforms,

Table 1
Protein identification by MS

Spot number	Protein name[a]	Accession number[b]	Theoretical pI/MW (kDa)	Observed pI/MW (kDa)	MASCOT score[c]	Sequence coverage (%)	COG[d]
1	**P146 adhesin like-protein**	MHP0663	**8.82/148.8**	**6.0/72.8**	**13**	**15**	**NC**
2	**P216 surface protein**	MHP0496	**8.50/216.5**	**8.8/77.7**	**8**	**1**	**NC**
3	**P216 surface protein**	MHP0496	**8.50/216.5**	**9.0/77.7**	**10**	**5**	**NC**
4	**P76 membrane protein**[e]	MHP0497	**8.67/159.7**	**9.1/73.1**	**28**	**1**	**NC**
5	**P97 adhesin protein**	MHP0198	**9.02/122.8**	**8.8/65.4**	**24**	**1**	**NC**
6	**P97 adhesin protein**	MHP0198	**9.02/122.8**	**9.0/64.9**	**29**	**28**	**NC**
7	**P97 adhesin protein**	MHP0198	**9.02/122.8**	**9.2/64.7**	**18**	**21**	**NC**
8	**P97 adhesin protein**	MHP0198	**9.02/122.8**	**9.3/65.4**	**11**	**8**	**NC**
9	Heat shock protein 70	MHP0067	4.97/65.6	5.0/65.6	189	8	O
10	Heat shock protein 70	MHP0067	4.97/65.6	4.9/65.6	241	12	O
11	**Lppt protein**	MHP0372	**8.99/109.0**	**6.7/59.0**	**11**	**12**	**NC**
12	ATP synthase alpha chain	MHP0479	7.10/57.7	7.8/59.1	13	8	C
13	**P76 membrane protein**[e]	MHP0497	**8.67/159.7**	**8.7/58.4**	**13**	**13**	**NC**
14	**P76 membrane protein**[e]	MHP0497	**8.67/159.7**	**9.1/58.1**	**15**	**16**	**NC**
15	**Hypothetical protein**	MHP0662	**7.57/132.4**	**8.9/54.8**	**12**	**12**	**NC**
16	Elongation factor Tu	MHP0523	5.61/44.1	5.9/50.8	19	44	J
17	Elongation factor Tu	MHP0523	5.61/44.1	6.0/50.6	31	51	J
18	**P46 surface antigen precursor**	MHP0513	**7.60/45.8**	**5.8/50.8**	**13**	**20**	**NC**
19	Phosphoglycerate kinase	MHP0490	6.00/44.3	6.4/48.5	16	17	G
20	Acetate kinase	MHP0508	6.73/44.0	7.2/4.7	12	13	C
21	Pyruvate dehydrogenase E1-alpha subunit	MHP0115	5.23/42.4	5.4/45.0	15	24	C
22	Pyruvate dehydrogenase E1-alpha subunit	MHP0115	5.23/42.4	5.5/44.6	19	33	C
23	Aminopeptidase	MHP0129	6.21/39.3	6.5/45.1	11	26	G
24	Aminopeptidase	MHP0129	6.21/39.3	6.8/45.3	13	17	G
25	Conserved hypothetical protein	MHP0377	6.81/39.2	7.3/44.4	29	26	NC
26	Conserved hypothetical protein	MHP0377	6.81/39.2	7.6/44.2	12	18	NC
27	DNA-directed RNA polymerase alpha subunit	MHP0168	5.46/37.4	5.7/42.3	15	11	K
28	Elongation factor EF-Ts	MP06150	5.72/34.0	6.0/40.2	44	3	J
29	Pyruvate dehydrogenase E1-beta subunit	MHP0116	6.21/36.8	6.5/41.7	19	20	C
30	Pyruvate dehydrogenase E1-beta subunit	MHP0116	6.21/36.8	6.7/41.0	16	32	C
31	Phosphate acetyltransferase	MHP0509	6.25/35.1	6.8/43.2	28	6	C
32	Glyceraldehyde 3-phosphate dehydrogenase	MHP0035	6.67/37.1	7.0/43.0	25	2	G
33	Glyceraldehyde 3-phosphate dehydrogenase	MHP0035	6.67/37.1	7.3/43.0	120	9	G
34	Thioredoxin reductase	MHP0098	6.97/33.9	7.6/40.8	8	2	O
35	L-Lactate dehydrogenase	MHP0137	7.63/34.3	8.1/38.5	16	15	C
36	L-Lactate dehydrogenase	MHP0137	7.63/34.3	7.3/38.2	27	28	C
37	Purine-nucleoside phosphorylase	MHP0084	8.75/25.6	9.5/30.0	15	16	F
38	Peptide chain release factor 1	MHP0139	7.75/40.8	6.7/31.0	10	15	J
39	Adenine phosphoribosyltransferase	MHP0114	5.34/18.6	5.66/27.1	25	41	F
40	Conserved hypothetical protein	MHP0335	6.40/24.6	6.6/26.4	16	19	NC
41	Thymidine phosphorylase	MHP0083	8.72/47.0	6.8/29.5	10	2	F
42	**P146 adhesin like-protein**	MHP0663	**8.82/148.8**	**5.7/74.4**	**9**	**7**	**NC**
43	**P146 adhesin like-protein**	MHP0663	**8.82/148.8**	**5.8/74.3**	**10**	**7**	**NC**
44	**P146 adhesin like-protein**	MHP0663	**8.82/148.8**	**5.9/74.2**	**13**	**11**	**NC**
45	Heat shock protein 70	MHP0067	4.97/65.6	5.0/65.6	68	37	O

Table 1 (*Continued*)

Spot number	Protein name[a]	Accession number[b]	Theoretical pI/MW (kDa)	Observed pI/MW (kDa)	MASCOT score[c]	Sequence coverage (%)	COG[d]
46	Heat shock protein 70	MHP0067	4.97/65.6	5.0/65.2	46	29	O
47	Heat shock protein 70	MHP0067	4.97/65.6	5.1/63.2	49	24	O
48	Heat shock protein 70	MHP0067	4.97/65.6	5.2/63.1	35	36	O
49	Trigger factor	MHP0149	5.51/50,533	5.8/56.0	14	12	O
50	**Lppt protein**	MHP0372	**8.99/109.0**	**6.2/54.5**	**17**	**13**	**NC**
51	**Lppt protein**	MHP0372	**8.99/109.0**	**6.5/53.9**	**11**	**14**	**NC**
52	Elongation factor Tu	MHP0523	5.61/44.1	5.9/50.6	37	31	J
53	**P46 surface antigen precursor**	MHP0513	**7.60/45.8**	**5.7/46.7**	**19**	**16**	**NC**
54	Pyruvate dehydrogenase E1-alpha subunit	MHP0115	5.23/42.4	5.2/44.5	37	20	C
55	Pyruvate dehydrogenase E1-alpha subunit	MHP0115	5.23/42.4	5.3/44.0	40	27	C
56	Pyruvate dehydrogenase E1-alpha subunit	MHP0115	5.23/42.4	5.4/43.8	31	26	C
57	**Hypothetical protein**	MHP0662	**7.57/132.4**	**5.9/44.8**	**10**	**11**	**NC**
58	Phosphate acetyltransferase	MHP0509	6.25/35.1	6.1/42.2	11	24	C
59	Phosphate acetyltransferase	MHP0509	6.25/35.1	6.2/42.1	11	24	C
60	Aminopeptidase	MHP0129	6.21/39.3	6.3/43.6	28	26	G
61	Phosphate acetyltransferase	MHP0509	6.25/35.1	6.4/42.0	12	15	C
62	Phosphate acetyltransferase	MHP0509	6.25/35.1	6.6/41.9	11	12	C
63	Pyruvate dehydrogenase E1-beta subunit	MHP0116	6.21/36.8	6.1/40.3	11	8	C
64	Pyruvate dehydrogenase E1-beta subunit	MHP0116	6.21/36.8	6.2/40.4	14	13	C
65	Pyruvate dehydrogenase E1-beta subunit	MHP0116	6.21/36.8	6.3/40.1	49	46	C
66	Pyruvate dehydrogenase E1-beta subunit	MHP0116	6.21/36.8	6.4/40.2	33	20	C
67	Pyruvate dehydrogenase E1-beta subunit	MHP0116	6.21/36.8	6.5/39.8	35	29	C
68	Adenine phosphoribosyltransferase	MHP0114	5.34/18.6	5.5/26.9	42	43	F
69	Adenine phosphoribosyltransferase	MHP0114	5.34/18.6	5.8/27.7	34	33	F
70	L-Lactate dehydrogenase	MHP0137	7.63/34.3	6.5/37.0	24	15	C
71	Hexulose-6-phosphate synthase	MHP0438	6.02/24.9	6.6/27.3	18	31	G
72	Thiol peroxidase	MHP0096	5.15/18.7	5.3/18.5	18	21	O
73	Peptide methionine sulfoxide reductase	MHP0529	6.51/16.7	6.6/16.5	18	26	O

Protein spots from 2DE (Fig. 1) were sequenced by MS/MS and identified by searching *M. hyopneumoniae* strains 7448 translated databases using MASCOT search engine. Proteins with putative post-translational modifications are indicated in bold.

[a] Protein identification according to SABIA database (Almeida et al., 2004).

[b] CDS access number in the SABIA database (Almeida et al., 2004).

[c] MASCOT score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event.

[d] COG database functional classes: (C) energy production and conversion; (F) nucleotide transport and metabolism; (G) carbohydrate transport and metabolism; (J) translation, ribosomal structure and biogenesis; (K) transcription; (O) post-translational modification, protein turnover, chaperones; (NC) proteins not classified.

[e] The MHP0497 CDS in the SABIA database (http://www.genesul.lncc.br/finalMP) corresponds to the P76 membrane protein precursor (with 159 kDa), while the spots 4, 13 and 14 correspond to proteolitically processed isoforms of this protein.

double spots or fragmentations (see Fig. 1A and B), representative of extense and variable post-translational modifications. For instance, we were able to demonstrate spot trains with the same MW but with distinct pI corresponding to the P97 adhesin (spots 5–8). Moreover, we have identified pI variants for the P146 (spots 42–44), P216 (spots 2 and 3) and LPPT (spots 50 and 51) surface proteins.

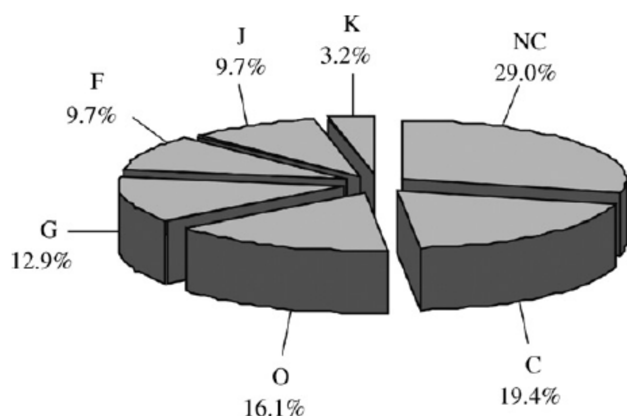

Fig. 2. COG database functional classification of identified *M. hyopneumoniae* 7448 proteins. Percentages of identified proteins in a given class, represented by a sector in the circle, are indicated. (O) Post-translational modification, protein turnover, chaperones; (C) energy production and conversion; (G) carbohydrate transport and metabolism, (J) translation, ribosomal structure and biogenesis, (K) transcription and (F) nucleotide transport and metabolism; (NC) proteins not classified.

According to the Clusters of Orthologous Groups (COG) functional classification (Table 1), most of the identified proteins (42%) are related to cell metabolism (Fig. 2), but we also identified several proteins possibly involved in cytoadherence, including P76, P97, P146, P216 and LPPT. Three of the identified protein products corresponded to hypothetical or conserved hypothetical coding DNA sequences (CDS) in the SABIA *M. hyopneumoniae* 7448 database (MHP0662, MHP0377 and MHP0335), experimentally validating them. The experimentally estimated MW and p*I*s of the MHP0377 and MHP0335 encoded proteins are in good agreement with the theoretical predictions. For the MHP0662 product, however, observed MW and p*I* were distinct from the predicted ones, suggesting post-translational modifications.

3.3. Identification of antigenic proteins

In order to identify novel antigenic proteins from *M. hyopneumoniae*, immunoblot assays of 2DE using the serum of an immunized pig were performed (Fig. 3). This serum allowed the establishment of IgG recognition patterns comprising 23 and 25 spots for IPG 3–10 and IPG 4–7, respectively. The comparison of the immunoblots with the *M. hyopneumoniae* proteomic map allowed the unambiguous identification of five highly antigenic proteins, namely HSP70, EF-TU, PDHB, p76 and a surface antigen precursor

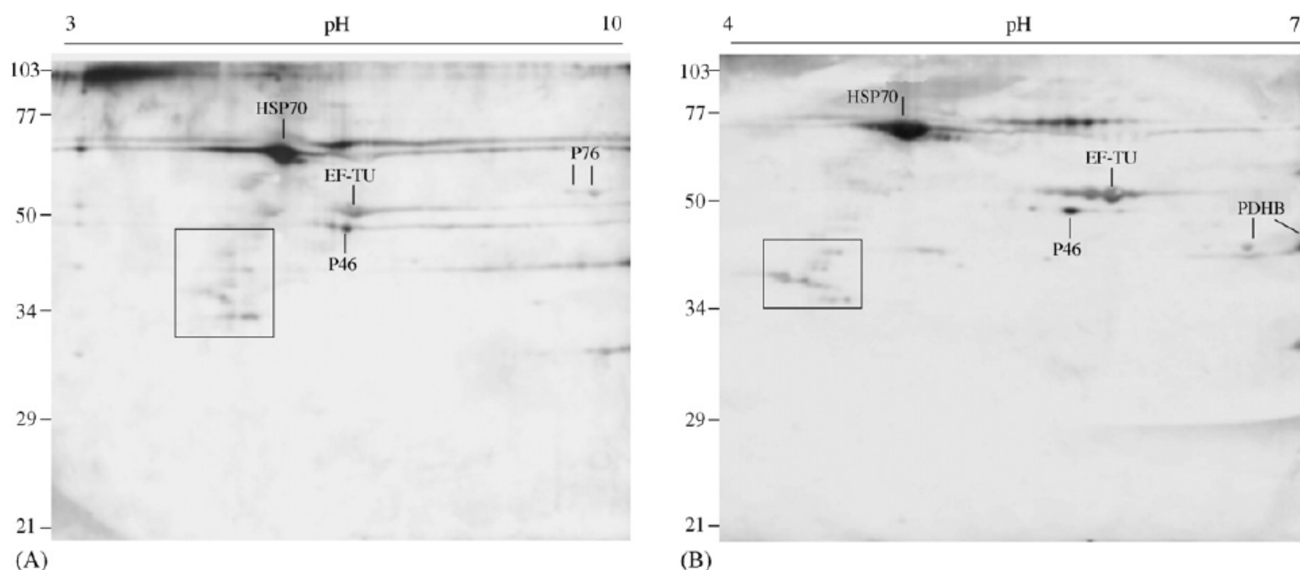

Fig. 3. Identification of antigenic proteins from *M. hyopneumoniae* 7448. Proteins resolved by 2DE as in Figs. 1 and 3 were electroblotted onto PVDF membranes (Amersham), and probed with an anti-*M. hyopneumoniae* hyperimmune pig antiserum (1:2000 dilution). Anti-pig IgG alkaline phosphatase-labeled secondary antibody (1:2000 dilution) was used to develop antigen-antibody reactions. (A) Strain 7448, isoelectric focusing (IEF) in pH gradient of 3–10; (B) strain 7448, IEF in pH gradient of 4–7. Molecular weight marker is shown on the left of the gel and the acid to alkaline gradient is from left to right. The group of at least eight antigenic spots, not detected in the corresponding Coomassie Blue stained 2DE gels, is indicated by rectangles in A and B.

(p46). From these, only p46 has been previously reported as an *M. hyopneumoniae* immunogenic protein (Okada et al., 2005). Interestingly, a group of at least eight antigenic spots (indicated in Fig. 3) in the pH and MW ranges of 4.2–5.7 and 30–40 kDa, respectively, were not detected in the corresponding Coomassie or silver stained 2DE gels, probably due to the low concentration of these proteins in the extract.

4. Discussion

We described a preliminary proteome map of the *M. hyopneumoniae* pathogenic strain 7448, whose entire genome sequence has been recently reported (Vasconcelos et al., 2005). The 2DE allowed the resolution of more than 350 protein spots in the pH range of 3–10, and 280 protein spots in the pH range of 4–10, corresponding to a coverage of more than 50 and 30%, respectively, of the 671 predicted protein gene products from the *M. hyopneumoniae* 7448 genome, even considering that some different spots may correspond to a single gene in cases of post-translational modification. This level of proteome coverage was similar to the observed in proteomic studies of other mycoplasmas (Regula et al., 2000; Wasinger et al., 2000; Ueberle et al., 2002; Jaffe et al., 2004).

So far, MW and/or tryptic peptide sequence MS data were obtained for 73 spots, and comparison with the DNA sequence-derived predictions allowed the assignment of these proteins to 31 genes. Under our experimental conditions, 22 of the identified proteins are involved in essential processes, such as cell metabolism, cellular processes and information storage and processing, most of them also identified in proteomes of other organisms, from eubacteria to archaea and eukaryotes (Brocchieri and Karlin, 2005). The other nine gene-assigned proteins, including adhesins (e.g., P76, P97 and P146), antigens (e.g., P36 and P46) and conserved hypothetical and hypothetical proteins, were not classifiable by COG. We were not able to detect proteins from some other important categories, such as signal transduction or intracellular trafficking and coenzyme transport, a situation also reported for *Mycoplasma penetrans* (Ferrer-Navarro et al., 2006), but contrary to that found in the *Mycoplasma pneumoniae* (Regula et al., 2000) and *Mycoplasma genitalium* proteomes (Wasinger et al., 2000). Transcriptomic analyses, as those made by Madsen et al. (2006a,b), may help to complement our knowledge on the *M. hyopneumoniae* repertoire of expressed genes under standard or modified culture conditions.

In *Mollicutes*, the necessary close contact with the host is favored by the absence of a cell wall and may be enhanced by the expression of specific cytoadherence proteins (Razin, 1999). We were able to detect protein spots corresponding to five proteins associated with cytoadherence in *M. hyopneumoniae* (P76, P97, P146, P216 and LPPT), which are, therefore, expressed even in the used laboratory culture conditions. This may be consequence of a constitutive mode of expression for these adhesins, which would render the bacterium ready for adhesion and infection even without contact with the host. Alternatively, the expression of these proteins may be induced by Friis medium components, such as regulatory proteins and signaling molecules from pig serum.

We have found several cases in which more than one spot was assigned to the same protein, including those of P46, P76, P97, P146, P216 and LPPT surface proteins and of the now validated MHP0662 CDS product. These variants or isoforms may be attributed to biologically important post-translational modifications, ranging from chemical modifications to proteolytic cleavage. We were able to confirm the occurrence of at least some of the P76 proteolytic cleavage products previously described by Burnett et al. (2006). Proteolytic processing was also previously reported for P97 (Djordjevic et al., 2004), and, although our current MS data allowed us to identify only four P97 p*I* variants, the concomitant occurrence of P97 MW variants in the *M. hyopneumoniae* 7448 proteome was confirmed by 2DE immunoblots probed with a monoclonal antibody anti-P97 (our unpublished results).

M. hyopneumoniae presents a relatively small spectrum of antigenic proteins, some of which have been previously described, including P36, P46, P97 and NrdF (Thacker, 2004). Although restricted, this antigenic repertoire still presents several unidentified members, especially those that are not surface proteins. Our 2DE immunoblot experiments associated with MS analysis allowed soluble *M. hyopneumoniae* proteins resolved in a wide range of p*I* (pH 3–10) and MW (15–216 kDa) to be probed with a serum of an

immunized pig. Four novel *M. hyopneumoniae* antigens were identified, namely HSP70, EF-TU, pdhB and P76, and the recently described P46 antigenicity (Okada et al., 2005) was confirmed. HSP70 was described as a T cell reactive antigen in *Francisella tularensis* (Elkins et al., 2003) and as an immunogenic protein in *Mycobacterium tuberculosis* (Tobian et al., 2005). The EF-TU and PDHB antigenic characters were previously demonstrated in *Mollicutes* (Olson et al., 1991; Alonso et al., 2002; Lopez et al., 2005) and *Bacillus stearothermophilus* (De Berardinis and Haigwood, 2004), respectively.

Immunological characterization of *M. hyopneumoniae* HSP70 and EF-TU using recombinant proteins have shown that these proteins elicit specific humoral responses in swines with EP (our unpublished results), suggesting their possible diagnostic value. These proteins, as well as pyruvate dehydrogenase (PDH), are usually regarded as cytoplasmic, and, in this case, the elicitation of specific immune responses against them would occur only upon their release during infection, due to the death of *M. hyopneumoniae* cells. However, a cell surface location cannot be discarded for these antigens, since, EF-TU, PDH and HSP70 presence on cell surface has been described for some bacteria (see, for example, Young and Bernlohr, 1991; Wallbrandt et al., 1992; Porcella et al., 1996; Macellaro et al., 1998; Marques et al., 1998; Layh-Schmitt et al., 2000; Dallo et al., 2002).

The now available *M. hyopneumoniae* 7448 proteome map will be a useful reference for future comparative studies and the associated identification of novel antigenic proteins will further increase the repertoire of antigens available for the formulation of EP immunodiagnostic reagents and vaccines. Besides, proteomic analyses of the same strain under different culture conditions and of different pathogenic and non-pathogenic strains will allow the identification of differentially expressed or processed proteins. Once identified, it will be possible to experimentally address the function of these proteins, which may lead to the elucidation of specific metabolic processes and/or mechanisms of virulence.

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4. ARTIGO 2

O ARTIGO 2 (*Comparative proteomics analysis of pathogenic and non-pathogenic strains from the swine pathogen* Mycoplasma hyopneumoniae) é um manuscrito submetido para publicação no periódico *Molecular and Cellular Proteomics* em dezembro de 2008. O material suplementar deste manuscrito está no ANEXO I.

O manuscrito descreve uma análise proteômica comparativa entre três cepas de *M. hyopneumoniae*, uma não-patogênica (cepa J) e duas cepas patogênicas (7448 e 7422). Na comparação por 2DE foi possível identificar diferença no nível de expressão entre as cepas em pelo menos 67 spots protéicos, incluindo proteínas envolvidas em citoaderência, super expressas nas cepas patogênicas. Para uma análise mais ampla dos perfis protéicos das três cepas foi utilizada uma estratégia baseada em LC-MS/MS. Nas três cepas 231 proteínas foram identificadas, correspondendo a cerca de 35% da capacidade codificante do genoma de *M. hyopneumoniae*, e pelo menos 36 produtos de genes hipotéticos foram experimentalmente validados. Quando analisada a redundância da identificação entre as cepas J, 7448 e 7422, cerca de 81 proteínas foram identificadas em apenas uma cepa, 54 proteínas foram identificadas em duas cepas e 96 proteínas foram identificadas nas três cepas. A classificação funcional das proteínas identificadas sugere diferenças fisiológicas entres as cepas não-patogênicas e patogênicas. Também a aplicação de uma proteômica quantitativa *label-free* (o *exponentially modified protein abundance index*) demonstrou diferenças significativas nos níveis de expressão de pelo menos 64 proteínas, incluindo a proteína de membrana envolvida em adesão P97. Por fim, uma análise imunológica baseada em 2DE utilizando um anticorpo monoclonal contra a repetição R1 da proteína P97, foi capaz de identificar um padrão de clivagem proteolítica diferencial entre as três cepas de *M. hyopneumoniae*.

Comparative proteomic analysis of pathogenic and non-pathogenic strains from the swine pathogen *Mycoplasma hyopneumoniae*

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Running Title: Comparative proteomics of *Mycoplasma hyopneumoniae*

ABBREVIATIONS

2DE – two-dimensional gel electrophoresis

BCIP - 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt

CapLC – capillary liquid chromatography

CDS – coding DNA sequence

COG – clusters of orthologous groups

emPAI – exponentially modified protein abundance index

EP – enzootic pneumonia

ICEH – integrative conjugal element of *M. hyopneumoniae*

IgG – immunoglobulin G

MudPIT – multidimensional protein identification technology

NBT – nitro blue tetrazolium chloride

NPS-RP-HPLC – nonporous reversed-phase HPLC

PVDF – polyvinylidene difluoride

SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SUMMARY

Following the previous report of a proteomic survey of the pathogenic 7448 strain, *Mycoplasma hyopneumoniae* swine pathogen, we performed comparative protein profiling of three *M. hyopneumoniae* strains, namely the non-pathogenic J strain and the pathogenic strains 7448 and 7422. In 2DE comparisons, we were able to identify differences in expression levels between strains for 67 proteins, including cases of over-expression of cytoadherence-related proteins in pathogenic strains. 2DE immunoblot analyses allowed the identification of differential proteolytic cleavage patterns of the P97 adhesin in the three strains. For more comprehensive protein profiling, an LC-MS/MS strategy based on a modified multidimensional protein identification technology was used. Overall, 231 different proteins were identified, corresponding to 35% of the *M. hyopneumoniae* genome coding capacity, and experimental validation for at least 36 hypothetical genes was provided. Partially overlapping profiles of identified proteins were observed for strains J, 7448, and 7422, with 81 proteins identified only in one strain, 54 proteins identified in two of them, and 96 proteins identified in all strains. The functional classification of identified proteins was suggestive of physiological differences between the non-pathogenic and the pathogenic strains, with the first being richer in proteins classified as involved with cellular processes, and the latter being richer in proteins of the class of poorly characterized proteins (which includes adhesion molecules). Also, application of the exponentially modified protein abundance index demonstrated significant differences in the expression levels of proteins detected in more than one strain, confirming over-expression in one or two of the strains for 64 proteins.

Keywords: *Mycoplasma hyopneumoniae*; comparative proteomics; shotgun proteomics; emPAI; adhesins; virulence.

INTRODUCTION

Mycoplasma hyopneumoniae is a highly infectious swine pathogen and is the causative agent of enzootic pneumonia (EP), a disease characterized by a sporadic, dry, and non-productive cough, retarded growth, and inefficient food conversion (1). Despite presenting with low direct mortality rates, EP is responsible for major economical losses in pig industry as it causes increased susceptibility to secondary respiratory infections, due to the *M. hyopneumoniae*-associated deactivation of mucociliary functions.

The *M. hyopneumoniae* genomes of a non-pathogenic (J) and two pathogenic strains (strains 7448 and 232) (2, 3) have been sequenced. Comparative genomic analyses have been carried out in order to provide insights into evolutionary aspects of mycoplasma reduced genomes (4) and *M. hyopneumoniae* virulence determinants (5, 6).

So far, the study of virulence factors in *M. hyopneumoniae* has been centered on the characterization of adhesion mediating molecules, especially the P97 adhesin (see, for example, 7, 8). However, the mechanisms of *M. hyopneumoniae* pathogenicity suggest the existence of several other classes of yet unidentified virulence factors, including, for instance, genes/proteins involved in secretion and/or trafficking of molecules between host and pathogen cells, or evasion and/or modulation of the host immune system (6). The observed presence of a putative integrative conjugal element (ICEH) in three *M. hyopneumoniae* pathogenic strains, but not in a non-pathogenic one, is also suggestive of the involvement of potentially mobile genetic elements in *M. hyopneumoniae* virulence (6, 9).

For many species, differences in pathogenicity or other biological features between strains correspond to significant differences at the genomic level. For example, speciation and diversity of strains have been achieved by horizontal gene transfer of DNA encoding

novel genes that are probably required for niche specific survival (10). In the case of *M. hyopneumoniae*, no extensive genomic differences have been detected among the genomes of different strains, even in the comparison of the non-pathogenic strain J with the pathogenic strains 7448 and 232 (2, 5). In this context, it can be assumed that differences between pathogenic determinants are not predominantly at the genomic level, and may be associated with differences at the expression level of genes encoding virulence factors. Therefore, comparative transcriptomic and proteomic analyses of relevant strains have the potential to discover gene products that play a role in *M. hyopneumoniae* pathogenesis**.**

Transcriptomic studies aimed at identifying differentially expressed genes have been recently published (11-15). However, these studies were unable to identify genes specifically related to virulence, even when analyzing infection conditions (13). The *M. hyopneumoniae* proteomic studies performed so far were mainly prospective, and resulted in the 2DE mapping of the *M. hyopneumoniae* strain 7448 (16) and in evidence of post-translational modifications of several *M. hyopneumoniae* proteins, including P97 (8, 16). More comprehensive and comparative proteomic approaches are expected to provide an overview of the *M. hyopneumoniae* repertoire of virulence-related proteins.

Here, we describe the comparison of the proteomes of the avirulent J strain and the virulent 7422 and 7448 strains by a modified multidimensional protein identification technology (MudPIT) approach. We experimentally confirmed the functionality of approximately 35% of the *M. hyopneumoniae* predicted protein-encoding genes and report abundance data for these proteins based on the exponentially modified protein abundance index (emPAI). This and complementary 2DE and immunoblotting analyses provide the first comprehensive comparative proteomic analysis of *M. hyopneumoniae* strains, and demonstrate the over-expression of virulence factors specifically in the pathogenic strains.

EXPERIMENTAL PROCEDURES

Bacterial strains, cultivation, and cell protein extracts

M. hyopneumoniae strain J (ATCC 25934), a nonpathogenic strain with reduced adhesion capacity to porcine cilia, was acquired from American Type Culture Collection by the Empresa Brasileira de Pesquisa Agropecuária-Centro Nacional de Pesquisa de Suínos e Aves (EMBRAPA-CNPSA), in Concórdia (Santa Catarina, Brazil). *M. hyopneumoniae* pathogenic strain 7448 was isolated from infected swine from Lindóia do Sul (Santa Catarina, Brazil) (2). *M. hyopneumoniae* pathogenic strain 7422, a field isolate from Concórdia (Santa Catarina, Brazil), was obtained from the EMBRAPA-CNPSA collection. Isolation and cultivation were performed in standard conditions, as described by Friis (1975) (17), with cells grown in 2 L of medium until a density of 10^8 CFU mL^{-1}.

For protein extract preparation, cells were harvested by centrifugation at 18,000 × g for 10 min and resuspended in 1 mL of 25 mM Tris–HCl, pH 7.2. Cell suspensions were then lysed by sonication (25 Hz in a VC601 Sonics and Materials Inc. sonicator) in an ice bath by five 30 s cycles with a 1 min interval between pulses. Proteins were quantified using the Bradford method (Bio-Rad Protein Assay, Bio-Rad). For each strain, protein extracts were prepared from three identical and independent cultures (biological replicates) and then mixed into a single protein sample.

Two-dimensional gel electrophoresis and gel image analysis

Protein samples were solubilized in isoelectric focusing (IEF) buffer containing 7 M urea, 2 M thio-urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT), and 0.2% (v/v) ampholytes pH 3–10 (Bio-Rad, Hercules, US). The 17 cm immobilized pH gradient (IPG)

strips (pH 3–10 or 4–7, Bio-Rad, Hercules, US) were passively rehydrated for 16 h with 300 µL of cell extract samples containing 1–2 mg of protein. IEF was performed in a Protean IEF cell system (Bio-Rad, Hercules, US) with up to 50,000 VH at a maximum voltage of 10,000 V. Strips were equilibrated for 15 min in equilibration buffer I (30%, v/v, glycerol, 6 M urea, 1% DTT, a trace of bromophenol blue) and for 15 min in equilibration buffer II (equilibration solution I with DTT replaced by 4% iodoacetamide). In the second dimension, IPG strips were run vertically onto SDS-PAGE 12% gels using PROTEAN® II xi 2D Cell (Bio-Rad, Hercules, US). For each protein sample, three independent gels were run (technical replicates). Gels were stained with 0.1% Coomassie Brilliant Blue G (Acros, Geel, Belgium) and scanned with a computer-assisted G-800 densitometer (Bio-Rad, Hercules, US). The 2DE image analyses were carried out using the PDQuest 8.0 software package (18). After background subtraction and spot detection and match, one standard gel, representative of a given strain sample, was defined. Spots in the standard gels were then matched to the previously reported *M. hyopneumoniae* 7448 proteomic maps (16) for protein assignment, and compared to each other in order to identify proteins differentially expressed between strains.

Liquid chromatography tandem mass spectrometry

A modified MudPIT strategy was used, which consisted of a liquid chromatography (LC) separation (reversed-phase HPLC, NPS-RP-HPLC) coupled with tandem mass spectrometry (MS/MS). MS/MS analyses were performed in an electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) Ultima API mass spectrometer (Micromass, Manchester, UK) coupled to a capillary liquid chromatography system (CapLC, Waters, Milford, US). A nanoflow ESI source was used with a lockspray source for lockmass

measurement during all the chromatographic runs. Samples of about 10 mg of each *M. hyopneumoniae* strain protein extract were digested with trypsin (Promega, Madison, US) and the resulting peptide mixture was desalted using an OASIS® HLB Cartridge column (Waters, Milford, US). The peptides were separated in a Nanoease C18 (75 µm ID) capillary column by elution with a water/acetonitrile 0.1% formic acid gradient. Data were acquired in data-dependent mode (DDA), and multiple charged peptide ions (+2 and +3) were automatically mass selected and dissociated in MS/MS experiments. Typical LC and ESI conditions were flow of 200 nl/min, nanoflow capillary voltage of 3.5 kV, block temperature of 100°C, and cone voltage of 100 V. For each protein sample, three independent LC-MS/MS were performed.

Data processing and bioinformatics analyses

The MS/MS spectra were processed using Proteinlynx v. 2.0 software (Waters, Milford, US) and the generated PKL files were used to perform database searches using the MASCOT software v. 2.2 (Matrix Science, London, UK) against the non-redundant NCBI database (6,666,652 sequences and 2,285,042,942 residues, at Aug 3, 2008). Search parameters allowed a maximum of one missed cleavage, the carbamidomethylation of cysteine, the possible oxidation of methionine, peptide tolerance of 0.2 Da, and MS/MS tolerance of 0.1 Da. The significance threshold was set at $p < 0.05$, and identification required that each protein contained at least one peptide with an expected value <0.05. For each protein match identified by MASCOT, the software calculated the corresponding exponentially modified protein abundance index (emPAI) (19) as the transformed ratio of the number of experimentally observed peptides to the total number of peptides that can theoretically be detected within the operating mass range and retention range of the

instrument. In the comparisons of emPAI values of a given protein between strains, differences were considered significant when there was at least a twofold difference between the calculated emPAIs (19).

The Clusters of Orthologous Groups (COG) classification of each of the identified proteins was obtained from the Southern Genome Investigation Program *M. hyopneumoniae* Genome Database (http://www.genesul.lncc.br).

The Compute p*I*/Mw tool from the ExPASy Proteomics Server (http://ca.expasy.org/) was used to estimate p*I* and MW corresponding to proteins/polypeptides represented in 2DE spots.

Immunoblotting

Proteins were resolved by 2DE and electroblotted onto PVDF membranes (GE Healthcare, Chalfont St. Giles, UK). Blotted membranes were blocked with 5% (w/v) skim milk powder in PBS (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) and then incubated with an anti-P97 monoclonal antibody (F1B6, purchased from Iowa University) (20) diluted 1:400 in blocking solution. Membranes were washed three times in PBS for 10 min, incubated with a secondary antibody (anti-mouse IgG alkaline phosphatase conjugate, Sigma-Aldrich, St. Louis, US) diluted 1:2000, washed, and developed with NBT/BCIP (Sigma-Aldrich, St. Louis, US). For each protein sample, three independent immunoblot experiments were performed (technical replicates).

RESULTS

2DE comparative analysis of M. hyopneumoniae *strains J, 7448, and 7422*

For a comparative investigation of the repertoires of protein gene products in *M. hyopneumoniae* strains J, 7448, and 7422, the respective protein extracts were resolved by 2DE and analyzed using the previously established proteomic maps of *M. hyopneumoniae* 7448 as a reference (16). The average spot matching rate for the replicate gels of each sample was 0.92, meaning that 92% of the spots in each gel were also found in the corresponding replicates, thus validating the obtained 2DE results as reproducible and reliable.

Thirty-eight spots (17 identified, corresponding to two different proteins) of 2DE in the pH 4-7 range (Fig. 1), and 29 spots (10 identified, corresponding to four different proteins) of 2DE in the pH 3-10 range (Fig. 2) presented significant qualitative (presence versus absence) or quantitative differences between the three *M. hyopneumoniae* strains under the same culture conditions. In the 2DE analyses of pathogenic strains (7448 and 7422), we identified as over-expressed surface and/or cytoadhesion-related proteins (P46, P97, and P146), and a protein previously described as a hypothetical one (product of the MHP0662 CDS) (2). The number of P97 and P146 p*I* isoforms observed in the different strains was also distinct, which denotes differences in post-translational processing. Two proteins, namely P216, an adhesin, and thiol peroxidase (TPx), and an oxidative stress-related protein described as a virulence factor for other pathogenic bacteria (21, 22), showed over-expression in the J strain.

Comparative shotgun proteomics of M. hyopneumoniae

Since 2DE does not favor the identification of less abundant protein species or proteins with extremes of p*I*, we performed a complementary shotgun LC–MS/MS analysis using a modified MudPIT approach for a more comprehensive comparative analysis of the

protein expression profiles of *M. hyopneumoniae* J, 7448, and 7422 strains. Protein extracts from *in vitro* cultured strains were individually digested with trypsin and each of the resulting peptide mixtures were independently analyzed three times by LC-MS/MS. A >93% concordance in the repertoire of identified proteins was obtained between the three independent LC-MS/MS analyses of each sample. Combined results from the LC-MS/MS experiments of the three strains resulted in a pool of 231 unique protein identifications (from 953 unique peptide sequences). Totals of 164, 154, and 158 proteins were respectively identified for stains J, 7448, and 7422 (Supplementary Material Tables 1, 2, and 3, respectively). The genome-wide coverage of the obtained proteomic data corresponds to 35% of the *M. hyopneumoniae* genome theoretical coding capacity, and provided experimental validation for 39 genes previously regarded as hypothetical (2).

The Venn diagram presented in Fig. 3 summarizes data from Table 4 (Supplementary Material), which describes the protein sets that are exclusive to one strain, shared by two strains, or common to all of them. In total, 96 of the identified proteins, including 12 products from hypothetical genes, were shared by the three strains. On the other hand, 17 proteins were shared by J and 7448 strains, 21 proteins were shared by J and 7422 strains, and 16 proteins were shared by the pathogenic strains, while 81 proteins (more than 35% of the total of identified proteins) were detected in a single strain, with 25 from the 7448 strain, 30 from the J strain, and 26 from the 7422 strain.

To extend our comparative analysis to those proteins shared by two or three strains, we also compared the relative abundance of the LC-MS/MS identified proteins in *M. hyopneumoniae* strains J, 7448, and 7422 by their emPAI values. Hence, we were able to infer that the concentration of at least 64 proteins differed significantly among the analyzed *M. hyopneumoniae* strains (Supplementary Material Table 4, highlighted in gray), as

graphically represented in Fig. 4. In the comparison between the non-pathogenic strain J and the pathogenic strain 7448 (Fig. 4A), 30 proteins were relatively over-expressed in the J strain, while 8 were over-expressed in the 7448 strain. When compared to the 7422 strain (Fig. 4B), strain J presented 15 proteins as relatively over-expressed, while 19 were classified as over-expressed in the 7442 strain. Finally, in the comparison between the two pathogenic strains (Fig. 4C), strain 7448 presented 8 proteins with a relative over-expression, and while 27 were classified as over-expressed in the 7442 strain.

In an attempt to correlate the proteomic profile of each strain to possible functional/physiological features, the identified proteins of the non-pathogenic and pathogenic *M. hyopneumoniae* strains were categorized into COG classes and comparatively analyzed as summarized in Fig. 5. According to the COG functional classification, most of the identified proteins in the pathogenic 7448 and 7422 strains were assigned to the poorly characterized proteins (Pc) major class, which includes 19 proteins possibly involved in cytoadherence, such as P76 and P97, and 30 hypothetical proteins. In 8 COG functional classes, namely D (Cell division and chromosome partitioning), F (Nucleotide transport and metabolism), K (Transcription), L (DNA replication, recombination and repair), M (Cell wall/membrane biogenesis), P (Inorganic ion transport and metabolism), Q (secondary metabolites biosynthesis, transport, and catabolism), and R (General function prediction only), more proteins were detected in the pathogenic strains than in the non-pathogenic one. In the other 5 COG functional classes, namely C (Energy production and conversion), G (Energy production and conversion), J (Translation, ribosomal structure, and biogenesis), O (Post-translational modification, protein turnover, and chaperones), and S (Function unknown), more proteins were detected in the non-

pathogenic J strain, with Isp (information storage and processing) being the most prominent major class with 48 identified proteins including 22 ribosomal proteins.

Comparative analysis of P97 post-translational modifications

As different p*I* P97 isoforms were detected by 2DE and shotgun proteomics in *M. hyopneumoniae* strains J, 7448, and 7422, we further investigated the P97 isoform profiles of each strain by 2DE immunoblotting using a monoclonal antibody directed against the P97 R1 region. As shown in Fig. 6 (A-C), three main P97 isoforms were identified in all strains, one corresponding to the mature P97 adhesin (a polypeptide with 94,412 kDa, p*I* 9.02, indicated as P97 in the Fig. 6) and two isoforms (indicated as *a* and *b* in the Fig. 6) with different MW (between 60 and 90 kDa) and/or p*I* (between 7.1 and 9.8). In the 7448 and 7422 pathogenic strains, two and four additional low MW species were detected, respectively, without any correspondence to the J non-pathogenic strain.

The two additional P97 isoforms found in the 7448 strain (Fig. 6B, spots 1 and 2) presented the same theoretical MW (33 kDa) and p*I*s of 5.6 and 5.7, respectively. The four additional P97 isoforms of the 7422 strain (Fig. 6C, spots 1-4) presented p*I*s of 5.4 (spots 1 and 3) and 5.6 (spots 2 and 4) and MWs of 34 kDa (spots 1 and 2) and 30 kDa (spots 3 and 4). The P97-derived low p*I* and low MW spots detected in the 7448 and 7422 strains were consistently observed in the corresponding biological and technical replicates of each strain.

An *in silico* P97 amino acid sequence analysis (data not shown) failed to predict any P97-derived polypeptides including the R1 repeat that would correspond to the observed MW and p*I*. This suggests that the additional peptides observed in the 7448 and 7442 strains are a result of not only proteolytic cleavage, which explains their lower MW,

but also post-translational amino acid modifications that would explain their reduced p*I*s in comparison to the theoretically predicted ones.

DISCUSSION

We performed a comprehensive and comparative proteomic survey of three strains of the swine pathogen *M. hyopneumoniae*, which was the first study of this scope for a species of this genus of genome-reduced bacteria. Overall, our comparative analyses, including 2DE, immunoblotting, and LC-MS/MS experiments, are suggestive of differential gene expression between *M. hyopneumoniae* strains under culture conditions, which in part is likely related to virulence.

The high degree of similarity in gene content among the genomes of different *M. hyopneumoniae* strains, which have more than 84% of their genes in common (2), suggests that many of the observed physiological differences between strains, including their virulence, are due to differential gene expression. Indeed, in our 2DE, we are able to identify differences in the expression level and the p*I* and/or MW of several proteins, including surface antigenic proteins related to *M. hyopneumoniae* pathogenicity.

Our LC-MS/MS approach, complementary to the 2DE and immunoblot analyses, resulted in the identification of 35% of the predicted *M. hyopneumoniae* genome protein products. Such coverage is comparable to other recent bacterial proteomic studies, like those of *Halobacterium salinarum* (23, 24), *Natronomonas pharaonis* (25), and *Haloferax volcanii* (26), which ranged from 29 to 33%. Since neither a detergent nor another solubilization agent was used, the analyzed *M. hyopneumoniae* samples consist mostly of cytosolic components. Therefore, complementary proteome data is expected to be obtained from future analyses of samples enriched in secreted or membrane-bound proteins.

Differential proteomic studies employing label-free quantification rely on the comparison of peptide abundance as a measure of the corresponding protein level in multiple LC/MS/MS analyses (27). The emPAI values of proteins in one sample can be compared to those in another sample, and outliers from the emPAI correlation between the two samples can be assigned as over or under-expressed proteins (19). Although the emPAI may not define the absolute abundance of different proteins, this parameter is useful in estimating the relative abundance of a given protein in different mixtures (19, 28, 29), which here were samples representative of different *M. hyopneumoniae* strains. A further validation of the emPAI comparative analyses was their consistent concordance with several cases of differential protein expression demonstrated by 2DE.

The comparative emPAI analysis resulted in evidence of pathogenic-strain-specific over-expression of several protein groups. For instance, heat-shock proteins and proteins involved in pyruvate metabolizing pathways, such as acetate kinase, pyruvate dehydrogenase E1-alpha subunit, pyruvate dehydrogenase E1-beta subunit, pyruvate kinase, and lactate dehydrogenase, were assigned as over-expressed for the 7448 and 7422 strains. These results are consistent with the idea that a functional heat shock response, already detected in *M. hyopneumoniae* and other related species, is important for responding to temperature stress during host pyrexia (11, 30, 31). Also, the capacity to use glucose and other alternative carbon sources, such as glycerol and fructose also present in *Mycoplasma pneumoniae* and other *Mycoplasma* species (32, 33), would be important for *M. hyopneumoniae* to cope with predicted changes in the availability of carbon sources in its habitat.

Another important group of proteins with differential expression between strains is that of redox balancing proteins. The response of the swine immune system to the presence

of *M. hyopneumoniae* in the consequent inflammatory process that includes the production of superoxides (34) and a mechanism to evade that response would be quite adaptive for the pathogen. In agreement with that, most of differentially expressed oxidative stress response proteins were significantly more represented in the pathogenic strains. *M. hyopneumoniae* lacks antioxidants such as catalase and superoxide dismutase (2), but, from a repertoire of five detected oxidative stress response proteins, four, namely thioredoxin, NADH oxidase, methionine sulfoxide reductase, and thioredoxin reductase, were differentially expressed and over-represented in samples from at least one of the pathogenic strains in comparison to the J strain.

Antigenic surface proteins constituted a well represented group among the LC MS/MS identified proteins with 13 members including P97 and its two P97-like orthologs and the two P102 orthologs (2). P97, P102, and at least some of the other identified antigenic surface proteins are involved in the adherence of *M. hyopneumoniae* to the mucosa of the distal portion of the respiratory tract of swine, which is a fundamental step in the development of EP (7, 35). Our samples were from *in vitro* cultures, which did not have any contact with host tissue, and the large representation of surface adhesion-related proteins could be a result of induction of their expression by Friis medium components, such as proteins and signaling molecules from pig serum. An alternative explanation would be a somewhat “constitutive” mode of expression of the repertoire of adhesins, which would render the bacterium competent for infection and adhesion prior to direct interaction with the swine respiratory epithelium.

In the comparative emPAI analysis however, only P97 isoforms, of all the identified surface and/or adhesion-related proteins, showed significant differences in their expression levels and were more prominent in both the pathogenic strains than in the J

strain. Therefore, the adhesion-deficient (36) J strain is not significantly devoid of adhesins, but our 2DE and immunoblot results suggest both qualitative and quantitative underrepresentation of at least three important adhesins, namely P97, P146, and P216, in comparison to the 7448 and 7422 strains.

Overall, the non-pathogenic strain protein expression profile is suggestive of a non-infective proliferate lifestyle with most of the identified proteins assigned to the COG Isp major class, which is comprised of translation, ribosomal structure, and biogenesis; transcription; and DNA replication, recombination, and repair categories. For the pathogenic 7448 and 7422 strains, most of the identified proteins were assigned to the poorly characterized proteins (Pc) major class, which is comprised of proteins with only general function prediction and unknown function, including proteins known to be involved in cytoadherence, and several hypothetical proteins, at least six of which were predicted as surface proteins and four of which were predicted as secreted proteins (our unpublished data). The similar yet contrasting distribution of the expressed proteins in the avirulent versus virulent strains suggests that the differential presence/absence or abundance of expressed proteins may be related to their ability to infect a suitable swine host, provided that experimental support to the previous assumptions is based on genomic analyses (2).

The P97 adhesin is considered an important virulence factor for *M. hyopneumoniae* and variation in the number of tandem amino acid repeats in the R1 region of this protein has been correlated to the bacterial capacity of adhesion (37). The 121 kDa P97 precursor is generated by peptide-signal cleavage, possibly in concert with P97 translocation to the membrane (20). It has also been demonstrated that mature P97 is further proteolytically processed to generate smaller derived polypeptides containing the R1 repeat (8). Further

extending these previous observations regarding P97 post-translational processing, we were able to demonstrate differences in P97 proteolytic cleavage between strains, generating different numbers of R1-containing polypeptides in the pathogenic 7448 and 7422 strains that were not observed in the non-pathogenic J strain. Besides, the P97-derived low MW polypeptides detected by 2DE immunoblotting in the pathogenic strains presented p*I*s lower than those predicted for any theoretical P97-derived polypeptides including the R1 repeat, which is evidence that these 30-34 kDa P97 proteolytic products also undergo some sort of post-translational p*I*-decreasing amino acid modification (e.g., acetylation, carbamylation, or phosphorylation). Initially, it was assumed that, as a classical transmembrane protein, P97 would be translocated to the cell surface through the general secretory pathway. There it would remain attached to the cell membrane by its transmembrane domain and would expose its cilium-binding motif to the extracellular milieu. However, the previous demonstration that P97 proteolytic cleavage may separate its transmembrane and cilium-binding domains (20, 38) and that different protein fragments including the R1-containing cilium binding motif are also generated (8) already pointed to an alternative mechanism for regulating the exposure of the P97 cilium-binding domain in the *M. hyopneumoniae* cell surface. Our results add further complexity to this scenario as they show that the repertoire of proteolytic P97 peptides including the adhesion-related R1 repeat vary between strains with different virulence properties. Therefore, further experiments will be necessary to investigate which P97 fragments are actually presented on the cell surface, how do they reach and stay in that site, and what would be the actual contribution of each of them to the adhesion of *M. hyopneumoniae* to the swine respiratory epithelium.

Besides P97, there are other high-MW *M. hyopneumoniae* proteins which undergo proteolytic processing (16, 39, 40), suggesting that this is an important post-translational process in the physiology of this species. This has not been described for any other *Mycoplasma* species (or at least not to this extent), although the protease repertoires of these species do not differ significantly from that found in the *M. hyopneumoniae* genome (2, 3). Of the total of 13 proteases found in the *M. hyopneumoniae* genome (41), 8 had their expression confirmed by our shotgun proteomic analysis, but, again, this did not significantly differ from the repertoire of proteases expressed in other mycoplasmas (42-45). Therefore, assuming a common repertoire and expression pattern of proteases in different *Mycoplasma* species, the apparent larger number of proteins post-translationally processed by proteolysis in *M. hyopneumoniae* may be evidence of particular regulatory mechanisms and/or recent mutations leading to susceptibility to proteolytic processing in some key target proteins.

In conclusion, approximately one-third of the total proteome of *M. hyopneumoniae* was identified in a comprehensive inter-strain analysis, which showed that the repertoires of identified proteins of the two pathogenic strains are as different from each other as they are from the non-pathogenic one. This is indicative of significant inter-strain variability for this species not necessarily related to pathogenicity, although some potentially virulence-related differences in protein expression have been detected. Our results improve the current annotation of *M. hyopneumoniae* genome by a careful analysis of high-scoring proteins coded by genes previously regarded as hypothetical and provide further insights into *M. hyopneumoniae* biology. The current characterized fraction of the *M. hyopneumoniae* proteome and its future expansion through proteomic analyses of changing growth conditions and various stress challenges will serve as a useful research resource

complementary to the increasing amount of data from comparative genomic and transcriptomic analyses and subsidiary to functional studies of specific genes and proteins.

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Fig. 1. 2DE proteome profiling the three *M. hyopneumoniae* strains with IEF at pH 4–7. Proteins samples (2 mg) from the *M. hyopneumoniae* strains J (A), 7448 (B), and 7422 (C) were separated by IEF using 17 cm pH 4–7 IPG strips, followed by SDS-PAGE on 12% gels, stained with Coomassie Brilliant Blue G. The apparent molecular weights are shown on the left of the gel and the acid-to-alkaline gradient is from left to right. The rectangle delimited areas (numbered *d1*-*d5*) in the gels and panels in (D) show gel regions in which spots corresponding to differentially expressed proteins were identified. Spots corresponding to proteins identified by matching to the previously reported *M. hyopneumoniae* 7448 proteome maps (16) were named according to the predicted gene products. Spots corresponding to proteins so far unidentified in proteome maps were numbered.

Fig. 2. 2DE proteome profiling of the three *M. hyopneumoniae* strains with IEF at pH 3–10. Proteins samples (2 mg) from the three *M. hyopneumoniae* strains J (A), 7448 (B), and 7422 (C) were separated by IEF using 17 cm pH 3–10 IPG strips, followed by SDS-PAGE on 12% gels, stained with Coomassie brilliant blue G. The approximate molecular weights are shown on the left of the gel and the acid-to-alkaline gradient is from left to right. The rectangle delimited areas (numbered *d1*-*d6*) in the gels and panels in (D) show gel regions in which spots corresponding to differentially expressed proteins were identified. Spots corresponding to proteins identified by matching to the previously reported *M. hyopneumoniae* 7448 proteome maps (16) were named according to the predicted gene products or by the corresponding CDS number. Spots corresponding to proteins so far unidentified in proteome maps were numbered.

Fig. 3. Venn diagram of the protein sets. Venn diagram obtained from the comparison of LC-MS/MS identified protein repertoires of *M. hyopneumoniae* strains J, 7448, and 7422.

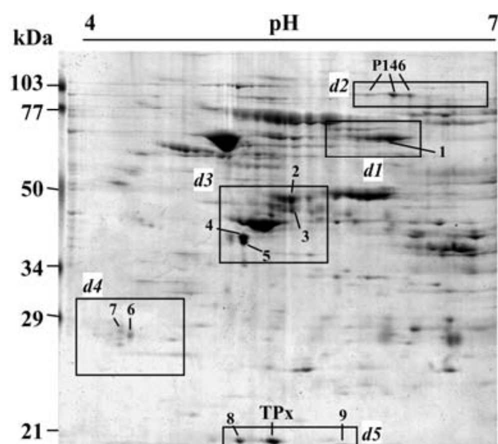
Fig. 4. Comparative analyses of *M. hyopneumoniae* strains J, 7448, and 7422 LC-MS/MS proteomes based on emPAI relative abundance. (A-C) Bivariant plots of emPAI values of proteins from: strain J against strain 7448 (A); strain J against strain 7422 (B); and strain 7448 against strain 7422 (C). Plotted protein emPAI values are in Table 4 (Supplementary Material). Proteins outside the V-shaped shaded area (marked in gray) were assumed to be over-expressed in the strain whose emPAI values are assigned to the proximal axis. For over-expression assumption, a two-fold or higher difference between the emPAI values for a given protein in the two compared strains was required.

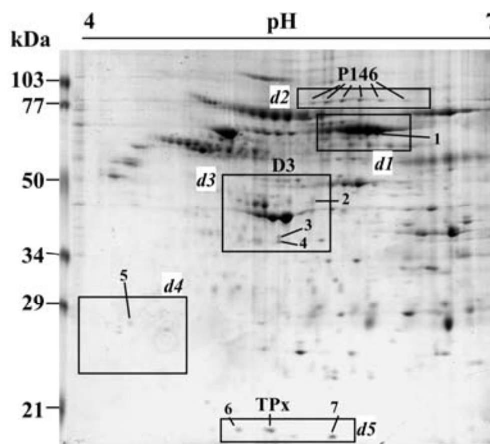
Fig. 5. COG functional classification of identified proteins from *M. hyopneumoniae* strains J, 7448, and 7422. Histogram representations of identified proteins belonging to the COG functional classes as follows: Major class Isp, information storage and processes: (J) Translation, ribosomal structure and biogenesis, (K) Transcription, (L) DNA replication, recombination, and repair; Major class Cp, cellular processes: (D) Cell division and chromosome partitioning, (O) Post-translational modification, protein turnover, and chaperones, (M) Cell envelope biogenesis, outer membrane, (N) Cell motility and secretion, (P) Inorganic ion transport and metabolism; Major class Me, metabolism: (C) Energy production and conversion, (G) Carbohydrate transport and metabolism, (E) Amino acid transport and metabolism, (F) Nucleotide transport and metabolism, (H) Coenzyme metabolism, (I) Lipid metabolism, (Q) Secondary metabolites biosynthesis, transport, and

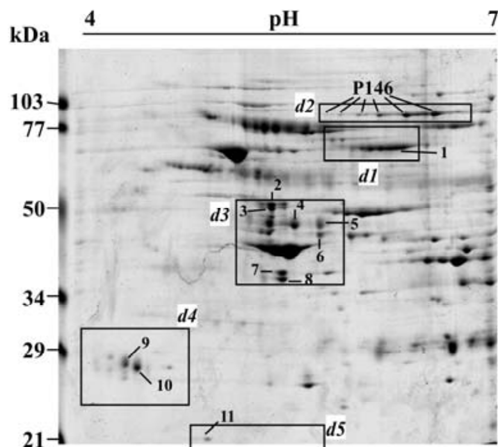
catabolism; Major class Pc, poorly characterized: (R) General function prediction only, (S) Function unknown.

Fig. 6. 2DE immunoblotting analyses of P97. Proteins samples from the *M. hyopneumoniae* strains J (C), 7448 (B), and 7422 (A) were resolved by 2DE as described in Fig. 1 and 2, electroblotted onto PVDF membranes, and probed with the anti-P97 monoclonal antibody F1B6 (1:400 dilution). Anti-mouse IgG alkaline phosphatase-labeled secondary antibody (1:2000 dilution) was used to develop antigen-antibody reactions. The approximate molecular weights are shown on the left of the gel and the acid-to-alkaline gradient is from left to right. Mature P97 (*P97*), its two main proteolytic products (*a* and *b*), and the P97 pathogenic-strain-specific, low-MW proteolytic products (*1-4*) are indicated.

Fig. 1







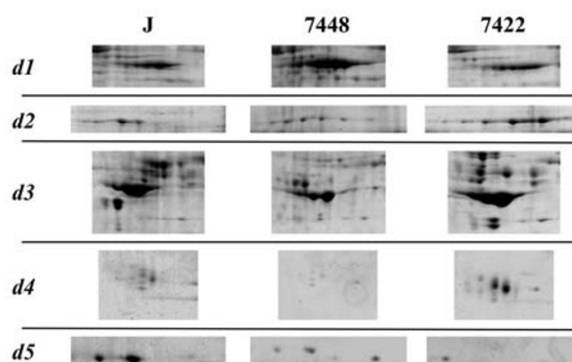
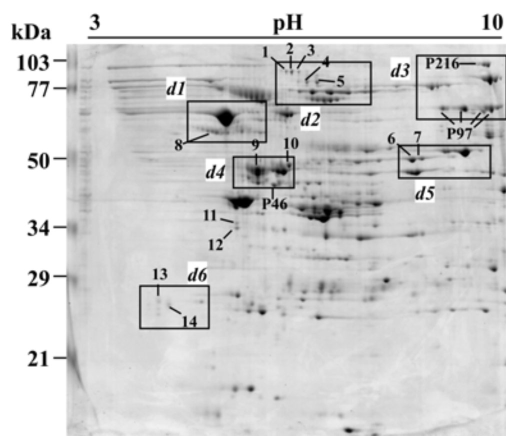


Fig. 2

A



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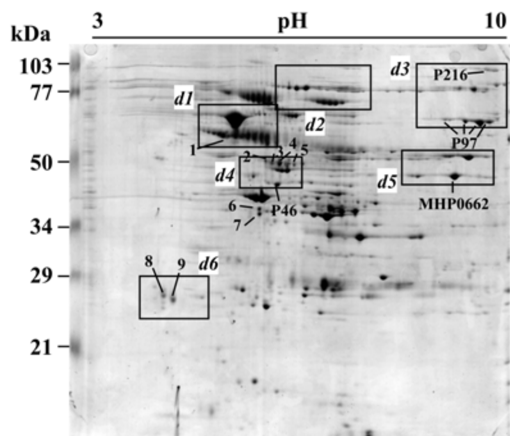
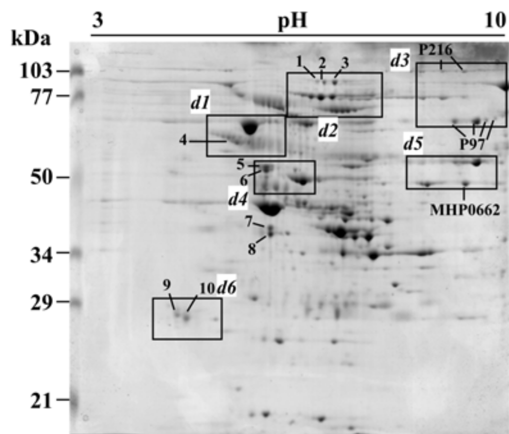


C



D

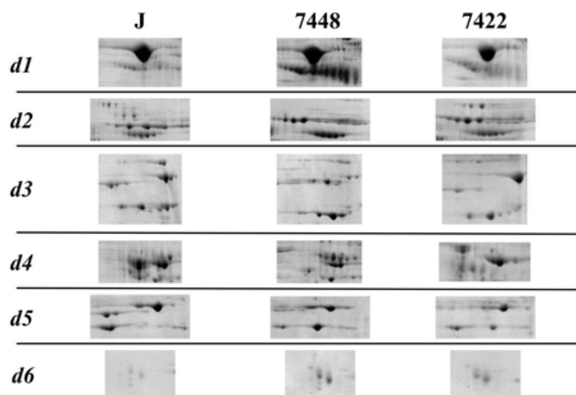


Fig. 3

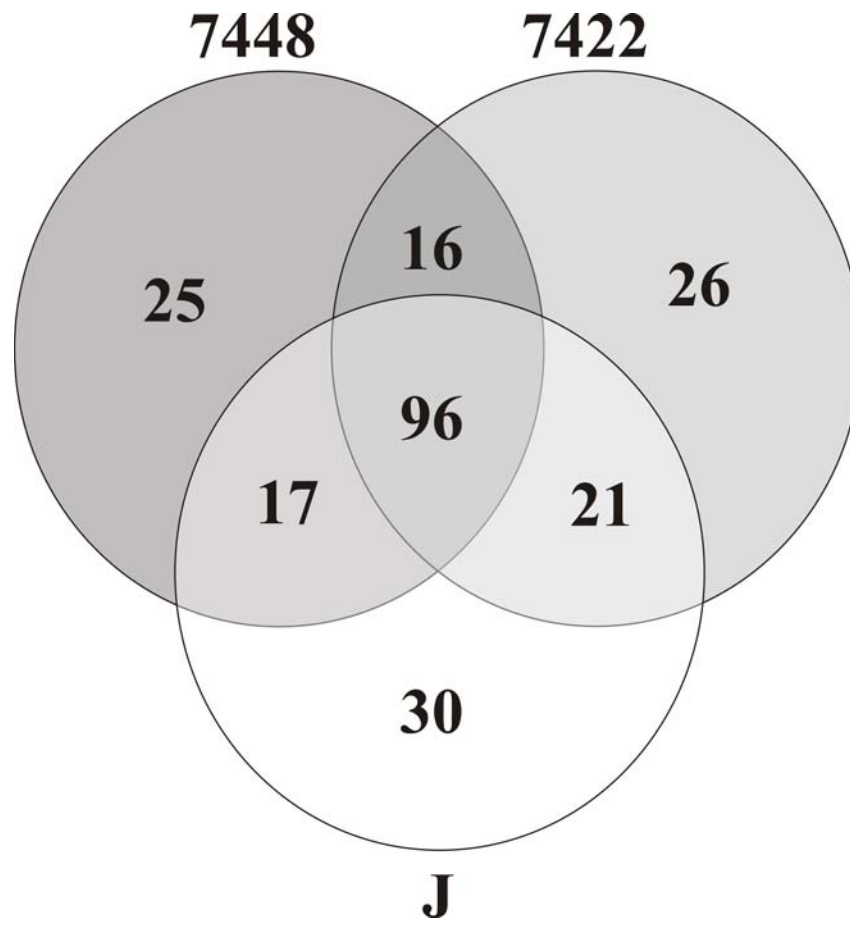


Fig. 4

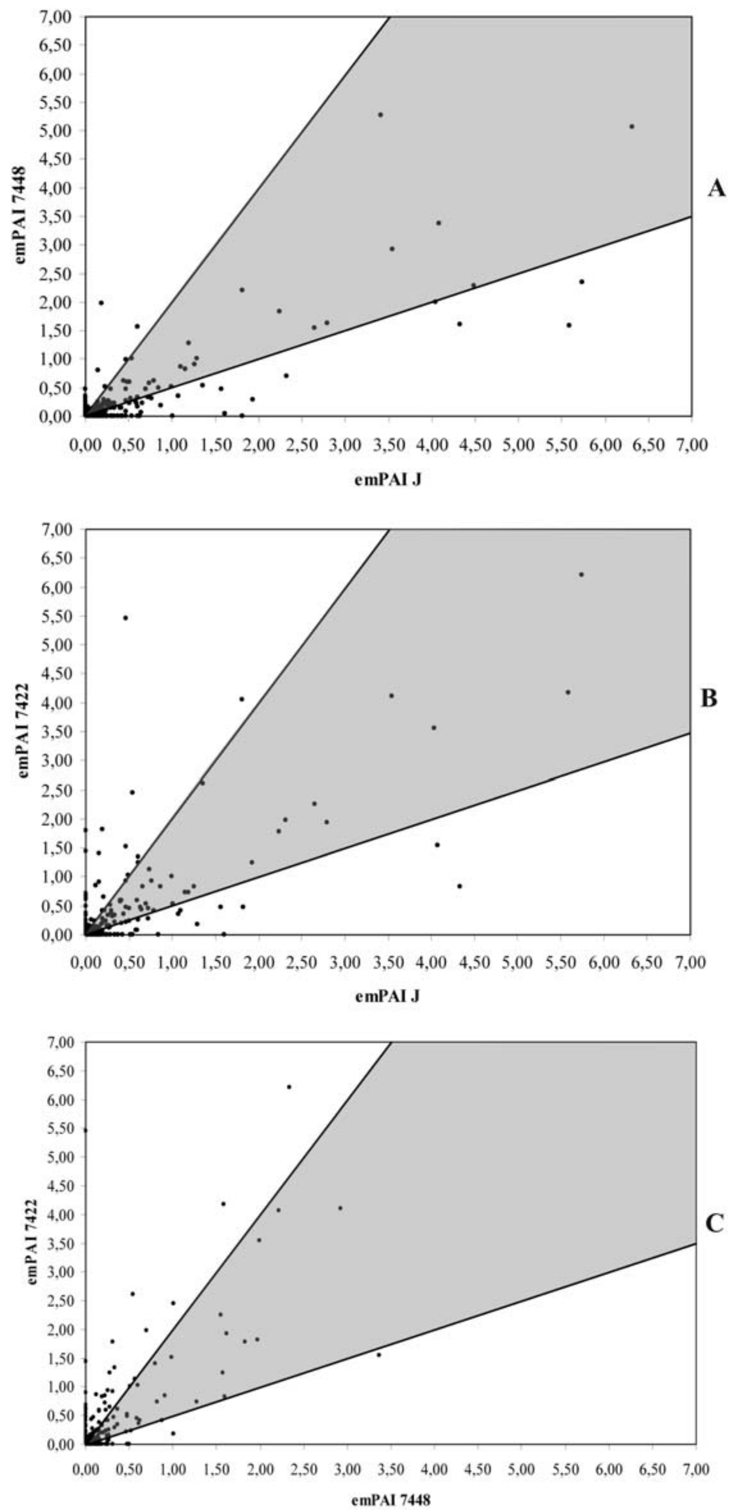


Fig. 5

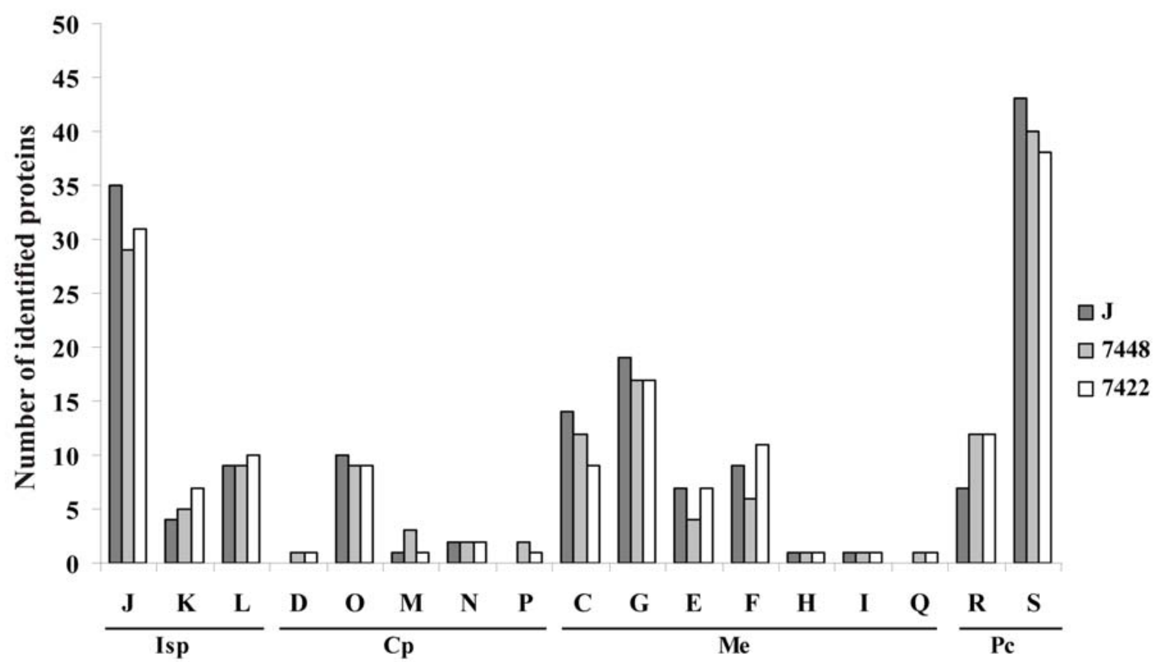
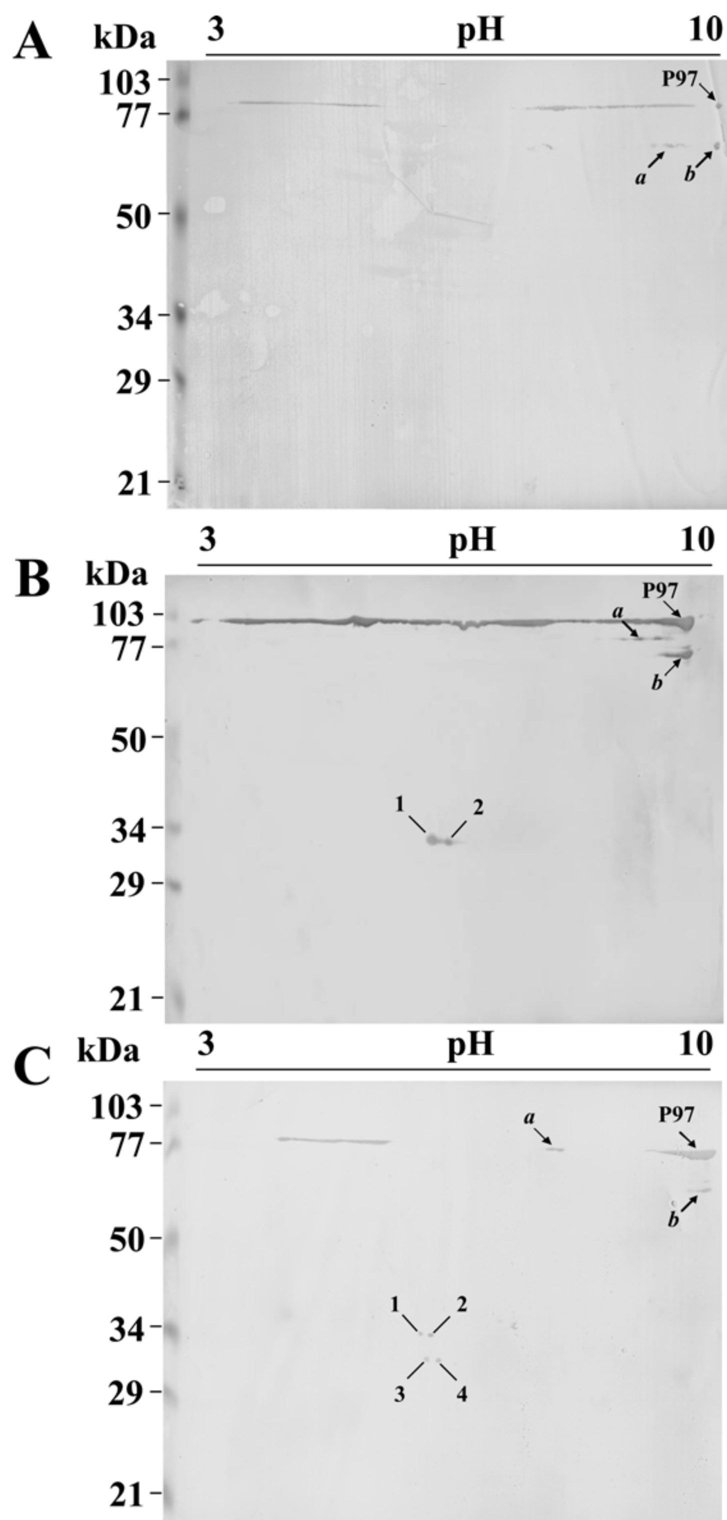


Fig. 6



5. DISCUSSÃO GERAL

A bactéria *M. hyopneumoniae* faz parte de um grupo de organismos que vem recebendo uma atenção cada vez maior desde meados da década de 1990. Este foco direto sobre os micoplasmas se deve ao seqüenciamento do genoma de várias espécies deste grupo e à discussão do conceito de genoma mínimo (HUTCHISON *et al.*, 1999). A partir do primeiro genoma totalmente seqüenciado de micoplasmas (*M. genitalium*) (FRASER *et al.*, 1995) mais 11 genomas de diferentes espécies e cepas do gênero *Mycoplasma* foram seqüenciados (HIMMELREICH *et al.*, 1996; CHAMBAUD *et al.*, 2001; SASAKI *et al.*, 2002; PAPAZISI *et al.*, 2003; JAFFE *et al.*, 2004; MINION *et al.*, 2004; WESTBERG *et al.*, 2004; VASCONCELOS *et al.*, 2005; SIRAND-PUGNET *et al.*, 2007; DYBVIG *et al.*, 2008). Estes projetos de seqüenciamento de genomas geraram um número de informações extremamente importante e complexo sobre a biologia e bioquímica destas bactérias, sua interação com seus hospedeiros e sua função em vários nichos ecológicos, principalmente como parasitas importantes para uma série de organismos, inclusive de seres humanos (MAZEL *et al.*, 1997; JEFFERY *et al.*, 2006). Entretanto, o seqüenciamento de genomas, apesar de extremamente importante, ainda deixa importantes lacunas no entendimento da biologia dos organismos e sua interação com habitat e nichos, principalmente para organismos infecciosos ou parasitas (PAINE & FLOWER, 2002).

Nos últimos anos, análises de genômica funcional foram realizadas para diversos organismos que tiveram os seus genomas seqüenciados (MEDINI *et al.*, 2008). No gênero *Mycoplasma*, quase todos os organismos que tiveram seu genoma desvendado possuem algum tipo de análise de genômica funcional, como, por exemplo, análises de proteínas específicas (DJORDJEVIC *et al.*, 2004; WILTON *et al.*, 2009), análises transcriptômicas (MADSEN *et al.*, 2006, 2008; SCHAFER *et al.*, 2007) e análises proteômicas (REGULA

et al., 2000; JAFFE *et al.*, 2004). Projetos de re-anotação dos genomas também foram impulsionados por análises de genômica funcional, principalmente no caso de análises comparativas baseadas em biologia computacional, como é o caso da re-anotação do genoma de *M. pneumoniae* (DANDEKAR *et al.*, 2000). Para *M. hyopneumoniae* uma abordagem por genômica funcional tem sido realizada através de estudos transcriptômicos (MADSEN *et al.*, 2006 e 2008; SCHAFER *et al.*, 2007) e/ou de estudos de caracterização de proteínas específicas (DJORDJEVIC *et al.*, 2004; WILTON *et al.*, 2009).

As análises baseadas em 2DE permitiram uma resolução de cerca de 350 spots protéicos na faixa de pH de 3-10 e algo em torno de 280 spots protéicos na faixa de pH 4-7, o que corresponde a mais de 50 e 30%, respectivamente, do proteoma deduzido de *M. hyopneumoniae* cepa 7448. Estes níveis de cobertura são condizentes com os observados em outros estudos proteômicos de espécies do genêro *Mycoplasma* (REGULA *et al.*, 2000; WASINGER *et al.*, 2000; UEBERLE *et al.*, 2002; JAFFE *et al.*, 2004).

Nós identificamos algumas proteínas que possivelmente sofreram algum tipo de modificação pós-tradudicional, que altera o MW e/ou o pI, incluindo algumas proteínas de superfície (P46, P76, P97, P216 e LPPT) e um produto gênico de um gene hipotético (MHP0662). Estas variantes ou isoformas podem ser atribuídas a importantes modificações pós-traducionais, desde clivagem proteolítica (que altera o MW) até modificações químicas (que altera o pI). Em *M. hyopneumoniae*, as modificações pós-traducionais estão se mostrando como um possível passo na patogenicidade, principalmente na adesão, pois, além do nosso trabalho outros trabalhos já demonstraram clivagens proteolíticas para outras três adesinas, a P97 (DJORDJEVIC *et al.*, 2004), a P76 (BRUNETT *et al.*, 2006) e a P216 (WILTON *et al.*, 2009).

O repertório de proteínas antigênicas caracterizadas e disponíveis para utilização tanto para o imunodiagnóstico, quanto para a vacinação é ainda bastante restrito, sendo as mais conhecidas a P36, a P46, a P97 e a NrdF (THACKER, 2004). Nossas análises imunológicas utilizando anti-soros de suínos imunizados com extratos protéicos de *M. hyopneumoniae* identificaram pelo menos cinco proteínas antigênicas. Quatro destas proteínas foram identificadas pela primeira vez como sendo proteínas antigênicas em *M. hyopneumoniae*, uma proteína de choque térmico 70 (HSP70), um fator de alongamento Tu (EF-TU), a subunidade E1-beta da piruvato desidrogenase (PDHB) e a proteína de membrana P76. Também foi possível comprovar a antigenicidade da proteína P46, já descrita por OKADA *et al.* (2005). A HSP70 já foi anteriormente descrita como um antígeno de *Francisella tularensis* (ELKINS *et al.*, 2003) e como um importante imunógeno de *Mycobacterium tuberculosis* (TOBIAN *et al.*, 2005). Já o EF-TU já foi descrito como antígeno em algumas espécies de Mollicutes (OLSON *et al.*, 1991; ALONSO *et al.*, 2002; LOPEZ *et al.*, 2005) e a PDHB é uma proteína antigênica em *Bacillus stearothermophilus* (DE BERARDINIS & HAIGWOOD, 2004). Estas proteínas antigênicas são candidatas naturais para a clonagem e a produção das moléculas recombinantes (DNA ou proteínas), a serem avaliadas como componentes de uma possível vacina recombinante para a PES e/ou como reagentes para imunodiagnóstico. Isto pode ser ilustrado pela caracterização imunológica preliminar que foi feita em nosso grupo (SCHUCK, 2007). Nestes estudos as proteínas HPSP70 e EF-TU expressas de forma heteróloga demonstraram uma resposta humoral específica em suínos com PES. Do mesmo modo, o estudo funcional destes genes e de seus produtos pode prover melhor entendimento dos mecanismos moleculares envolvidos na infecção e na sobrevivência do *M. hyopneumoniae* no hospedeiro.

Utilizando uma estratégia comparativa baseada em 2DE, análises imunológicas e LC-MS/MS pôde-se identificar aproximadamente 35% do proteoma predito de *M. hyopneumoniae*, o que significou um total de 231 proteínas identificadas nas três cepas estudadas. Esse grau de cobertura é condizente com alguns trabalhos utilizando estratégias de proteômica *shotgun* para bactéria, como os realizados para de *Halobacterium salinarum* (KLEIN *et al.*, 2005; TEBBE *et al.*, 2005), *Natronomonas pharaonis* (KONSTANTINIDIS *et al.*, 2007) e *Haloferax volcanii* (KIRKLAND *et al.*, 2008).

Estratégias baseadas em 2DE e MS (normalmente MALDI-TOF) são utilizadas amplamente na literatura no estudo de proteomas dos mais diversos organismos (PAPASOTIRIOU *et al.*, 2008) inclusive de espécies de *Mycoplasma* (FERRER-NAVARRO *et al.*, 2006). Entretanto já foi demonstrado que é possível identificar maior número de proteínas utilizando as técnicas de MDLC do que as técnicas de eletroforese bidimensional (GARBIS *et al.*, 2005). De acordo com o apresentado, a análise do proteoma de *M. hyopneumoniae* por técnicas baseadas em LC-MS/MS permitiu uma maior reprodutibilidade dos dados e maior capacidade na resolução de misturas protéicas complexas, além de detectar proteínas em menor abundância na amostra total e extremos de hidrofobicidade, pI e massa molecular, o que são condições limitantes para a técnica de eletroforese (SDS-PAGE 2D) como já demonstrados em vários estudos baseados nessa estratégia (GRIFFIN & AEBERSOLD, 2001; STRITTMATTER *et al.*, 2003; MAYNARD *et al.*, 2004; GARBIS *et al.*, 2005; TOLL *et al.*, 2005; WEI *et al.*, 2005).

Utilizando o emPAI pode-se identificar pelo menos 64 proteínas com diferenças significativas nos níveis de expressão entre as três cepas de *M. hyopneumoniae*. Um dos maiores desafios da análise proteômica atualmente é correlacionar os dados de identificação de proteínas e peptídeos com o grau de expressão destes em uma dada

condição fisiológica testada, o que é chamado de proteômica quantitativa (BANTSCHEFF *et al.*, 2007). A maioria dos métodos de proteômica quantitativa utiliza marcações com isótopos estáveis para criar uma *tag* de massa que pode ser reconhecido pelo espectrômetro de massas e ao mesmo tempo fornece a base para a quantificação (GYGI *et al.*, 1999; ODA *et al.*, 1999; PASA-TOLIC *et al.*, 1999). Mais recentemente estratégias alternativas vêm emergindo, chamadas de quantificação *label-free*, que correlacionam a intensidade do sinal dos peptídeos proteolíticos ou o número de eventos de seqüenciamento de peptídeos como quantificação relativa ou absoluta de uma dada proteína (BONDARENKO *et al.*, 2002; ISHIHAMA *et al.*, 2005). Uma das estratégias *label-free* é o emPAI, que é um cálculo para quantificação relativa de proteínas de uma mistura baseada na cobertura de peptídeos identificados no banco de dados (ISHIHAMA *et al.*, 2005). Embora o emPAI não seja utilizado (normalmente) para designar a abundância absoluta para diferente proteínas, este parâmetro é útil para mensurar a abundância relativa de proteínas em diferentes misturas complexas quando comparadas. (ISHIHAMA *et al.*, 2005; BARRIOS-LLERENA *et al.*, 2006; GRAHAM *et al.*, 2007).

A análise comparativa utilizando os valores de emPAI resultou na evidência de expressão diferencial de alguns grupos de proteínas. Por exemplo, proteínas de choque térmico e proteínas envolvidas na rota metabólica do piruvato foram identificadas como super expressas nas cepas patogênicas. Estes resultados são consistentes com a idéia de uma resposta funcional contra choque térmico e estresse respiratório, já detectados em *M. hyopneumoniae* e outras espécies relacionadas, é um passo importante para a vida parasitaria destes organismos (WEINER *et al.*, 2003; MADSEN *et al.*, 2006; MUSATOVOVA *et al.*, 2006). Também consistente com esta hipótese outro grupo de proteínas super expressas nas cepas 7448 e 7422 estão envolvidas no equilíbrio redox da

célula (como por exemplo, tioredoxina, NADH oxidase, metionina sulfoxide redutase e tioredoxina redutase). O que é uma estratégia contra as respostas de geração de espécies reativas de oxigênio que o sistema imune do suíno em uma infecção (SARRADELL *et al.*, 2003).

O grupo de antígenos de superfície foi bem representado nas análises por LC-MS/MS com 13 membros identificados incluindo ortólogos da P97 e ortólogos da P102. Entretanto, de todas as proteínas de superfície e/ou proteínas envolvidas em adesão apenas o produto gênico de um dos ortólogos da P97 mostrou uma diferença significativa no nível de expressão, sendo mais expressas em ambas as cepas patogênicas do que na cepa não-patogênica. Análises imunológicas demonstramos que existe padrão de clivagem proteolítica diferencial entre as três cepas de *M. hyopneumoniae* para esta proteína. Além da P97 outras proteínas de possivelmente envolvidas em adesão foram descritas como alvos para processamento proteolítico (BURNETT *et al.*, 2006; WILTON *et al.*, 2009) o que sugere que este seja um passo importante na fisiologia desta espécie.

6. CONCLUSÕES

- O protocolo de 2DE desenvolvido permite uma boa resolução e reprodutibilidade para extratos protéicos de diferentes cepas de *Mycoplasma hyopneumoniae*. Os mapas proteômicos de *M. hyopneumoniae* (pH 4-7 e 3-10) produzidos servem como base para análises comparativas utilizando 2DE. Os perfis protéicos de 2DE são bastante similares entres as três cepas analisadas, entretanto, diferenças tanto qualitativas quanto quantitativas em *spots* protéicos foram identificadas. Estes resultados sugerem que mesmo sob condições de cultura *in vitro* há um perfil de expressão diferencial entre as cepas de *M. hyopneumoniae*.
- O perfil de proteínas antigênicas de *M. hyopneumoniae* foi ampliado por uma análise baseada em 2DE e western blot, onde quatro novos antígenos foram descritos (HSP70, EF-TU, PDHB e P76). Entretanto as análises demonstraram que existem outras proteínas com caráter antigênico em *M. hyopneumoniae* que não puderam ser identificadas.
- *M. hyopneumoniae* possui um perfil de expressão de proteases bastante grande para um organismo de genoma reduzido ou em processo de redução e a regulação da expressão gênica de forma pós-traducional é utilizada para produtos gênicos de várias classes funcionais. Pois foram identificados diferentes *spots* protéicos com o MW e/ou o pI alterados dos preditos. Outro exemplo é o da proteína P97, pois possui um padrão de clivagem proteolítica diferencial entre as três cepas analisadas, o que sugere que este seja um passo importante na biologia desta espécie e/ou um fator de virulência.
- O protocolo de LC-MS/MS desenvolvido permite uma maior cobertura de identificações de proteínas de diferentes cepas de *M. hyopneumoniae*. Pelo menos 231 CDSs das cepas J, 7448 e 7422 foram experimentalmente validadas pela identificação de seus produtos protéicos, incluindo pelo menos 30 provenientes de genes hipotéticos. O

índice emPAI utilizado é confiável para identificação de proteínas diferencialmente expressas baseadas em dados de LC-MS/MS.

- Pelo menos 64 proteínas são diferencialmente expressas entre pelo menos duas das três cepas analisadas. Sugerindo uma significativa variabilidade entre os perfis protéicos das três cepas, não necessariamente relacionado à patogenicidade, entretanto, alguns fatores potencialmente envolvidos em virulência e/ou patogenicidade foram identificados como super expressos nas cepas patogênicas (proteínas de choque térmico e proteínas envolvidas na rota metabólica do piruvato; proteínas antioxidantes e a proteína P97).

7. PERSPECTIVAS

- Realizar análises proteômicas comparativas de diferentes cepas de *M. hyopneumoniae* em diferentes condições. Por exemplo, em condições de estresse térmico, estresse oxidativo e, principalmente, sob condições de infecção. Estes dados podem ser comparados aos dados transcriptômicos já realizados para estas condições.
- Realizar análises proteômicas direcionadas à identificação dos componentes de membrana de *M. hyopneumoniae*, na tentativa de identificar proteínas possivelmente envolvidas em adesão a células do hospedeiro e/ou de caráter antigênico e/ou outros processos importantes para a relação entre a bactéria e seu hospedeiro (como por exemplo, imunomodulação, interação com sistema imune do hospedeiro, tráfego intercelular, e conjugação).
- Realizar estudos funcionais para a caracterização das proteínas sugeridas como diferencialmente expressas na análise proteômica comparativa, em diferentes condições fisiológicas e/ou entre diferentes cepas. Dentre estes, estudos para a caracterização do perfil de proteases de *M. hyopneumoniae* e seus alvos diretos, visto que a clivagem proteolítica vem sendo demonstrada como um processo celular bastante comum e importante na fisiologia deste organismo.

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ANEXOS

ANEXO I

O ANEXO I é o material suplementar do ARTIGO 2. Neste anexo estão apresentados os dados de identificação das proteínas nas análises de proteômica *shotgun*. Na *table* 1 a 3 são apresentados o número de acesso no *GenBank*, a descrição da proteína identificada, o *score* do software MASCOT, a cobertura de seqüência e a classificação no COG para cada proteína identificada das cepas J, 7448 e 7422, respectivamente. Na *table* 4 são apresentados o número de acesso no *GenBank*, a descrição da proteína identificada e valor de emPAI para cada proteína identificada das três cepas, além de identificar as proteínas diferencialmente expressas (marcadas em cinza).

Table 1

Identification of J strain proteins by LC-MS/MS. Protein identified by a liquid chromatography (LC) separation (reversed-phase HPLC) coupled with a tandem mass spectrometry (MS/MS) by searching *M. hyopneumoniae* strain J protein databases using MASCOT search engine.

Accession number[1]	Protein description[2]	MASCOT score[3]	Sequence coverage (%)	COG[4]
gi|71893362|ref|YP_278808.1|	glucose-inhibited division protein A	16	3.3	D
gi|71893368|ref|YP_278814.1|	hypothetical protein MHJ_0009	252	10.8	S
gi|71893370|ref|YP_278816.1|	heat shock protein	172	20.0	O
gi|71893373|ref|YP_278819.1|	fructose-bisphosphate aldolase	288	49.1	G
gi|71893387|ref|YP_278833.1|	isoleucyl-tRNA synthetase	78	12.2	J
gi|71893390|ref|YP_278836.1|	glyceraldehyde 3-phosphate dehydrogenase	476	37.8	G
gi|71893407|ref|YP_278853.1|	ATP synthase gamma chain	23	6.0	C
gi|71893408|ref|YP_278854.1|	ATP synthase subunit B	230	30.1	C
gi|71893411|ref|YP_278857.1|	elongation factor Ts	79	22.1	J
gi|71893422|ref|YP_278868.1|	molecular chaperone DnaK	1508	38.5	O
gi|71893426|ref|YP_278872.1|	bacterial nucleoid DNA-binding protein	272	43.8	L
gi|71893430|ref|YP_278876.1|	elongation factor EF-2	161	19.1	K
gi|71893431|ref|YP_278877.1|	30S ribosomal protein S7	46	24.4	J
gi|71893437|ref|YP_278883.1|	NADH oxidase	940	35.2	R
gi|71893438|ref|YP_278884.1|	thymidine phosphorylase	85	24.4	F
gi|71893439|ref|YP_278885.1|	purine-nucleoside phosphorylase	239	22.0	F
gi|71893450|ref|YP_278896.1|	hypothetical protein MHJ_0091	42	17.0	S
gi|71893452|ref|YP_278898.1|	thiol peroxidase	242	38.4	O
gi|71893454|ref|YP_278900.1|	thioredoxin reductase	367	27.5	O
gi|71893457|ref|YP_278903.1|	ATP-dependent protease binding protein	144	16.6	O
gi|71893458|ref|YP_278904.1|	triosephosphate isomerase	13	9.0	G
gi|71893462|ref|YP_278908.1|	protein P97	258	18.8	S
gi|71893463|ref|YP_278909.1|	DNA gyrase subunit B	166	16.3	L
gi|71893464|ref|YP_278910.1|	6-phosphofructokinase	101	20.2	G
gi|71893467|ref|YP_278913.1|	adenine phosphoribosyltransferase	861	52.1	F
gi|71893468|ref|YP_278914.1|	pyruvate dehydrogenase E1-alpha subunit	1149	41.2	C
gi|71893469|ref|YP_278915.1|	pyruvate dehydrogenase	1731	55.4	C
gi|71893472|ref|YP_278918.1|	hypothetical protein MHJ_0115	29	35.4	S
gi|71893476|ref|YP_278922.1|	50S ribosomal protein L20	30	25.6	J
gi|71893479|ref|YP_278925.1|	pyruvate kinase	102	26.7	G
gi|71893482|ref|YP_278928.1|	aminopeptidase	48	4.0	G
gi|71893484|ref|YP_278930.1|	50S ribosomal protein L21	138	17.0	J
gi|71893485|ref|YP_278931.1|	50S ribosomal protein L27	29	36.9	J
gi|71893486|ref|YP_278932.1|	lipase-esterase	86	13.2	R
gi|71893489|ref|YP_278935.1|	L-lactate dehydrogenase	290	45.1	C
gi|71893490|ref|YP_278936.1|	hypothetical protein MHJ_0134	56	3.0	S
gi|71893491|ref|YP_278937.1|	peptide chain release factor 1	68	7.0	J
gi|71893500|ref|YP_278946.1|	hypothetical protein MHJ_0144	73	23.8	S
gi|71893501|ref|YP_278947.1|	trigger factor	312	27.7	O
gi|71893513|ref|YP_278959.1|	phosphopentomutase	48	3.0	G
gi|71893521|ref|YP_278967.1|	30S ribosomal protein S11	70	34.3	J

gi|71893522|ref|YP_278968.1|	30S ribosomal protein S13	99	15.3	J
gi|71893528|ref|YP_278974.1|	50S ribosomal protein L15	42	17.2	J
gi|71893531|ref|YP_278977.1|	50S ribosomal protein L6	284	33.5	J
gi|71893532|ref|YP_278978.1|	30S ribosomal protein S8	83	16.0	J
gi|71893534|ref|YP_278980.1|	50S ribosomal protein L5	130	23.3	J
gi|71893535|ref|YP_278981.1|	50S ribosomal protein L24	87	15.0	J
gi|71893541|ref|YP_278987.1|	50S ribosomal protein L22	58	22.4	J
gi|71893542|ref|YP_278988.1|	30S ribosomal protein S19	52	28.9	J
gi|71893543|ref|YP_278989.1|	50S ribosomal protein L2	248	33.0	J
gi|71893545|ref|YP_278991.1|	50S ribosomal protein L4	74	18.0	J
gi|71893546|ref|YP_278992.1|	50S ribosomal protein L3	58	18.7	J
gi|71893550|ref|YP_278996.1|	protein P97	1638	29.1	S
gi|71893551|ref|YP_278997.1|	protein P102	693	15.5	S
gi|71893558|ref|YP_279004.1|	cell division protein	149	24.6	O
gi|71893559|ref|YP_279005.1|	lysyl-tRNA synthetase	109	8.0	J
gi|71893560|ref|YP_279006.1|	hydrolase of the HAD family	28	12.0	R
gi|71893564|ref|YP_279010.1|	oligopeptide ABC transporter system permease	127	16.9	E
gi|71893566|ref|YP_279012.1|	oligopeptide ABC transporter ATP-binding protein	55	16.7	E
gi|71893567|ref|YP_279013.1|	oligopeptide ABC transporter ATP-binding protein	39	18.6	E
gi|71893568|ref|YP_279014.1|	hypothetical protein MHJ_0212	424	19.0	S
gi|71893569|ref|YP_279015.1|	lipoprotein	106	14.6	S
gi|71893571|ref|YP_279017.1|	ribonucleotide-diphosphate reductase alpha subunit	160	21.5	F
gi|71893572|ref|YP_279018.1|	hypothetical protein MHJ_0216	106	36.7	S
gi|71893573|ref|YP_279019.1|	ribonucleotide-diphosphate reductase beta subunit	64	34.0	F
gi|71893574|ref|YP_279020.1|	serine hydroxymethyltransferase	14	8.0	E
gi|71893575|ref|YP_279021.1|	methylmalonate-semialdehyde dehydrogenase	86	22.7	C
gi|71893579|ref|YP_279025.1|	myo-inositol catabolism protein	25	16.0	G
gi|71893589|ref|YP_279035.1|	protein-export membrane protein	29	12.0	N
gi|71893592|ref|YP_279038.1|	hypothetical protein MHJ_0236	45	25.0	S
gi|71893595|ref|YP_279041.1|	TRSE-like protein	23	2.0	N
gi|71893598|ref|YP_279044.1|	phosphopyruvate hydratase	313	37.9	G
gi|71893599|ref|YP_279045.1|	seryl-tRNA synthetase	39	21.0	J
gi|71893600|ref|YP_279046.1|	hypothetical protein MHJ_0244	62	33.0	S
gi|71893601|ref|YP_279047.1|	triacylglycerol lipase	26	23.0	R
gi|71893602|ref|YP_279048.1|	lipoate-protein ligase A	100	17.7	H
gi|71893604|ref|YP_279050.1|	DNA polymerase III subunits gamma and tau	14	5.0	L
gi|71893605|ref|YP_279051.1|	hypothetical protein MHJ_0249	56	27.0	S
gi|71893610|ref|YP_279056.1|	hypoxanthine-guanine phosphoribosyltransferase	132	21.9	F
gi|71893614|ref|YP_279060.1|	DNA ligase	13	5.0	L
gi|71893620|ref|YP_279066.1|	P97 - like	36	16.0	S
gi|71893621|ref|YP_279067.1|	phenylalanyl-tRNA synthetase alpha chain	26	15.0	J
gi|71893622|ref|YP_279068.1|	phenylalanyl-tRNA synthetase beta subunit	152	23.1	J
gi|71893628|ref|YP_279074.1|	CTP synthetase	49	17.2	F
gi|71893639|ref|YP_279085.1|	hypothetical protein MHJ_0283	65	23.1	S
gi|71893643|ref|YP_279089.1|	30S ribosomal protein S6	148	19.1	J
gi|71893644|ref|YP_279090.1|	hypothetical protein MHJ_0288	95	20.5	S
gi|71893650|ref|YP_279096.1|	permease	15	2.0	G
gi|71893700|ref|YP_279146.1|	hypothetical protein MHJ_0347	26	21.0	S

gi|71893701|ref|YP_279147.1|	P60-like lipoprotein	42	8.1	S
gi|71893706|ref|YP_279152.1|	amino acid permease	20	4.0	E
gi|71893715|ref|YP_279161.1|	lipoprotein	168	11.4	S
gi|71893721|ref|YP_279167.1|	Lppt protein	628	24.0	S
gi|71893722|ref|YP_279168.1|	hypothetical protein MHJ_0369	452	22.6	S
gi|71893724|ref|YP_279170.1|	PTS system enzyme IIB component	201	14.0	G
gi|71893726|ref|YP_279172.1|	hypothetical protein MHJ_0373	1042	43.1	S
gi|71893727|ref|YP_279173.1|	lipoprotein	494	24.3	S
gi|71893733|ref|YP_279179.1|	thioredoxin	174	30.9	O
gi|71893755|ref|YP_279201.1|	hypothetical protein MHJ_0404	18	6.0	S
gi|71893760|ref|YP_279206.1|	methionine--tRNA ligase	40	14.0	J
gi|71893765|ref|YP_279211.1|	asparaginyl-tRNA synthetase	99	10.7	J
gi|71893767|ref|YP_279213.1|	ATP-dependent helicase PcrA	177	19.5	L
gi|71893768|ref|YP_279214.1|	hypothetical protein MHJ_0417	67	15.0	S
gi|71893769|ref|YP_279215.1|	Holliday junction DNA helicase motor protein	19	4.0	L
gi|71893777|ref|YP_279223.1|	transketolase	288	28.0	G
gi|71893779|ref|YP_279225.1|	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	19	7.3	J
gi|71893787|ref|YP_279233.1|	3-hexulose-6-phosphate synthase	172	22.1	G
gi|71893793|ref|YP_279239.1|	hypothetical protein MHJ_0442	16	3.0	S
gi|71893796|ref|YP_279242.1|	hypothetical protein MHJ_0445	175	15.6	S
gi|71893803|ref|YP_279249.1|	acyl carrier protein phosphodiesterase	73	14.9	I
gi|71893807|ref|YP_279253.1|	50S ribosomal protein L1	197	45.9	J
gi|71893808|ref|YP_279254.1|	50S ribosomal protein L11	128	32.9	J
gi|71893812|ref|YP_279258.1|	leucyl aminopeptidase	128	32.7	E
gi|71893814|ref|YP_279260.1|	hypothetical protein MHJ_0463	127	15.7	S
gi|71893816|ref|YP_279262.1|	hypothetical protein MHJ_0465	32	15.4	S
gi|71893820|ref|YP_279266.1|	phosphoenolpyruvate-protein phosphotransferase	248	36.7	G
gi|71893826|ref|YP_279272.1|	ATP synthase subunit B	127	18.3	C
gi|71893827|ref|YP_279273.1|	ATP synthase subunit A	46	2.9	C
gi|71893830|ref|YP_279276.1|	hypothetical protein MHJ_0479	31	9.7	S
gi|71893831|ref|YP_279277.1|	hypothetical protein MHJ_0480	85	12.2	S
gi|71893837|ref|YP_279283.1|	hypothetical protein MHJ_0486	78	13.0	S
gi|71893838|ref|YP_279284.1|	phosphoglycerate kinase	252	22.3	G
gi|71893842|ref|YP_279288.1|	mannose-6-phosphate isomerase	154	40.4	G
gi|71893844|ref|YP_279290.1|	P216 surface protein	2746	46.5	S
gi|71893845|ref|YP_279291.1|	P76 membrane protein precursor	1931	30.4	S
gi|71893854|ref|YP_279300.1|	dihydrolipoamide acetyltransferase	269	23.5	C
gi|71893855|ref|YP_279301.1|	dihydrolipoamide dehydrogenase	271	10.2	C
gi|71893856|ref|YP_279302.1|	acetate kinase	290	26.4	C
gi|71893857|ref|YP_279303.1|	phosphate acetyltransferase	316	28.7	C
gi|71893862|ref|YP_279308.1|	46K surface antigen precursor	514	42.0	S
gi|71893873|ref|YP_279319.1|	oligoendopeptidase F	122	18.1	E
gi|71893875|ref|YP_279321.1|	elongation factor Tu	1178	55.2	J
gi|71893876|ref|YP_279322.1|	heat shock ATP-dependent protease	437	22.4	O
gi|71893879|ref|YP_279325.1|	deoxyribose-phosphate aldolase	219	33.0	F
gi|71893880|ref|YP_279326.1|	DNA gyrase subunit A	125	20.5	L
gi|71893883|ref|YP_279329.1|	glucose-6-phosphate isomerase	96	5.8	G
gi|71893886|ref|YP_279332.1|	ribosome recycling factor	34	9.8	J

gi|71893908|ref|YP_279354.1|	PTS system galactitol-specific enzyme IIB component	28	13.2	G
gi|71893920|ref|YP_279366.1|	hypothetical protein MHJ_0571	17	1.9	S
gi|71893921|ref|YP_279367.1|	dihydrolipoamide dehydrogenase	89	23.3	C
gi|71893934|ref|YP_279380.1|	translation initiation factor IF-2	20	4.0	J
gi|71893937|ref|YP_279383.1|	glycerol-3-phosphate dehydrogenase	18	6.8	R
gi|71893941|ref|YP_279387.1|	ATP-binding protein	171	21.9	L
gi|71893944|ref|YP_279390.1|	phosphoglyceromutase	93	22.2	G
gi|71893952|ref|YP_279398.1|	hypothetical protein MHJ_0603	14	5.0	S
gi|71893954|ref|YP_279400.1|	inorganic pyrophosphatase	43	10.8	C
gi|71893955|ref|YP_279401.1|	ABC transporter xylose-binding lipoprotein	575	34.2	R
gi|71893956|ref|YP_279402.1|	sugar ABC transporter ATP-binding protein	88	13.5	R
gi|71893966|ref|YP_279412.1|	DNA-directed RNA polymerase beta' subunit	143	23.3	K
gi|71893968|ref|YP_279414.1|	50S ribosomal protein L7/L12	94	24.0	J
gi|71893971|ref|YP_279417.1|	lipoprotein	51	13.2	S
gi|71893972|ref|YP_279418.1|	DNA adenine methylase	23	2.0	L
gi|71893979|ref|YP_279425.1|	5'-nucleotidase precursor	101	19.7	F
gi|71893984|ref|YP_279430.1|	O-sialoglycoprotein endopeptidase	101	25.2	O
gi|71893985|ref|YP_279431.1|	transcription antitermination protein NusG	25	4.0	K
gi|71893998|ref|YP_279444.1|	30S ribosomal protein S9	26	24.2	J
gi|71893999|ref|YP_279445.1|	50S ribosomal protein L13	30	13.2	J
gi|71894003|ref|YP_279449.1|	glucose-inhibited division protein B	17	12.0	M
gi|71894004|ref|YP_279450.1|	prolipoprotein p65	1431	37.2	S
gi|71894005|ref|YP_279451.1|	ABC transporter ATP-binding protein P115-like	108	20.1	D
gi|71894010|ref|YP_279456.1|	hypothetical protein MHJ_0662	896	20.2	S
gi|71894011|ref|YP_279457.1|	adhesin like-protein P146	1015	21.7	S
gi|71894015|ref|YP_279461.1|	transcription elongation factor	59	32.5	K
gi|71894021|ref|YP_279467.1|	hypothetical protein MHJ_0673	183	10.6	S

[1] CDS access number in the NCBI database (http://www.ncbi.nlm.nih.gov).
[2] Protein identification according to NCBI database (http://www.ncbi.nlm.nih.gov).
[3] MASCOT score is – 10 x log(P), where P is the probability that the observed match is a random event.
[4] COG database functional classes: (J) Translation, ribosomal structure and biogenesis, (K) Transcription, (L) DNA replication, recombination and repair, (D) Cell division and chromosome partitioning, (O) Posttranslational modification, protein turnover, chaperones, (M) Cell envelope biogenesis, outer membrane, (N) Cell motility and secretion, (C) Energy production and conversion, (G) Carbohydrate transport and metabolism, (E) Amino acid transport and metabolism, (F) Nucleotide transport and metabolism, (H) Coenzyme metabolism, (I) Lipid metabolism, transport and catabolism,(R) General function prediction only, and (S) Function unknown.

Table 2

Identification of 7448 strain proteins by LC-MS/MS. Protein identified by a liquid chromatography (LC) separation (reversed-phase HPLC) coupled with a tandem mass spectrometry (MS/MS) by searching *M. hyopneumoniae* strain 7448 protein databases using MASCOT search engine.

Accession number[1]	Protein description[2]	MASCOT score[3]	Sequence coverage (%)	COG[4]
gi|72080345|ref|YP_287403.1|	glucose-inhibited division protein A	15	3.1	D
gi|72080348|ref|YP_287406.1|	DHH family phosphoesterase	32	9.1	R
gi|72080351|ref|YP_287409.1|	hypothetical protein MHP7448_0009	76	18.4	S
gi|72080353|ref|YP_287411.1|	heat shock protein	54	18.4	O
gi|72080356|ref|YP_287414.1|	fructose-bisphosphate aldolase	94	30.7	G
gi|72080361|ref|YP_287419.1|	ABC transporter ATP-binding protein	55	13.3	R
gi|72080365|ref|YP_287423.1|	ABC transporter ATP-binding protein	26	1	R
gi|72080373|ref|YP_287431.1|	lipoprotein signal peptidase	23	10	M
gi|72080374|ref|YP_287432.1|	isoleucyl-tRNA synthetase	39	17.8	J
gi|72080377|ref|YP_287435.1|	glyceraldehyde 3-phosphate dehydrogenase	575	16.1	G
gi|72080379|ref|YP_287437.1|	VACB-like ribonuclease II	14	1	K
gi|72080383|ref|YP_287441.1|	GTP-binding protein Obg	17	4.8	R
gi|72080395|ref|YP_287453.1|	ATP synthase subunit B	94	9.6	C
gi|72080397|ref|YP_287455.1|	30S ribosomal protein S2	173	7.3	J
gi|72080398|ref|YP_287456.1|	elongation factor Ts	30	13.5	J
gi|72080406|ref|YP_287464.1|	hypothetical protein MHP7448_0064	40	13.3	S
gi|72080408|ref|YP_287466.1|	excinuclease ABC subunit C	29	11	L
gi|72080409|ref|YP_287467.1|	molecular chaperone DnaK	969	36.5	O
gi|72080413|ref|YP_287471.1|	bacterial nucleoid DNA-binding protein	66	14.6	L
gi|72080417|ref|YP_287475.1|	elongation factor EF-2	134	22.2	K
gi|72080419|ref|YP_287477.1|	30S ribosomal protein S12	51	5.8	J
gi|72080424|ref|YP_287482.1|	NADH oxidase	478	16.5	R
gi|72080428|ref|YP_287486.1|	translocase	18	4.2	N
gi|72080434|ref|YP_287492.1|	hypothetical protein MHP7448_0092	28	12.5	S
gi|72080438|ref|YP_287496.1|	thiol peroxidase	107	40.2	O
gi|72080440|ref|YP_287498.1|	thioredoxin reductase	125	28.5	O
gi|72080443|ref|YP_287501.1|	ATP-dependent protease binding protein	37	5.5	O
gi|72080448|ref|YP_287506.1|	protein P97	344	19.3	S
gi|72080449|ref|YP_287507.1|	DNA gyrase subunit B	36	20	L
gi|72080451|ref|YP_287509.1|	6-phosphofructokinase	29	25.2	G
gi|72080454|ref|YP_287512.1|	adenine phosphoribosyltransferase	597	41.4	F
gi|72080455|ref|YP_287513.1|	pyruvate dehydrogenase E1-alpha subunit	1541	34.5	C
gi|72080456|ref|YP_287514.1|	pyruvate dehydrogenase	1185	62	C
gi|72080461|ref|YP_287519.1|	hypothetical protein MHP7448_0121	17	6.3	S
gi|72080466|ref|YP_287524.1|	pyruvate kinase	50	4.2	G
gi|72080471|ref|YP_287529.1|	50S ribosomal protein L21	32	17.2	J
gi|72080472|ref|YP_287530.1|	50S ribosomal protein L27	92	22.6	J
gi|72080473|ref|YP_287531.1|	lipase-esterase	80	20.8	R
gi|72080475|ref|YP_287533.1|	hexosephosphate transport protein	38	7.1	P
gi|72080476|ref|YP_287534.1|	L-lactate dehydrogenase	388	43.5	C
gi|72080487|ref|YP_287545.1|	hypothetical protein MHP7448_0148	33	8.3	S

gi|72080488|ref|YP_287546.1|	trigger factor	171	18.6	O
gi|72080489|ref|YP_287547.1|	hypothetical protein MHP7448_0150	18	4.7	S
gi|72080500|ref|YP_287558.1|	phosphopentomutase	16	6	G
gi|72080507|ref|YP_287565.1|	DNA-directed RNA polymerase alpha subunit	59	10.8	K
gi|72080508|ref|YP_287566.1|	50S ribosomal protein L11	93	32	J
gi|72080508|ref|YP_287566.1|	30S ribosomal protein S11	42	35.1	J
gi|72080509|ref|YP_287567.1|	30S ribosomal protein S13	67	14.6	J
gi|72080515|ref|YP_287573.1|	50S ribosomal protein L15	66	9	J
gi|72080519|ref|YP_287577.1|	30S ribosomal protein S8	78	16.8	J
gi|72080521|ref|YP_287579.1|	50S ribosomal protein L5	82	37.8	J
gi|72080522|ref|YP_287580.1|	50S ribosomal protein L24	87	15	J
gi|72080525|ref|YP_287583.1|	50S ribosomal protein L29	78	22	S
gi|72080528|ref|YP_287586.1|	50S ribosomal protein L22	50	15.3	J
gi|72080530|ref|YP_287588.1|	50S ribosomal protein L2	137	11.3	J
gi|72080532|ref|YP_287590.1|	50S ribosomal protein L4	47	12.1	J
gi|72080533|ref|YP_287591.1|	50S ribosomal protein L3	36	12.2	J
gi|72080537|ref|YP_287595.1|	protein P97	1550	26.3	S
gi|72080538|ref|YP_287596.1|	protein P102	575	25.1	S
gi|72080540|ref|YP_287598.1|	alanine--tRNA ligase	15	2.3	J
gi|72080545|ref|YP_287603.1|	cell division protein	130	11.7	O
gi|72080546|ref|YP_287604.1|	lysyl-tRNA synthetase	104	4.9	J
gi|72080556|ref|YP_287614.1|	lipoprotein	36	3.1	S
gi|72080560|ref|YP_287618.1|	ribonucleotide-diphosphate reductase alpha subunit	45	22.4	F
gi|72080562|ref|YP_287620.1|	ribonucleotide-diphosphate reductase beta subunit	33	6.5	F
gi|72080564|ref|YP_287622.1|	methylmalonate-semialdehyde dehydrogenase	346	38.2	C
gi|72080568|ref|YP_287626.1|	myo-inositol catabolism protein	91	11.7	G
gi|72080573|ref|YP_287631.1|	periplasmic sugar-binding proteins	51	27.4	G
gi|72080574|ref|YP_287632.1|	myo-inositol 2-dehydrogenase	20	3.2	R
gi|72080580|ref|YP_287638.1|	protein-export membrane protein	35	5	N
gi|72080583|ref|YP_287641.1|	hypothetical protein MHP7448_0244	54	9.9	S
gi|72080589|ref|YP_287647.1|	phosphopyruvate hydratase	141	35.4	G
gi|72080590|ref|YP_287648.1|	seryl-tRNA synthetase	31	7.5	J
gi|72080591|ref|YP_287649.1|	hypothetical protein MHP7448_0252	20	18	S
gi|72080593|ref|YP_287651.1|	lipoate-protein ligase A	14	6.9	H
gi|72080601|ref|YP_287659.1|	hypoxanthine-guanine phosphoribosyltransferase	30	34.3	F
gi|72080607|ref|YP_287665.1|	cation-transporting P-type ATPase	37	22.4	P
gi|72080611|ref|YP_287669.1|	protein P97-like	26	19	S
gi|72080613|ref|YP_287671.1|	phenylalanyl-tRNA synthetase beta subunit	43	14.8	J
gi|72080628|ref|YP_287686.1|	hypothetical protein MHP7448_0289	23	4	S
gi|72080634|ref|YP_287692.1|	30S ribosomal protein S6	51	16.2	J
gi|72080635|ref|YP_287693.1|	hypothetical protein MHP7448_0297	32	23.7	S
gi|72080644|ref|YP_287702.1|	ABC transporter ATP-binding protein	55	17.8	R
gi|72080647|ref|YP_287705.1|	NADH-dependent flavin oxidoreductase	28	24.6	C
gi|72080652|ref|YP_287710.1|	ABC transporter ATP-binding protein	15	3.2	R
gi|72080653|ref|YP_287711.1|	ABC transporter ATP-binding protein	55	18.3	R
gi|72080687|ref|YP_287745.1|	hypothetical protein MHP7448_0351	20	6.3	S
gi|72080688|ref|YP_287746.1|	hypothetical protein MHP7448_0352	29	13.7	S
gi|72080689|ref|YP_287747.1|	P60-like lipoprotein	44	4.1	S

gi|72080690|ref|YP_287748.1|	HIT-like protein	25	44.5	F
gi|72080692|ref|YP_287750.1|	hypothetical protein MHP7448_0356	29	15.2	S
gi|72080702|ref|YP_287760.1|	lipoprotein	151	16.3	S
gi|72080703|ref|YP_287761.1|	lipoprotein	28	20.3	S
gi|72080708|ref|YP_287766.1|	Lppt protein	115	17.8	S
gi|72080709|ref|YP_287767.1|	hypothetical protein MHP7448_0373	452	14.8	S
gi|72080711|ref|YP_287769.1|	PTS system enzyme IIB component	177	14.6	G
gi|72080713|ref|YP_287771.1|	hypothetical protein MHP7448_0377	434	30	S
gi|72080714|ref|YP_287772.1|	lipoprotein	17	3.5	S
gi|72080720|ref|YP_287778.1|	thioredoxin	272	44.5	O
gi|72080725|ref|YP_287783.1|	hypothetical protein MHP7448_0391	18	6.8	S
gi|72080726|ref|YP_287784.1|	S-adenosyl-methyltransferase	24	9.5	M
gi|72080735|ref|YP_287793.1|	asparaginyl-tRNA synthetase	69	18.5	J
gi|72080737|ref|YP_287795.1|	ATP-dependent helicase PcrA	49	5.2	L
gi|72080739|ref|YP_287797.1|	Holliday junction DNA helicase motor protein	15	18.1	L
gi|72080758|ref|YP_287816.1|	hypothetical protein MHP7448_0425	16	4.2	S
gi|72080761|ref|YP_287819.1|	transketolase	122	23.9	G
gi|72080771|ref|YP_287829.1|	3-hexulose-6-phosphate synthase	78	19.8	G
gi|72080786|ref|YP_287844.1|	acyl carrier protein phosphodiesterase	57	21.6	I
gi|72080791|ref|YP_287849.1|	50S ribosomal protein L1	19	7.4	J
gi|72080796|ref|YP_287854.1|	leucyl aminopeptidase	80	20.5	E
gi|72080798|ref|YP_287856.1|	hypothetical protein MHP7448_0466	133	15.7	S
gi|72080804|ref|YP_287862.1|	phosphoenolpyruvate-protein phosphotransferase	49	9.2	G
gi|72080810|ref|YP_287868.1|	ATP synthase subunit A	66	2.9	C
gi|72080813|ref|YP_287871.1|	hypothetical protein MHP7448_0482	58	27	S
gi|72080814|ref|YP_287872.1|	hypothetical protein MHP7448_0483	61	12.2	S
gi|72080820|ref|YP_287878.1|	hypothetical protein MHP7448_0489	38	14.2	S
gi|72080821|ref|YP_287879.1|	phosphoglycerate kinase	46	23.5	G
gi|72080825|ref|YP_287883.1|	mannose-6-phosphate isomerase	92	28	G
gi|72080827|ref|YP_287885.1|	putative p216 surface protein	1560	21.6	S
gi|72080828|ref|YP_287886.1|	p76 membrane protein precursor	1366	30.8	S
gi|72080837|ref|YP_287895.1|	dihydrolipoamide acetyltransferase	198	37.7	C
gi|72080839|ref|YP_287897.1|	acetate kinase	101	37.6	C
gi|72080840|ref|YP_287898.1|	phosphate acetyltransferase	441	28.7	C
gi|72080844|ref|YP_287902.1|	46K surface antigen precursor	900	26.5	S
gi|72080845|ref|YP_287903.1|	xylose ABC transporter ATP-binding protein	17	4.7	G
gi|72080852|ref|YP_287910.1|	oligoendopeptidase F	117	7.5	E
gi|72080854|ref|YP_287912.1|	elongation factor Tu	1229	48.3	J
gi|72080855|ref|YP_287913.1|	heat shock ATP-dependent protease	160	23.1	O
gi|72080859|ref|YP_287917.1|	DNA gyrase subunit A	14	5.8	L
gi|72080862|ref|YP_287920.1|	glucose-6-phosphate isomerase	49	5.8	G
gi|72080865|ref|YP_287923.1|	ribosome recycling factor	90	18.4	J
gi|72080873|ref|YP_287931.1|	spermidine/putrescine ABC transporter ATP-binding	27	14.6	E
gi|72080899|ref|YP_287957.1|	dihydrolipoamide dehydrogenase	47	4.2	C
gi|72080904|ref|YP_287962.1|	30S ribosomal protein S4	103	19	J
gi|72080909|ref|YP_287967.1|	5'-3' exonuclease	31	7.5	L
gi|72080914|ref|YP_287972.1|	transcription elongation factor NusA	41	16.2	K
gi|72080920|ref|YP_287978.1|	ATP binding protein	95	8.9	L

gi|72080923|ref|YP_287981.1|	phosphoglyceromutase	74	17.8	G
gi|72080929|ref|YP_287987.1|	hypothetical protein MHP7448_0601	15	9.7	S
gi|72080931|ref|YP_287989.1|	inorganic pyrophosphatase	42	10.3	C
gi|72080932|ref|YP_287990.1|	ABC transporter xylose-binding lipoprotein	372	15.2	R
gi|72080933|ref|YP_287991.1|	sugar ABC transporter ATP-binding protein	75	11.1	R
gi|72080944|ref|YP_288002.1|	DNA-directed RNA polymerase beta' subunit	15	2.7	K
gi|72080949|ref|YP_288007.1|	lipoprotein	61	15.5	S
gi|72080954|ref|YP_288012.1|	ABC transporter ATP-binding - Pr1-like	19	1.5	Q
gi|72080957|ref|YP_288015.1|	5'-nucleotidase precursor	20	7	F
gi|72080975|ref|YP_288033.1|	excinuclease ABC subunit B	26	20	L
gi|72080977|ref|YP_288035.1|	30S ribosomal protein S9	49	13.6	J
gi|72080982|ref|YP_288040.1|	glucose-inhibited division protein B	19	12.4	M
gi|72080983|ref|YP_288041.1|	prolipoprotein p65	939	35.7	S
gi|72080986|ref|YP_288044.1|	XAA-PRO aminopeptidase	89	23.8	E
gi|72080989|ref|YP_288047.1|	hypothetical protein MHP7448_0662	626	21.9	S
gi|72080990|ref|YP_288048.1|	adhesin like-protein P146	559	28.4	S
gi|72080999|ref|YP_288057.1|	valyl-tRNA synthetase	21	3.8	J

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[4] COG database functional classes: (J) Translation, ribosomal structure and biogenesis, (K) Transcription, (L) DNA replication, recombination and repair, (D) Cell division and chromosome partitioning, (O) Posttranslational modification, protein turnover, chaperones, (M) Cell envelope biogenesis, outer membrane, (N) Cell motility and secretion, (P) Inorganic ion transport and metabolism,(C) Energy production and conversion, (G) Carbohydrate transport and metabolism, (E) Amino acid transport and metabolism, (F) Nucleotide transport and metabolism, (H) Coenzyme metabolism, (I) Lipid metabolism, (Q) Secondary metabolites biosynthesis, transport and catabolism,(R) General function prediction only, and (S) Function unknown.

Table 3

Identification of 7422 strain proteins by LC-MS/MS. Protein identified by a liquid chromatography (LC) separation (reversed-phase HPLC) coupled with a tandem mass spectrometry (MS/MS) by searching *M. hyopneumoniae* strain 7448 and J protein databases using MASCOT search engine.

Accession number[1]	Protein description[2]	MASCOT score[3]	Sequence coverage (%)	COG[4]
gi|72080345|ref|YP_287403.1|	glucose-inhibited division protein A	42	25.2	D
gi|72080347|ref|YP_287405.1|	putative MgpA like-protein	60	9.2	R
gi|72080348|ref|YP_287406.1|	DHH family phosphoesterase	74	14.1	R
gi|72080351|ref|YP_287409.1|	hypothetical protein MHP7448_0009	75	24.8	S
gi|72080353|ref|YP_287411.1|	heat shock protein	118	12.8	O
gi|72080356|ref|YP_287414.1|	fructose-bisphosphate aldolase	326	13.6	G
gi|72080363|ref|YP_287421.1|	ABC transporter ATP-binding protein	14	4.5	R
gi|72080370|ref|YP_287428.1|	amidase	44	20.7	J
gi|72080377|ref|YP_287435.1|	glyceraldehyde 3-phosphate dehydrogenase	567	36.6	G
gi|72080379|ref|YP_287437.1|	VACB-like ribonuclease II	28	17.2	K
gi|72080395|ref|YP_287453.1|	ATP synthase subunit B	216	31	C
gi|72080397|ref|YP_287455.1|	30S ribosomal protein S2	533	34	J
gi|72080398|ref|YP_287456.1|	elongation factor Ts	70	30.7	J
gi|72080401|ref|YP_287459.1|	DNA primase	22	2	L
gi|72080409|ref|YP_287467.1|	molecular chaperone DnaK	543	24.5	O
gi|72080413|ref|YP_287471.1|	bacterial nucleoid DNA-binding protein	38	57.3	L
gi|72080417|ref|YP_287475.1|	elongation factor EF-2	364	27.6	K
gi|72080419|ref|YP_287477.1|	30S ribosomal protein S12	34	20.1	J
gi|72080424|ref|YP_287482.1|	NADH oxidase	1376	41.3	R
gi|72080425|ref|YP_287483.1|	thymidinephosphorylase	21	4	F
gi|72080426|ref|YP_287484.1|	purine-nucleoside phosphorylase	186	10.8	F
gi|72080428|ref|YP_287486.1|	translocase	30	25.3	N
gi|72080436|ref|YP_287494.1|	hypothetical protein MHP7448_0094	38	20.7	S
gi|72080438|ref|YP_287496.1|	thiolperoxidase	13	14	O
gi|72080440|ref|YP_287498.1|	thioredoxin reductase	117	25.9	O
gi|72080441|ref|YP_287499.1|	outer membrane protein - P95	17	1	S
gi|72080444|ref|YP_287502.1|	triosephosphate isomerase	35	9.1	G
gi|72080447|ref|YP_287505.1|	protein P102	64	16.9	S
gi|72080448|ref|YP_287506.1|	protein P97	303	21.2	S
gi|72080449|ref|YP_287507.1|	DNA gyrase subunit B	95	21.4	L
gi|72080451|ref|YP_287509.1|	6-phosphofructokinase	68	13.4	G
gi|72080454|ref|YP_287512.1|	adenine phosphoribosyltransferase	495	47.9	F
gi|72080455|ref|YP_287513.1|	pyruvate dehydrogenase E1-alpha subunit	2274	39.3	C
gi|72080456|ref|YP_287514.1|	pyruvate dehydrogenase	2031	57.2	C
gi|72080461|ref|YP_287519.1|	hypothetical protein MHP7448_0121	17	6	S
gi|72080466|ref|YP_287524.1|	pyruvate kinase	224	28.8	G
gi|72080469|ref|YP_287527.1|	aminopeptidase	111	24.2	G
gi|72080471|ref|YP_287529.1|	50S ribosomal protein L21	90	16.2	J
gi|72080472|ref|YP_287530.1|	50S ribosomal protein L27	130	22.6	J
gi|72080473|ref|YP_287531.1|	lipase-esterase	52	27.9	R
gi|72080475|ref|YP_287533.1|	hexosephosphate transport protein	62	30.1	P

gi|72080476|ref|YP_287534.1|	L-lactate dehydrogenase	441	28.9	C
gi|72080477|ref|YP_287535.1|	hypothetical protein MHP7448_0138	80	16.5	S
gi|72080487|ref|YP_287545.1|	hypothetical protein MHP7448_0148	27	20.4	S
gi|72080488|ref|YP_287546.1|	trigger factor	347	22.5	O
gi|72080492|ref|YP_287550.1|	guanylate kinase	32	33	F
gi|72080495|ref|YP_287553.1|	GTP-binding protein	26	12.6	R
gi|72080500|ref|YP_287558.1|	phosphopentomutase	48	23.6	G
gi|72080507|ref|YP_287565.1|	DNA-directed RNA polymerase alpha subunit	296	14.2	K
gi|72080508|ref|YP_287566.1|	30S ribosomal protein S11	122	46.3	J
gi|72080509|ref|YP_287567.1|	30S ribosomal protein S13	44	21.9	J
gi|72080518|ref|YP_287576.1|	50S ribosomal protein L6	70	42.5	J
gi|72080519|ref|YP_287577.1|	30S ribosomal protein S8	31	8.4	J
gi|72080521|ref|YP_287579.1|	50S ribosomal protein L5	46	16.7	J
gi|72080522|ref|YP_287580.1|	50S ribosomal protein L24	53	13.1	J
gi|72080525|ref|YP_287583.1|	50S ribosomal protein L29	78	22.1	S
gi|72080528|ref|YP_287586.1|	50S ribosomal protein L22	122	34.7	J
gi|72080530|ref|YP_287588.1|	50S ribosomal protein L2	113	27.3	J
gi|72080532|ref|YP_287590.1|	50S ribosomal protein L4	86	18.4	J
gi|72080533|ref|YP_287591.1|	50S ribosomal protein L3	116	12.6	J
gi|72080537|ref|YP_287595.1|	protein P97	1199	26.9	S
gi|72080538|ref|YP_287596.1|	protein P102	451	24.9	S
gi|72080539|ref|YP_287597.1|	hypothetical protein MHP7448_0200	18	4	S
gi|72080540|ref|YP_287598.1|	alanine--tRNAligase	20	2	J
gi|72080545|ref|YP_287603.1|	cell division protein	335	20.7	O
gi|72080546|ref|YP_287604.1|	lysyl-tRNA synthetase	110	19.2	J
gi|72080547|ref|YP_287605.1|	hydrolase of the HAD family	22	5.4	R
gi|72080551|ref|YP_287609.1|	oligopeptide ABC transporter system permease	17	4.4	E
gi|72080554|ref|YP_287612.1|	oligopeptideABCtransporterATP-bindingprotein	17	7	E
gi|72080560|ref|YP_287618.1|	ribonucleotide-diphosphate reductase alpha subunit	38	7.7	F
gi|72080562|ref|YP_287620.1|	ribonucleotide-diphosphate reductase beta subunit	244	13.6	F
gi|72080564|ref|YP_287622.1|	methylmalonate-semialdehyde dehydrogenase	445	33.1	C
gi|72080568|ref|YP_287626.1|	myo-inositol catabolism protein	303	14.9	G
gi|72080573|ref|YP_287631.1|	periplasmic sugar-binding proteins	84	16.7	G
gi|72080582|ref|YP_287640.1|	aspartyl-tRNA synthetase	80	17.6	J
gi|72080583|ref|YP_287641.1|	hypothetical protein MHP7448_0244	46	9.9	S
gi|72080586|ref|YP_287644.1|	TRSE-likeprotein	23	3	N
gi|72080589|ref|YP_287647.1|	phosphopyruvate hydratase	224	20.8	G
gi|72080591|ref|YP_287649.1|	hypothetical protein MHP7448_0252	29	16.9	S
gi|72080592|ref|YP_287650.1|	triacylglycerol lipase	38	12.8	R
gi|72080593|ref|YP_287651.1|	lipoate-protein ligase A	40	12.6	H
gi|72080596|ref|YP_287654.1|	hypothetical protein MHP7448_0257	84	34.6	S
gi|72080597|ref|YP_287655.1|	recombination protein RecR	34	34.5	L
gi|72080601|ref|YP_287659.1|	hypoxanthine-guanine phosphoribosyltransferase	17	16.3	F
gi|72080605|ref|YP_287663.1|	DNA ligase	37	5.6	L
gi|72080611|ref|YP_287669.1|	protein P97-like	56	11.6	S
gi|72080613|ref|YP_287671.1|	phenylalanyl-tRNA synthetase beta subunit	89	13.2	J
gi|72080618|ref|YP_287676.1|	transcriptional regulator	89	9.6	K
gi|72080619|ref|YP_287677.1|	CTP synthetase	30	19.8	F

gi|72080635|ref|YP_287693.1|	hypothetical protein MHP7448_0297	39	32.6	S
gi|72080643|ref|YP_287701.1|	ABCtransporterATP-bindingprotein	18	1	R
gi|72080650|ref|YP_287708.1|	glycine cleavage system H protein	16	11.8	E
gi|72080682|ref|YP_287740.1|	hypothetical protein MHP7448_0346	15	2	S
gi|72080688|ref|YP_287746.1|	hypothetical protein MHP7448_0352	38	14.8	S
gi|72080689|ref|YP_287747.1|	P60-like lipoprotein	140	17.3	S
gi|72080690|ref|YP_287748.1|	HIT-like protein	48	19.1	F
gi|72080702|ref|YP_287760.1|	lipoprotein	562	33.4	S
gi|72080703|ref|YP_287761.1|	lipoprotein	82	13.6	S
gi|72080708|ref|YP_287766.1|	Lppt protein	26	14.1	S
gi|72080709|ref|YP_287767.1|	hypothetical protein MHP7448_0373	389	20.3	S
gi|72080711|ref|YP_287769.1|	PTS system enzyme IIB component	156	14.6	G
gi|72080713|ref|YP_287771.1|	hypothetical protein MHP7448_0377	1003	50	S
gi|72080720|ref|YP_287778.1|	thioredoxin	351	44.5	O
gi|72080725|ref|YP_287783.1|	hypothetical protein MHP7448_0391	18	6	S
gi|72080726|ref|YP_287784.1|	S-adenosyl-methyltransferase	14	9	M
gi|72080735|ref|YP_287793.1|	asparaginyl-tRNA synthetase	99	20.7	J
gi|72080737|ref|YP_287795.1|	ATP-dependent helicase PcrA	130	10.7	L
gi|72080761|ref|YP_287819.1|	transketolase	212	30.6	G
gi|72080771|ref|YP_287829.1|	3-hexulose-6-phosphate synthase	129	22	G
gi|72080787|ref|YP_287845.1|	acyl carrier protein phosphodiesterase	57	25.4	I
gi|72080791|ref|YP_287849.1|	50S ribosomal protein L1	155	28.1	J
gi|72080792|ref|YP_287850.1|	50S ribosomal protein L11	117	26.2	J
gi|72080795|ref|YP_287853.1|	hypothetical protein MHP7448_0463	17	2	S
gi|72080796|ref|YP_287854.1|	leucyl aminopeptidase	125	24.5	E
gi|72080798|ref|YP_287856.1|	hypothetical protein MHP7448_0466	115	17.5	S
gi|72080801|ref|YP_287859.1|	ABC transporter atp-binding protein	46	16.9	R
gi|72080804|ref|YP_287862.1|	phosphoenolpyruvate-protein phosphotransferase	260	17	G
gi|72080820|ref|YP_287878.1|	hypothetical protein MHP7448_0489	83	20.7	S
gi|72080825|ref|YP_287883.1|	mannose-6-phosphate isomerase	126	17.6	G
gi|72080827|ref|YP_287885.1|	putative p216 surface protein	2622	34.4	S
gi|72080828|ref|YP_287886.1|	p76 membrane protein precursor	1885	27.3	S
gi|72080832|ref|YP_287890.1|	oligopeptide ABC transporter ATP-binding protein	20	5.2	E
gi|72080833|ref|YP_287891.1|	oligopeptide ABC transporter ATP binding protein	40	18.6	E
gi|72080837|ref|YP_287895.1|	dihydrolipoamide acetyltransferase	120	22.6	C
gi|72080838|ref|YP_287896.1|	dihydrolipoamide dehydrogenase	429	17.1	C
gi|72080839|ref|YP_287897.1|	acetate kinase	510	39.3	C
gi|72080840|ref|YP_287898.1|	phosphate acetyltransferase	823	39.7	C
gi|72080844|ref|YP_287902.1|	46K surface antigen precursor	1275	39.9	S
gi|72080852|ref|YP_287910.1|	oligoendopeptidase F	193	10.6	E
gi|72080854|ref|YP_287912.1|	elongation factor Tu	1388	63.7	J
gi|72080855|ref|YP_287913.1|	heat shock ATP-dependent protease	263	25.6	O
gi|72080858|ref|YP_287916.1|	deoxyribose-phosphate aldolase	54	24.9	F
gi|72080859|ref|YP_287917.1|	DNA gyrase subunit A	27	17.9	L
gi|72080860|ref|YP_287918.1|	methionine sulfoxide reductase B	101	42.8	O
gi|72080862|ref|YP_287920.1|	glucose-6-phosphate isomerase	68	28	G
gi|72080865|ref|YP_287923.1|	ribosome recycling factor	202	23	J
gi|72080909|ref|YP_287967.1|	5'-3' exonuclease	54	14.6	L

gi|72080912|ref|YP_287970.1|	translation initiation factor IF-2	25	21.7	J
gi|72080918|ref|YP_287976.1|	tryptophanyl-tRNA synthetase	124	39.3	J
gi|72080920|ref|YP_287978.1|	ATP binding protein	298	25	L
gi|72080923|ref|YP_287981.1|	phosphoglyceromutase	123	21	G
gi|72080932|ref|YP_287990.1|	ABC transporter xylose-binding lipoprotein	607	15.2	R
gi|72080933|ref|YP_287991.1|	sugar ABC transporter ATP-binding protein	87	25.8	R
gi|72080944|ref|YP_288002.1|	DNA-directed RNA polymerase beta' subunit	53	17	K
gi|72080945|ref|YP_288003.1|	DNA-directed RNA polymerase beta subunit	43	16.1	K
gi|72080946|ref|YP_288004.1|	50S ribosomal protein L7/L12	104	27.3	J
gi|72080949|ref|YP_288007.1|	lipoprotein	75	20.8	S
gi|72080952|ref|YP_288010.1|	ABC transporter ATP-binding protein - Pr2	30	10.2	Q
gi|72080957|ref|YP_288015.1|	5'-nucleotidase precursor	55	15.5	F
gi|72080959|ref|YP_288017.1|	segregation and condensation protein A	32	22.6	L
gi|72080974|ref|YP_288032.1|	leucyl-tRNAsynthetase	15	1	J
gi|72080977|ref|YP_288035.1|	30S ribosomal protein S9	91	10.6	J
gi|72080978|ref|YP_288036.1|	50S ribosomal protein L13	28	22.2	J
gi|72080983|ref|YP_288041.1|	prolipoprotein p65	1485	27.6	S
gi|72080987|ref|YP_288045.1|	hypothetical protein MHP7448_0660	30	16.9	S
gi|72080989|ref|YP_288047.1|	hypothetical protein MHP7448_0662	399	22.4	S
gi|72080990|ref|YP_288048.1|	adhesin like-protein P146	533	27.7	S
gi|72080995|ref|YP_288053.1|	transcription elongation factor	188	30.6	K

[1] CDS access number in the NCBI database (http://www.ncbi.nlm.nih.gov).
[2] Protein identification according to NCBI database (http://www.ncbi.nlm.nih.gov).
[3] MASCOT score is – 10 x log(*P*), where *P* is the probability that the observed match is a random event.
[4] COG database functional classes: (J) Translation, ribosomal structure and biogenesis, (K) Transcription, (L) DNA replication, recombination and repair, (D) Cell division and chromosome partitioning, (O) Posttranslational modification, protein turnover, chaperones, (M) Cell envelope biogenesis, outer membrane, (N) Cell motility and secretion, (P) Inorganic ion transport and metabolism,(C) Energy production and conversion, (G) Carbohydrate transport and metabolism, (E) Amino acid transport and metabolism, (F) Nucleotide transport and metabolism, (H) Coenzyme metabolism, (I) Lipid metabolism, (Q) Secondary metabolites biosynthesis, transport and catabolism,(R) General function prediction only, and (S) Function unknown.

Table 4
The emPAI value comparison and differentially expressed proteins. The complete list of emPAI values for each protein from the three *M. hyopneumoniae* strains and differentially expressed proteins inferred by emPAI analysis.

Accession number[1]	Protein description[2]	emPAI value[3]		
		J	7448	7422
	Proteins identified only in strain J			
gi|71893407|ref|YP_278853.1|	ATP synthase gamma chain	0.08	-	-
gi|71893826|ref|YP_279272.1|	ATP synthase subunit B	0.24	-	-
gi|71894005|ref|YP_279451.1|	ABC transporter ATP-binding protein P115-like	0.03	-	-
gi|71893706|ref|YP_279152.1|	amino acid permease	0.08	-	-
gi|71893574|ref|YP_279020.1|	serine hydroxymethyltransferase	0.10	-	-
gi|71893566|ref|YP_279012.1|	oligopeptide ABC transporter ATP-binding protein	0.20	-	-
gi|71893650|ref|YP_279096.1|	permease	0.06	-	-
gi|71893908|ref|YP_279354.1|	PTS system galactitol-specific enzyme IIB component	0.54	-	-
gi|71893760|ref|YP_279206.1|	methionyl-tRNA synthetase	0.05	-	-
gi|71893779|ref|YP_279225.1|	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	0.08	-	-
gi|71893491|ref|YP_278937.1|	peptide chain release factor 1	0.12	-	-
gi|71893431|ref|YP_278877.1|	30S ribosomal protein S7	0.19	-	-
gi|71893621|ref|YP_279067.1|	phenylalanyl-tRNA synthetase alpha chain	0.19	-	-
gi|71893542|ref|YP_278988.1|	30S ribosomal protein S19	0.34	-	-
gi|71893476|ref|YP_278922.1|	50S ribosomal protein L20	0.38	-	-
gi|71893985|ref|YP_279431.1|	transcription antitermination protein NusG	0.15	-	-
gi|71893604|ref|YP_279050.1|	DNA polymerase III subunits gamma and tau	0.04	-	-
gi|71893972|ref|YP_279418.1|	DNA adenine methylase	0.05	-	-
gi|71893984|ref|YP_279430.1|	O-sialoglycoprotein endopeptidase	0.23	-	-
gi|71893937|ref|YP_279383.1|	glycerol-3-phosphate dehydrogenase	0.11	-	-
gi|71893796|ref|YP_279242.1|	hypothetical protein MHJ_0445	0.01	-	-
gi|71893793|ref|YP_279239.1|	hypothetical protein MHJ_0442	0.03	-	-
gi|71893768|ref|YP_279214.1|	hypothetical protein MHJ_0417	0.06	-	-
gi|71893816|ref|YP_279262.1|	hypothetical protein MHJ_0465	0.06	-	-
gi|71893920|ref|YP_279366.1|	hypothetical protein MHJ_0571	0.06	-	-
gi|71894021|ref|YP_279467.1|	hypothetical protein MHJ_0673	0.06	-	-
gi|71893568|ref|YP_279014.1|	hypothetical protein MHJ_0212	0.17	-	-
gi|71893639|ref|YP_279085.1|	hypothetical protein MHJ_0283	0.17	-	-
gi|71893472|ref|YP_278918.1|	hypothetical protein MHJ_0115	0.29	-	-
gi|71893572|ref|YP_279018.1|	ribonucleotide reductase stimulatory protein	0.43	-	-
	Proteins identified only in strain 7448			
gi|72080647|ref|YP_287705.1|	NADH-dependent flavin oxidoreductase	-	0.07	-
gi|72080873|ref|YP_287931.1|	spermidine/putrescine ABC transporter ATP-binding	-	0.06	-
gi|72080986|ref|YP_288044.1|	XAA-PRO aminopeptidase	-	0.08	-
gi|72080845|ref|YP_287903.1|	xylose ABC transporter ATP-binding protein	-	0.08	-
gi|72080999|ref|YP_288057.1|	valyl-tRNA synthetase	-	0.03	-
gi|72080904|ref|YP_287962.1|	30S ribosomal protein S4	-	0.48	-
gi|72080914|ref|YP_287972.1|	transcription elongation factor NusA	-	0.05	-
gi|72080975|ref|YP_288033.1|	excinuclease ABC subunit B	-	0.04	-
gi|72080408|ref|YP_287466.1|	excinuclease ABC subunit C	-	0.05	-
gi|72080373|ref|YP_287431.1|	lipoprotein signal peptidase	-	0.25	-

gi|72080607|ref|YP_287665.1|	cation-transporting P-type ATPase	-	0.03	-
gi|72080954|ref|YP_288012.1|	ABC transporter ATP-binding - Pr1-like	-	0.05	-
gi|72080652|ref|YP_287710.1|	ABC transporter ATP-binding protein	-	0.04	-
gi|72080653|ref|YP_287711.1|	ABC transporter ATP-binding protein	-	0.04	-
gi|72080383|ref|YP_287441.1|	GTP-binding protein Obg	-	0.07	-
gi|72080361|ref|YP_287419.1|	ABC transporter ATP-binding protein	-	0.08	-
gi|72080365|ref|YP_287423.1|	ABC transporter ATP-binding protein	-	0.08	-
gi|72080644|ref|YP_287702.1|	ABC transporter ATP-binding protein	-	0.08	-
gi|72080574|ref|YP_287632.1|	myo-inositol 2-dehydrogenase	-	0.08	-
gi|72080434|ref|YP_287492.1|	hypothetical protein MHP7448_0092	-	0.03	-
gi|72080692|ref|YP_287750.1|	hypothetical protein MHP7448_0356	-	0.05	-
gi|72080687|ref|YP_287745.1|	hypothetical protein MHP7448_0351	-	0.06	-
gi|72080758|ref|YP_287816.1|	hypothetical protein MHP7448_0425	-	0.11	-
gi|72080628|ref|YP_287686.1|	hypothetical protein MHP7448_0289	-	0.23	-
gi|72080489|ref|YP_287547.1|	hypothetical protein MHP7448_0150	-	0.24	-
Proteins identified only in strain 7422				
gi|72080795|ref|YP_287853.1|	hypothetical protein MHP7448_0463	-	-	0.04
gi|72080974|ref|YP_288032.1|	leucyl-tRNA synthetase	-	-	0.04
gi|72080441|ref|YP_287499.1|	outer membrane protein-P95	-	-	0.04
gi|72080952|ref|YP_288010.1|	ABC transporter ATP-binding protein - Pr2	-	-	0.05
gi|72080945|ref|YP_288003.1|	DNA-directed RNA polymerase beta subunit	-	-	0.05
gi|72080987|ref|YP_288045.1|	hypothetical protein MHP7448_0660	-	-	0.05
gi|72080833|ref|YP_287891.1|	oligopeptide ABC transporter ATP binding protein	-	-	0.05
gi|72080447|ref|YP_287505.1|	protein P102	-	-	0.06
gi|72080401|ref|YP_287459.1|	DNA primase	-	-	0.07
gi|72080370|ref|YP_287428.1|	glutamyl-tRNA amidotransferase subunit A	-	-	0.07
gi|72080682|ref|YP_287740.1|	hypothetical protein MHP7448_0346	-	-	0.07
gi|72080832|ref|YP_287890.1|	oligopeptide ABC transporter ATP-binding protein	-	-	0.08
gi|72080801|ref|YP_287859.1|	ABC transporter atp-binding protein	-	-	0.09
gi|72080959|ref|YP_288017.1|	segregation and condensation protein A	-	-	0.12
gi|72080539|ref|YP_287597.1|	hypothetical protein MHP7448_0200	-	-	0.13
gi|72080347|ref|YP_287405.1|	MgpA like-protein	-	-	0.13
gi|72080495|ref|YP_287553.1|	GTP-binding protein	-	-	0.15
gi|72080363|ref|YP_287421.1|	ABC transporter ATP-binding protein	-	-	0.19
gi|72080643|ref|YP_287701.1|	ABC transporter ATP-binding protein	-	-	0.19
gi|72080492|ref|YP_287550.1|	guanylate kinase	-	-	0.22
gi|72080582|ref|YP_287640.1|	aspartyl-tRNA synthetase	-	-	0.24
gi|72080618|ref|YP_287676.1|	transcriptional regulator	-	-	0.34
gi|72080597|ref|YP_287655.1|	recombination protein RecR	-	-	0.49
gi|72080918|ref|YP_287976.1|	tryptophanyl-tRNA synthetase	-	-	0.63
gi|72080650|ref|YP_287708.1|	glycine cleavage system H protein	-	-	0.68
gi|72080860|ref|YP_287918.1|	methionine sulfoxide reductase B	-	-	1.44
Proteins identified in strains J and 7448				
gi|71893589|ref|YP_279035.1|	protein-export membrane protein SecD	0.05	0.05	-
gi|72080556|ref|YP_287614.1|	lipoprotein	0.23	0.05	-
gi|71893954|ref|YP_279400.1|	inorganic pyrophosphatase	0.15	0.15	-
gi|71893827|ref|YP_279273.1|	ATP synthase subunit A	0.17	0.17	-
gi|72080899|ref|YP_287957.1|	dihydrolipoamide dehydrogenase	0.84	0.50	-

gi|71893838|ref|YP_279284.1|	phosphoglycerate kinase	0.33	0.15	-
gi|71893387|ref|YP_278833.1|	isoleucyl-tRNA synthetase	0.03	0.08	-
gi|71893599|ref|YP_279045.1|	seryl-tRNA synthetase	0.10	0.10	-
gi|71893643|ref|YP_279089.1|	30S ribosomal protein S6	0.24	0.11	-
gi|71893528|ref|YP_278974.1|	50S ribosomal protein L15	0.52	0.31	-
gi|71893769|ref|YP_279215.1|	Holliday junction DNA helicase motor protein	0.13	0.19	-
gi|71894003|ref|YP_279449.1|	glucose-inhibited division protein B	0.14	0.14	-
gi|71893457|ref|YP_278903.1|	ATP-dependent protease binding protein	0.12	0.12	-
gi|72080714|ref|YP_287772.1|	lipoprotein	1.61	0.05	-
gi|72080813|ref|YP_287871.1|	hypothetical protein MHP7448_0482	0.10	0.10	-
gi|72080929|ref|YP_287987.1|	hypothetical protein MHP7448_0601	0.10	0.10	-
gi|72080814|ref|YP_287872.1|	hypothetical protein MHP7448_0483	0.23	0.23	-
	Proteins identified in strains J and 7422			
gi|71893595|ref|YP_279041.1|	TRSE-like protein	0.03	-	0.03
gi|71893628|ref|YP_279074.1|	CTP synthetase	0.13	-	0.05
gi|71893614|ref|YP_279060.1|	DNA ligase	0.04	-	0.06
gi|71893934|ref|YP_279380.1|	translation initiation factor IF-2	0.10	-	0.07
gi|72080425|ref|YP_287483.1|	thymidine phosphorylase	0.17	-	0.07
gi|72080436|ref|YP_287494.1|	hypothetical protein MHP7448_0094	0.08	-	0.08
gi|72080554|ref|YP_287612.1|	oligopeptide ABC transporter ATP-binding protein	0.60	-	0.08
gi|72080487|ref|YP_287545.1|	hypothetical protein MHP7448_0148	0.14	-	0.10
gi|71893601|ref|YP_279047.1|	triacylglycerol lipase	0.16	-	0.11
gi|72080551|ref|YP_287609.1|	oligopeptide ABC transporter system permease	0.08	-	0.12
gi|71893879|ref|YP_279325.1|	deoxyribose-phosphate aldolase	0.29	-	0.14
gi|72080547|ref|YP_287605.1|	hydrolase of the HAD family	0.25	-	0.15
gi|71893458|ref|YP_278904.1|	triosephosphate isomerase	0.19	-	0.19
gi|71893999|ref|YP_279445.1|	50S ribosomal protein L13	0.31	-	0.31
gi|72080995|ref|YP_288053.1|	transcription elongation factor	0.19	-	0.41
gi|71893439|ref|YP_278885.1|	purine-nucleoside phosphorylase	0.63	-	0.47
gi|71893531|ref|YP_278977.1|	50S ribosomal protein L6	1.82	-	0.47
gi|71893968|ref|YP_279414.1|	50S ribosomal protein L7/L12	1.01	-	0.54
gi|72080469|ref|YP_287527.1|	aminopeptidase	0.42	-	0.59
gi|72080838|ref|YP_287896.1|	dihydrolipoamide dehydrogenase	0.15	-	0.90
gi|72080596|ref|YP_287654.1|	hypothetical protein MHP7448_0257	0.47	-	5.46
	Proteins identified in strains 7448 and 7422			
gi|72080690|ref|YP_287748.1|	HIT-like protein	-	0.27	0.26
gi|72080573|ref|YP_287631.1|	periplasmic sugar-binding proteins	-	0.16	0.37
gi|72080540|ref|YP_287598.1|	alanine--tRNA ligase	-	0.03	0.03
gi|72080419|ref|YP_287477.1|	30S ribosomal protein S12	-	0.22	0.22
gi|72080397|ref|YP_287455.1|	30S ribosomal protein S2	-	0.31	1.79
gi|72080379|ref|YP_287437.1|	VACB-like ribonuclease II	-	0.06	0.16
gi|72080507|ref|YP_287565.1|	DNA-directed RNA polymerase alpha subunit	-	0.22	0.72
gi|72080909|ref|YP_287967.1|	5'-3' exonuclease	-	0.24	0.14
gi|72080726|ref|YP_287784.1|	S-adenosyl-methyltransferase	-	0.10	0.10
gi|72080428|ref|YP_287486.1|	preprotein translocase SecA subunit	-	0.04	0.09
gi|72080475|ref|YP_287533.1|	hexosephosphate transport protein	-	0.09	0.19
gi|72080348|ref|YP_287406.1|	DHH family phosphoesterase	-	0.09	0.09
gi|72080406|ref|YP_287464.1|	hypothetical protein MHP7448_0064	-	0.02	0.02

gi|72080703|ref|YP_287761.1|	lipoprotein	-	0.09	0.04
gi|72080461|ref|YP_287519.1|	hypothetical protein MHP7448_0121	-	0.13	0.13
gi|72080525|ref|YP_287583.1|	50S ribosomal protein L29	-	0.36	0.62
	Proteins identified in all strains			
gi|71893408|ref|YP_278854.1|	ATP synthase subunit B	0.33	0.22	0.22
gi|71893521|ref|YP_278967.1|	30S ribosomal protein S11	0.59	0.23	0.59
gi|71893522|ref|YP_278968.1|	30S ribosomal protein S13	0.47	0.47	0.21
gi|71893532|ref|YP_278978.1|	30S ribosomal protein S8	0.23	0.52	0.23
gi|71893998|ref|YP_279444.1|	30S ribosomal protein S9	1.07	0.36	0.35
gi|71893787|ref|YP_279233.1|	3-hexulose-6-phosphate synthase	0.73	0.33	0.28
gi|72080844|ref|YP_287902.1|	46K surface antigen precursor	3.55	2.92	4.11
gi|71893807|ref|YP_279253.1|	50S ribosomal protein L1	0.87	0.19	0.83
gi|71893808|ref|YP_279254.1|	50S ribosomal protein L11	0.76	0.31	0.92
gi|71893543|ref|YP_278989.1|	50S ribosomal protein L2	0.66	0.22	0.84
gi|71893484|ref|YP_278930.1|	50S ribosomal protein L21	1.57	0.48	0.48
gi|71893541|ref|YP_278987.1|	50S ribosomal protein L22	0.40	0.16	0.58
gi|71893535|ref|YP_278981.1|	50S ribosomal protein L24	0.47	0.99	1.52
gi|71893485|ref|YP_278931.1|	50S ribosomal protein L27	0.61	0.33	1.34
gi|71893546|ref|YP_278992.1|	50S ribosomal protein L3	0.44	0.61	0.36
gi|71893545|ref|YP_278991.1|	50S ribosomal protein L4	0.79	0.62	0.42
gi|71893534|ref|YP_278980.1|	50S ribosomal protein L5	0.61	1.57	1.24
gi|72080957|ref|YP_288015.1|	5'-nucleotidase precursor	0.20	0.12	0.07
gi|71893464|ref|YP_278910.1|	6-phosphofructokinase	0.09	0.09	0.09
gi|71893955|ref|YP_279401.1|	ABC transporter xylose-binding lipoprotein	2.32	0.70	1.98
gi|71893856|ref|YP_279302.1|	acetate kinase	0.54	1.01	2.45
gi|71893803|ref|YP_279249.1|	acyl carrier protein phosphodiesterase	0.30	0.15	0.37
gi|71893467|ref|YP_278913.1|	adenine phosphoribosyltransferase	9.76	8.65	9.43
gi|72080990|ref|YP_288048.1|	adhesin like-protein P146	1.15	0.82	0.73
gi|71893765|ref|YP_279211.1|	asparaginyl-tRNA synthetase	0.15	0.25	0.06
gi|72080920|ref|YP_287978.1|	ATP binding protein	0.10	0.12	0.23
gi|71893767|ref|YP_279213.1|	ATP-dependent helicase PcrA	0.13	0.05	0.13
gi|71893426|ref|YP_278872.1|	bacterial nucleoid DNA-binding protein	4.33	1.60	0.83
gi|71893558|ref|YP_279004.1|	cell division protein	0.21	0.28	0.65
gi|71893854|ref|YP_279300.1|	dihydrolipoamide acetyltransferase	1.19	1.27	0.73
gi|71893880|ref|YP_279326.1|	DNA gyrase subunit A	0.12	0.03	0.03
gi|71893463|ref|YP_278909.1|	DNA gyrase subunit B	0.05	0.05	0.05
gi|71893966|ref|YP_279412.1|	DNA-directed RNA polymerase beta' subunit	0.04	0.02	0.04
gi|72080417|ref|YP_287475.1|	elongation factor EF-2	0.29	0.48	0.51
gi|71893411|ref|YP_278857.1|	elongation factor Ts	0.10	0.14	0.14
gi|71893875|ref|YP_279321.1|	elongation factor Tu	6.31	5.07	8.40
gi|71893373|ref|YP_278819.1|	fructose-bisphosphate aldolase	0.99	0.51	1.01
gi|71893883|ref|YP_279329.1|	glucose-6-phosphate isomerase	0.10	0.10	0.10
gi|71893362|ref|YP_278808.1|	glucose-inhibited division protein A	0.07	0.12	0.10
gi|71893390|ref|YP_278836.1|	glyceraldehyde 3-phosphate dehydrogenase	2.24	1.83	1.78
gi|71893876|ref|YP_279322.1|	heat shock ATP-dependent protease	0.26	0.19	0.34
gi|72080353|ref|YP_287411.1|	heat shock protein	1.93	0.28	1.24
gi|72080351|ref|YP_287409.1|	hypothetical protein MHP7448_0009	0.42	0.15	0.19
gi|72080477|ref|YP_287535.1|	hypothetical protein MHP7448_0138	0.03	0.17	0.08

gi|72080583|ref|YP_287641.1|	hypothetical protein MHP7448_0244	0.19	0.19	0.19
gi|72080591|ref|YP_287649.1|	hypothetical protein MHP7448_0252	0.51	0.23	0.23
gi|72080635|ref|YP_287693.1|	hypothetical protein MHP7448_0297	0.07	0.07	0.07
gi|72080688|ref|YP_287746.1|	hypothetical protein MHP7448_0352	0.06	0.10	0.06
gi|72080709|ref|YP_287767.1|	hypothetical protein MHP7448_0373	0.51	0.59	0.45
gi|72080713|ref|YP_287771.1|	hypothetical protein MHP7448_0377	5.74	2.34	6.21
gi|72080725|ref|YP_287783.1|	hypothetical protein MHP7448_0391	0.20	0.20	0.20
gi|72080798|ref|YP_287856.1|	hypothetical protein MHP7448_0466	0.27	0.27	0.11
gi|72080820|ref|YP_287878.1|	hypothetical protein MHP7448_0489	0.20	0.15	0.20
gi|72080989|ref|YP_288047.1|	hypothetical protein MHP7448_0662	1.10	0.87	0.42
gi|71893610|ref|YP_279056.1|	hypoxanthine-guanine phosphoribosyltransferase	0.61	0.16	0.25
gi|71893812|ref|YP_279258.1|	leucyl aminopeptidase	0.06	0.07	0.13
gi|72080473|ref|YP_287531.1|	lipase-esterase	0.27	0.27	0.16
gi|71893602|ref|YP_279048.1|	lipoate-protein ligase A	0.18	0.08	0.18
gi|72080702|ref|YP_287760.1|	lipoprotein	0.47	0.26	0.93
gi|72080949|ref|YP_288007.1|	lipoprotein	0.04	0.04	0.15
gi|71893489|ref|YP_278935.1|	L-lactate dehydrogenase	1.81	2.21	4.06
gi|72080708|ref|YP_287766.1|	Lppt protein	0.58	0.27	0.07
gi|71893559|ref|YP_279005.1|	lysyl-tRNA synthetase	0.18	0.08	0.08
gi|72080825|ref|YP_287883.1|	mannose-6-phosphate isomerase	0.34	0.19	0.33
gi|72080564|ref|YP_287622.1|	methylmalonate-semialdehyde dehydrogenase	0.15	0.80	1.41
gi|72080409|ref|YP_287467.1|	molecular chaperone DnaK	4.08	3.37	1.54
gi|71893579|ref|YP_279025.1|	myo-inositol catabolism protein	0.06	0.13	0.25
gi|71893437|ref|YP_278883.1|	NADH oxidase	4.49	2.28	9.77
gi|71893873|ref|YP_279319.1|	oligoendopeptidase F	0.30	0.23	0.41
gi|71893844|ref|YP_279290.1|	P216 surface protein	2.65	1.55	2.25
gi|72080689|ref|YP_287747.1|	P60-like lipoprotein	0.19	0.08	0.27
gi|72080828|ref|YP_287886.1|	p76 membrane protein precursor	2.80	1.62	1.93
gi|71893622|ref|YP_279068.1|	phenylalanyl-tRNA synthetase beta subunit	0.16	0.08	0.06
gi|71893857|ref|YP_279303.1|	phosphate acetyltransferase	5.59	1.58	4.18
gi|71893820|ref|YP_279266.1|	phosphoenolpyruvate-protein phosphotransferase	0.65	0.07	0.43
gi|71893944|ref|YP_279390.1|	phosphoglycero mutase	0.14	0.14	0.12
gi|71893513|ref|YP_278959.1|	phosphopentomutase	0.11	0.07	0.07
gi|71893598|ref|YP_279044.1|	phosphopyruvate hydratase	0.70	0.48	0.53
gi|72080983|ref|YP_288041.1|	prolipoprotein p65	4.04	1.99	3.55
gi|72080538|ref|YP_287596.1|	protein P102	1.26	0.91	0.84
gi|72080448|ref|YP_287506.1|	protein P97	0.19	1.97	1.81
gi|72080537|ref|YP_287595.1|	protein P97	0.22	0.25	0.42
gi|72080611|ref|YP_287669.1|	protein P97-like	0.04	0.03	0.04
gi|72080711|ref|YP_287769.1|	PTS system enzyme IIB component	1.36	0.54	2.61
gi|72080456|ref|YP_287514.1|	pyruvate dehydrogenase	18.34	10.49	37.38
gi|72080455|ref|YP_287513.1|	pyruvate dehydrogenase E1-alpha subunit	6.60	7.50	15.18
gi|71893479|ref|YP_278925.1|	pyruvate kinase	0.47	0.09	0.47
gi|71893571|ref|YP_279017.1|	ribonucleotide-diphosphate reductase alpha subunit	0.20	0.04	0.06
gi|72080562|ref|YP_287620.1|	ribonucleotide-diphosphate reductase beta subunit	0.12	0.12	0.86
gi|71893886|ref|YP_279332.1|	ribosome recycling factor	0.74	0.57	1.13
gi|71893956|ref|YP_279402.1|	sugar ABC transporter ATP-binding protein	0.06	0.06	0.06
gi|71893452|ref|YP_278898.1|	thiol peroxidase	1.29	1.01	0.18

gi|71893733|ref|YP_279179.1|	thioredoxin	3.41	5.27	7.41
gi|71893454|ref|YP_278900.1|	thioredoxin reductase	0.32	0.46	0.73
gi|71893777|ref|YP_279223.1|	transketolase	0.40	0.15	0.60
gi|71893501|ref|YP_278947.1|	trigger factor	0.49	0.60	1.02

[1] CDS access number in the NCBI database (http://www.ncbi.nlm.nih.gov).
[2] Protein identification according to NCBI database (http://www.ncbi.nlm.nih.gov).
[3] The exponentially modified protein abundance index value (emPAI) is the transformed ratio of the number of experimentally observed peptides to the total number of peptides calculated by MASCOT software (Matrix Science, London, UK). The proteins with a difference of two fold or more in emPAI (marked in gray) were considered differentially expressed.

ANEXO II

Artigo "Swine and poultry pathogens: the complete genome sequences of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae*" de autoria de Vasconcelos, A.T., Ferreira, H.B., Bizarro, C.V., Bonatto, S.L., Carvalho, M.O., **Pinto, P.M.**, *et al.*, publicado no periódico *Journal of Bacteriology*, v. 187, pp. 5568-5577 no ano de 2005.

Este artigo foi um esforço do consórcio nacional brasileiro de seqüenciamento de genomas e da rede sul de análises de genomas. Este artigo descreve o genoma de uma cepa de *M. synoviae* e duas cepas de *M. hyopneumoniae*. Além desta descrição foi realizada a primeira análise genômica comparativa de genomas de *Mycoplasma* spp.

A minha participação foi na construção de bibliotecas genômicas para o fechamento do genoma, anotação e re-anotação das CDSs dos três genomas e na redação de relatórios para a redação do manuscrito.

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Swine and Poultry Pathogens: the Complete Genome Sequences of Two Strains of *Mycoplasma hyopneumoniae* and a Strain of *Mycoplasma synoviae*†

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This work reports the results of analyses of three complete mycoplasma genomes, a pathogenic (7448) and a nonpathogenic (J) strain of the swine pathogen *Mycoplasma hyopneumoniae* and a strain of the avian pathogen *Mycoplasma synoviae*; the genome sizes of the three strains were 920,079 bp, 897,405 bp, and 799,476 bp, respectively. These genomes were compared with other sequenced mycoplasma genomes reported in the literature to examine several aspects of mycoplasma evolution. Strain-specific regions, including integrative and conjugal elements, and genome rearrangements and alterations in adhesin sequences were observed in the *M. hyopneumoniae* strains, and all of these were potentially related to pathogenicity. Genomic comparisons

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† Supplemental material for this article may be found at http://jb.asm.org/.

revealed that reduction in genome size implied loss of redundant metabolic pathways, with maintenance of alternative routes in different species. Horizontal gene transfer was consistently observed between *M. synoviae* and *Mycoplasma gallisepticum*. Our analyses indicated a likely transfer event of hemagglutinin-coding DNA sequences from *M. gallisepticum* to *M. synoviae*.

Mycoplasmas comprise a group of more than 180 species of wall-less bacteria that are obligate parasites of a wide range of organisms including humans, plants, and animals (46). Mycoplasmas typically exhibit strict host tissue specificities, probably due to their nutritional requirements (45), a direct consequence of the genome reduction that likely occurred as a consequence of the metabolic complementarity of their hosts (3). The evolutionary dynamics of these organisms involved population bottlenecks and asexual reproduction leading to accumulation of deleterious mutations, which resulted in further genome contraction (58). A predictable consequence of this process is preservation of a minimal genome comprising essential genes to maintain basic core functions and adaptation to specific environments.

Two species, *Mycoplasma hyopneumoniae* and *Mycoplasma synoviae*, have a significant adverse economic impact on animal production. The former is the infective agent of enzootic pneumonia in pigs, which results in deactivation of mucociliary functions (15) and increased susceptibility to secondary infections (12). The latter is responsible for respiratory tract disease and synovitis in chickens and turkeys. It can be transmitted vertically through contaminated eggs (28), resulting in considerable losses due to reduced egg production and meat quality as well as a lowered rate of viable hatchings. Thus, knowledge of their respective biological characteristics seems of paramount importance.

The genomes of several mycoplasmas have been sequenced and analyzed in recent years (11, 22, 25–27, 35, 44, 50, 61), but comparative analyses of species belonging to the Pneumoniae and Hominis clades have not been undertaken. In addition, interstrain, whole-genome comparisons have not yet been carried out, although the genes involved in DNA repair, including those of the organisms herein studied, have recently been analyzed (10).

Here we report the complete genome sequences of a pathogenic (7448) and a nonpathogenic (J [ATCC 25934]) strain of *M. hyopneumoniae* and the complete genome of *M. synoviae* strain 53. Comparative analyses of the *M. hyopneumoniae* strains allowed the identification of strain-specific regions that might be related to their variable pathogenicity. A detailed phylogenetic analysis of several mycoplasma species belonging to the Pneumoniae and Hominis clades was also carried out, comparing metabolic pathways and genes involved in the adhesion process. Comparisons of *M. gallisepticum* and *M. synoviae* genomes pointed to the evolutionary origin of the hemagglutinin gene family and showed evidence of horizontal transfer of other gene clusters.

MATERIALS AND METHODS

Bacterial strains. *M. hyopneumoniae* strain J (ATCC 25934) was acquired from American Type Culture Collection by CNPSA, EMBRAPA (Concórdia, Santa Catarina, Brazil). This is a nonpathogenic strain with a reduced adhesion capacity to porcine cilia (62–64). *M. hyopneumoniae* strain 7448 was isolated from an infected swine in Lindóia do Sul, Santa Catarina, Brazil. Specific-pathogen-free pigs inoculated with strain 7448 consistently produced the characteristic symptoms of enzootic pneumonia. *M. synoviae* was isolated from a broiler breeder in the state of Paraná in Brazil (19).

Genome sequencing, assembling, and annotation. Genomes were sequenced using the shotgun sequencing strategy (20). Sequencing, assembling, annotation, and comparative in silico analyses were carried out by the Brazilian National Genome Sequencing Consortium and the Southern Network for Genome Analysis (PIGS), involving a total of 28 sequencing laboratories, one bioinformatics center, and three coordinating laboratories. Template preparation was performed using standard protocols. DNA sequencing reactions were performed using the DYEnamic ET dye terminator cycle sequencing (MegaBACE) kit and run on MegaBACE 1000 capillary sequencers (Amersham Biosciences). Approximately 10,000 reads per genome with phred scores of >20 were generated from both ends of plasmid clones ranging from 2.0 to 4.0 kb, providing an approximately 13-fold genome coverage. Sequences were assembled using phred/phrap/consed (http://www.phrap.org). Sequencing gaps were closed using the information generated by autofinisher, while our recently developed strategy of PCR-assisted contig extension (PACE) (8) was used for physical gap closure. Annotation was carried out using the System for Automated Bacterial Integrated Annotation (SABIÁ) (2), developed to integrate public-domain and purpose-built software for the automated identification of genome landmarks, including tRNA and rRNA sequences, repetitive elements, and coding DNA sequences (CDSs) (which indicate regions likely to encode proteins). Paralogous gene families were defined using a cutoff E value of 10^{-5} with at least 60% query coverage and 50% identity.

Phylogenetic reconstructions and comparative analyses. Maximum likelihood (ML) phylogenies, based on individual orthologous proteins, were generated using ProtML (Molphy package [http://www.ism.ac.jp/ismlib/softother.e.html]) and TREE-PUZZLE 5.1 (51). A data set concatenating all proteins in a single sequence unit was analyzed using neighbor-joining (NJ) distance trees with MEGA 2.1 (30), maximum parsimony using PAUP* 4.0b10 (55), and by ML using ProtML, all with confidence estimates based on 100 bootstrap replicates. A bootstrap gene tree was calculated following 500 random, protein resamplings and concatenation, with subsequent analysis by NJ based on ML distances using the Molphy package. Divergence times were estimated by the linearized tree method using MEGA 2.1 and r8s 1.6 (http://ginger.ucdavis.edu/r8s), assuming 450 million years before the present (MYBP) as the time of divergence of the phytoplasmas from mycoplasmas (32). Orthologous clusters were identified using the bidirectional best hit method (43). Clustering of hemagglutinin CDSs was performed with Tribe-MCL (18), based on data from allXall National Center for Biotechnology Information (NCBI) BLASTp searches. Global genome alignments were carried out using Mauve (13). Genome rearrangements between *M. hyopneumoniae* strains J, 7448, and 232 were identified by combined analyses with GRIL (14) and Artemis (49). Genome duplications were inferred from self-BLAST searches. Visualization of local similarities between CDSs of complete mollicute genomes was carried out with PhyloGrapher (www.atgc.org/PhyloGrapher). Horizontal gene transfer (HGT) was initially detected with allXall BLAST searches in available mollicute genomes, supplemented by a compositional and codon bias scan. Further alignment was carried out with Mauve for genomes with best hits in the initial search. Phylogenetic distances between regions sharing at least 300 nucleotides, with Mauve alignment, were identified in each genome and were compared against distance estimates based on 16S rRNA sequence data. An HGT event was considered plausible when the estimated distance of the aligned region was lower than the estimated 16S rRNA distance.

IGR analysis. All intergenic regions (IGRs) were extracted from the genome data of *Mycoplasma hyopneumoniae* strains J and 7448 and compared using MUMmer. The search for putative regulatory signals upstream of *M. hyopneumoniae* CDSs employed two different methodologies. Initially, a clusterization of the upstream region of *M. hyopneumoniae* genes was carried out (first 50 bases upstream of the translation starting point), on the basis of similarity, and employing the BLASTCLUST software (ftp://ftp.ncbi.nlm.nih.gov/blast/). Sequences were subsequently submitted to analysis with GLAM software (http://zlab.bu.edu/glam/), aiming to find conserved patterns among the initially clusterized sequences. A second strategy involved search of fuzzy motifs with the Self-Organizing Map, a neural-network algorithm generated by SOMBRERO software (http://bioinf.nuigalway.ie/sombrero/index.html).

TABLE 1. General characteristics of the genomes of *M. hyopneumoniae* strains J and 7448 and *M. synoviae*[a]

Characteristic	Mhy-J	Mhy-P	Msy
Total length (base pairs)	897,405	920,079	799,476
G+C content (%)	28	28	28
Total no. of CDSs	679	681	694
Genome constituting coding regions (%)	88	88	91
Average CDS length (base pairs)	1,178	1,190	1,058
No. of known proteins	412	421	464
No. of conserved hypothetical proteins	109	105	167
No. of hypothetical proteins	158	155	63
No. of rRNAs			
16S	1	1	2
23S	1	1	2
5S	1	1	3
No. of tRNAs	30	30	34
No. of insertion sequences[b]			
IS*3*	2	0	0
tMH	(4)	2 (8)	0
ISMhp1	14	11	13

[a] Abbreviations: Mhy-J, *M. hyopneumoniae* strain J; Mhy-P, *M. hyopneumoniae* strain 7448; Msy, *M. synoviae*.

[b] Number of copies of different insertion sequence families. The number of defective copies is shown in parentheses.

Nucleotide sequence accession numbers. Sequence data reported in this paper were deposited in GenBank (accession nos. AE017243, AE017244, and AE017245). Sequence and annotation data are available at http://www.brgene.lncc.br/finalMS (*M. synoviae*), http://www.genesul.lncc.br/finalMH (*M. hyopneumoniae* strain J), and http://www.genesul.lncc.br/finalMP (*M. hyopneumoniae* strain 7448).

RESULTS AND DISCUSSION

Features of the *M. hyopneumoniae* strain J, *M. hyopneumoniae* strain 7448, and *M. synoviae* genomes. The main features of the three newly sequenced genomes are shown in Table 1. Clustering analysis of *M. synoviae*, *M. hyopneumoniae* strains J, 7448, and 232 (35), and eight other mycoplasma genomes (*Mycoplasma pneumoniae* M129, *Mycoplasma pulmonis* UAB CTIP, *Mycoplasma penetrans* HF-2, *Mycoplasma genitalium* G37, *Mycoplasma gallisepticum* R, *Mycoplasma mycoides* subsp. *mycoides* SC PG1, *Mycoplasma mobile* 163K, and *Ureaplasma urealyticum* serovar 3) revealed 235 orthologous clusters. A comparison of the number of CDSs in all sequenced mycoplasma genomes is shown in Table 2.

Genome-specific regions and rearrangements in *M. hyopneumoniae* strains. Comparison of the three *M. hyopneumoniae* strains provided evidence of intraspecific rearrangements, resulting in strain-specific gene clusters (Fig. 1). This was the case for a 16-kb region of *M. hyopneumoniae* strain J, containing 15 CDSs, most of which encoded type III restriction-modification (R-M) system components and putative transposases. *M. hyopneumoniae* strain 7448 contained a specific 22.3-kb region similar to the integrative conjugal element (ICEF) of *Mycoplasma fermentans* (7), which was designated ICEH (for integrative conjugal element of *M. hyopneumoniae*). ICEH contained 14 CDSs, four of which similar to *tra* genes, usually associated with bacterial conjugative plasmids, and another encoding a single-strand binding protein (SSB), an essential protein for the transfer process. Direct repeat sequences (TAGATTTTT), generated by target site duplications, flanked ICEH. This target site was localized in the homologous region of *M. hyopneumoniae* strain J, pointing to the mobility of this element. Evidences for the presence of circular extrachromosomal forms of ICEH in *M. hyopneumoniae* strain 7448 and another unrelated, pathogenic Brazilian field isolate of *M. hyopneumoniae* were obtained by an inverted PCR assay (results not shown), indicating that this element might be functionally active in these isolates. Moreover, we also observed a region similar to ICEF in the genome of *M. hyopneumoniae* strain 232. It has recently been demonstrated that some pathogenic bacteria use the type IV secretion system, composed of subunits related to the conjugation machinery, for the delivery of effector molecules to host cells (16), and that this system may be involved in pathogenesis (52). However, the involvement of ICEH in pathogenesis through delivery of effector molecules into cells remains to be explored. Other strain-specific differences included an inverted region of 243,104 bp in *M. hyopneumoniae* strain 232 (Fig. 1) and less drastic rearrangements between *M. hyopneumoniae* strains at five genomic regions (Fig. 1; see Tables S1 to S3 in the supplemental mate-

TABLE 2. Comparison of the total number of CDSs in all sequenced mycoplasma genomes and number of exclusive CDSs per species[a]

Organism[b]	GenBank accession no.	No. of CDSs				
		Total	Known	Conserved hypothetical	Hypothetical	Exclusive
Mhy-J	AE017243	679	412	109	158	67
Mhy-P	AE017244	681	421	105	155	53
Mhy-232	AE017332	691	327	227	137	59
Msy	AE017245	694	464	167	63	150
Mpu	NC002771	782	695	86	204	204
Mmo	NC006908	633	571	25	37	121
Mpn	NC000912	689	428	188	73	169
Mge	NC000908	484	318	164	2	8
Mga	NC004829	726	452	152	122	164
Uur	NC002162	614	323	107	184	164
Mpe	NC004432	1,037	647	190	200	433
Mmy	NC005364	1,016	833	26	157	491

[a] Data derived from the GenBank database.

[b] Abbreviations: Mhy, *Mycoplasma hyopneumoniae* (strains J, 7448 [P], and 232); Msy, *Mycoplasma synoviae*; Mge, *Mycoplasma genitalium*; Mpn, *Mycoplasma pneumoniae*; Mga, *Mycoplasma gallisepticum*; Mpu, *Mycoplasma pulmonis*; Mpe, *Mycoplasma penetrans*; Mmy, *Mycoplasma mycoides*; Mmo, *Mycoplasma mobile*; Uur, *Ureaplasma urealyticum*.

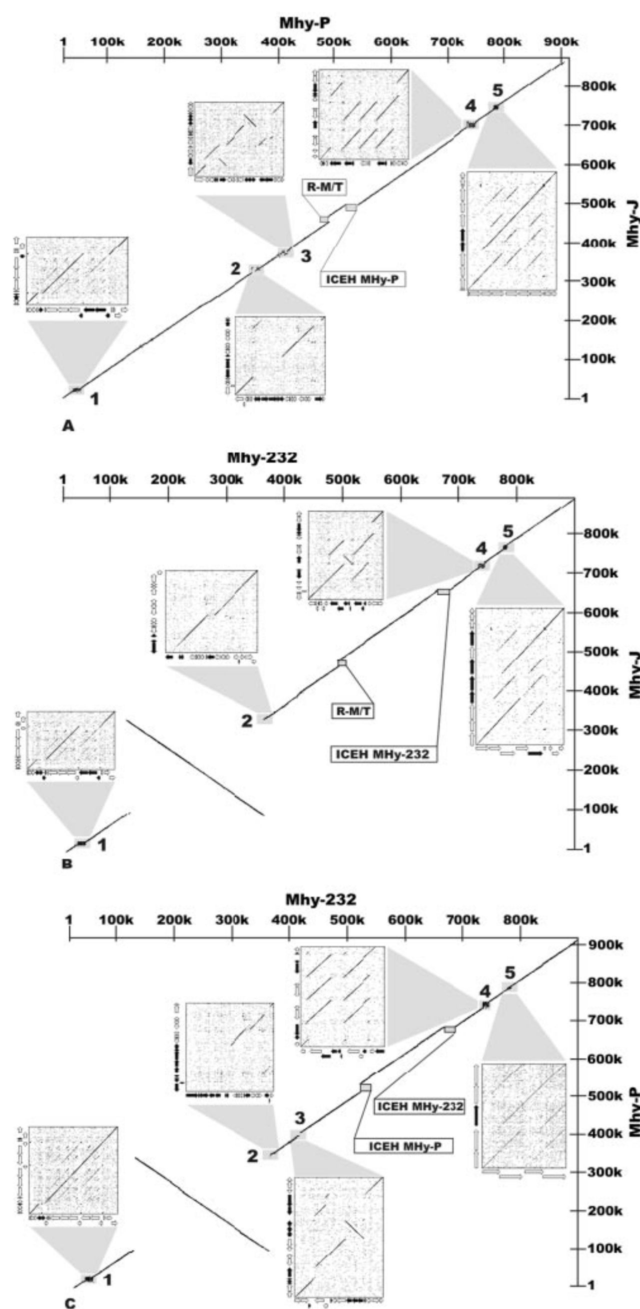

FIG. 1. Comparison of the genomes of *M. hyopneumoniae* strains J, 7448, and 232. Genome-specific regions (ICEH and R-M/T) and rearranged regions (regions 1 to 5) are indicated; CDSs located in these regions are described in Tables S1 to S3 in the supplemental material). CDSs that are not identical in two genomes or that have undergone rearrangements are represented by small black arrows. Mhy-J, *M. hyopneumoniae* strain J; Mhy-P, *M. hyopneumoniae* strain 7448; Mhy-232, *M. hyopneumoniae* strain 232. The axes show the scales, in kilobases.

rial). The positions of regions 1, 2, 4, and 5 involved in rearrangements are conserved in all three strains. Region 3 is syntenic in *M. hyopneumoniae* strains J and 232. In *M. hyopneumoniae* strain J, ABC transporter-encoding region 1 lacks two CDSs that are present in *M. hyopneumoniae* strains 7448 (MHP0023 and MHP0024) and 232 (mhp025 and mhp026), which probably originated by duplication followed by divergence. Region 2 is characterized, in the three strains, by the presence of several unique CDSs. This region also contains short translocations and duplications. Region 3 shows short unique insertions in *M. hyopneumoniae* strain 7448 in comparison to strain J or 232 and a duplication of approximately 2.2 kb containing a hypothetical CDS and a serine protease-encoding CDS. Probably, this duplication was followed by deletion of part of the serine protease sequence. A third rearrangement involved a translocation that displaced the DNA segment containing the complete or partial serine protease-encoding CDS by approximately 300 kb in the two strains. This region was syntenic in both *M. hyopneumoniae* strains J and 232. Region 4, in the three genomes, shows short unique insertions and translocations of CDS-containing segments, probably mediated by a transposable element, as indicated by the presence of an ISMhp1 transposon-like element in this region. Region 5 presents a series of imperfect duplications of hypothetical CDSs, generating seven copies in *M. hyopneumoniae* strain J, five in strain 7448, and up to four copies in strain 232.

A comparison of 362 orthologous IGRs of *M. hyopneumoniae* strains showed that 21 to 29% of them shared identical sequences, 43 to 45% differed by 1 to 5 bp, 13 to 15% by 6 to 10 bp, and 13 to 20% by more than 10 bp. Moreover, in 34% of them, repeated sequences varied in length (see Table S4 in the supplemental material), as was the case for CDSs encoding DnaJ, thymidine phosphorylase, RpoB, a p97-like protein, methyltransferase, serine protease, and p65. This variation has been reported in both intergenic and coding regions in several genomes (31, 33, 47) and is associated with polymerase slippage during replication (57). The intergenic regions identified in the *M. hyopneumoniae* genomes present a higher A+T content (about 80%) than coding sequences (about 70%). It has been recently shown, in 152 genomes, that AT content is higher in the 200 bp upstream of translation start codons than in the 200 bp downstream (56). Only a few promoters have been identified and analyzed in mycoplasmas (36, 59, 60), and these studies identified strong consensus sequences at −10 regions but only weak consensus sequences at −35 regions. We searched for putative regulatory signals upstream of *M. hyopneumoniae* CDSs and found 17 significant clusters, representing only a small fraction of the CDSs of the *M. hyopneumoniae* genome (70 CDSs). The physiological relevance of genomic rearrangements, nucleotide substitutions, and changes in the lengths of IGRs in *M. hyopneumoniae* remains to be elucidated.

Genomic comparisons between pathogenic (*M. hyopneumoniae* strains 7448 and 232) and nonpathogenic (*M. hyopneumoniae* strain J) strains revealed important aspects related to pathogenicity. Among previously described adhesion-related proteins (29, 48) (Table 3), p97 is regarded as the major cell adhesion determinant, although other proteins, derived from either a mycoplasma or host, might also participate in membrane anchorage (17). In the three strains, the p97 CDS is linked to p102 CDS, comprising a two-CDS operon (p97-p102 operon I), as well as two other operons that are clearly related (p97-p102 operons II and III) that exhibit >80% identity between orthologous deduced amino acid sequences. The p97 orthologous CDSs in the p97-p102 operon I of *M. hyopneu-*

TABLE 3. CDSs encoding adhesion-related proteins in different mycoplasma species or strains

Protein	Presence of CDS encoding protein in mycoplasma[a]												
	Hominis group						Pneumoniae group					Mmy	Other
	Mhy-J	Mhy-P	Mhy-232	Msy	Mpu	Mmo	Mpn	Mge	Mga	Mpe	Uur		
Tip organelle-related proteins													
P1, MgPa	●	●	●	●	●	●	●	●	●	●	●	●	● (Mpi)
GapA													
p30, p32							●	●	●				
Orf6, MgpC							●	●	●				
CrmA													
Hmw1							●	●	●	?	?		
Hmw2					●		●	●	?				
Hmw3							●	●					
Other adhesion-related proteins													
LppS	●	●	●		●								● (Mco)
LppT	●	●	●										●
p97	●	●	●										
p102	●	●	●										
p216	●	●	●										
p146	●	●	●										
p110 (yx1)	●	●	●	●					●				
p140/p110							●	●					
p76	●	●	●										
p29													● (Mfe)
p65 (Mpn)							●	●					
p65 (Mhy)	●	●	●	●		●					●		
p40													● (Mag)
p50													● (Mho)
p69								●					
Vaa				●				●	●			●	● (Mho)
PvpA									●				

[a] Presence of CDSs encoding proteins related to the tip organelle or other previously described adhesion-related proteins are indicated by dots. Question marks indicate putative orthologous CDSs with relatively low similarity (between 40 and 55% at the amino acid level). Species names are indicated in parentheses in the case of CDSs identified in mycoplasmas whose genomes had not been previously sequenced (Mag, *Mycoplasma agalactiae*; Mco, *Mycoplasma conjunctivae*; Mfe, *M. fermentas*; Mho, *Mycoplasma hominis*; Mpi, *Mycoplasma pirum*). Orthologs were identified by BLAST. Additional abbreviations of mycoplasma species described in Table 2, footnote *b*.

moniae strains 7448, 232, and J code for proteins with 10, 15, and 9 of the previously described R1 tandem repeats, respectively; all three strains had more than the minimum number of CDSs (8 CDSs) required for cilium binding (34). This indicates that other adhesion determinants, with different characteristics in *M. hyopneumoniae* strains 7448, 232, and J, must be responsible for their different adhesion properties. Differences between the encoded adhesins and other putative virulence factors in *M. hyopneumoniae* strains 7448, 232, and J include variations in the number of amino acid repeats between orthologous proteins (Ta-

TABLE 4. CDSs encoding proteins showing differences between *M. hyopneumoniae* strains J, 7448, and 232 with respect to repetitive amino acid sequences[a]

CDS product[b]	Mhy-J CDS	Mhy-P CDS	Mhy-232 CDS
Known adhesins			
p97 adhesin	MHJ0194	MHP0198	mhp183
p76 membrane protein precursor	MHJ0494	MHP0497	mhp494
p216 surface protein	MHJ0493	MHP0496	mhp493
p146 adhesin-like protein	MHJ0663	MHP0663	mhp684
Membrane or putative membrane proteins			
p95 outer membrane protein	MHJ0096	MHP0099	mhp280
Hypothetical protein	MHJ0441	MHP0443	mhp445
Hypothetical protein	MHJ0444	MHP0445	mhp447
Hypothetical protein	MHJ0350	MHP0355	mhp366
Hypothetical protein	MHJ0032	MHP0036	mhp037
Hypothetical protein	MHJ0662	MHP0662	mhp683
Hypothetical protein	MHJ0442	MHP0444	mhp446
Other			
Conserved hypothetical protein	MHJ0089	MHP0092	mhp287

[a] Abbreviations: Mhy-J, *M. hyopneumoniae* strain J; Mhy-P, *M. hyopneumoniae* strain 7448; Mhy-232, *M. hyopneumoniae* strain 232.
[b] The membrane character for hypothetical proteins was established by PSORT.

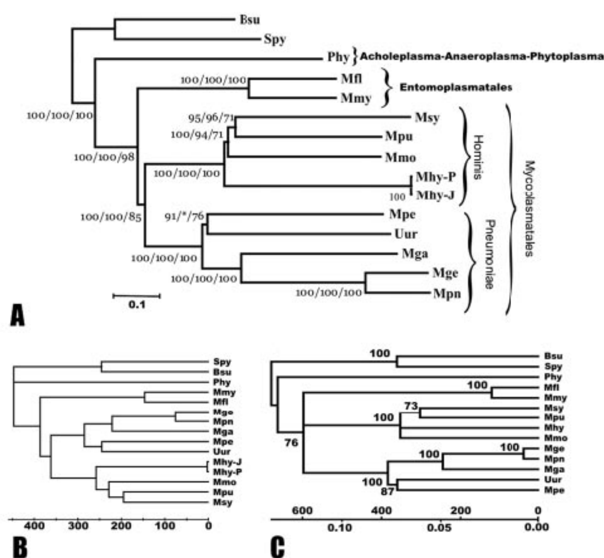

FIG. 2. Phylogenetic analysis of *Mollicutes*. (A) Phylogenetic tree of the *Mollicutes* based on the concatenated alignment of deduced amino acid sequences of 146 CDSs. Bootstrap support values (based on 100 replicates) are indicated near each node for neighbor joining with JTT distance, maximum parsimony, and TREE-PUZZLE maximum likelihood, respectively (the asterisk indicates an unconfirmed node). The main taxonomic groups within *Mollicutes* are shown on the right. The *Mollicutes* phytoplasma ("*Candidatus* Phytoplasma asteris" OY strain, AP006628) (Phy) from the Acholeplasma-Anaeroplasma-Phytoplasma clade, *Mesoplasma florum* (NC_006055) (Mfl) from the *Entomoplasmatales*, as well as *Bacillus subtilis* (NC000964) (Bsu) and *Streptococcus pyogenes* (NC002737) (Spy) were included as outgroups. (B) Phylogenetic tree with branch lengths proportional to divergence times based on a 146-CDS data set estimated with the r8s program. (C) Linearized tree based on 16S rRNA sequences estimated by NJ with Kimura's two-parameter distance estimate and 100 bootstrap replicates. Divergence times were estimated assuming a divergence rate of 1% per 50 million years. Differences between estimates were due to the different calibration methods. The abbreviations of the mycoplasma species are explained in Table 2, footnote *b*.

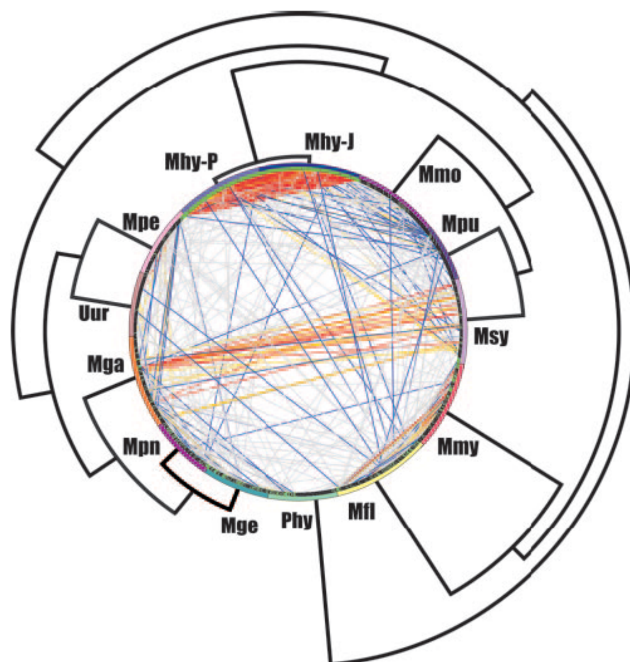

FIG. 3. Similarity relationships between 12 *Mollicute* genomes. Connecting lines inside the circle denote BLAST matches between nodes, with each node representing a protein sequence. Connected nodes are shown in green. The color of the connecting line indicates the degree of similarity between nodes as follows: red, a score between 0.9 and 1.0; yellow, a score between 0.8 and 0.9; blue, a score between 0.7 to 0.8; and light gray, a score of <0.7 (PhyloGrapher software). The order of nodes, or protein sequences, in the graph was arranged according to the topology (shown outside the circle) grouping the 12 mollicute genomes. The abbreviations of the mycoplasma species are explained in Table 2, footnote *b*. Phy, Phytoplasma.

ble 4). These insertions/deletions result from variations in the number of tandem nucleotide repeats within coding regions, which is indicative of a molecular mechanism generating functional and/or antigenic variants. This variation in surface proteins is likely to be a key determinant of different pathogenic properties of each *M. hyopneumoniae* strain.

Phylogenetic reconstructions. Phylogenetic relationships were established on the basis of a maximum of 206 single-copy CDSs (comprising ~86,000 aligned deduced amino acid positions). The results of phylogenetic analyses based on individual orthologous proteins were subsequently compared to the ML, maximum parsimony, and distance topologies based on the concatenated amino acid sequence alignment of a single sequence unit. In contrast to the highly incongruent topologies resulting from separate analyses of individual orthologous CDSs, the concatenated alignment generated a single, highly supported tree (Fig. 2A). Phylogenies were also estimated with nucleotide data using a wide variety of methods, based on either gene content or gene order. Only the tree generated with the concatenated protein sequence unit was presented, as all methods gave essentially the same results. A tentative time frame of genome evolution was estimated (Fig. 2B and C), indicating that the *Mycoplasmatales* and *Entomoplasmatales* orders split into three clades between 600 to 400 MYBP, while most of the species diverged some 400 to 300 MYBP, approximately at the time of the emergence and diversification of tetrapods (4). *M. pneumoniae* and *M. genitalium* diverged more recently, between 75 to 35 MYBP, and *M. hyopneumoniae* strains J and 7448 diverged about 2 MYBP.

HGT. We analyzed the possibility of HGT in view of its putative role in determining the characteristics of prokaryotic genomes (41) and the transfer of genes related to pathogenesis (21). A comparison of mollicute genomes using both parametric and phylogenetic strategies showed that HGT was most likely to have occurred between *M. synoviae* and *M. gallisepticum* (Fig. 3). Fourteen putative transferred regions were identified (Table 5), the largest comprising 5.9 kb and encompassing not only hypothetical CDSs but also CDSs coding for an ABC transporter, a signal peptidase I, and a putative EF-G elongation factor. This region was almost identical in both genomes, indicating a recent transfer event. Additionally, another region containing a relevant, pathogenicity-related CDS (coding for a putative sialidase) might also have been horizontally transferred (Table 5). The presence of a putative sialidase in both genomes is noteworthy, since this enzyme had been identified only in *Mycoplasma alligatoris* elsewhere among the

TABLE 5. Horizontally transferred regions between *M. synoviae* and *M. gallisepticum*

Species and region	Region			Product
	Start	End	Length	
M. synoviae				
1	41155	47098	5943	ABC transporter, signal peptidase I, elongation factor EF-G, conserved hypothetical proteins
2	80242	80947	705	Transposase
3	185374	186994	1620	Conserved hypothetical protein
4	221238	224926	3688	Putative sialidase, glyceraldehyde 3-phosphate dehydrogenase
5	311024	311541	517	Transposase
6	320763	325527	4764	Conserved hypothetical proteins
7	533311	534827	1516	Conserved hypothetical proteins (compositional bias)
8	535709	537101	1392	Conserved hypothetical proteins (compositional bias)
9	537179	537545	366	Conserved hypothetical proteins (compositional bias)
10	546230	547733	1503	Conserved hypothetical proteins (compositional bias)
11	549723	554763	5040	Conserved hypothetical proteins (compositional bias)
12	693080	693615	535	Conserved hypothetical protein (transposase)
13	693616	695386	1770	Conserved hypothetical proteins (transposase)
14				Hemagglutinin cluster (codon bias)[a]
Total			29359 (3.21)[b]	
M. gallisepticum				
1	356997	362986	5989	ABC transporter, signal peptidase I, elongation factor EF-G, conserved hypothetical proteins
2	372501	373205	704	Transposase
3	370747	372370	1623	Conserved hypothetical protein
4	771294	774961	3667	Putative sialidase, glyceraldehyde 3-phosphate dehydrogenase
5	400149	400682	533	Transposase
6	365139	369905	4766	Conserved hypothetical proteins
7	969572	971087	1515	Conserved hypothetical proteins
8	951348	952740	1392	Conserved hypothetical proteins
9	950904	951270	366	Conserved hypothetical proteins
10	947932	949436	1504	Conserved hypothetical proteins
11	349890	354930	5040	Conserved hypothetical proteins
12	398943	399492	549	Conserved hypothetical protein (transposase)
13	401897	403633	1736	Conserved hypothetical protein (transposase)
14				Three hemagglutinin CDSs[a]
Total			29384 (2.58)	

[a] Due to the large extent of recombination in hemagglutinin clusters, a single region of horizontal transfer could not be identified. However, region 14 in this study contains CDSs listed in Table 1 at http://www.brgene.ncc.br/finalMS/table1 as cluster number 2.

[b] The genome percentage is shown in parentheses after the total length.

mycoplasmas (6). Sialidase cleaves terminal sialic acid residues from sialoglycoconjugates, generating free sialic acid (a likely nutrient). The *M. synoviae* genome region (216417 to 224099) contains five CDSs encoding enzymes involved in sialic acid catabolism (sialic acid lyase, *N*-acetylmannosamide kinase, *N*-acetylmannosamine-6-phosphate epimerase, glucosamine-6-phosphate isomerase, and *N*-acetylglucosamine-6-phosphate deacetylase). Some of these enzymes are found in selected mycoplasmas (e.g., *M. hyopneumoniae*, *M. mycoides*, and *M. penetrans*), but none of them contains the CDS cluster found in *M. synoviae*. This suggests that sialic acid could be a substrate for *M. synoviae* growth, a hypothesis that can be addressed experimentally.

Hemagglutinins play a fundamental role in the pathogenesis of *M. synoviae* and *M. gallisepticum*, and their genes could have been transferred between these species (5, 40). However, the organization of hemagglutinin genes differs sharply in *M. synoviae* and *M. gallisepticum*, the former with a single locus (1) comprising 70 CDSs (see Fig. 1 in http://www.brgene.lncc.br/finalMS/fig1) and the latter containing 43 genes organized in five loci (44). Whole-genome alignments of *M. synoviae* and *M. gallisepticum* allowed the identification of three *M. gallisepticum* hemagglutinin genes showing a strong similarity to the *M. synoviae* hemagglutinin CDS cluster. Using the Tribe-MCL algorithm, based on similarity data of the whole set of hemagglutinin CDSs of *M. gallisepticum* and *M. synoviae*, three hemagglutinin CDS groups could be identified (see Table 1 at http://www.brgene.lncc.br/finalMS/table1). The first one contained 41 *M. synoviae* CDSs, the second contained 35 *M. gallisepticum* CDSs, and the third contained 29 *M. synoviae* CDSs and the 3 *M. gallisepticum* highly homologous CDSs found in whole-genome alignments. These results, and additional phylogenetic and codon usage analyses, support the postulation of a likely transfer event of hemagglutinin genes from *M. gallisepticum* to *M. synoviae*.

Comparative genomics and evolution. A deeper understanding of the genomic diversity and evolution of *Mycoplasmatales* is now possible by analyzing the available sequenced genomes of species belonging to the Hominis clade (*M. pulmonis*, *M. hyopneumoniae*, *M. synoviae*, and *M. mobile*) and the Pneu-

FIG. 4. Schematic representation of C_1-THF metabolism in mycoplasmas. FolA, dihydrofolate reductase; FolD, methylenetetrahydrofolate dehydrogenase (NADP+); Fmt, methionyl-tRNA formyltransferase; FthS, formate-dihydrofolate ligase; GlyA, glycine hydroxymethyltransferase; ThyA, thymidylate synthase; ThyX, thymidylate synthase (FAD).

moniae clade (*M. genitalium*, *M. pneumoniae*, *M. gallisepticum*, *U. urealyticum*, and *M. penetrans*). Apparently, the evolutionary reduction of mycoplasma genomes resulted in preservation of a minimum set of essential metabolic capabilities, rather than a minimum set of specific genes or pathways. The one-carbon pool tetrahydrofolate (C_1-THF) metabolism clearly illustrates the alternative retention of redundant metabolic pathways (Fig. 4). The C_1-THF metabolism, conserved in both Hominis and Pneumoniae clades, results in formylation of the methionyl-tRNA initiator (Met-tRNAi), as well as de novo dTMP synthesis. *M. hyopneumoniae* is the only exception, because it is apparently unable to formylate Met-tRNAi due to the absence of both methionyl-tRNA formyltransferase (Fmt) and peptide deformylase (Def).

M. hyopneumoniae is also devoid of enzymes involved in the C_1-THF metabolism, except for glycine hydroxymethyltransferase (GlyA). Since all mycoplasmas, except *M. hyopneumoniae*, possess methylenetetrahydrofolate dehydrogenase (NADP+) (FolD), the activities of GlyA and formate-dihydrofolate ligase (FthS) may be considered metabolically redundant (Fig. 4). *M. synoviae*, *M. pulmonis*, *M. pneumoniae*, *M. genitalium*, and *M. gallisepticum* have retained GlyA but lost FthS, while the opposite has occurred in *U. urealyticum*. In both cases, a minimum set of metabolic interconversions essential for Met-tRNAi formylation and dTMP synthesis has been conserved, although the pathway itself has not. *M. penetrans*, which has the largest known mycoplasma genome, is the only species that has retained a redundant C_1-THF pathway.

Formylation of initiator Met-tRNA provides selectivity for the initiation factor IF2 and also blocks the binding of the elongation factor EF-Tu to the initiator tRNA (39, 54). We noticed the absence of FolD, Fmt, and Def in a recently sequenced plant-pathogenic phytoplasma (42). As far as we know, *M. hyopneumoniae* and *Phytoplasma* sp. are the first naturally occurring eubacteria potentially unable to formylate methionyl-tRNA$_f^{Met}$. The physiological consequences, if any, to bacterial growth behavior remain to be studied. Interestingly, disruption of the *fmt* gene severely impairs growth of *Escherichia coli* (23). This could be related to the higher binding affinity of IF-2 to fMet-tRNA$_f^{Met}$ than to Met-tRNA$_f^{Met}$ (24). However, a *fmt*-deficient strain of *Pseudomonas aeruginosa* can carry out formylation-independent initiation of protein synthesis, conceivably because IF-2 has a dual substrate specificity (38, 53).

Genome reduction resulted in a complex pattern of losses and retentions in the purine and pyrimidine metabolic pathways. Except for *M. pneumoniae* and *M. genitalium*, no other pair of mycoplasma species shares an identical set of pyrimidine metabolism pathways. Uridine kinase was found to be specific for the Pneumoniae clade, while 5′-nucleotidase is restricted to the Hominis clade. The lack of both enzymes, as previously reported for *M. penetrans* (50), was also observed in *M. synoviae*. Although UMP may be derived from either uracil or carbamoyl phosphate in *M. penetrans* (50), *M. synoviae* relies exclusively on uracil for UMP synthesis. The lack of both enzymes also implies that *M. penetrans* and *M. synoviae* cannot metabolize cytidine or uridine, only their deoxy derivatives. Thymidylate synthase (ThyA) and dihydrofolate reductase (FolA) are functionally coupled, since ThyA-mediated, de novo dTMP synthesis requires a continuous reduction of dihydrofolate reductase to THF (9). Interestingly, a novel class of flavin-dependent thymidylate synthases (ThyX) that preserve THF in its reduced form is widely distributed in bacterial genomes (37). Mycoplasmas lacking ThyA (*M. hyopneumoniae*, *U. urealyticum*, and *M. mycoides*) might have an alternative mechanism for de novo dTMP synthesis involving an unknown ThyX activity; alternatively, the dTMP pool could be exclusively dependent on thymidylate kinase, which is present in all mycoplasmas sequenced so far.

Different mechanisms of cell adhesion have evolved among mycoplasmas. Recent studies (29, 44) have shown that, in one branch of the Pneumoniae clade (leading to *M. pneumoniae-M. genitalium-M. gallisepticum*), mechanisms promoting attachment to host cells are mediated by proteins forming part of a tip organelle. The organization of operons encoding the major cytadhesins in these species (P1, MgPa, and GapA) and their cytadherence-related molecules have been evolutionarily conserved. This major adhesion mechanism is also conserved in *M. penetrans*, despite the facts that it diverged from *M. pneumoniae* and *M. genitalium* some 200 MYBP and that it switched from a mammal host to a bird host. Conversely, in all genomes of the more distantly related *M. penetrans-U. urealyticum* branch of the Pneumoniae clade and in the Hominis clade, MgPa-like protein CDSs were identified, including two in *M. hyopneumoniae* and four in *M. synoviae*. However, CDSs encoding most of the other components of the tip organelle were not found. These species also showed other exclusive or partially shared cell adhesion-related CDSs (Table 3), suggesting that cell adhesion mechanisms have followed different evolutionary pathways. The diversity of adhesion determinants is indicative of the plasticity of these small genomes (our data) (48), occasionally shuffled by HGT or intraspecific recombination, to generate smaller and adaptive arrangements to specific hosts or microhabitats.

Conclusions. Comparative analyses of mycoplasma genomes allowed the identification of adaptive mechanisms accounting

for perpetuation across a wide host range. Despite their small genomes, mycoplasmas showed a heterogeneous gene composition, with several species-specific genes whose identification and functional characteristics might be helpful for the prevention and treatment of diseases caused by these bacteria. Our analyses confirmed the occurrence of high rates of genomic rearrangements in mycoplasmas, which was demonstrated by the presence of strain-specific regions in the three *M. hyopneumoniae* strains; some of these regions were probably involved in rearrangements or pathogenesis. In fact, a variety of CDSs coding for putative, outer membrane proteins, or adhesins, were found to contain motifs of repetitive sequences which might have a role in their biological function or in antigenic variation. The presence of an ICEH element restricted to pathogenic strains suggests its possible role in pathogenicity. For the first time, phylogenetic relationships of all sequenced mycoplasma genomes were established on the basis of a concatenated data set, which resulted in a single, highly supported tree. These data were used for estimating a time frame of genome evolution, which added new insights to the evolution of the *Mycoplasmatales-Entomoplasmatales* group. Our studies provide evidence pointing to HGT as the process that provided *M. synoviae* and *M. gallisepticum* with the capacity of infecting the same host.

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ANEXO III

Artigo "Molecular analysis of an Integrative Conjugative Element, ICEH, present in the chromosome of different strains of *Mycoplasma hyopneumoniae*" de autoria de **Pinto, P.M.**, De Carvalho, M.O., Alves-Junior, L., Brocchi, M., Schrank, I.S. publicado no periódico *Genetics and Molecular Biology*, v. 30, pp. 256-263 no ano de 2007.

Este artigo relata a análise de uma região, de 23 kb, específica da cepa patogênica 7448 (VASCONCELOS *et al.*, 2005), o ICEH (elemento integrativo conjugativo de *M. hyopneumoniae*). Análises computacionais deste elemento sugerem que o ICEH possui uma evolução mais acelerada, pois mesmo presente em cepas do mesmo organismo o elemento é mais divergente do que outros *loci* de *M. hyopneumoniae*. Interessantemente, uma forma extracromossomal do ICEH foi identificada na cepa 7448, sugerindo um possível envolvimento na mobilização e/ou transferência de CDSs para novos organismos.

A minha participação foi toda a parte experimental e na redação e revisão do manuscrito.

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Research Article

Molecular analysis of an Integrative Conjugative Element, ICEH, present in the chromosome of different strains of *Mycoplasma hyopneumoniae*

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Abstract

Diversification of bacterial species and pathotypes is largely caused by lateral gene transfer (LGT) of diverse mobile DNA elements such as plasmids, phages, transposons and genomic islands. Thus, acquisition of new phenotypes by LGT is very important for bacterial evolution and relationship with hosts. This paper reports a 23 kb region containing fourteen CDSs with similarity to the previous described Integrative Conjugal Element of *Mycoplasma fermentans* (ICEF). This element, named ICEH, is present as one copy at distinct integration sites in the chromosome of 7448 and 232 pathogenic strains and is absent in the type strain J (non-pathogenic). Notable differences in the nucleotide composition of the insertion sites were detected, and could be correlated to a lack of specificity of the ICEH integrase. Although present in strains of the same organism, the ICEH elements are more divergent than the typical similarity between other chromosomal locus of *Mycoplasma hyopneunomiae*, suggesting an accelerated evolution of these constins or an ongoing process of degeneration, while maintaining conservation of the *tra* genes. An extrachromosomal form of this element had been detected in the 7448 strain, suggesting a possible involvement in its mobilization and transference of CDSs to new hosts.

Key words: LGT, *Mycoplasma*, constins.

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Introduction

In recent years, lateral gene transfer (LGT), the intraspecies and interspecies exchange of genetic information, has been substantially correlated to changes in genome content and with the evolution of bacteria, disseminating key traits within and among bacterial species (Boucher *et al.*, 2003; Lerat *et al.*, 2005). With the increase in genome sequence information associated with microarray technology, it is now possible to trace the presence or absence of genes in closely related bacterial genomes that could determine a sudden adaptation of bacteria to new environmental conditions. (Calcutt *et al.*, 2002; Porwollik and McClelland, 2003; Ochman *et al.*, 2005).

A number of phenotypes has been associated with this type of dissemination during conjugation of chromosomal regions: the sucrose metabolism in *Salmonella enterica* Seftemberg via cTn*scr*94 (Hochhut *et al.*, 1997); the symbiosis traits associated with the 500 kb symbiosis island in *Mesorhizobium loti* (Sullivan and Ronson, 1998); the degradation of chlorocatechol mediated by gene products resident on the 105 kb *clc* element of *Pseudomonas putida* (Ravatn *et al.*, 1998); the tetracycline resistant element Tn916 of *Bacteroides* (Salyers *et al.*, 1995); the large STX unit of *Vibrio cholerae* (Hochhut and Waldor, 1999); and the antibiotic resistance gene cluster from *Salmonella* (Doublet *et al.*, 2005). The term constin has been recently coined to describe this diverse group of conjugative, self-transmissible, integrating elements (Hochhut *et al.*, 2001). Despite the existence of a diversity of LGT mechanisms, only evolutionarily relevant events of transfer persist within the bacteria genome (Jain *et al*., 2003).

Mycoplasma hyopneumoniae is recognized as a potent pathogen causing mycoplasmal pneumonia in swine after colonising the respiratory tract and by inducing an inflammatory response (Maes *et al.*, 1996). This chronic

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worldwide disease causes important economic losses, including those in Brazil (Sobestiansky *et al.*, 2001). With the completion of genome sequence of 10 different species of Mycoplasmas, the search for horizontally acquired genes revealed the presence of constins only in *M. fermentans* (Calcutt *et al.*, 2002) and *M. hyopneumoniae* (Vasconcelos *et al.*, 2005). During a survey of specific sequences, a region containing fourteen CDS (≈ 23 kb) with similarity to the previous described Integrative Conjugal Element of *M. fermentans* (ICEF) (Calcutt *et al.*, 2002) was found in the Brazilian field isolate (7448) strain (Vasconcelos *et al.*, 2005). Recently, subtractive hybridization analysis indicated the presence of coding sequences related to ICEF in *Mycoplasma bovis* and *M. agalactiae* genomes (Marenda *et al.*, 2005).

Scrutiny of the complete genome from three strains (7448, J and 232) of *M. hyopneumoniae* (Vasconcelos *et al.*, 2005; Minion *et al.*, 2004) revealed the presence of a constin element in only two of them. This genetic element is present as one copy at distinct integration sites in the chromosome of 7448 and 232 strains and is absent in the type strain J. An extrachromosomal form of this element can be detected in the 7448 strain by the inverse PCR method (Vasconcelos *et al.*, 2005).

Methods

Sequence analysis

Sequence data from *Mycoplasma hyopneumoniae* strains analyzed in this paper were obtained from GenBank accession no. NC_006360 for strain 232, NC_007295 for strain J and NC_007332 for strain 7448.

Similarity searches were performed using a locally installed NCBI BLAST 2.2.12 (Altschul *et al.*, 1997) against custom built databases of known constins and the *nr* database (downloaded in 05/09/2005) from NCBI. Initial analysis of sequence families was done using the Tribe-MCL software (Enright *et al.*, 2002) and the Bidirectional Best Hits (BBH) (Tatusov *et al.*, 2000) approach was used to find putative ortologues among the analyzed constins. Sequence annotation was manually curated with the help of similarity searches against the PFAM (Sonnhammer *et al.*, 1997), PRODOM (Bru *et al.*, 2005) and INTERPRO (Mulder *et al.*, 2005) databases. Genometrics indexes were obtained with the EMBOSS suite of programs (Rice *et al.*, 2000). Phylogenetic analyses were performed with MEGA 3 (Kumar *et al.*, 2004), and multiple sequence alignments were generated with CLUSTALX (Thompson *et al.*, 1997). JTT distance was employed for both the neighbor-joining and parsimony tree reconstruction analysis, and a value of 1000 replications was used for bootstrap analysis. The software TOPALi (Milne *et al.*, 2004) was used for the recombination analysis of the *traE* genes, and PDM and HMM analysis were done with default settings.

Bacterial strains and culture conditions

M. hyopneumoniae strain J (ATCC 25934), a non-pathogenic strain with reduced capacity to adhere to porcine cilia (Zielinski and Ross 1990; Zielinski and Ross, 1993; Zhang *et al.*, 1995) was acquired from American Type Culture Collection by CNPSA, EMBRAPA (Concórdia, SC, Brazil). *M. hyopneumoniae* strain 7448 was isolated from an infected swine in Lindóia do Sul, Santa Catarina, Brazil, as described by Vasconcelos *et al.* (2005).

PCR analysis and DNA sequencing

PCR amplification of the target sequences was performed in a DNA thermal Cycler (MJ Research). The PCR mixture contained the reaction buffer (50 mm KCl, 10 mm Tris-Cl pH 8.3, 1.5 mm $MgCl_2$), 200 mm of each dNTPs, 20 pmol of each primer, and 1U of *Taq* polymerase (CenBiot enzymes, Centro de Biotecnologia, UFRGS). Assays were carried out using 50 ng of genomic DNA from both *M. hyopneumoniae* strains (J and 7448) and distilled water for a total of 25 µL. The reaction mixture was subjected to PCR under the following conditions: heat denaturation at 94 °C for 5 min. and then an additional 30 cycles with heat denaturation at 94 °C for 1 min., annealing at 60.6 °C for 1 min., and DNA extension at 68 °C for 1 min. After the last cycle, samples were maintained at annealing temperature for 5 min. followed by 68 °C for 8 min. PCR products are resolved in a 1.2% gel electrophoresis, stained with ethidium bromide and visualized by UV illumination. PCR amplification was performed with primers ICEH1-F (5'TTTTTCATTGCCTAAATCTTGTTT3') and ICEH2-R (5'AAATCACAAACTTAAAAATGCCAAT3') (Invitrogen, USA). Before the sequencing reaction, the amplification products (300 ng) were purified using GFX kit (Amersham Biosciences, GE Healthcare) or treated with 3.3U of exonuclease III and 0.2U shrimp alkaline phosphatase (SAP) (Amersham Biosciences, GE Healthcare) at 37 °C during 30 min. After this period, the enzymes were inactivated by incubation at 80 °C during 15 min. Amplification products were sequenced using the DYEnamic ET dye terminator cycle sequencing (MegaBACE) kit and run on MegaBACE 1000 capillary sequencers (Amersham Biosciences, GE Healthcare). The generated sequences were assembled and a quality score of 20 was considered for the final assembly. The derived sequence was searched using the BLASTn software using the complete *M. hyopneumoniae* strain 7448 genome as the database to identify the element in the chromosome.

Results and Discussion

General characteristics

Although conjugative elements have been described for members of many bacterial genera, indigenous self-transmissible molecules have been reported only in *M. fermentans* so far in Mycoplasma. During an ongoing anal-

ysis of genes encoding surface components of *M. fermentans*, genetic elements designated ICEF were found (Calcutt *et al*., 2002). Two kinds of elements that differ regarding the presence or absence of a few CDSs were characterized by those authors and called Type I and II. The CDSs present in these elements suggested that they belong to the constin family. The sequence comparisons of three *M. hyopneumoniae* genomes carried out recently (Vasconcelos *et al*., 2005) also reveal the presence of putative ICE elements in two of these strains that were called ICEH. Interestingly, these elements were present in the two pathogenic strains (7448 and 232) but were absent from the non-pathogenic one (J strain).

Unlike ICEF elements I and II, which consist of twenty one and nineteen CDSs, respectively, the ICEH7448 elements consist of 17 CDSs and ICEH232 of 22 CDSs. However, despite these differences, the organization of these elements is very similar.

The *M. hyopneumoniae* elements ICEH7448 and ICEH232 are 22,816 and 21,061 nucleotides in length, respectively. The average length of the ICEH CDSs is 1,218 bp in ICEH7448 and 1,187 bp in ICEH232. Most of the CDSs (14 ICEMH7448 and 12 in ICEMH232) are considered hypothetical since no significant similarity was found with the available databases. Conversely, some CDSs present similarity to *tra* genes that are associated with the conjugative plasmids of bacteria (Snyder and Champness, 2002) such as *traK, traI, traE* and a CDS encoding for a single strand binding protein (Ssb) that is essential for the transfer process. The ICEF element presents two *tra* (*tra*G and *tra*E) genes, with an average similarity of 30% to *tra* genes found on ICEH232 (*tra*G and *tra*E) and 29% to *tra* gene of ICEH7448 (only *traE*). The ICEH232 element has three *tra* genes, with one *tra*G and two copies of the *traE* gene. In the *M. hyopneumoniae* 7448 ICEH element, we found only one *tra*E gene with an average similarity of 44% to the *tra*E genes found in the ICEH232. A characteristic that is shared by both ICEH and ICEF type elements is the lack of a recognizable gene encoding for an integrase function. Calcutt *et al*. (2002) suggested that the integrase function is possibly mediated by the hypothetical CDSs present in both type I and II ICEF elements. This observation may also be true for the ICEH elements because they contain hypothetical CDSs as well (Table I). The AT content of some CDSs in the ICEH element is higher than the overall percentage for the *M. hyopneumoniae* genome. For instance, CDSs ICE_MH7448-ORF09 and ICE_MH7448-ORF017 exhibit 76.13% and 76.06% AT content respectively, suggesting a heterologous origin for these ORFs in the ICEH element.

A complete reannotation analysis of the elements ICEH7448, ICEH232 and ICEF was also conducted. From these analyses, we have attributed functions to seven previously not annotated CDSs, including a CDS in ICEH7448 that was not recognized initially and was identified as a putative helicase. Table 1 shows the CDSs annotated in this way and they are marked with asterisks. Note the identification of a transposase-like CDS in the element ICEH232, but not in ICEF and ICEH7448. However, other ICEs have already been described which contain CDSs related to transposases, like the ICE element from *Streptococcus thermophilus* (Pavlovic *et al.* 2004). The role of transposases residing inside mobile genomic islands is not clear. However it is possible that such elements could help the transfer process of the ICEs acting as triggers for the activation of the damage-repair machinery found in the host cells. It has already been suggested that the damage-repair machinery is important for the integration of the ICEs in the chromosome of the host cells (Auchtung *et al.*, 2005).

The ICEH7448 element is inserted at the position 517,895 of the genome of *M. hyopneumoniae* 7448, unlike ICEH232 which is found at position 654,116 of the *M. hyopneumoniae* 232 genome from the replication origin. This indicates that these elements may insert themselves in different genomic regions. We were unable to determine with accuracy the presence of direct or inverted repeats flanking both elements. However, ICEH7448 is flanked by a duplication of part of the YP_287818.1 CDS, which is located downstream from the element. Nevertheless, considering that the duplicated gene is not exactly at the terminus of the element, we cannot conclude that this duplication is result of the ICEH7448 insertion.

The difference in number of CDSs present in distinct ICE elements suggests that they are able to acquire or lose CDSs over time. For instance, the CDSs AAN85219.1-AAN85220.1 and AAN85229.1, although present in the ICEF-I element are not present in the ICEF-II element, possibly indicating a very recent acquisition of new sequences by ICEF-I. Thus, it is possible that these elements may work as carriers of CDSs that, when mobilized to a new host, could confer new properties.

Genometric analyses have been conducted on the genomes of *M. hyopneumoniae* 7448 and 232 strains. Interestingly, the ICEH7448 element was found in an island of high AT content, relative to the genome, while the ICEH232 element was located in a high GC island content. The observed deviations on the GC content of the insertion sites indicates that DNA structural features derived from compositional constraints may not be a major determinant for the element insertion site determination. Individually, the CDSs of the ICEH232 element display a slightly higher GC content. The element positions and the graph of GC% cumulative skew are depicted in Figure 1. The variability in base composition is not limited to the element as a whole when compared to the genome. Analysis of the genometric properties of the ICE elements revealed that specific regions contain deviations of sequence properties. The *traE* gene sequence is peculiar in the sense that, in all elements, this gene presents a higher AT content than the other CDSs of the element. This internal variation in base composition

Table 1 - Annotated CDS of the elements ICEH7448, ICEH232 and ICEF.

NCBI Ids	CDS	Annotation
AAN85213.1	ICEF_ORF-01	Hypothetical
AAN85214.1	ICEF_ORF-02	putative relaxase
AAN85215.1	ICEF_ORF-02_2	Hypothetical
-	ICEF_ORF-03	putative TraG (contains a frameshift)
AAN85216.1	ICEF_ORF-04	Hypothetical
AAN85217.1	ICEF_ORF-05	putative methyltransferase *
AAN85218.1	ICEF_ORF-06	hypothetical
AAN85219.1	ICEF_ORF-07	hypothetical
AAN85220.1	ICEF_ORF-08	hypothetical
AAN85221.1	ICEF_ORF-08_2	hypothetical
AAN85222.1	ICEF_ORF-09	SSB protein
AAN85223.1	ICEF_ORF-10	hypothetical
AAN85224.1	ICEF_ORF-11	P57 lipoprotein
AAN85225.1	ICEF_ORF-12	putative phosphatidate transferase *
AAN85226.1	ICEF_ORF-13	hypothetical
AAN85227.1	ICEF_ORF-14	TraE/TrsE family NTPase
AAN85228.1	ICEF_ORF-15	hypothetical
-	ICEF_ORF-16	hypothetical (contains a frameshift)
-	ICEF-ORF16A	hypothetical (contains a frameshift)
AAN85229.1	ICEF_ORF-17	hypothetical
AAN85230.1	ICEF_ORF-18	hypothetical
AAN85231.1	ICEF_ORF-19	hypothetical
YP_115644.1	ICE_MH232_1-ORF01	putative DNA processing protein *
YP_115645.1	ICE_MH232_1-ORF02	hypothetical
YP_115646.1	ICE_MH232_1-ORF03	hypothetical
YP_115647.1	ICE_MH232_1-ORF04	TraE/TrsE family NTPase
YP_115648.1	ICE_MH232_1-ORF05	hypothetical
YP_116031.1	ICE_MH232_4-ORF06	hypothetical
YP_116032.1	ICE_MH232_4-ORF07	SSB protein *
YP_116033.1	ICE_MH232_4-ORF08	hypothetical
YP_116034.1	ICE_MH232_4-ORF09	hypothetical
YP_116035.1	ICE_MH232_4-ORF010	TraG *
YP_116036.1	ICE_MH232_4-ORF011	hypothetical
YP_116037.1	ICE_MH232_4-ORF012	hypothetical
YP_116038.1	ICE_MH232_4-ORF013	hypothetical
YP_116039.1	ICE_MH232_4-ORF014	hypothetical
YP_116040.1	ICE_MH232_4-ORF015	TraE/TrsE family NTPase*
YP_116041.1	ICE_MH232_4-ORF016	TraE/TrsE family NTPase
YP_116042.1	ICE_MH232_4-ORF017	hypothetical
YP_116043.1	ICE_MH232_4-ORF018	hypothetical
YP_116044.1	ICE_MH232_4-ORF019	hypothetical
YP_116045.1	ICE_MH232_4-ORF020	hypothetical
YP_116046.1	ICE_MH232_4-ORF021	hypothetical
YP_116047.1	ICE_MH232_4-ORF022	putative transposase *
MHP0414	ICE_MH7448-ORF02	hypothetical
MHP0415	ICE_MH7448-ORF03	hypothetical
MHP0416	ICE_MH7448-ORF04	hypothetical
MHP0417	ICE_MH7448-ORF05	hypothetical
MHP0418	ICE_MH7448-ORF06	TraE/TrsE family NTPase
MHP0419	ICE_MH7448-ORF07	hypothetical
MHP0420	ICE_MH7448-ORF08	hypothetical
MHP0421	ICE_MH7448-ORF09	hypothetical
MHP0422	ICE_MH7448-ORF10	SSB protein
MHP0423	ICE_MH7448-ORF11	hypothetical
MHP0424	ICE_MH7448-ORF12	hypothetical
MHP0425	ICE_MH7448-ORF13	hypothetical
MHP0426	ICE_MH7448-ORF14	hypothetical
-	ICE_MH7448-ORF15	putative helicase *
MHP0427	ICE_MH7448-ORF16	hypothetical
MHP0428	ICE_MH7448-ORF17	hypothetical
-	ICE_MH7448-ORF18	hypothetical

could explain a high expression for the *traE* genes. High codon usage bias has already been linked to a high expression profile in genes with distinctive base composition (Wang *et al.*, 2005). This is important as the *traE* genes are fundamental in the processes of element transfer, and an indication that the selection for optimum codon usage is a process that can exist in constins in a marked way.

ICEH elements

It was not possible to determine a single value for similarity among the studied elements, as the sequences are heterogeneous regarding their local similarities scores. The similarity rates range from regions with none (even between ICEH232 and ICEH7448) to regions with 67% of similarity (the *traE* genes for instance). Figures 2 and 3 demonstrate the similarity between the ICEH232 and ICEH7448 elements. Figure 2 exhibits a nucleic acid word match, while Figure 3 is a TBLASTX comparison based on the 3-frame translated amino acid sequence of all CDSs in the elements. The notable divergence between the ICEH elements, present in different strains of the same organism, could suggest an ongoing process of degeneration or accelerated evolution. These questions can be resolved when more sequences of ICEH constins become available.

While searching for CDSs related to ICEs in the chromosomes of *M. hyopneumoniae*, strains J, 7448 and 232, a

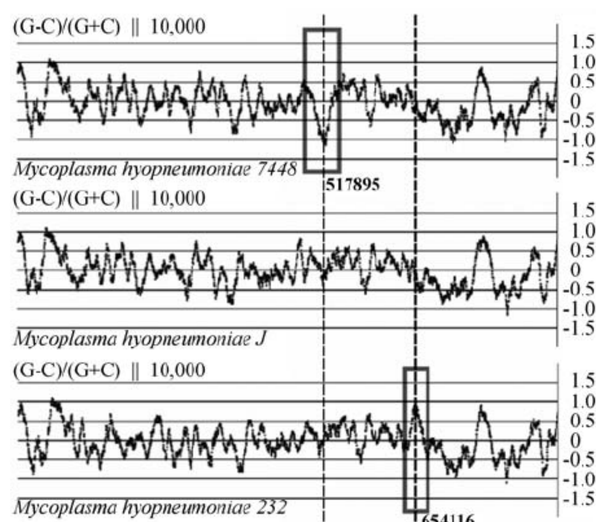

Figure 1 - GC% Skew graph showing the differential regions between the strains 7448, J and 232 of *Mycoplasma hyopneumoniae*. The dotted lines indicate the position of the ICEH7448 and ICEH232, respectively.

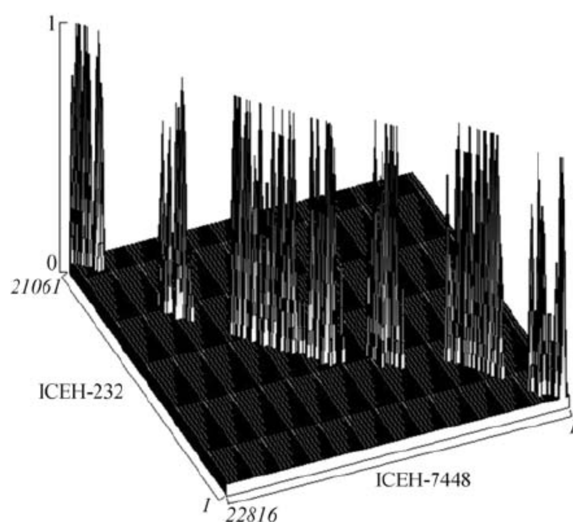

Figure 2 - Word density 3D plot of the ICEH-232 and ICEF-7448 sequences. The peaks correspond to the word density match (number of nucleotide local sequence similarities in a region) between the sequences of the elements, where more elevated peaks denote higher similarity. Location of the peaks in the base plan is determined by the coordinates of the word matches between the two sequences, represented in the X-Z axis.

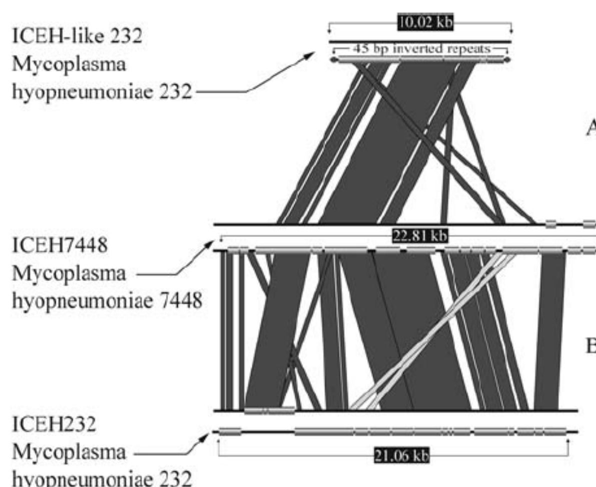

Figure 3 - TBLASTX (translated search of nucleotide sequences) similarity of the elements ICEH-like MH232, ICEH7448 and ICEH232. Dark gray bars denote direct sequence similarity between elements; light gray bars connect reverse sequence similarity between elements. CDS are represented by boxes. Inverted repeats of the ICEH-like elements are represented by arrows. The CDS represented are listed in Table 1 in order of appearance in the figure, except for the ICEH232 element that have the matches flipped for better visualization and the CDS are in inverted order as represented in the table. A: Comparison between the ICEH232-like element and ICEH232 element. B: Comparison between the ICEH232 element and ICEH7448 element.

specific region of about 10 kb that has significant similarity to the genes *traE* and two other hypothetical proteins have been found. We named these regions ICEH-like MHJ, ICEH-like 7448 and ICEH-like 232. Figure 3 A depicts the ICEH-232 region organization. It is interesting to note that this ICE-like region does not only have sequence similarity, but the gene order is also conserved, indicating a common origin with the ICEH elements. Additionally these regions are flanked by inverted repeats of 45 nucleotides in length and show a conservation of 71% between them. This element is present in the three *M. hyopneumoniae* strains sequenced to date with a highly conserved gene content, gene order and structure, except for the ICEH-like MH232 which has an additional CDS located upstream from the SMF DNA processing protein without any similarity to known genes. It is possible that these ICEH-like elements could be the product of an ancestral ICE integration event that suffered an irregular excision leaving parts of them inserted in the chromosome. However, phylogeny analyses of the *traE* genes suggest that these regions are maintained at approximately the same evolutionary rate as the other genes in the chromosome, with lower divergence between the *traE* genes found in the ICEH-like region or in the ICEH232 and ICEH7448 elements (Figure 4 B). These might be an indication that the ICEH-like elements have acquired new functions during evolution, which differ from those assigned to the ICEH elements that assume an independent behavior in the host.

Comparisons between mycoplasma ICE elements

Two clustering experiments have been also conducted, using the CDSs available from six elements (ICEH-like MHJ, ICEH7448, ICEH232, pSKU146, pBJS-O and ICESt1) sequenced so far, one with the BBH strategy and another employing the MCL algorithm. Both methods found a small number of putative ortologous genes among the analysed sequences, and the *traE* gene was the most conserved of all. It is interesting to note that although present in the same species, the two ICEH elements are less

conserved in relation to each other than the pSKU146 and pBJS-O elements. These two latter elements, although present in different species, are more conserved and syntenic. This could be explained by the fact that the ICEH elements diverged before the pSKU146 and pBJS-O elements, as evidenced by the phylogenetic analysis of 3 conserved ortologous CDSs of these elements (Figure 4 A). Although the phylogenetic tree of the elements is congruent with their host species, it disagrees for the *traE* gene, the most conserved putative ortologous gene. This result might indicate a process of CDS exchange by horizontal gene transfer between these elements or that this gene is under more stringent selective pressures. These analyses also indicate that the *traE* gene of ICE clearly diverged before the same genes found in the more recent ICEs such as pSKU146 and pBJS-O.

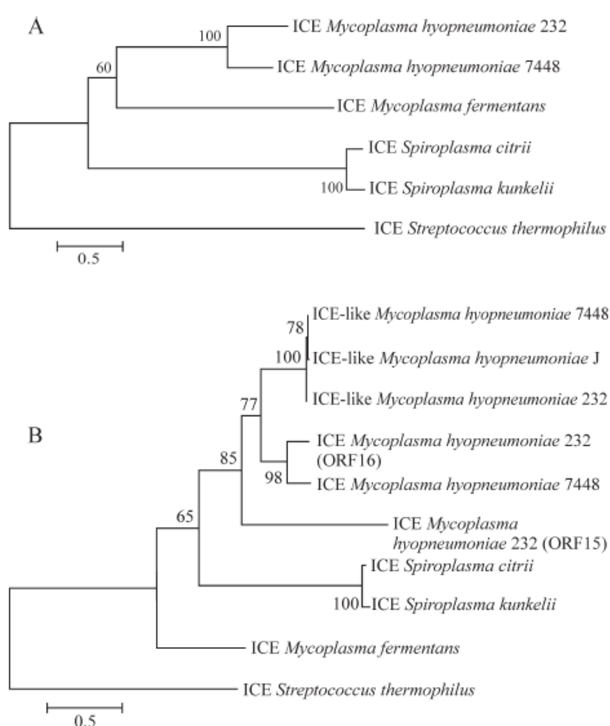

Figure 4 - Phylogenetic analysis of the ICE elements and TraE genes A: Neighbor-Joining tree calculated using the JTT distance of three concatenated ortologous CDS between the six analyzed elements.(The parsimony tree had the same topology of the Neighbor-joining analysis and was omitted from the figure). B: Neighbor-Joining tree calculated using the JTT distance of 10 TraE genes found in the elements ICEH-Like MH232, ICEH-Like MH7448, ICEH-Like MHJ, ICEH7448, ICEH232, pkstu, pksc and ICESt1.

Curiously, a duplication of the *traE* genes in the MH232 ICE element has been identified. From the phylogenetic analysis (Figure 4 B), it is possible to assume that the CDS15 originated by duplication of CDS16, which is the putative ortholog of the unique *traE* gene from ICEH7448. This observation is corroborated by the best bidirectional hit observed among these genes. Apparently, CDS15 is under a process of degeneration as it is the more distinct *traE* gene among the other homologous genes found in the ICEH elements. An analysis of recombination done on the most conserved *traE* genes, from the elements ICEH-Like MH232, ICEH-like MH7448, ICEH MH232 and ICEH MH7448, showed that a probable recombination event has occurred in an ancestral form of these genes, originating a mosaic structure comprising two phylogenetically distinct domains (Figure 5).

Evidence for a circular intermediate ICEH

Extrachromosomal forms of the integrative conjugative element have been shown in *M. fermentans* (Calcutt *et al.*, 2002) and *M. agalactiae* (Marenda *et al.*, 2006). A model has been proposed where the element is excised, at low frequency, from the chromosome, circularized as a nonreplicative intermediate, and transferred by conjugation to a recipient. Finally, in the recipient cell, the element is integrated into the chromosome (Calcutt *et al.*, 2002).

To investigate the possible presence of extrachromosomal forms of ICEH in *M. hyopneumoniae,* two outwardly facing PCR primers have been designed, each one annealing at regions located near the ends of the element. The presence of a circular form should yield a fragment of about 600 bp after amplification by PCR. PCR reactions were carried out using 50 ng of genomic DNA from both *M. hyopneumoniae* strains (J and 7448). Figure 6 shows that a product of 633 bp was observed in *M. hyopneumoniae* strain 7448 and as expected, no product was observed in strain J. The PCR product was sequenced and perfectly aligned to the extremities of the ICEH, as expected if this element has a circular form (data not shown). This data indicates the presence of an ICEH circular intermediate in *M. hyopneumoniae* strain 7448, possibly gener-

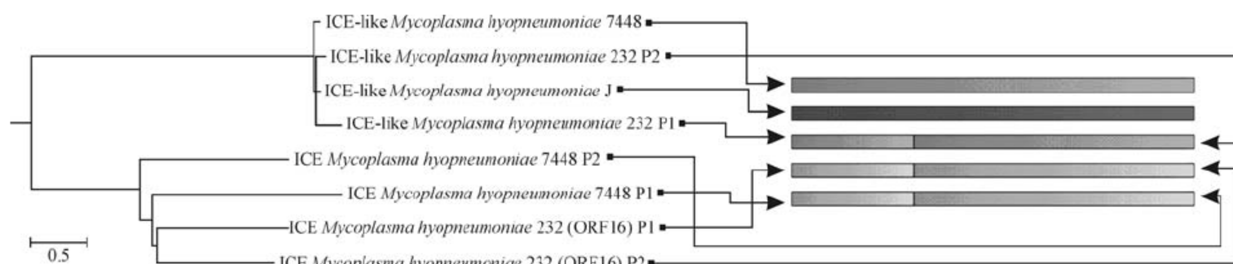

Figure 5 - Recombination tree based on the *traE* sequences on the left is based on the representation of the *traE* mosaic structure.

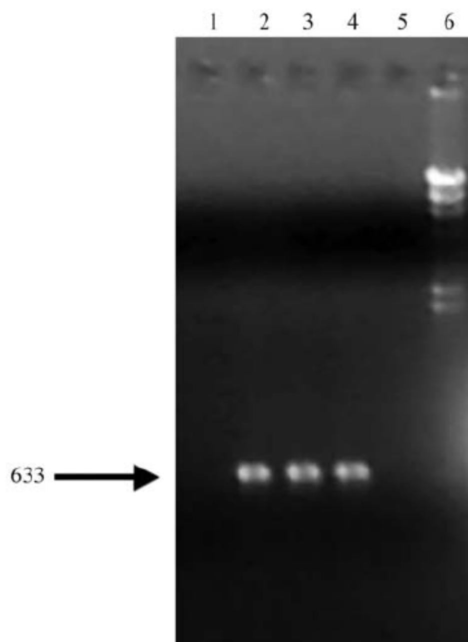

Figure 6 - Identification of extrachromosomal copy of ICEH. Genomic DNA from *M. hyopneumoniae* strains J (5) and 7448 (2-4) were used in PCR reactions with primers ICEH1-F and ICEH2-R. (1) negative control and (6) Lambda DNA digested with *Hin*dIII. Arrow shows the 633 amplicom from different DNA samples of *M. hyopneumoniae* strain 7448.

ated by the excision from genome, like occurred in *M. fermentans* (Calcutt *et al.*, 2002) and *M. agalactiae* (Marenda *et al.*, 2006).

The circular conformation of the excised ICEH has important implications for the biology of the element, since in this form, the constin can remain more stable, increasing the probability of horizontal transfers. As constins do not rely on transposases, which remain associated with the insertion sequence DNA molecule during the course of the excision/insertion process, a linear form would be quickly degraded by exonucleases, compromising the relocation to a different host or locus. Additionally, circular DNA forms are structurally more stable than linear ones, helping the element to maintain its structural integrity over the transfer process.

Concluding Remarks

Our analyses indicate the presence of putative integrative conjugal elements (ICE) in pathogenic strains of *M. hyopneumoniae*. It was also demonstrated that this element exists in an extra-chromosomal form. These results suggest that ICE is a mobile DNA that is probably involved in genomic recombination events and in pathogenicity. Nevertheless, the transference of ICEH between cells remains to be demonstrated experimentally. Genomic sequence analysis also indicated the existence of ICE-like elements in *M. hyopneumoniae* which are probably derived from an ancestral ICEH integration event that suffered an irregular excision. The ICEs are not confined to *M. hyopneumoniae*. Sequences related to these elements were also found in other species such as *M. fermentans*, *M. bovis* and *M. agalactiae*. Interestingly, some CDSs of these elements have similarities to Type IV secretion machinery present in different bacterial species. This system is involved in the secretion of DNA and proteins to the extra-cellular milieu or into eukaryotic cells which have a central role in pathogenicity (reviewed by Christie *et al.*, 2005). This observation reinforces the role of ICEs in pathogenicity. However, there is no evidence that links them to protein secretion.

Acknowledgments

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Internet Resources

ProDom, http://prodom.prabi.fr/prodom/current/html/home.php (November 15, 2005).

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InterPro - The Integrated Resource of Protein Domains and Protein Families, http://www.ebi.ac.uk/interpro/ (November 12, 2005).

National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov.

National Center for Biotechnology Information (NCBI) Microbial Genomes, http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi (September 05, 2006).

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ANEXO IV

Paulo Marcos Pinto

Curriculum Vitae

Março de 2009.

Dados Pessoais

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Sexo masculino

Filiação Paulino Flores Pinto e Maria de Lourdes Ziani
Nascimento 23/04/1981 - Porto Alegre/RS - Brasil
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Formação Acadêmica/Titulação
2005 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Análise estrutural e funcional de genomas de diferentes cepas de *Mycoplasma hyopneumoniae*
Orientador: Prof Dr. Henrique Bunselmeyer Ferreira
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2002 - 2003 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Estudo do gene srg do fungo *Metarhizium anisopliae*: um novo gene relacionado ao radical superóxido., Ano de obtenção: 2003
Orientador: Prof. Dr. Augusto Schrank
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

1998 - 2001 Graduação em Ciências Biológicas.
Universidade Federal de Santa Maria, UFSM, Santa Maria, Brasil
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Formação complementar

2003 - 2003 Curso de curta duração em Métodos em Proteômica.
Universidade de Buenos Aires, UBA, Argentina.

2004 - 2004 Curso de curta duração em Tools for the genetic analysis of bacteria in the.
XXIV Reunião de Genética de Microorganismos, REGEM, Brasil.

2004 - 2004 Curso de curta duração em Bioinformática.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.

2006 - 2006 Curso de curta duração em Theoretical and Practical Course in Bioinformatic.
The International Centre for Genetic Engineering and Biotechnology, (ICGEB), Itália.
Bolsista do(a): The International Centre for Genetic Engineering and Biotechnology

2008 - 2008 Curso de curta duração em Introduction to Mass Spectrometry and Proteomics. Waters Corporation, WATERS, Grã-Bretanha

Atuação profissional
1. Universidade Federal de Santa Maria - UFSM
Vínculo institucional
1999 - 2002 Vínculo: Outro , Enquadramento funcional: Bolsista de Iniciação Científica , Carga horária: 20, Regime: Parcial
1999 - 1999 Vínculo: Outro , Enquadramento funcional: Bolsista de Iniciação Científica (FAPERGS) , Carga horária: 20, Regime: Parcial
2000 - 2002 Vínculo: Outro , Enquadramento funcional: Bolsista de Iniciação Científica (PIBIC-CNPq) , Carga horária: 20, Regime: Parcial

Atividades
03/1999 - 12/2001 Pesquisa e Desenvolvimento, Universidade Federal de Santa Maria
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2002 - 2003 Vínculo: Estudante de Mestrado , Enquadramento funcional: Estudante de Mestrado , Carga horária: 40, Regime: Dedicação Exclusiva
2003 - 2005 Vínculo: Bolsista DTI , Enquadramento funcional: Bolsista DTI , Carga horária: 40, Regime: Dedicação Exclusiva
2005 - Atual Vínculo: Estudante de Doutorado , Enquadramento funcional: Doutorando , Carga horária: 40, Regime: Dedicação Exclusiva
Atividades
03/2002 – Atual Projetos de pesquisa, Centro de Biotecnologia
03/2002 – Atual Pesquisa e Desenvolvimento, Centro de Biotecnologia
Linhas de Pesquisa:
Genômica , Proteômica , Biologia Molecular e Celular de Microorganimos

Linhas de pesquisa
1. Biologia Molecular e Celular de Microorganimos
2. Genômica
3. Proteômica

Projetos

2004 – Atual Estudo proteômico de *M. hyopneumoniae*
Descrição: Análise proteômica prospectiva e comparativa de cepas patogênicas e não patogênicas de *M. hyopneumoniae*.
Situação: Em Andamento Natureza: Pesquisa
Alunos envolvidos: Doutorado (1);
Integrantes: Paulo Marcos Pinto (Responsável);
Financiador(es):

2003 – Atual Produção, caracterização de proteínas recombinantes e desenvolvimento de testes imunodiagnósticos e vacinas para *Mycoplasma hyopneumoniae* - Caracterização de isolados de campo de *M. hyopneumoniae*.
Descrição: O presente projeto tem como objetivo geral a expressão, purificação e análise do potencial de antígenos de *M. hyopneumoniae* para o desenvolvimento de teste imunodiagnóstico e vacina, bem como a análise da possível variabilidade antigênica em isolados de *M. hyopneumoniae*. Os objetivos específicos são: Produção e caracterização de proteínas recombinantes de *M. hyopneumoniae* com potencial para utilização em imunodiagnóstico e vacinação 1. Clonagem de seqüências (ORFs) de *M. hyopneumoniae* 7448, selecionadas por apresentarem características de localização em membrana, em vetores de expressão. 2. Produção e purificação das proteínas expressadas em *Escherichia coli*. 3. Utilização dos antígenos recombinantes para a formulação de reagentes para diagnóstico imunológico e/ou vacinação. a. Formulação de vacinas com diferentes adjuvantes para teste de inocuidade. b. Estudo da inocuidade das vacinas preparadas com as suspensões de antígenos fornecidas pela Rede Genoma PIGS em animais de laboratório (coelhos) e na espécie alvo (suínos). c. Análise da resposta imunológica em suínos. Análise da variabilidade das seqüências (ORFs) que codificam as proteínas selecionadas em diferentes isolados de *M. hyopneumoniae* 1. Isolamento de *M. hyopneumoniae* 2. Construção de bibliotecas shotgun dos diferentes isolados e seqüenciamento visando à análise da possível conservação e/ou variabilidade das seqüências selecionadas e também do elemento integrativo e conjugativo (ICEH). 3. Caracterização dos isolados quanto à presença dos antígenos por western blot.
Situação: Em Andamento Natureza: Pesquisa
Integrantes: Paulo Marcos Pinto (Responsável);
Financiador(es):

2003 – Atual Projeto de Investigação de Genomas do Sul (PIGS)
Descrição: The program is supported by a Network of Bioinformatics Laboratories, Sequencing Laboratories, Associated Laboratories and Diagnosis and Vaccine Development Laboratories located mainly at the States of Paraná, Santa Catarina and Rio Grande do Sul. The Network receives financial support from the Brazilian Ministry of Science and Technology (MCT) and the Rio Grande do Sul State Secretary of Science and Technology - Rio Grande do Sul State Foundation for Research (SCT - FAPERGS). The genome of Mycoplasma hyopneumoniae, a bacterium that causes enzootic pneumonia in swine will be the first genome to be analysed by the PIGS
Situação: Em Andamento Natureza: Pesquisa
Integrantes: Paulo Marcos Pinto (Responsável);
Financiador(es):

2002 – 2003 Caracterização molecular do processo de infecção de fungos entomopatogênicos
Descrição: *Metarhizium anisopliae* é considerado um dos organismos mais promissores no controle de carrapatos e de insetos praga sendo utilizado como biopesticida no Brasil e outros países. O processo de infecção do fungo nos hospedeiros ocorre por penetração direta da cutícula. A elucidação dos mecanismos moleculares deste processo tem se mostrado um modelo importante para o estudo das relações patógeno-hospedeiro e de processos celulares básicos em organismos eucarióticos. Além disso, tem sido demonstrada a aplicabilidade da identificação e da expressão de genes isolados no aumento da eficiência do processo de infecção, melhorando seu potencial de utilização em biocontrole. Nosso grupo tem contribuído na caracterização de genes e proteínas que participam do processo de infecção de *M. anisopliae*. Nosso modelo de estudo são hospedeiros praga na agropecuária (carrapatos) e na agricultura (hemípteros). Utilizamos duas abordagens: (i) a caracterização de genes de enzimas hidrolíticas que degradam os componentes da cutícula dos hospedeiros (quitinases, proteases e lipases) e (ii) estratégias mais globais com a caracterização de genes diferencialmente expressos em condições de infecção utilizando metodologias de bibliotecas de subtração e estudos proteômicos. Objetivamos a caracterização funcional e o estudo da regulação da expressão destes e de outros genes alvo. Para isso, estamos desenvolvendo vetores de expressão para *M. anisopliae* e um sistema eficiente de transformação, utilizando o sistema *Agrobacterium tumefaciens*. Estas ferramentas estão sendo utilizadas para a disrupção ou superexpressão de genes alvo para determinar a sua participação no processo de infecção
Situação: Concluído Natureza: Pesquisa
Integrantes: Paulo Marcos Pinto (Responsável);
Financiador(es):

Áreas de atuação
1.Genética Molecular e de Microorganismos
2.Biologia Molecular
3.Proteômica

Idiomas
Inglês Compreende Bem, Fala Bem, Escreve Razoavelmente, Lê Bem
Espanhol Compreende Razoavelmente, Fala Razoavelmente, Escreve Pouco, Lê Razoavelmente

Prêmios e títulos
2006 Trabalho Destaque da XXXV Reunião Anual da SBBq, SBBq
2004 Trabalho Destaque da XXIV Reunião de Genética de Microrganismos,

Produção em C, T & A
Produção bibliográfica
Artigos completos publicados em periódicos
1. PINTO, P. M; KLEIN, C. C; ZAHA, A.; FERREIRA, H. B. Comparative proteomics analysis of pathogenic and non-pathogenic strains from the swine pathogen *Mycoplasma hyopneumoniae*. Mol. Cell. Proteomics, submitted.

2. PINTO, P. M; KLEIN, C. C; ZAHA, A.; FERREIRA, H. B. Comparing three

Mycoplasma *hyopneumoniae* proteomes. Activity Report (LNLS), 07: 1-2, 2008.

3. PINTO, P. M., CARVALHO, M. O., ALVES-JUNIOR, L., BROCCHI, M., SCHANK, I. Molecular analysis of an Integrative Conjugative Element, ICEH, present in the chromosome of different strains of *Mycoplasma hyopneumoniae*. Genetics and Molecular Biology., v.30, p.256 - 263, 2007.

4. PINTO, P. M., KLEIN, C. C., ZAHA, Arnaldo, FERREIRA, H. B. MudPIT based proteomics analyses of the swine pathogen *Mycoplasma hyopneumoniae*. Activity Report (Laboratório Nacional de Luz Síncrotron). , v.06, p.1 - 2, 2007.

5. PINTO, P. M., CHEMALE, G., CASTRO, L. A., COSTA, A. P. M., kich, J. D., VAINSTEIN, M. H., ZAHA, Arnaldo, FERREIRA, H. B. Proteomic survey of the pathogenic *Mycoplasma hyopneumoniae* strain 7448 and identification of novel post-translationally modified and antigenic proteins. Veterinary Microbiology (Amsterdam). , v.121, p.83 - 93, 2007.

6. PINTO, P. M., CHEMALE, G., CASTRO, L. A., COSTA, A. P. M., ZAHA, Arnaldo, VAINSTEIN, M. H., FERREIRA, H. B. Proteomic study of different *Mycoplasma hyopneumoniae* strains/isolates. Activity Report (Laboratório Nacional de Luz Síncrotron). , v.04, p.1 - 2, 2005.

7. VASCONCELOS, Ana Tereza Ribeiro, el al., PINTO, P. M., ZAHA, Arnaldo Swine and poultry pathogens: the complete genome sequences of two strains of *Mycoplasma hyopneumoniae* and a strain of *Mycoplasma synoviae*.. Journal of Bacteriology. , v.187, p.5568 - 5577, 2005.

8. STAATS, C. C., SILVA, M. S. N., PINTO, P. M., VAINSTEIN, M. H., SCHRANK, A. The *Metarhizium anisopliae* trp1 gene : cloning and regulatory analysis. Current Microbiology. , v.49, p.66 - 70, 2004.

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1. MACHADO, C. X., Schuck, D., PINTO, P. M., ZAHA, Arnaldo, FERREIRA, H. B. CHARACTERIZATION OF THE THIOL-PEROXIDASE FROM THE SWINE PATHOGEN *MYCOPLASMA HYOPNEUMONIAE* In: XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular (SBBq), 2008, Águas de Lindóia. **XXXVII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular (SBBq)**. , 2008.

2. PINTO, P. M., KLEIN, C. C., ZAHA, Arnaldo, FERREIRA, H. B. Comparative proteomics analisys of three *Mycoplasma hyopneumoniae* strains In: 18ª RAU - Reunião Anual de Usuários do LNLS, 2008, Campinas. **18ª RAU - Reunião Anual de Usuários do LNLS**. , 2008. v.2008.

3. MACHADO, C. X., Schuck, D., PINTO, P. M., COSTA, A. P. M., ZAHA, Arnaldo, FERREIRA, H. B. CLONING AND EXPRESSION OF A THIOL-PEROXIDASE FROM THE SWINE PATHOGEN *MYCOPLASMA HYOPNEUMONIAE* In: X IUBMB Conference and XXXVI Reunião Anual da SBBq, 2007, Salvador. **X**

IUBMB Conference and XXXVI Reunião Anual da SBBq. , 2007.

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Orientações e Supervisões
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15. **DNA mitocondrial de plantas: estrutura e expressão.**, 1999. (Oficina)
16. **Introdução à Sintemática Filogenética**, 1999. (Oficina)
17. **45o. Congresso Nacional de Genética**, 1999. (Congresso)
18. **Semana Acadêmica da Biologia**, 1999. (Seminário)
19. **Seminário Estadual: Biotecnologia e Produtos Transgênicos**, 1999. (Seminário)
20. **Introdução ao estudo dos QTLs (locos de caracteres quantitativos)**, 1999. (Oficina)
21. **Seminário Regional de Educação Ambiental do Pró-Guaíba**, 1998. (Seminário)
22. **Aula Inaugural ENTENDO A PROFISSÃO**, 1998. (Encontro)

Bancas
Participação em banca de trabalhos de conclusão
Graduação
1. PINTO, P. M. Participação em banca de Gabriel da Luz Wallau. **Análise da atividade do elemento de transposição mariner em populações de *Drosophila simulans* originárias da América do Sul**, 2008 (Ciências Biológicas) Universidade Federal de Santa Maria.