UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Identificação de proteínas de superfície de *Mycoplasma hyopneumoniae 7448*

Tese de Doutorado

Luciano Antonio Reolon

Porto Alegre, abril de 2015

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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 a obtenção do grau de Doutor em Ciências.

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ÍNDICE

ABREVIATURAS

1DE	eletroforese unidimensional
2DE	eletroforese bidimensional
A	adenina
C	citosina
CHAPS	3-[(3-colamidopropil)-imetilamonio]-1-propanosulfonato
CTAB	brometo de cetil-trimetilamônio
DNA	ácido desoxirribonucleico
ELISA	ensaio imunosorbente ligado à enzima
G	guanina
LP	lipoproteína
MS	espectrometria de massas
NBD	domínio de ligação à nucleotídeos
pb	pares de bases
PES	pneumonia enzoótica suína
RNA	ácido ribonucleico
SDS	dodecil sulfato de sódio
SVM	*support vector machines*
T	timina
TM	transmembrana
U	uracila

RESUMO

A caracterização do repertório de proteínas expostas na superfície celular do *Mycoplasma hyopneumoniae*, agente etiológico da pneumonia enzootica suína (PES), é essencial para o entendimento dos processos fisiológicos associados a capacidade de infecção bacteriana, sobrevivência no hospedeiro e patogênese. Análises *in silico* indicam que aproximadamente um terço dos genes bacterianos codificam proteínas de superfície. Entretanto, até momento poucas proteínas de *M. hyopneumoniae* tiveram sua expressão e localização na superfície celular confirmadas experimentalmente, fazendo-se necessária a prospecção experimental destas proteínas utilizando ferramentas de proteômica, aumentando assim a confiabilidade dos dados obtidos *in silico*. Neste contexto, nós desenvolvemos uma abordagem experimental baseada na marcação *in vivo* da superfície celular com biotina seguida pela identificação por espectrometria de massas, associada à predições *in silico*, que nos possibilitou a identificação de proteínas expostas na superfície do *M. hyopneumoniae*. Como resultado, obtivemos 167 identificações proteicas, correspondendo a 59 proteínas não reduntantes, na abordagem proteômica experimental. A análise *in silico* resultou na identificação de 292 proteínas transmembrana e de 25 lipoproteínas. A análise comparativa revelou que 39 proteínas (66%) identificadas experimentalmente por espectrometria de massas como expostas na superfície celular, também foram preditas *in silico* com tal, sendo representadas principalmente por proteínas relacionadas com adesão, lipoproteínas e proteínas hipotéticas. As outras 20 proteínas (34%) correspondem principalmente a proteínas tradicionalmente relacionadas ao metabolismo, porém com várias delas previamente descritas atuando na superfície celular, participando de processos como interação patógeno-hospedeiro. Os resultados obtidos nesta tese fornecem uma visão geral da composição proteíca da superfície celular do *M. hyopneumoniae*, permitindo a seleção de alvos para futuros estudos funcionais visando melhorar o entendimento dos processos de patogenicidade bacteriana, além do desenvolvimento de drogas, vacinas e testes diagnósticos mais eficientes.

ABSTRACT

The characterization of the repertoire of proteins exposed on the cell surface by *Mycoplasma hyopneumoniae*, the etiological agent of enzootic pneumonia (EP) in pigs, is critical to understanding physiological processes associated with bacterial infection capacity, survival and pathogenesis. It is predicted that about a third of the genes in the *M. hyopneumoniae* genome code for surface proteins, but so far, just a few of them have experimental confirmation of their expression and surface localization. An experimental proteomic survey of a surface protein enriched sample is necessary to better define the *M. hyopneumoniae* set of proteins exposed to the host, adding confidence to *in silico* predictions. In this work, we developed an experimental approach based on cell surface labeling followed by mass spectrometry coupled to an *in silico* analysis, which enabled us to survey the surface exposed proteins in *M. hyopneumoniae*. A total of 167 protein identifications corresponding to 59 different protein species were identified in proteomic approach. An *in silico* survey of *M. hyopneumoniae* transmembrane proteins and lipoproteins results in the prediction of 292 and 25 proteins, respectively. A comparative analysis revealed that 39 proteins (66%) experimentally identified in surface were also *in silico* predicted as transmembrane proteins or lipoproteins and are represented mainly by adhesion related proteins, lipoproteins and hypothetical proteins. The other 20 proteinas (34%) comprise mainly proteins traditionally related to metabolism, but some of them were previously suggested to be involved in bacterial-host interactions and pathogenicity of *Mycoplasma* species. The obtained results provided a better picture of the *M. hyopneumoniae* cell surface that will help in the understanding of processes important for bacterial pathogenesis, selection of targets for further functional studies and development of more efficient drugs, vaccines and diagnostic tools for EP treatment, prevention and control.

1. INTRODUÇÃO

1.1 Gênero *Mycoplasma*

As bactérias do gênero *Mycoplasma* pertencem à classe Mollicutes, ordem Mycoplasmatales, família Mycoplasmataceae, tendo como principais características a ausência de parede celular e tamanho diminuto, fazendo com que sejam classificadas entre os menores organismos auto-replicáveis conhecidos (RAZIN, 1998). A ausência de parede celular impede que se corem no teste de Gram, sendo então consideradas bactérias Gram-negativas (RAZIN, 1998). Entretanto estudos demonstraram que, filogeneticamente, os micoplasmas estão relacionados com bactérias Gram-positivas, compartilhando um ancestral comum com os gêneros *Bacillus, Clostridium*, *Lactobacillus* e *Streptococcus* (WOLF *et al*., 2004) e evoluindo, especificamente, através de eventos de degeneração ou redução de genomas de bactérias Gram-positivas com genoma rico em A+T (WOESE, 1987).

O tamanho do genoma dos organismos pertencentes ao gênero *Mycoplasma* varia de 580 kb (*Mycoplasma genitalium*) até 1.358 kb (*Mycoplasma penetrans*) (FRASER *et al*., 1995; SASAKI *et al*., 2002) e apresenta um baixo conteúdo de G+C. Este genoma atipicamente rico em A+T, (WOESE, 1987), pode ter contribuído para o fato de micoplasmas utilizarem o códon UGA para codificar triptofano (KANNAN & BASEMAN, 2000).

A redução do genoma ocasionou a perda de algumas vias metabólicas características da maioria das bactérias, tais como vias *de novo* de síntese de purinas (MITCHELL & FINCH, 1977), o ciclo do ácido carboxílico completo (MANOLUKAS *et al.*, 1988, POLLAK, 1992) e um sistema de cadeia transportadora de elétrons mediada por citocromo (FRASER *et al.*, 1995). Portanto, os micoplasmas necessitam obter

algumas macromoléculas essenciais para seu crescimento, tais como colesterol, ácidos graxos, aminoácidos, vitaminas, coenzimas, precursores de ácidos nucléicos e íons do hospedeiro ou do ambiente (RAZIN, 1998).

A necessidade de nutrientes específicos e não usuais, associados à natureza fastidiosa característica dos micoplasmas, fazem com que o cultivo e o estabelecimento de meios mínimos para o crescimento bacteriano seja dificultado. Para atender tal necessidade utiliza-se, comumente, meios de cultura suplementados com soro animal e extrato de levedura, o que dificulta a identificação exata dos componentes destes meios e compromete a utilização destes cultivos para estudos bioquímicos e fisiológicos (FRIIS, 1975; RAZIN, 1998).

Micoplasmas apresentam-se amplamente distribuídos no reino animal. Na sua maioria atuam como patógenos colonizando um número variado de hospedeiros, como animais, plantas e insetos (PITCHER & NICHOLAS, 2005), não tendo sido descrito até o momento nenhuma espécie de vida livre. Embora normalmente sejam patógenos extracelulares encontrados aderidos à superfície extracelular de células e tecidos do hospedeiro, já foram descritas algumas espécies colonizando o interior de células eucarióticas (BASEMAN *et al*., 1993; LO *et al*., 1993).

1.2 *Mycoplasma hyopneumoniae*

Pertencente ao gênero *Mycoplasma*, *M. hyopneumoniae* é o agente etiológico da pneumonia enzoótica suína (PES), infectando rebanhos do mundo todo (MINION, 2004). MARE & SWITZER (1965) e GOODWIN *et al.* (1967) foram os primeiros pesquisadores a realizar o isolamento de micoplasma de pulmões com sinais clínicos de pneumonia e, posteriormente, a realizar a reprodução experimental da doença. *M. hyopneumoniae*

infectando um único hospedeiro, o suíno. Contudo, os mecanismos relacionados a esta especificidade permanecem desconhecidos.

Até o momento, seis linhagens de *M. hyopneumoniae* tiveram seu genoma completamente sequenciados (232, J, 7448, 7422, 168 e 168-L), fazendo com que seja a espécie de micoplasma mais vezes sequenciada (MINION *et al.*, 2004; VASCONCELOS *et al.*, 2005; LIU *et al.*, 2011, LIU *et al.*, 2013, SIQUEIRA *et al*., 2013). Como característica geral, estes genomas apresentam variação entre 892.758 pb a 925.576 pb, sendo que o conteúdo G+C perfaz apenas aproximadamente 28% deste genoma. A linhagem *M. hyopneumoniae* 7448 apresenta 681 prováveis regiões codificadoras, para o qual a função foi assinalada apenas para aproximadamente 60% delas (421 proteínas) (VASCONCELOS *et al.*, 2005).

Estes dados têm possibilitado a expansão de estudos de nosso grupo visando um melhor entendimento da biologia molecular, do metabolismo e dos mecanismos de patogenicidade e de virulência deste microorganismo (FERREIRA & CASTRO, 2007; PINTO *et al*., 2007; MACHADO *et al.*, 2009; PINTO *et al*., 2009; SIQUEIRA *et al.*, 2011; MOITINHO-SILVA *et al*., 2012; WEBER *et al*., 2012; SILVA *et al.*, 2012; SIQUEIRA *et al.*, 2013; REOLON *et al*., 2014; SIQUEIRA *et al.*, 2014).

1.3 Pneumonia enzoótica suína

A PES é uma doença infecciosa crônica de abrangência mundial (MINION *et al.*, 2004), caracterizada por uma broncopneumonia catarral associada a hepatização dos pulmões (SOBESTIANSKY *et al*., 1999), a qual é transmitida por contato direto, indireto ou por aerossóis expelidos durante os acessos de tosse. A alta morbidade e baixa mortalidade também são características da PES, ocasionando perdas econômicas

decorrentes da queda da produtividade que podem chegar a 20% sobre a conversão alimentar e 30% sobre o ganho de peso do animal (SOBESTIANSKY *et al*., 1999).

A adesão do *M. hyopneumoniae* ao epitélio ciliar do trato respiratório do suíno (Figura 1) é essencial para a colonização e o estabelecimento da doença, resultando em ciliostase, lesões e morte das células epiteliais, conduzindo a um estado de inflamação aguda da traquéia e dos brônquios (BASEMAN *et al.*, 1993; ZIELINSKI & ROSS, 1993; DEBEY & ROSS, 1994; RAZIN, 1999; RUIZ *et al.*, 2002). A perda de função do elevador mucociliar, o qual constitui o principal mecanismo de defesa inespecífico do trato respiratório, acaba predispondo o animal a patógenos secundários como *Pasteurella multocida* ou *Bordetella bronchiseptica* (CIPRIAN *et al*., 1988; THACKER *et al*., 1999).

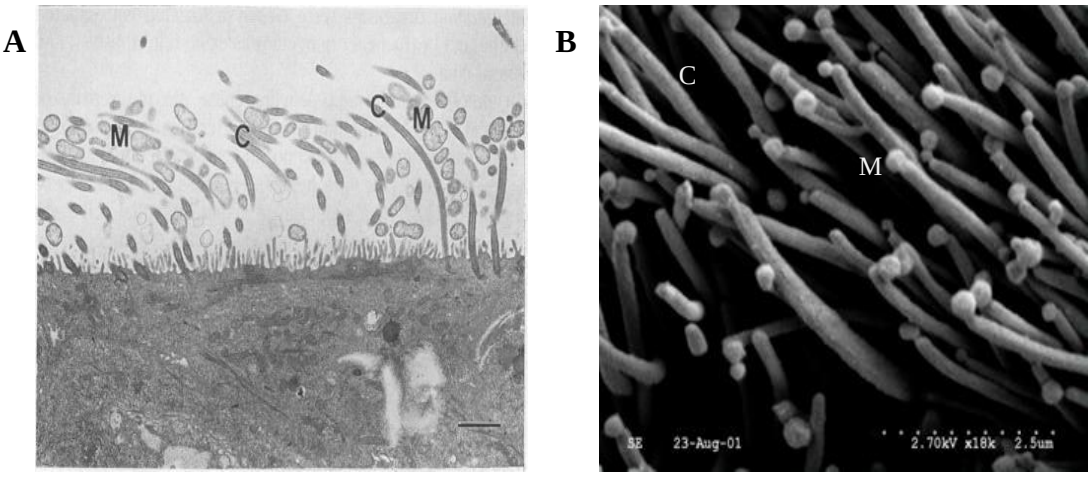

Figura 1 – Micrografia eletrônica (A) de transmissão e (B) de varredura, mostrando o *M. hyopneumoniae* (M) aderido ao epitélio ciliar (C) do trato respiratório de suíno. Fonte: DEBEY & ROSS, 1994; RUIZ *et al*., 2002.

O controle da doença consiste basicamente em medidas que visem a otimização das práticas de manejo e o acondicionamento dos rebanhos (MAES *et al.*, 2008), associadas a estratégias de medicação profilática (VICCA *et al*., 2004) e vacinação. No entanto, a re-infecção é relatada como um fator que compromete a eliminação do

patógeno (HEGE *et al.*, 2002), sendo ocasionada por via aérea ou pela adição ao rebanho de suínos erroneamente diagnosticados sorologicamente como negativos (MAES *et al.*, 2008).

As bacterinas, preparadas a partir de extratos celulares de *M. hyopneumoniae*, constituem a principal forma de imunização para o combate da PES (MEYNS *et al.*, 2006). No entanto, apesar de conferirem certo grau de proteção contra a doença reduzindo os sinais clínicos, estas vacinas apresentam altos custos e apenas diminuem o grau de severidade dos sintomas e lesões ocasionadas pela PES (HAESEBROUCK *et al*., 2004).

Para o diagnóstico da infecção por *M. hyopneumoniae,* o teste mais sensível é a cultura de isolados de tecido pulmonar em meio Friis Agar (FRIIS, 1975; SORENSEN *et al.*, 1997), sendo este considerado o padrão ouro. Entretanto, este teste apresenta algumas dificuldades, especialmente devido à natureza fastidiosa do microorganismo (podendo o crescimento demorar semanas) e às características morfológicas das colônias (sendo a identificação correta das colônias dependente da experiência do analista). Além disso, pode haver dificuldade no correto diagnóstico devido à possibilidade de contaminações ou de co-cultivo de *Mycoplasma hyorhinis* e *Mycoplasma flocculare* (MAES *et al.*, 2006), espécies frequentemente encontradas no trato respiratório dos suínos que apresentam semelhante morfologia, crescimento e antigenicidade do *M. hyopneumoniae* (THACKER *et al.*, 1999). Assim outros métodos baseados na identificação sorológica ou molecular surgem como alternativa ao cultivo de *M. hyopneumoniae*. Entre estes, destaca-se o ensaio imunosorbente ligado à enzima (ELISA) como o mais comumente utilizado na rotina de monitoramento (SIBILA *et al.,* 2009). Contudo, embora apresente uma alta especificidade, possui uma sensibilidade que oscila entre 35 a 63% (ERLANDSON *et al*., 2005).

A análise da literatura mostra que diversas revisões bibliográficas têm avaliado a disponibilidade e o futuro da utilização de novos antígenos para a confecção de vacinas e de testes diagnósticos (SIBILA *et* al., 2009; SIMIONATTO *et al.*, 2013). Estas revisões indicam que a maior parte das vacinas utilizadas e testadas até o momento possuem na sua formulação antígenos relacionados com os mecanismos de adesão (KING *et al.*, 1997; CHEN *et al.*, 2006a; OKAMBA *et al.*, 2007), de choque térmico (CHEN *et al.*, 2003) e de metabolismo (FAGAN *at al.*, 1997; FAGAN *at al.*, 2001; CHEN *et al.*, 2006b). No entanto, embora seja evidente os avanços e contribuições destas vacinas, a proteção conferida por elas aos rebanhos é apenas parcial, fazendo-se necessário expandir os estudos em busca de novas estratégias, como vacinas utilizando tecnologia do DNA recombinante e novos antígenos proteicos com potencial para a vacinação (SIMIONATTO *et al.*, 2013). Desta forma, investir na identificação das proteínas presentes na superfície do *M. hyopneumoniae* pode representar um caminho promissor.

1.4 Superfície da célula bacteriana

Embora classificado como uma bactéria Gram-negativa, *M. hyopneumoniae* possui organização estrutural do envoltório celular bastante peculiar. Diferentemente da maioria dos membros desta classe, não possui a estrutura tradicional das Gram-negativas: uma membrana externa (*OM*, do inglês, *outer membrane*), uma membrana interna (*IM*, do inglês, *inner membrane*) e uma escassa parede de peptideoglicano (GLAUERT & THORNLEY, 1969). Assim, por não possuir parede celular, a única estrutura encontrada é uma membrana trilaminar simples, composta basicamente por lipídeos (fosfolipídeos e colesterol) e proteínas de membrana.

As proteínas de membrana representam aproximadamente um terço das proteínas encontradas nas células bacterianas (WALLIN & von HEIJNE, 1998), desempenhando

funções bastante diversas, como sinalização celular, tráfego de moléculas, adesão celular e evasão ou modulação da resposta imune do hospedeiro (ROTTEN, 2003; YOU *et al*., 2006; MACHER & YEN, 2007). Embora algumas das características físico-químicas destas proteínas, como a alta hidrofobicidade, impõem uma série de dificuldades para seu isolamento e estudo, as proteínas de membrana têm despertado o interesse da comunidade científica. Estas proteínas, na sua grande parte, encontram-se expostas na superfície bacteriana, atuando na interface entre a bactéria e o ambiente ou hospedeiro. Portanto, tornam-se excelentes alvos para estudos que visem aprofundar o conhecimento sobre a fisiologia e os mecanismos de patogênese bacteriana, além de possuírem potencial para o desenvolvimento de vacinas e de testes diagnósticos.

Com raras exceções, as proteínas associadas diretamente à membrana bacteriana podem ser classificadas em duas grandes classes: proteínas transmembrana, as quais encontram-se associadas à membrana através de estruturas como a α-hélice, e lipoproteínas, que se ligam de forma covalente à membrana. A seguir, iremos discutir separadamente estas duas classes.

1.4.1 Proteínas de superfície bacteriana

1.4.1.1 Proteínas transmembrana

Proteínas transmembrana são proteínas anfipáticas, possuindo regiões hidrofóbicas e hidrofílicas (ALBERTS *et al.*, 2008). Suas regiões hidrofóbicas atravessam a membrana e interagem com as caudas hidrofóbicas dos fosfolipídeos, enquanto suas regiões hidrofílicas ficam expostas à água no interior ou exterior da célula bacteriana.

A maior parte das proteínas de membrana bacteriana possuem a estrutura secundária na conformação α-hélice. Esta estrutura é caracterizada pela presença de

regiões altamente hidrofóbicas, contendo de 15 a 20 aminoácidos que se inserem na membrana em uma conformação de α-hélice (SCHNEIDER *et al.*, 2007). As proteínas transmembrana de α-hélice podem ter uma organização simples com apenas uma ou poucas estruturas atravessando a membrana (Figura 2a), sendo então denominadas proteínas de α-hélice de passagem simples (em inglês, *simple pass*), ou, apresentando uma estrutura oligomérica complexa com muitas hélices atravessando a membrana (Figura 2b), denominadas proteínas de α-hélice de passagem múltipla (em inglês, *multi pass*) (SCHNEIDER *et al.*, 2007). Poucas proteínas transmembrana de α-hélice são encontradas na sua forma monomérica, sendo que a oligomerização é a base para a execução de várias funções essenciais para as células, incluindo eventos de sinalização celular, transferência de íons, catálise e conversão de energia (UBARRETXENA-BELANDIA & ENGELMAN, 2001).

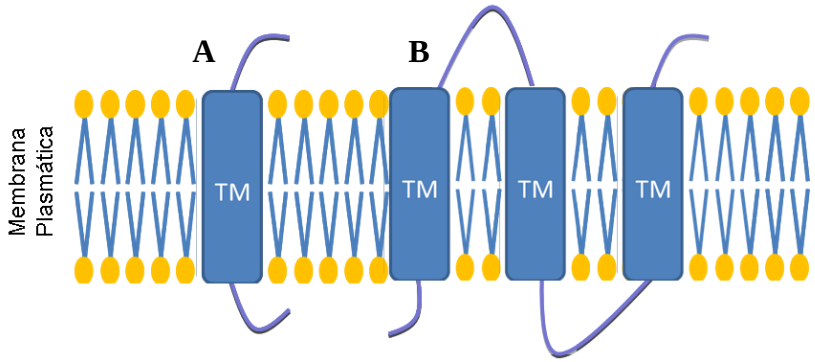

Figura 2 – Representação esquemática de proteínas transmembranas. A) α-hélice de passagem única (*simple pass*); B) α-hélice de passagem múltipla (*multi pass*). Figura do autor.

1.4.1.2 Lipoproteínas de membrana

As lipoproteínas são proteínas que caracteristicamente sofrem modificações lipídicas que facilitam sua inserção e ancoramento em substratos hidrofóbicos, como as membranas biológicas (KOVACS-SIMON *et al.*, 2011). São inicialmente sintetizadas como prolipoproteínas, possuindo um peptídeo sinal N-terminal de aproximadamente 20 aminoácidos (INOUYE *et al.*, 1977). Posteriormente a prolipoproteína é transformada na

proteína madura através da clivagem do peptídeo sinal pela enzima sinal peptidase II (PAETZEL *et al.*, 2002). A análise de diferentes sequencias de lipoproteínas bacterianas revelou a presença de uma estrutura comum na região carboxi-terminal do peptídeo sinal, denominadas *lipobox*, [LVI][ASTVI][GAS]C. Esta estrutura é modificada no decorrer do processo de biossíntese das lipoproteinas (SANKARAN & WU, 1994), sendo o *lipobox* utilizado para a predição *in silico* de lipoproteínas.

Acredita-se que a maior parte das lipoproteínas de *Mycoplasma* spp. são expostas no meio extracelular com os grupamentos *acyl* ancorando a molécula na membrana citoplasmática (BROWNING *et al.*, 2011). Embora sejam muito abundantes em membranas de *Mycoplasma* spp. quando comparada com outras eubactérias (RAZIN, 1998), poucas tem sua função definida e, aparentemente, atuam de forma semelhante às proteínas periplásmicas encontradas nas bactérias Gram negativas (BROWNING *et al.*, 2011).

As lipoproteínas bacterianas podem desempenhar diversas funções nas células, incluindo transporte de nutrientes, adesão e transdução de sinal (SUTCLIFFE & RUSSELL, 1995; KOVACS-SIMON *et al.*, 2011). Alguns genes que codificam lipoproteínas estão organizados em forma de *operon* com genes que codificam transportadores, como a lipoproteína OppA de *Mycoplasma hominis* (HENRICH *et al.*, 1999) e a lipoproteína MHP379 de *M. hyopneumoniae* cepa 232 (SCHIMIDT *et al.*, 2007), sugerindo uma função potencial destas lipoproteínas no transporte de nutrientes para as células.

Além disso, lipoproteínas de micoplasma também podem estar envolvidas no processo de patogênese. Já foi demonstrado que diversas lipoproteínas sofrem variação antigênica (descritas adiante, no item 2.2.1.2), auxiliando a bactéria na evasão do sistema imune do hospedeiro.

1.5 Processos celulares envolvendo proteínas de superfície *M. hyopneumoniae* - transporte

O transporte celular, representado por um conjunto de mecanismos que regula e passagem de solutos como íons e outras pequenas moléculas através da membrana plasmática, é essencial para todos os organismos vivos, posibilitando a célula, por exemplo, importar nutrientes e excretar substâncias (ALBERTS *et al.*, 2012). A mediação do transporte é realizada por proteínas presentes na membrana celular, envolvendo interações entre estas proteínas e o soluto.

Como descrito anteriormente, a redução do genoma de espécies de *Mycoplasma* ocasionou a perda de algumas importantes rotas metabólicas, fazendo com que estas bactérias necessitem importar parte dos seus nutrientes (RAZIN, 1998). A análise do genoma de *M. hyopneumoniae* cepas 7448 e J (VASCONCELOS *et al*., 2005) mostra que aproximadamente 13% das regiões codificantes codificam para proteínas classificadas como transportadores. Destas, mais de 85% correspondem a transportadores tipo ABC (*ATP-binding cassette*) envolvidos no transporte de aminoácidos, glicerol, açucares, oligopeptídeos e poliaminas (NICOLAS *et al*., 2007).

Os transportadores ABC são uma das principais famílias de transportadores, sendo caracterizados como um grande grupo de complexos protéicos localizados na membrana plasmática que acoplam o transporte de substâncias à hidrólise de ligações fosfato do ATP (HIGGINS, *et al*., 1992, ter BEEK, *et al.*, 2014). Em procariotos, os transportadores ABC estão localizados na membrana plasmática e o ATP é hidrolisado na face citoplasmática. A arquitetura molecular dos transportadores ABC consiste basicamente de dois domínios transmembrana (TM), e duas subunidades ligantes de nucleotídeos (NBDs, do inglês, *nucleotide-binding domains*) (Figura 4).

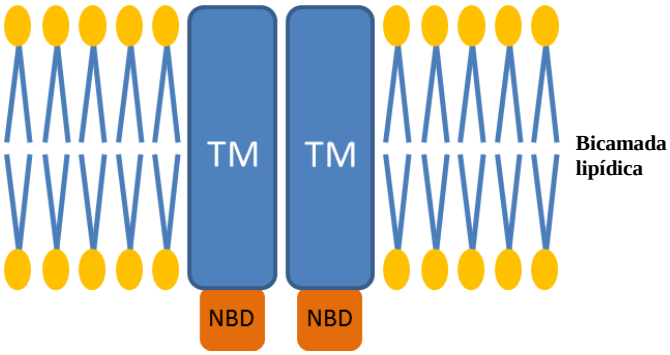

Figura 4 – Representação da arquitetura molecular de transportadores ABC. TM – domínio transmembrana; NBD – subunidade ligante de nucleotídeo. Figura do autor.

Transportadores ABC também podem estar relacionados à patogenia em algumas espécies de micoplasmas, como o transportador de glicerol (*gtsABC*) de *Mycoplasma mycoides* subsp. *mycoides*, encontrado na cepa virulenta e ausente na avirulenta (ABDO *et al.*, 2000), indicando que a habilidade de importar glicerol via *gtsABC* é aparentemente importante para a patogenicidade do organismo. Além disso, diversos componentes de transportadores ABC identificados no genoma de micoplasmas mostraram propriedades imunogênicas, estimulando resposta imune específica (PINTO *et al*., 2007, NICOLAS *et al*., 2007).

1.6 Interações entre *M. hyopneumoniae* e o hospedeiro: fatores de virulência associados à superfície bacteriana

A virulência, definida como a habilidade de uma bactéria em produzir uma doença, pode ser medida pelas suas taxas de mortalidade e/ou pela sua habilidade de invasão e colonização, sendo dependente de uma série de fatores (CASADEVALL & PIROFSKI, 2001; FERREIRA & CASTRO, 2007). Estes diversos fatores podem ser divididos em dois grandes grupos, compreendendo os relacionados ao hospedeiro, como a resposta imune e o estado imunológico, e os relacionados à bactéria, especialmente os chamados fatores de virulência. Estes últimos fatores habilitam a bactéria a se estabelecer

e se desenvolver em um determinado hospedeiro, estando envolvidos na adesão, colonização, invasão, inibição da resposta imune e produção de toxinas.

Ferreira & Castro (2007) realizaram um estudo global dos fatores de virulência baseados em análise do genoma de *M. hyopneumoniae*, resultando na identificação de uma série de potenciais fatores de virulência. Entre eles, destacam-se as proteínas envolvidas com a adesão e as proteínas antigênicas.

1.6.1 Adesão

A aderência do micoplasma às células do hospedeiro é fundamental para o estabelecimento da infecção (BASEMAN *et al.*, 1992; ZIELINSKI & ROSS, 1993; RAZIN, 1999), pois mutantes que apresentam deficiência na adesão perdem a capacidade de infectar o hospedeiro, sendo esta totalmente revertida com a reversão para o modelo selvagem (RAZIN *et al.*, 1998). *M. hyopneumoniae* adere a superfície mucosa da traquéia, brônquios e bronquíolos de suínos (Figura 1) (DEBEY & ROSS, 1994; RUIZ *et al.*, 2002) com o auxilio de proteínas e de carboidratos presentes na sua superfície, denominadas adesinas (ZIELINSKI & ROSS, 1993).

Existem dois tipos principais de aderência descritos para micoplasmas, ambos utilizando como base as adesinas. Algumas espécies, como *Mycoplasma pneumoniae* e *Mycoplasma genitalium*, possuem organelas especiais semelhantes a pontas (*tips*, em inglês), constituindo uma estrutura de adesão multiproteica que permite ao micoplasma aderir à superfície do hospedeiro (LAYH-SCHMITT & HARKENTHAL, 1999; MUSATOVOVA *et al.*, 2003). Diferentemente, outras espécies, entre elas o *M. hyopneumoniae*, não produzem todas as proteínas necessárias para a formação completa da organela (VASCONCELOS *et al.*, 2005), tendo portanto, as adesinas que estão distribuídas pela membrana celular como mediadoras do processo de adesão.

Embora seja um processo multifatorial que envolve outras proteínas bacterianas acessórias e receptores do hospedeiro além das adesinas, a revisão de literatura mostra que o conhecimento sobre o processo de adesão em *M. hyopneumoniae* tem como base os estudos das adesinas. A adesina P97 é a mais estudada, sendo considerada um importante fator de virulência de *M. hyopneumoniae*. Estudos utilizando anticorpos monoclonais mostraram a importância da P97 na adesão bacteriana às células do epitélio ciliar (ZHANG *et al.*, 1995). Posteriormente, foi identificado que a extremidade C-terminal da P97 possui duas regiões de aminoácidos repetidos, denominadas R1 e R2 (HSU *et al.*, 1997), sendo que a R1 é a região de ligação ao epitélio ciliar do suíno (HSU & MINION *et al.*, 1998), e que ambas (R1 e R2) desempenham papel central na ligação a heparina e a fibronectina (DEUTSCHER *et al.*, 2010). Análise da topologia revela que a P97 (Figura 5), assim como outras adesinas, possuem como característica uma região transmembrana localizada próxima da extremidade N-terminal, e uma cauda extracelular, na qual é possível identificar regiões repetidas na porção C-terminal.

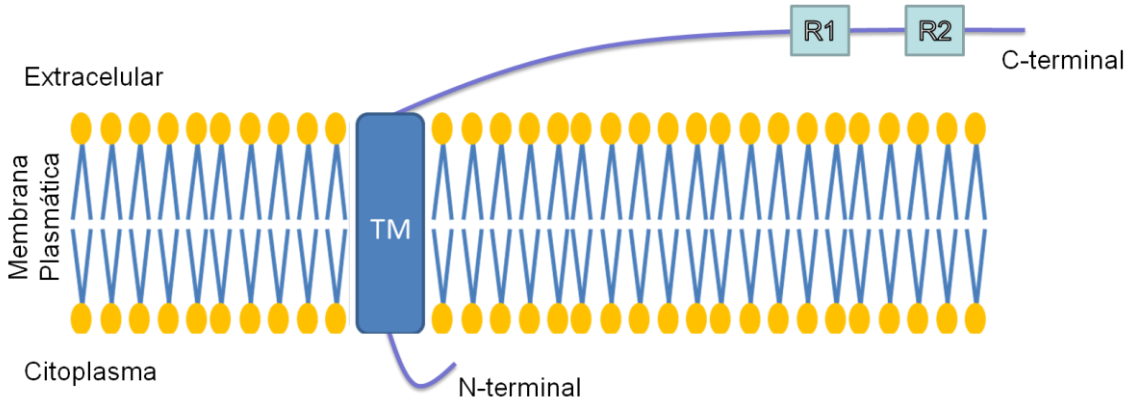

Figura 5 – Representação topológica da adesina P97 de *Mycoplasma hyopneumoniae* 7448. As caixas indicam as regiões repetidas R1 e R2. TM representa o segmento transmembrana da proteína. Figura do autor.

Além da proteína P97, *M. hyopneumoniae* possui outras adesinas potenciais, previamente descritas e listadas na Tabela 1. Estudos demonstraram (Tabela 1) a habilidade de algumas destas proteínas, como a P116, a P216 e a P102, em se ligarem a

componentes do epitélio ciliar do hospedeiro, como a fibronectina, o plasminogênio e a heparina.

A Tabela 1 mostra que algumas proteínas, tais como a P102, a P95 e a P60, aparecem na coluna Referência como "não determinado". Isto ocorre pois ainda não existem estudos funcionais que permitam a classificação definitiva das mesmas como adesinas. Estão neste grupo por serem proteínas que demonstraram, através de estudos de bioinformática, grande similaridade e homologia com outras proteínas previamente descritas como envolvidas na adesão em outras espécies.

Tabela 1 – Principais proteínas conhecidas relacionadas com adesão em *M. hyopneumoniae*.

Nome	Ligante	Referência
P97-like	Heparina, fibronectina, plasminogênio	Seymour *et al*., 2011(35)
P116 ou P102-like	Epitélio ciliar, fibronectina, plasminogênio	Seymour *et al*., 2010 (33)
P97	Epitélio ciliar, heparina, fibronectina	Deutscher *et al*., 2010 (34)
P102	Não determinado	Não determinado
P216	Epitélio ciliar, heparina	Wilton *et al*., 2009 (32)
P159, P110 ou P76	Heparina	Burnett *et al*., 2006 (31)
P97	Epitélio ciliar, heparina	Jenkins *et al*., 2006
P102	Heparina, fibronectina, plasminogênio	Seymour *et al*., 2012 (36)
Proteína hipotética	Epitélio ciliar, glicosaminoglicana	Bogema *et al.,* 2011
P146	Epitélio ciliar, heparina, plasminogênio	Bogema *et al*., 2012 (37)
Proteína hipotética	Epitélio ciliar, heparina	Deutscher *et al*., 2012
Proteína hipotética	Epitélio ciliar, heparina	Deutscher *et al*., 2012
P95	Não determinado	Não determinado
P60	Não determinado	Não determinado

Adaptado de Siqueira *et al*., 2013.

1.6.2 Proteínas antigênicas

Antígenos ou proteínas antigênicas possuem como característica principal a capacidade de estimular o sistema imune do hospedeiro a produzir anticorpos, fazendo com que a resposta imune se inicie sempre que houver o contato com estas proteínas. São

particularmente interessantes, uma vez que podem auxiliar no entendimento de patologias, além de serem excelentes alvos para a produção de vacinas e testes diagnósticos (ALBERTS *et al.*, 2002). Em *M. hyopneumoniae*, diversas proteínas, classicamente não relacionadas com adesão e que desenvolvem resposta imune em hospedeiros, tiveram sua atividade antigênica experimentalmente comprovada, entre elas a proteína de choque térmico (HSP70), o fator de alongamento da tradução Tu (EF-TU), a piruvato desidrogenase B (PDHB) e a lactato desidrogenase (P36) (PINTO *et al*., 2007).

Micoplasmas, assim como muitos outros parasitas, são confrontados com ambientes complexos e desafiadores dos hospedeiros e necessitam se adaptar para sobreviver. A utilização de conjuntos de genes que podem sofrer rápidas e reversíveis mudanças genéticas, produzindo um grande número de variantes de superfície celular, tem sido uma estratégia encontrada em diversos microorganismos (CITTI *et al*., 2010). Esta capacidade em modificar a estrutura e composição das proteínas expostas na superfície celular (variação antigênica) contribui para a constante mudança na configuração de antígenos e a consequente evasão do sistema imune do hospedeiro (RAZIN *et al.*, 1998; van der WOUDLE & BÄUMLER, 2004).

Neste contexto, as lipoproteínas variáveis (Vlps, do inglês *variable lipoproteins*) constituem uma das principais classes de proteínas que sofrem variação antigênica em micoplasmas. Foram inicialmente descritas e caracterizadas em *Mycoplasma hyorinis*, onde foi observado um processo de mutação espontâneo baseado em DNA *slippage* (“escorregamento”), gerando variações em lipoproteínas expostas na superfície bacteriana (ROSENGARTEN & WISE, 1990; YOGEV *et al*., 1991). Posteriormente, observou-se que outras espécies também compartilham estas características, como *Mycoplasma gallisepticum* (YOGEV *et al.*, 1994), *Mycoplasma synoviae*

(VASCONCELOS *et al*., 2005) e *Mycoplasma agalactiae*, (CHOPRA-DEWASTHALY *et al.*, 2008).

Em *M. hyopneumoniae,* existem diversas evidências que indicam a existência de variação antigênica utilizando mecanismos que envolvem modificações no DNA ou nas proteínas. Variações em sequencias repetidas de nucleotídeos em regiões codificantes do DNA podem ser observadas em diferentes cepas de *M. hyopneumoniae* (como 7448, 232 e J), resultando em alterações na composição e no número de aminoácidos repetidos em suas proteínas (VASCONCELOS *et al.*, 2005). Entretanto, a variação de nucleotídeos não ocorre apenas em regiões codificantes, tendo sido demonstrado a presença de variabilidade em regiões intergênicas, potencialmente realizando um processo "liga/desliga" dos genes associados (FERREIRA & CASTRO, 2007).

Além das variações em sequências de nucleotídeos, modificações pós-traducionais ocasionadas pelo processamento proteolítico em proteínas antigênicas de *M. hyopneumoniae* já foram descritas, sendo encontradas principalmente em adesinas e lipoproteínas (DJORDJEVIC *et al.*, 2004, BURNETT *et al.*, 2006, PINTO *et al.*, 2007).

1.7 Análise e identificação de proteínas de superfície

A era da genômica iniciou-se na década de 90, onde a rápida expansão dos projetos de sequenciamento forneceram para a comunidade científica um grande número de dados referentes as sequencias de DNA de diversos organismos. Com isso, fez-se necessário o desenvolvimento de tecnologia para a análise de grandes volumes de dados, surgindo diversas novas *"omics"*, sendo a proteômica uma das principais da chamada era pós-genômica (BROWER, 2001). Esta "nova ciência" que tinha como objetivo principal a identificação de todas as proteínas da célula, como também estabelecer onde elas se encontram, por quanto tempo e com quem interagem, desenvolveu-se e atualmente

permite a identificação e análise de forma individual das diferentes frações protéicas de um organismo.

A fração que corresponde às proteínas de superfície pode ser analisada através da utilização de duas abordagens principais: (1) *in vitro*, com a utilização de técnicas que permitem o enriquecimento e a separação de frações com proteínas de superfície, seguida de métodos de identificação e análise qualitativa e quantitativa destas frações; (2) *in silico*, com a utilização de recursos de bioinformática para a predição de características físico-químicas que possibilitem a inferência sobre a localização celular, topologia, estruturas tridimensionais, entre outros.

1.7.1 Análise de proteínas de superfície *in vitro*

Proteínas expostas na superfície celular, integrais ou associadas à membrana plasmática, são comprovadamente difíceis de analisar, uma vez que as técnicas de proteômica nem sempre funcionam para este tipo de proteína. Isto se deve especialmente a algumas características físico-químicas destas proteínas, tais como a baixa solubilidade e alta hidrofobicidade, associadas a dificuldades de isolamento e de obtenção de frações enriquecidas com proteínas de superfície (CORDWELL, 2006).

Recentes revisões (CORDWELL, 2006; RABILLOUD, 2009) descrevem de forma comparativa as principais tecnologias proteômicas disponíveis para o estudo de proteínas de superfície, pontuando as vantagens e desvantagens da cada uma delas. O desenho experimental utilizado na identificação de proteínas de superfície é dividido em duas etapas: obtenção de frações enriquecidas com proteínas de superfície; e identificação das proteínas oriundas das frações enriquecidas.

1.7.1.1 Obtenção de frações enriquecidas com proteínas de superfície

A primeira etapa da identificação *in vitro* de proteínas de membrana consiste em fracionar as proteínas totais das células, gerando frações ricas em proteínas de membrana. Diferentes são os métodos disponíveis para este fim, entre eles se destacam a solubilidade diferencial, a marcação de proteínas de superfície e a digestão enzimática de proteínas de superfície *in vivo*.

Os métodos de solubilidade diferencial são aqueles que utilizam detergentes para solubilizar proteínas com base nas suas características químicas (hidrofílicas e hidrofóbicas). Detergentes são substâncias anfifílicas, as quais possuem tanto grupos hidrofílicos quanto hidrofóbicos, sendo que os principais utilizados em proteômica (Figura 6) podem ser classificados em: aniônicos, como o dodecil sulfato de sódio (SDS); catiônicos, como o brometo de cetil-trimetilamônio (CTAB); zwiteriônico, como o 3-[(3-colamidopropil)-imetilamonio]-1-propanosulfonato (CHAPS); não iônico, como o Triton.

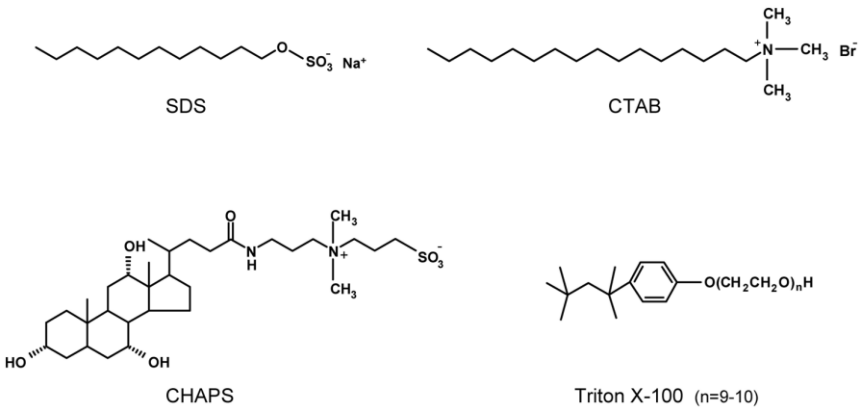

Figura 6 – Estrutura química de detergentes de diferentes classes utilizados em proteômica para a solubilização de proteínas hidrofóbicas. Fonte: Adaptado de G-Biosciences®, disponível em http://www.genotech.com/bulletins/detergent-handbook.pdf

Os detergentes pertencentes às classes não iônica e zwiteriônica têm se tornado popular em experimentos que buscam o enriquecimento de frações com proteínas de

membrana, uma vez que podem solubilizar as proteínas mantendo a sua função (KALIPATNAPU & CHATTOPADHYAY, 2005). Diversos estudos obtiveram sucesso utilizando Triton X-100, Triton X-114 e CHAPS e outros detergentes (CORDWELL, 2006).

Embora a solubilização diferencial possa remover grandes quantidades de proteínas hidrofílicas (citoplasmáticas) da amostra, apresenta como limitações o fato de não produzir uma fração pura, uma vez que apresenta grandes quantidades de contaminantes citosólicos. Também pode ocorrer a perda de proteínas solúveis associadas à membrana (não lipoproteínas), resultando numa fração difícil de ser submetida à eletroforese, uma vez que a presença dos detergentes influencia na migração das proteínas no gel (CORDWELL, 2006).

A marcação das proteínas da superfície celular pode ser feita utilizando-se reagentes como a biotina. Esta técnica consiste na marcação *in vivo* da superfície celular intacta com biotina, seguida de cromatografia de afinidade em coluna de avidina (SCHEURER *et al.*, 2005). Durante algum tempo, esta técnica apresentou algumas limitações, especialmente devido à permeabilidade da membrana, levando à entrada da biotina na célula e à marcação de proteínas intracelulares. Este problema foi solucionado a partir da síntese de um reagente biotinilante solúvel em água e que não consegue atravessar a membrana (GAUTHIER *et al.*, 2004).

Além do método tradicional de biotinilação, atualmente estão disponíveis alguns "kits" comerciais que utilizam marcadores de aminoácidos, como o ICAT™ e o iTRAQ™ (Applied Biosystems), permitindo a comparação entre a geração de picos de peptídeos oriundos de diferentes amostras, bem como a interpretação quantitativa dos dados (GYGI *et al.*, 2002). O uso destes marcadores também se mostrou bastante eficiente

na análise de mudanças da expressão protéica durante diferentes condições as quais a célula é submetida (GOODCHILD *et al.*, 2005).

No método de digestão enzimática de proteínas de superfície *in vivo*, a utilização de uma protease específica, como por exemplo a tripsina, para a digestão *in vivo* das porções protéicas expostas na superfície celular, seguida pela coleta dos peptídeos gerados e identificação em espectrometria de massas, é uma alternativa bastante interessante no estudo dos chamados "superficiomas" (OLAYA-ABRIL *et al*., 2013). Esta metodologia permite analisar e identificar proteínas altamente hidrofóbicas sem as tradicionais dificuldades, uma vez que os epitopos expostos na superfície bacteriana e que são hidrofílicos são identificados (CORDWELL, 2006). Porém, esta técnica pode apresentar alguns desafios, especialmente em relação à integridade celular, uma vez que a proteólise que ocorre na superfície celular pode desestabilizar a membrana, ocasionando a lise da célula e, consequentemente, a contaminação da amostra com proteínas citoplasmáticas, diminuindo assim a especificidade de técnica.

1.7.1.2 Métodos para a identificação de proteínas em amostras enriquecidas com proteínas de superfície

A segunda etapa da identificação *in vitro*, realizada após o fracionamento das proteínas e da obtenção de amostras enriquecidas com proteínas de superfície, consiste na obtenção de dados qualitativos e quantitativos destas amostras. Embora existam diversos métodos conhecidos e utilizados na identificação de proteínas em geral, poucos são aplicáveis para amostras enriquecidas com proteínas de superfície, principalmente, devido às características físico-químicas destas proteínas (anteriormente descritas). Os principais métodos utilizados para a identificação destas proteínas são os géis bidimensionais (2DE), a técnica denominada GeLC-MS/MS (*gel-based liquid*

chromatography-tandem mass spectrometry) e a tecnologia de identificação proteica multidimensional (MudPIT ou "*shotgun proteomics*").

Em técnicas baseadas em 2DE, amostras enriquecidas com proteínas de superfície são fracionadas em duas etapas, uma de separação pelo ponto isoelétrico (primeira dimensão), e outra pela massa (segunda dimensão) por 2DE, seguida da digestão das bandas do gel com proteases (por exemplo, tripsina) e na análise dos peptídeos gerados por espectrometria de massas (CORDWELL, 2006). Apesar de ter sido muito utilizada em diversos experimentos com proteínas de membrana (LAI et al., 2004; RHOMBERG *et al*., 2004; SINHA *et al*., 2005; TWINE *et al.*, 2005; XU *et al*., 2005), esta técnica apresenta diversas limitações, especialmente relacionadas à solubilização e resolução de proteínas com muitas regiões transmembrana.

A técnica denominada GeLC-MS/MS também tem sido utilizada em diversos experimentos de proteômica (PIERSMA *et al.*, 2013). Esta técnica consiste basicamente na separação eletroforética em gel unidimensional de poliacrilamida-dodecil sulfato de sódio (1DE *SDS-PAGE*) das proteínas oriundas de frações enriquecidas com proteínas de membrana, seguida pela excisão e digestão das bandas geradas (5 – 20 bandas) com a enzima tripsina no próprio gel. Posteriormente, é realizada a cromatografia líquida de fase reversa dos peptídeos gerados, associada a espectrometria de massas. Esta técnica tem se mostrado bastante útil especialmente devido às vantagens do detergente SDS, tais como a completa solubilização de diversas classes de proteínas, tolerância a sais, tampões e detergentes e boa matriz para a digestão protéica.

Na tecnologia de identificação proteica multidimensional MudPIT, a amostra enriquecida com peptídeos de membrana é submetida a uma cromatografia líquida bidimensional, na qual a primeira dimensão utiliza um forte trocador de cátions e a segunda dimensão uma cromatografia de fase reversa (SCHIRMER *et al*., 2003;

DELAHUNTY & YATES, 2007). Os peptídeos fracionados são então submetidos à espectrometria de massas. Esta técnica apresenta algumas vantagens quando comparada com os métodos baseados em géis bidimensionais, como a melhor identificação de proteínas com alto peso molecular, ponto isoelétrico extremo e pouca abundância. Como principais desvantagens, pode-se citar o fato de ser pouco eficiente para análises comparativas se não utilizado com algum tipo de marcação e a alta taxa de falsos positivos, ocasionado pela natureza do método.

1.7.2 Análise de proteínas de superfície *in silico* - Modelos e estratégias computacionais

Um dos primeiros modelos utilizados para a análise de proteínas de membrana *in silico,* mais especificamente regiões transmembrana, utilizava o chamado *plot* de hidrofobicidade e foi proposto em 1982 (KYTE & DOOLITTLE, 1982). A análise era relativamente simples, baseando-se na identificação de regiões altamente hidrofóbicas na sequencia de interesse. Como resultado, foi gerada, uma escala utilizada até hoje e amplamente aplicada para delinear o caráter hidrofóbico de aminoácidos e cadeias polipeptídicas nas sequências de interesse denominada GRAVY (do inglês, *Grand Average of Hydropathicity Index*). Este tipo de análise pode ser visualizado em forma de gráfico, onde a sequência de aminoácidos está representada no eixo x e o grau de hidrofobicidade no eixo y (Figura 7).

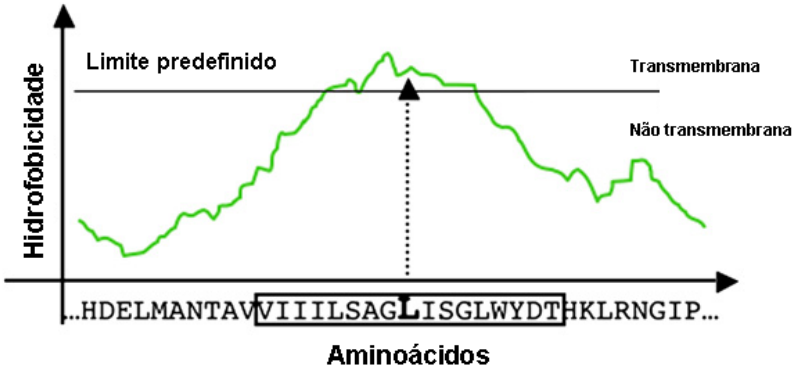

Figura 7 – Representação gráfica de gráfico (*plot*) de hidrofobicidade. Fonte: Kyte & Doolittle, 1982.

As limitações do uso das ferramentas de bioinformática baseadas apenas em análises de padrões e características simples (como a hidrofobicidade) em aminoácidos individuais, associada às observações de que não apenas dentro da região transmembrana, como também nos aminoácidos que a flanqueiam, podemos encontrar padrões peculiares como resíduos aromáticos (LANDOLT-MARTICORENA *et al*., 1993), evidenciaram a necessidade de desenvolvimento de novas estratégias. Neste contexto, o uso de estratégias de aprendizagem automática (*machine-learning*), como os modelos ocultos de Markov (HMM, do inglês *Hidden Markov Model*), as redes neurais artificiais (ANNs, do inglês *Artificial Neural Networks*) e as máquinas de vetores de suporte (SVM, do inglês *Support Vector Machines*), tornaram-se populares especialmente devido à capacidade destes modelos em identificar e modelar padrões complexos em dados biológicos (PRESNELL & COHEN, 1993; ROST et al., 1996; DIEDERICHS *et al*., 1998; REINHARDT & HUBBARD, 1998; HUA & SUN, 2001; JACOBONI *et al*., 2001; CAI *et al.*, 2003; YUAN *et al*., 2004; PARK *et al*., 2005).

Embora a utilização individual de modelos e estratégias computacionais tenha obtido resultados satisfatórios, atualmente entende-se que o melhor desenho experimental para a predição *in silico* de proteínas de membrana consiste em utilizá-los de forma combinada (Figura 8). Está ideia já foi amplamente testada, e as estatísticas demonstram que devido cada estratégia possuir pontos fortes e fracos, sua utilização em conjunto irá produzir uma predição superior quando comparada a utilização de cada uma de forma individual (HANSEN & SALAMON, 1990).

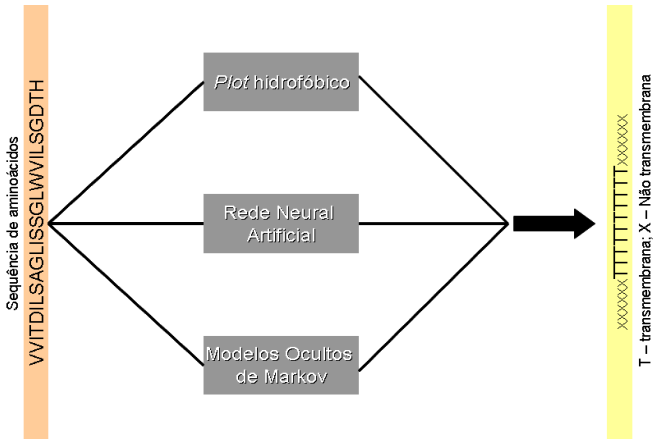

Figura 8 – Representação esquemática de um exemplo de análise combinada utilizando múltiplas estratégias de predição *in silico*. Fonte: elaborada pelo autor.

Atualmente, inúmeras ferramentas de bioinformática, baseadas nos modelos anteriormente descritos, estão disponíveis para a realização da predição *in silico* de proteínas de superfície. O desenvolvimento de programas (*softwares*) com uma interface simples e "amigável" permitiu que pesquisadores, mesmo com pouca experiência em programação computacional, pudessem adicionar análises *in silico* em seus estudos.

Diversas ferramentas, que se baseiam na predição de características comuns das proteínas de membrana, tais como a presença de região transmembrana ou região *lipobox* e presença de peptídeo sinal estão disponíveis para análise, conforme descrito na Tabela 2.

Tabela 2 – Programas de bioinformática comumente utilizados na predição de proteínas de membrana.

Programa	**URL**
Predição de região transmembrana	
DAS-TMWlter	http://mendel.imp.ac.at/sat/DAS/DAS
HMMTOP 2.0	http://www.enzim.hu/hmmtop/
MEMSAT3	http://bioinf.cs.ucl.ac.uk/psipred/
MINNOU	http://minnou.cchmc.org/

OrienTM	http://o2.biol.uoa.gr/orienTM/
PHDhtm	http://rostlab.org/predictprotein/submit_adv
Phobius	http://phobius.cgb.ki.se/
SOSUI	http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0
Split4	http://split.pmfst.hr/split/4/
TMAP	http://bioinfo.limbo.ifm.liu.se/tmap/index
TMHMM	http://www.cbs.dtu.dk/services/TMHMM/
TOP-PRED	http://bioweb.pasteur.fr/seqanal/interfaces/toppred

Predição de lipobox

LIPOPREDICT	http://lipopredict.cdac.in/
LIPO CBU	http://services.cbu.uib.no/tools/lipo
LipPred	http://www.ddg-pharmfac.net/lippred/LipPred

Predição de peptídeo sinal

LipoP 1.0	http://www.cbs.dtu.dk/services/LipoP/
SignalP 4.1	http://cbs.dtu.dk/services/SignalP/
Signal-BLAST	http://sigpep.services.came.sbg.ac.at/signalblast.html

2. JUSTIFICATIVA E OBJETIVOS

Considerando que:

- até o momento diversos aspectos envolvendo os mecanismos de patogenicidade do *M. hyopneumoniae*, como adesão, sinalização e modulação do sistema imune do hospedeiro ainda não foram totalmente elucidados;

- a PES é uma doença de abrangência mundial, que causa inúmeros prejuízos econômicos para os produtores;

- o controle da PES ainda é bastante precário, considerando especialmente as deficiências encontradas na vacinação e diagnóstico, além do alto custo envolvido na terapêutica medicamentosa;

- a definição do repertório de proteínas de superfície do *M. hyopneumoniae* é fundamental para o entendimento dos mecanismos de patogenicidade bacteriana, além de ser importante para o desenvolvimento de estratégias de controle da PES, tais como novas vacinas, testes diagnósticos e medicamentos.

Este trabalho tem como **objetivo geral** realizar a identificação e a análise do repertório de proteínas de superfície de *M. hyopneumoniae* 7448. Além disso, tem como **objetivos específicos:**

a) Desenvolver uma abordagem que permita a produção de frações protéicas enriquecidas com proteínas de superfície de *M. hyopneumoniae*.

b) Identificar as proteínas oriundas das frações enriquecidas com proteínas de superfície utilizando espectrometria de massas.

c) Utilizar ferramentas de bioinformática para realizar a predição *in silico* de proteínas transmembrana e de lipoproteínas do *M. hyopneumoniae*. Predizer e analisar a topologia das adesinas codificadas pelo genoma do *M. hyopneumoniae*.

d) Realizar um estudo comparativo entre o que foi identificado na superfície bacteriana nas análises por proteômica experimental e nas predições *in silico*.

3. RESULTADOS

Os resultados desta tese estão apresentados em dois capítulos, organizados da seguinte forma:

- **Capítulo 1**: Survey of surface proteins from the pathogenic *Mycoplasma hyopneumoniae* strain 7448 using a biotin cell surface labeling approach.

- **Capítulo 2:** Predições *in silico* de proteínas de superfície de *M. hyopneumoniae* 7448.

3.1 CAPÍTULO 1: Survey of surface proteins from the pathogenic *Mycoplasma hyopneumoniae* strain 7448 using a biotin cell surface labeling approach.

Neste capítulo estão descritos os resultados referentes à identificação e a análise do repertório de proteínas de superfície de *M. hyopneumoniae* 7448 (objetivo geral da tese), organizados na forma de artigo científico. Neste artigo, também estarão descritos os resultados correspondentes aos objetivos específicos (a), (b), (c) e (d).

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Autores: Luciano Antonio Reolon (LAR), Carolina Lumertz Martello (CLM), Irene Silveira Schrank (ISS) e Henrique Bunselmeyer Ferreira (HBF).

Contribuição dos autores: LAR realizou os cultivo de *M. hyopneumoniae*, a biotinilação e a purificação por cromatografia de afinidade das proteínas de superfície, as análises por espectrometria de massas, as predições *in silico* e a redação do manunscrito. CLM auxiliou nas predições *in silico*. ISS e HBF conceberam o estudo e participaram do seu design, implementação e coordenação.

O artigo está completamente reproduzido a seguir, com numeração de páginas original mantida. Os arquivos adicionais encontram-se descritos no Apêndice 1 desta tese.

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Survey of Surface Proteins from the Pathogenic *Mycoplasma hyopneumoniae* Strain 7448 Using a Biotin Cell Surface Labeling Approach

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Abstract

The characterization of the repertoire of proteins exposed on the cell surface by *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*), the etiological agent of enzootic pneumonia in pigs, is critical to understand physiological processes associated with bacterial infection capacity, survival and pathogenesis. Previous *in silico* studies predicted that about a third of the genes in the *M. hyopneumoniae* genome code for surface proteins, but so far, just a few of them have experimental confirmation of their expression and surface localization. In this work, *M. hyopneumoniae* surface proteins were labeled in intact cells with biotin, and affinity-captured biotin-labeled proteins were identified by a gel-based liquid chromatography-tandem mass spectrometry approach. A total of 20 gel slices were separately analyzed by mass spectrometry, resulting in 165 protein identifications corresponding to 59 different protein species. The identified surface exposed proteins better defined the set of *M. hyopneumoniae* proteins exposed to the host and added confidence to *in silico* predictions. Several proteins potentially related to pathogenesis, were identified, including known adhesins and also hypothetical proteins with adhesin-like topologies, consisting of a transmembrane helix and a large tail exposed at the cell surface. The results provided a better picture of the *M. hyopneumoniae* cell surface that will help in the understanding of processes important for bacterial pathogenesis. Considering the experimental demonstration of surface exposure, adhesion-like topology predictions and absence of orthologs in the closely related, non-pathogenic species *Mycoplasma flocculare*, several proteins could be proposed as potential targets for the development of drugs, vaccines and/or immunodiagnostic tests for enzootic pneumonia.

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Introduction

Mycoplasmas belong to the class Mollicutes and are among the smallest free-living organisms capable of self-replication. Evolutionarily related to Gram-positive bacteria, mycoplasmas have undergone extensive genome reduction, which led to simplification or loss of some metabolic pathways and structural cell components. They are unable to synthesize peptidoglycans or their precursors, and therefore, present no cell wall. The mycoplasma cell membrane is formed from proteins, phospholipids and cholesterol; cholesterol is an essential nutrient to bacterial growth and is responsible for membrane rigidness and stability [1,2].

M. hyopneumoniae is the etiological agent of enzootic pneumonia (EP), a chronic disease characterized by a dry and non-productive cough, most evident when pigs are roused, retarded growth, and inefficient food conversion [3]. In pigs, *M. hyopneumoniae* is found to be attached to the cilia of the tracheal epithelial cells, causing a reduction in ciliary action [4,5], and predisposing the swine to infection by other pathogens, such as *Pasteurella multocida* [6] and porcine reproductive and respiratory syndrome virus (PRRSV) [7].

M. hyopneumoniae adhesion to the swine tracheal epithelial cells is essential to disease establishment, and the characterization of adhesion-mediating molecules has been the focus of most studies on the bacterial mechanisms of virulence and pathogenesis. Bacterial adhesive capability is related to several proteins, such as the well-described P97 adhesin [8,9,10] and adhesin-like proteins, such as P216 [11], P159 [12], P102 [13], P146 [14] and P116 [15]. It has been suggested, however, that several other proteins that so far remain uncharacterized are involved in the *M. hyopneumoniae* cell adhesion process [12].

Membrane (integral or associated) proteins are directly exposed on the cell surface and play key roles in cell adhesion, and evasion and/or modulation of the host immune system, events which are important for environmental, bacterial and host cell interactions [16,17]. The identification of membrane proteins represents a great challenge, especially due to their mainly hydrophobic nature and selective loss during purification, which especially occurs in the precipitation and solubilization steps.

Several methods have been applied to the experimental identification of mycoplasma membrane proteins, especially methods involving selective solubilization the use of detergents, such as Triton X100 [18,19] and Triton X114 [20]. These methods yield enriched membrane protein fractions, including lipoproteins, but do not completely avoid contamination with cytosolic and ribosomal proteins [21]. Selective labeling using hydrophilic and membrane-impermeable reagents, such as Sulfo-NHS-Biotin, is an alternative way to reduce this contamination and allow the recovery of a more specific fraction of surface exposed proteins [22].

Here, we describe a proteomic approach, based on intact cell surface labeling and labeled protein purification, coupled to gel-based liquid chromatography-tandem mass spectrometry (GeLC-MS/MS), to identify *M. hyopneumoniae* surface exposed proteins. This strategy allowed to identify surface proteins possibly involved in pathogenesis, including some previously annotated as hypothetical proteins. Moreover, a comparative analysis was carried with the closely related non-pathogenic species *Mycoplasma flocculare* (*M. flocculare*), to verify which of the identified surface proteins are found only in the pathogenic counterpart. The potential of some of the identified *M. hyopneumoniae* surface exposed proteins as novel targets for the development of vaccines, diagnostic tests and therapeutic drugs is discussed.

Methods

Bacterial strain and culture conditions

The *Mycoplasma hyopneumoniae* pathogenic strain 7448 was isolated from an infected swine from Lindóia do Sul (Santa Catarina, Brazil), and cultured in Friis medium as previously described [23].

Biotin labeling and affinity recovery of labeled *M. hyopneumoniae* strain 7448 proteins

A cell pellet from 100 ml of fresh *M. hyopneumoniae* 7448 culture was collected by centrifugation at 3360 x g for 15 min. The pellet was washed three times with cold phosphate-buffered saline (PBS; pH 7.2) and resuspended in the same buffer with the addition of 1 mg of sulfosuccinimidyl biotin (EZ-Link Sulfo-NHS-Biotin; Pierce, USA)/ml, in a final concentration of 2 mM of biotin reagent, according to the manufacturer's instructions. The labeling reaction was performed as previously described [22] for 30 min at 4°C, and the residual sulfo-NHS-biotin was quenched by adding glycine to a final concentration of 100 mM. To remove all unspecific and inactivated Sulfo-NHS-Biotin, the cell suspension was washed twice with 100 mM glycine (final concentration) in PBS. Cells were then lysed by five rounds of sonication (30 s, 20 kHz, 1 min interval between rounds) and labeled proteins were recovered by affinity chromatography in a Monomeric Avidin Resin (Pierce, USA), with gravity flow, using phosphate-buffered saline (PBS, pH 7.0) + 1% Triton X-100 as an equilibration and wash buffer and the same buffer with the addition of biotin to a final concentration of 2 mM was used to block biotin non-reversible binding sites and to elute the bound biotinylated molecule. All steps were performed in the presence of protease inhibitors (Sigma-Aldrich, USA). Chromatography was monitored by measuring the absorbance at 280 nm. As a control, a cell pellet from 100 ml of another fresh *M. hyopneumoniae* 7448 culture was collected by centrifugation at 3360×g for 15 min, washed three times with cold phosphate-buffered saline (PBS; pH 7.2) and lysed by sonication (as described above) prior to biotin labeling of total proteins. After this, labeled proteins were recovered and treated as described above for protein labeling of intact cells. Crude protein extracts were produced in the same way, excluding biotin labeling steps.

Protein samples were concentrated, and salts and detergents were removed by a chloroform/methanol precipitation step and freeze dried until use. All the above procedures of cell culture, and protein labeling and affinity purification were performed in triplicate.

Electrophoretic prefractionation followed by liquid chromatography tandem mass spectrometry of *M. hyopneumoniae* biotin-labeled proteins

A gel-based liquid chromatography-tandem mass spectrometry (GeLC-MS/MS) [24] approach, using protein prefractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel digestion (IGD), followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS), was used for the identification of biotin-labeled proteins of *M. hyopneumoniae* strain 7448. The workflow for GeLC-MS/MS was performed as follows. Freeze-dried protein samples (obtained as described in section 2.2) were resuspended in PBS (pH 7.0) and protein concentration was determined using the Qubit Protein Assay Kit (Invitrogen, USA), according to the manufacturer's instructions. Samples corresponding to 15 μg of biotin-labeled proteins in PBS, after addition of urea to a final concentration of 8 M, were fractionated by SDS-PAGE on 4–10% Mini-PROTEAN tetra cell precast gels at 80 mV using Tris-SDS running buffer, and stained with Coomassie Brilliant-Blue G250 (Sigma-Aldrich, USA). For each protein sample resolved by SDS-PAGE the corresponding gel lane was divided into 20 slices of similar size (~15 mm^2 in area), which were manually excised from the gel and individually processed as follows. They were initially submitted to three washes with 400 μl of 50% acetonitrile and 50 mM ammonium bicarbonate pH 8.0 for 15 min, followed by one washing step with 400 μl of acetonitrile. Gel slices were then incubated with 200 μl of 10 mM DTT in 50 mM ammonium bicarbonate at room temperature for 60 min for reduction, and proteins were subsequently alkylated by incubation with 50 mM iodoacetamide in 50 mM ammonium bicarbonate for 45 min in the dark at room temperature. Gel slices were dried in a CentriVap centrifuge (Labconco, USA). For IGD, gel slices were covered with a trypsin solution (Promega, USA) (20 μg in 1 mL of ammonium bicarbonate 50 mM) and samples were incubated overnight at 37°C. Extraction of the resulting peptides from gel slices was carried out by two successive 1 h incubations with 50 μl of 50% acetonitrile and trifluoroacetic acid (TFA).

Peptides resulting from IGD were separated in a Nanoease C18 (75 μm ID) capillary column by elution with a water/acetonitrile 0.1% formic acid gradient in a capillary liquid chromatography system (Waters, Milford, US). Liquid chromatography was coupled online with an electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) Ultima API mass spectrometer (Micromass, UK). 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 included a flow of 200 nL/min, a nanoflow capillary voltage of 3.5 kV, a block temperature of 100°C, and a

cone voltage of 100 V. For each replicate, three independent LC-MS/MS measurements were performed. Mass spectrometry was performed in the Unidade de Química de Proteínas e Espectrometria de Massas (Uniprote-MS), Centro de Biotecnologia, UFRGS (Porto Alegre, Brazil).

Protein identification based on peptide MS/MS data was performed using Mascot software (Matrix Science, UK). All tandem mass spectra were searched against a database generated via an *in silico* digest of all proteins encoded by the *M. hyopneumoniae* 7448 genome with the following search parameters: trypsin was used as the cutting enzyme, mass tolerance for the monoisotopic peptide window was set to ±0.2 Da, the MS/MS tolerance window was set to ±0.2 Da, one missed cleavage was allowed, and carbamidomethyl and oxidized methionine were chosen as variable modifications.

In silico analyses of protein identified by GeLC-MS/MS

Cluster of Orthologous in Genomes (COG) annotations were assigned based on sequence similarity searches of CDS entries from *M. hyopneumoniae* strain 7448 (http://www.ncbi.nlm.nih.gov/genome, NC_007332) against the COG annotated proteins database (http://www.ncbi.nlm.nih.gov/COG) [25]. Lipoprotein (LP) *in silico* prediction was performed using LIPOPREDICT (http://www.lipopredict.cdac.in/) and LIPO CBU (http://services.cbu.uib.no/tools/lipo). Ortholog analysis was performed as previously described [26].

Results

GeLC-MS/MS analysis of *M. hyopneumoniae* cell surface labeled proteins

Biotin-labeled *M. hyopneumoniae* strain 7448 proteins were resolved by SDS-PAGE, and proteins from gel slices (Figure S1, lane 2 and 3) were subjected to nanoHPLC-nanoESI-Q-TOF-MS/MS identification. MS/MS analyses were performed in replicates (technical replicates) for each of the three biological samples, with virtually the same results. The GeLC-MS/MS identification of labeled intact cell (LIC) samples, resulted in a total of 165 protein identifications (Table S1), corresponding to 59 different protein species (Table S2), which represent approximately 10% of the total proteins encoded by the genome of *M. hyopneumoniae* strain 7448. In control samples from labeled lysed cells (LLC), 96 different proteins species were identified, including several typical intracellular proteins, such as ribosomal proteins, not identified in samples from intact cells (Table S3).

Considering the repertoire of 59 surface exposed proteins detected by GeLC-MS/MS of labeled intact cell (LIC) samples, 34 were identified from two or more gel slices, including well-known adhesion related proteins, such as P76, P97, P102, P146 and P216, along with 19 (32%) hypothetical proteins. Thirty five of these proteins (58.3%) were previously predicted as surface protein [26]. We also performed an *in silico* prediction of *M. hyopneumoniae* 7448 lipoproteins (LPs) (Table S4), and, from the total of 25 predicted LPs (summing those predicted by LIPOPREDICT and LIPO CBU tools), 16 (64%) were detected in our survey of surface exposed proteins. Among the MS-detected surface proteins predicted *in silico* as such or as LPs, there are well-known adhesion related proteins, such as P76, P97, P102, P146 and P216, 46 K surface antigen precursor, ABC transporter xylose-binding lipoprotein, and prolipoprotein p65, along with 16 hypothetical proteins.

A total of 21 proteins out of the 59 identified as surface exposed by GeLC-MS/MS of labeled intact cell (LIC) samples (35%) were not predicted *in silico* as such [26] or as lipoproteins (our results) (Table S2). This group includes proteins traditionally involved in intracellular processes. An additional analysis of these proteins reveled that at least for some of them, like pyruvate dehydrogenase subunits, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), L-lactate dehydrogenase, elongation factor Tu (Ef-Tu), molecular chaperone DnaK, and NADH-dependent flavin oxidoreductase, there are previous evidences in the literature (see Discussion) suggesting their surface localization in mycoplasmas or other pathogenic bacteria.

Functional classification of *M. hyopneumoniae* surface proteins from LIC samples identified by GeLC-MS/MS

In an attempt to infer potential physiological/functional features, the functional classification of the 59 proteins experimentally identified as surface exposed was performed based on the COG [25] (Figure 1, Table S2). According to COG, more than half of the GeLC-MS/MS identified *M. hyopneumoniae* surface proteins (35 out of 59; 58%) from LIC samples were classified as having unknown function, a class which included adhesion-related (6 proteins) and hypothetical proteins (19 proteins). Energy production and conversion was the second well-represented class (9 out of 59; 15%), followed by nucleotide transport and metabolism (5 out of 59; 8%), carbohydrate transport and metabolism (3 out of 59; 5%), general function prediction (3 out of 59; 5%), posttranslational modification/protein turnover/chaperones (2 out of 59; 2%), inorganic ion transport and metabolism (1 out of 59; 1.6%), translation, ribosomal structure and biogenesis (1 out of 59; 1.6%) and translation (1 out of 59; 1.6%).

Topology predictions of *M. hyopneumoniae* surface proteins from LIC samples identified by GeLC-MS/MS

In silico topology predictions were performed for the 59 proteins experimentally identified as surface exposed. These predictions (Table S2) showed that 19 of them had transmembrane domains (TM proteins), including the adhesion related proteins P76, P97, P102, P146 and P216, the transporter potassium uptake protein, and the cell division protein. Interestingly, other 10 of the 19 putative transmembrane proteins also presented adhesion-like predicted topologies, consisting of a transmembrane helix and a large tail exposed at the cell surface. These proteins are protein MHP7448_0372, 46 K surface antigen precursor, periplasmic sugar-binding protein, and 7 hypothetical proteins (MHP7448_0138, MHP7448_0352, MHP7448_0373, MHP7448_0467, MHP7448_0468, MHP7448_0629, and MHP7448_0661).

Considering the experimental demonstration of surface exposure, adhesin-like topology predictions and absence of orthologue in the closely related, non-pathogenic species *M. flocculare*, 12 of the proteins identified by GeLC-MS/MS in LIC samples could be selected as potential targets for the development of drugs, vaccines and/or immunodiagnostic tests (Table 1). These include proteins previously related to pathogenicity in bacteria (NADH-dependent flavin oxidoreductase, and 46 K surface antigen precursor), and several previously uncharacterized *M. hyopneumoniae* hypothetical proteins.

Discussion

Cell surface proteins are directly exposed to the environment and can mediate important pathogen-host interactions, such as adhesion, cell signaling and immune modulation, all of which are relevant for pathogenesis. For *M. hyopneumoniae*, it is predicted that about a third of the genes in its genome code for surface

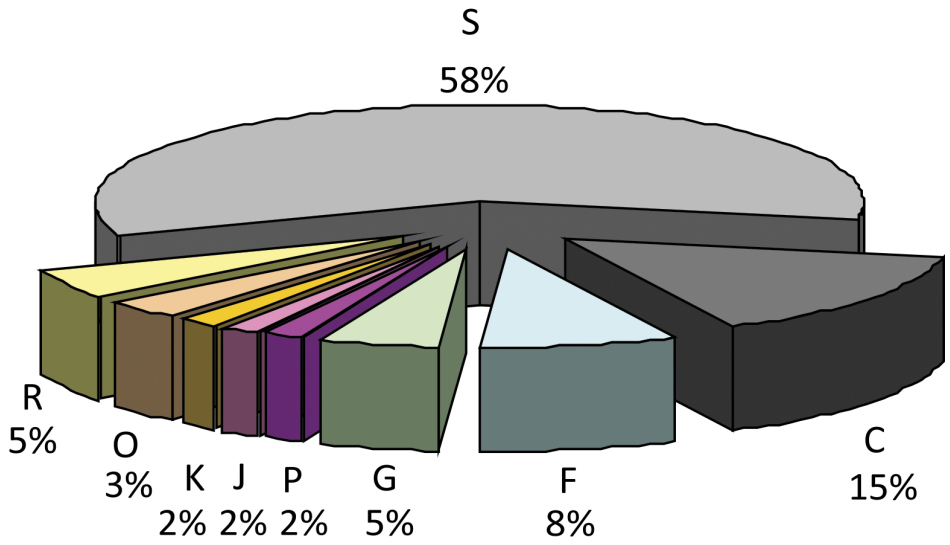

Figure 1. Functional analysis of *M. hyopneumoniae* surface proteins identified by GeLC-MS/MS in LIC samples. Percentages of proteins predicted in each functional category are indicated in the sectors of the circle. COG functional classes are as follows. Major class information storage and processes: (J) Translation, ribosomal structure and biogenesis, (K) Transcription; Major class cellular processes: (O) Post-translational modification, protein turnover, and chaperones, (P) Inorganic ion transport and metabolism; Major class Metabolism: (C) Energy production and conversion, (G) Carbohydrate transport and metabolism, (F) Nucleotide transport and metabolism; Major class poorly characterized: (R) General function prediction only, (S) Function unknown.
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proteins [26], but so far, just a few of them have experimental confirmation of their expression and surface localization. Here, we employed a biotin cell surface labeling approach followed by avidin affinity recovery of labeled proteins coupled to identification by GeLC-MS/MS to survey the exposed surface proteins from *M. hyopneumoniae*. Experimental demonstration of surface exposure, adhesion-like topology predictions and absence of orthologs in the closely related, non-pathogenic *M. flocculare* allowed to propose several proteins as candidates for the development of drugs, vaccines and/or immunodiagnostic tests for enzootic pneumonia.

Biotinylation has already been described as useful for the specific labeling of exposed tails of surface proteins [21,22]. The GeLC-MS/MS workflow was chosen for the qualitative identification of biotin-labeled *M. hyopneumoniae* surface proteins due to some advantages for hydrophobic protein analysis, including

Table 1. Adhesin topology prediction and additional criteria for the selection of the identified surface exposed proteins as potential targets for the development of drugs, vaccines and/or immunodiagnostic tests.

Locus Tag[1]	Protein product[2]	Protein name	Adhesin-like topology[3]	Absence in *M. flocculare*[4]	Studies suggested a surface localization[5]
MHP7448_0088	YP_287488.1	Hypothetical protein MHP7448_0088		•	
MHP7448_0138	YP_287535.1	Hypothetical protein MHP7448_0138	•		
MHP7448_0234	YP_287631.1	Periplasmic sugar-binding protein	•	•	[46]
MHP7448_0309	YP_287705.1	NADH-dependent flavin oxidoreductase		•	[48,49,50]
MHP7448_0324	YP_287719.1	Hypothetical protein MHP7448_0324		•	
MHP7448_0333	YP_287728.1	Hypothetical protein MHP7448_0333		•	
MHP7448_0352	YP_287746.1	Hypothetical protein MHP7448_0352	•		[29]
MHP7448_0372	YP_287766.1	Protein MHP7448_0372	•		
MHP7448_0373	YP_287767.1	Hypothetical protein MHP7448_0373	•		[30]
MHP7448_0513	YP_287902.1	46 K surface antigen precursor	•		
MHP7448_0629	YP_288014.1	Hypothetical protein MHP7448_0629	•		
MHP7448_0661	YP_288046.1	Hypothetical protein MHP7448_0661	•		

[1]Locus tag as defined for *M. hyopneumoniae* strain 7448 (http://www.ncbi.nlm.nih.gov/genome, NC_007332).
[2]Protein product according to NCBI database (http://www.ncbi.nlm.nih.gov).
[3]Topology prediction similar to well known adhesins (Table S2).
[4]No ortholog found closely related, non-pathogenic species *M. flocculare*.
[5]Published literature.
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complete solubilization by SDS and removal of detergent and salts [24]. The biotinylation-GeLC-MS/MS combined approach successfully enriched samples and identified *M. hyopneumoniae* surface proteins, with no ribosomal protein recovery and with the identification of few classical cytoplasm proteins (Table S2). Besides, a large proportion of adhesion related proteins and LPs was selectively recovered and identified from LIC samples, in contrast with labeled lysed cell (LLC) control samples (cells lysed prior to labeling), from which proteins involved in a broad range of cellular functions, localized in both cytoplasm and membrane were identified (Table S3).

The adherence of *M. hyopneumoniae* to tracheal host cells is a crucial step for colonization and establishment of disease in infected pigs [1,3,16]. Adhesins play key roles in the process of pathogen binding to a host cell, although cell adhesion is a multifactorial process that involves surface proteins from both bacterial and host cells [27]. Well-known *M. hyopneumoniae* adhesins, namely P97, P97-like, P216, P102, and P76 proteins, which have been associated with binding to porcine cilia, heparin, fibronectin and plasminogen [8,9,10,11,13], had their surface localization confirmed by GeLC-MS/MS identification in LIC samples (Table S2). Other well represented proteins in our proteomic survey were LPs, among which were ABC transporter xylose-binding lipoprotein, and P60-like lipoprotein. ABC transporter xylose-binding lipoprotein is similar to the outer membrane lipoprotein P48 of some *Mycoplasma* species [28], described as an immunomodulatory protein required for intracellular invasion. The P60-like lipoprotein ortholog of *Mycoplasma hominis* was previously described [29] as a surface protein associated with P80 protein, whose *M. hyopneumoniae* ortholog (MHP7448_352) was also identified in our survey.

The GeLC-MS/MS protein identification in LIC samples also revealed the presence of 19 different hypothetical proteins in the *M. hyopneumoniae* cell surface. This corresponds to 6.3% of the total of 298 *M. hyopneumoniae* 7448 CDS products annotated as hypothetical proteins [26]. The products of two of these CDSs (MHP7448_0373 and MHP7448_0662) have been recently associated with cell adhesion. The MHP7448_0373 hypothetical protein was described as a heparin binding protein in porcine cilia [30], and the MHP7448_0662 hypothetical protein, which is a paralog of P102 was described as an adhesion protein able to bind fibronectin extracellular compounds [31] overexpressed in pathogenic strains [32]. Furthermore, our topology analysis showed that five of the 19 identified hypothetical proteins have adhesin-like topologies (Table S2).

Some proteins traditionally related to cellular processes that occur mainly in the cytoplasm were also identified in the *M. hyopneumoniae* cell surface. At least some of them, namely pyruvate dehydrogenase, GAPDH, L-lactate dehydrogenase, Ef-Tu and DnaK, have also been described as bacterial surface components, with involvement in bacterium-host interaction and pathogenicity. Pyruvate dehydrogenase, an immunogenic *M. hyopneumoniae* protein [33,34], was described as a surface protein involved in the bacterial binding to the host extracellular matrix in *Mycoplasma pneumoniae* [35]. GAPDH, usually located in the cytoplasm and known to play a central role as a glycolytic enzyme, has been identified on the surface of pathogenic bacteria and was related to pathogenic processes [36], such as adhesion and host matrix binding [37], immunomodulation and immune evasion [38]. L-lactate dehydrogenase, also known as P36, was described as immunogenic for pigs infected with *M. hyopneumoniae* [39]. The translational factor Ef-Tu was also identified as an immunogenic cell surface protein in mycoplasmas [18]. The molecular chaperone DnaK has been shown to be surface accessible in *M. hyopneumoniae* [40] and was suggested as a surface or secreted protein of several other pathogenic bacteria, as *Bacillus anthracis* [41] and *Mycobacterium tuberculosis* [42].

The identification of the same protein species from two or more gel slices was taken as further evidence of proteolytic post-translational processing. This was observed for 34 of the 59 different protein species identified by GeLC-MS/MS in LIC samples, including adhesins and hypothetical proteins (Table S1). Proteolytic post-translational processing of *M. hyopneumoniae* proteins has been previously shown [43,44], and an alternative explanation based on protein degradation during sample processing was discarded in our survey due to the use of broad range protease inhibitors.

Nearly two thirds (39 out of 59) of the GeLC-MS/MS identified *M. hyopneumoniae* 7448 proteins in LIC samples was predicted *in silico* as surface proteins, considering the previous work by Siqueira *et al.* [26] and our complementary LP prediction. The extensive overlap between our proteomic approach and the *in silico* predictions is another indicator of the success of the selective surface biotin labeling, increasing the confidence of our results. Our proteomic results also contributed to better understanding of the *M. hyopneumoniae* 7448 repertoire of surface exposed proteins by also detecting, as discussed above, annotated (16) and hypothetical (5) proteins previously not predicted as bacterial surface components (Table S2). Considering sensitivity limitations of both labeling and MS detection [21], the identified proteins can be considered to be among the more abundant protein species on the *M. hyopneumoniae* 7448 cell surface.

The pathogenic *M. hyopneumoniae* and the non-pathogenic *M. flocculare* cohabit the swine respiratory tract and both species adhere to the cilia of tracheal epithelial cells, but just *M. hyopneumoniae* causes tissue damage [45]. Considering the extensive genetic similarity between *M. hyopneumoniae* and *M. flocculare* [26], we also surveyed the *M. flocculare* genome for orthologs of the *M. hyopneumoniae* 7448 proteins identified in LIC samples. Although *M. hyopneumoniae* 7448 and *M. flocculare* share nearly 90% of their predicted repertoire of surface proteins [26], 5 out of the 59 (8%) proteins identified in LIC samples are not found in *M. flocculare*. The *M. hyopneumoniae* proteins not shared with *M. flocculare* are three hypothetical proteins (MHP7448_0088, MHP7448_0324 and MHP7448_0333); a periplasmic sugar-binding protein that serves as primary receptor for diverse solutes in transport system, chemotaxis and signaling [46]; and a NAD-dependent flavin oxidoreductase. Interestingly, the involvement of oxidoreductase enzymes in pathogenicity has been described [47,48,49], and recent studies demonstrated that the *Mycobacterium avium* subsp. *paratuberculosis* NADH- flavin oxidoreductase from is essential for invasion of epithelial cells [50].

Besides the absence in *M. flocculare*, another aspect that calls attention to the periplasmic sugar-binding protein and to some other surface proteins identified in *M. hyopneumoniae* LIC samples was their adhesin-like topology. These criteria, along with previous studies of possible involvement of ortholog proteins in pathogen-host interactions, allowed us to propose at least 12 proteins (Table 1) as novel and promising targets for future studies.

Conclusion

The continuous development of immunization strategies, diagnosis tests and new drugs and antibiotics for disease treatment is essential for the control and prevention of EP [51]. Besides, some aspects of *M. hyopneumoniae* pathogenicity still need further clarification, including cell adhesion and immunomodulation [2,5,13]. Bacterial cell surface proteins play key roles in several

cellular events important for bacterial growth, colonization and host inception. The selective labeling of surface proteins and their identification by GeLC-MS/MS described here provided new insights about cell surface protein composition in *M. hyopneumoniae* and allowed to point out novel and promising targets for the development of vaccines, diagnostic tests and therapeutic drugs. Further insights on *M. hyopneumoniae* pathogenicity are expected when the approaches standardized here were coupled to quantitative proteomic strategies for the comparison of the surface protein content of pathogenic and non-pathogenic *M. hyopneumoniae* strains.

Supporting Information

Figure S1 Biotin labeling and affinity capture of M. hyopneumoniae proteins from labeled lysed cell (LLC) and labeled intact cell (LIC) samples. SDS-PAGE 10%, and stained with Coomassie Brilliant Blue. Lane M – marker, Precision Plus prestained protein standards (Bio-Rad). Lane 1– crude protein extracts from M. hyopneumoniae 7448 (15 μg). Lane 2– avidin affinity capture of proteins from M. hyopneumoniae LLC samples (15 μg). Lane 3 - avidin affinity capture of proteins from M. hyopneumoniae LIC samples (15 μg). Bands 1 to 20 were subjected to Nano-LC/MS/MS analysis.
(TIF)

Table S1 *Proteins identified by GeLC-MS/MS in labeled intact cell (LIC) samples*.
(XLSX)

Table S2 Different protein species identified by GeLC-MS/MS in labeled intact cell (LIC) samples.
(XLSX)

Table S3 Different protein species identified by GeLC-MS/MS in control samples from labeled lysed cells (LLC).
(XLSX)

Table S4 Lipoprotein predictions.
(XLSX)

Author Contributions

Conceived and designed the experiments: LAR ISS HBF. Performed the experiments: LAR CLM ISS HBF. Analyzed the data: LAR CLM ISS HBF. Contributed to the writing of the manuscript: LAR ISS HBF.

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3.2 CAPÍTULO 2: Analise *in silico* de proteínas de superfície de *M. hyopneumoniae*

Neste capítulo, encontram-se os materiais e métodos e os resultados referentes ao objetivo específico (c), no qual descrevemos as análises *in silico* de proteínas de superfície de *M. hyopneumoniae*. Estes resultados, obtidos pelo autor, fizeram parte do artigo científico "**New insights on the biology of swine respiratory tract mycoplasmas from a comparative genome analysis"**, publicado na revista BMC Genomics em 2013, apresentado integralmente no Apêndice 2 desta tese.

3.1.1 Materiais e métodos utilizados para a prospecção *in silico* de proteínas de superfície de *M. hyopneumoniae* 7448

Todas as regiões codificadoras do genoma do *M. hyopneumoniae* 7448 (http://www.ncbi.nlm.nih.gov/genome, NC_007332) foram submetidas a uma robusta análise *in silico*, utilizando programas de bioinformática que identificam domínios e motivos proteicos comumente encontrados em proteínas de superfície. A predição de topologia transmembrana foi realizada utilizando os softwares *TMHMM Server v. 2.0* (disponível em http://www.cbs.dtu.dk/services/TMHMM-2.0/), *SCAMPI* (disponível em http://scampi.cbr.su.se/) e *SVMtm Transmembrane Domain Predictor* (disponível em http://ccb.imb.uq.edu.au/svmtm/svmtm_predictor.shtml). *PSORTb 3.0.2* (disponível em http://www.psort.org/psortb/) e *CELLO* (disponível http://cello.life.nctu.edu.tw/) em foram utilizados para a predição de localização sub-celular. A predição de lipoproteínas foi realizada utilizando os softwares *LIPOPREDICT* (disponível em http://www.lipopredict.cdac.in/) e *LIPO CBU* (disponível em http://services.cbu.uib.no/tools/lipo). Sequencias sinal e vias não clássicas de secreção foram preditas utilizando o software *SignalP 4.1* (disponível em http://www.cbs.dtu.dk/services/SignalP/). Escalas de hidrofobicidade foram preditas

utilizando o *GRAVY Calculator* (disponível em http://www.gravy-calculator.de/). Proteínas foram consideradas como transmembrana (TM) e lipoproteína quando preditas em pelo menos 2 softwares diferentes. A classificação funcional das proteínas foi realizada com base na similaridade de sequência, utilizando como referência o banco de dados do *Cluster of Orthologous in Genomes* (COG) (http://www.ncbi.nlm.nih.gov/COG).

3.1.2 Predições *in silico* de proteínas de superficie de *M. hyopneumoniae 7448*

Na análise *in silico* de proteínas de superfície de *M. hyopneumoniae* 7448 foi possível observar que, do total de 716 regiões codificadoras, 292 (44,4%) correspondem a proteínas preditas como de superfície (Apêndice 2, Additional File 10B). Todas estas proteínas foram funcionalmente classificadas de acordo com a base de dados do COG (Apêndice 2, Additional File 9B). A maior parte destas proteínas (48,6%) não possuem classificação no COG. As demais proteínas, que possuem classificação funcional conhecida, distribuíram-se nas seguintes classes: (C) produção e conversão de energia (7 proteínas, 2,5%); (D) controle do ciclo celular, mitose e meiose (2 proteínas, 0,7%); (E) transporte e metabolismo de aminoácidos (19 proteínas, 6,5%); (F) transporte e metabolismo de nucleotídeos (5 proteínas, 1,7%); (G) transporte e metabolismo de carboidratos (20 proteínas, 6,8%); (H) transporte e metabolismo de coenzimas (2 proteínas, 0,7%); (I) transporte e metabolismo de lipídeos (3 proteínas, 1%); (J) tradução (3 proteínas, 1%); (K) transcrição (3 proteínas, 1%); (L) replicação, recombinação e reparo (13 proteínas, 4,4%); (M) biogênese de parede/membrana celular (5 proteínas, 1,7%); (O) modificações pós-traducionais e enovelamento proteico (8 proteínas, 2,7%); (P) transporte e metabolismo de íons inorgânicos (16 proteínas, 5,5%); (R) função geral (16 proteínas, 5,5%); (S) função desconhecida (8 proteínas, 2,7%); (T) transdução de sinal

(1 proteína, 0,3%); (U) tráfico intracelular e secreção (9 proteínas, 3,1%) e (V) mecanismos de defesa (20 proteínas, 6,8%).

4. DISCUSSÃO GERAL

Na ausência de parede celular, a superfície da membrana do *M. hyopneumoniae* representa o principal ponto de interação com o hospedeiro/ambiente. As proteínas presentes nesta superfície participam de importantes funções celulares como a sinalização celular, o tráfego de moléculas e o transporte de nutrientes. Além disto, possuem papel fundamental no processo patogênico, uma vez que a adesão, colonização e evasão do sistema imune do hospedeiro dependem diretamente da ação destas proteínas.

A composição global de proteínas expostas na superfície celular, recentemente denominada "superficioma" (CULLEN *et al*., 2005), tem chamado a atenção da comunidade científica, uma vez que, além de serem importantes para o entendimento da biologia celular, estas proteínas representam alvos terapêuticos ideais, tanto para a produção de fármacos e vacinas quanto para o desenvolvimento de testes diagnósticos. Desde então, inúmeros estudos investigando a superfície celular foram realizados utilizando como modelo de estudo bactérias (CULLEN *et al*., 2005; CACCIOTTO *et al*., 2010; ZHANG *et al*., 2013; KRASTEVA *et al*., 2014), parasitas (WAGNER *et al*., 2013; QUEIROZ *et al*., 2014) e células humanas, especialmente células tumorais (KOHNKE *et* al., 2009; RASMUSSEN & DITZEL, 2009; LARKIN & AUKIM-HASTIE, 2011; ORENTAS *et al*., 2012; CUNHA *et al*., 2013).

Considerando as deficiências na vacinação e no diagnóstico da PES, a identificação das proteínas de superfície de *M. hyopneumoniae* torna-se essencial. As vacinas tradicionalmente comercializadas são do tipo bacterinas, que são preparações de extratos celulares totais de *M. hyopneumoniae* acrescidas de adjuvantes (MEYNS *et al*., 2006). Estas vacinas, além de não serem totalmente efetivas (MAES *et al*., 2008), acabam tendo um custo muito elevado por exigirem grandes quantidades de cultivo bacteriano,

fato dificultado pela natureza fastidiosa do *M. hyopneumoniae* (FRIIS, 1975). O diagnóstico da PES possui como padrão ouro o cultivo do *M. hyopneumoniae* isolado do tecido pulmonar em meio Friis (FRIIS, 1975, SORENSEN *et al.*, 1997). Este cultivo pode demorar até 8 semanas, sendo muito suscetível ao co-isolamento ou contaminação com outras espécies, como *M. hyorhinis* e *M. flocculare* (MAES *et al.*, 1996). Portanto, têm-se buscado alternativas para o diagnóstico, sendo os testes baseados em ensaio imunosorbente ligado à enzima (ELISA) um dos mais utilizados (SIBILA *et al.*, 2009). Uma forma de melhorar a vacinação e o diagnóstico da PES é identificar novos antígenos na superfície bacteriana que possam ser utilizados no desenvolvimento de novas estratégias de vacinação.

No entanto, existem fatores que limitam e dificultam o estudo e a manipulação de proteínas de superfície. O principal está relacionado à presença de domínios hidrofóbicos que tornam as proteínas integrais de membrana pouco suscetíveis às análises utilizando métodos tradicionais de proteômica. Estas proteínas geralmente são pouco abundantes, por isso seu estudo requer técnicas para enriquecimento de frações (CORDWELL, 2006; RABILLOUD, 2009).

Antes de iniciarmos os experimentos que resultaram na identificação experimental de proteínas expostas na superfície do *M. hyopneumoniae*, realizamos a revisão e padronização dos protocolos de cultivo do microorganismo. Com este trabalho, foi possível estabelecer uma metodologia para o acesso rápido às curvas de crescimento do *M. hyopneumoniae* utilizando medidas de absorbância. Além disto, avaliamos a influência do soro animal, principal constituinte do meio de cultura, no conteúdo qualitativo e quantitativo das proteínas totais produzida pela bactéria nas diferentes condições. Os resultados deste trabalho, que encontram-se completamente descritos no Apêndice 3 desta tese, foram fundamentais para a execução dos demais experimentos,

visto que cultivos reprodutíveis e que assegurem a integridade celular são essenciais para o estudo de proteína de superfície bacteriana.

Após realizarmos a padronização dos cultivos de *M. hyopneumoniae*, propusemos estabelecer uma metodologia para a produção de frações enriquecidas com proteínas de superfície seguida da identificação destas proteínas por espectrometria de massas. Algumas estratégias já foram utilizadas com sucesso na identificação de proteínas de membrana/superfície de diversos organismos (CORDWELL, 2006). Inicialmente, optamos por analisar a eficiência de duas delas: a tripsinização *in vivo* e a solubilização diferencial com Triton X114.

A tripsinização *in vivo*, conhecida como "*shave and conquer peptidomic*", consiste basicamente na digestão *in vivo* dos peptídeos expostos na superfície bacteriana. Embora esta técnica tenha resultado na identificação de algumas proteínas que tradicionalmente estão relacionadas com processos celulares que ocorrem na superfície bacteriana (como adesinas e transportadores), também identificou um grande número de proteínas citoplasmáticas, especialmente proteínas ribossomais. Isto sugere que a tripsinização da superfície celular pode ter causado danos à membrana do *M. hyopneumoniae*, lisando a célula e ocasionando a contaminação da amostra com proteínas citoplasmáticas.

Como alternativa testamos a segunda estratégia, baseada na solubilização diferencial de proteínas utilizando o detergente Triton X-114, que já tinha sido previamente utilizada com sucesso para *Mycoplasma agalactiae* (CACCIOTTO *et al*., 2010). Porém, esta técnica mostrou-se bastante laboriosa, necessitando de grandes quantidades de cultivos bacterianos e exigindo inúmeras etapas de precipitação e trocas de tampão, necessárias para deixar a fração protéica, altamente hidrofóbica, compatível com a eletroforese e/ou tratamento com tripsina. Entretanto, mesmo realizando todas

estas etapas não obtivemos um resultado satisfatório. As amostras não foram bem resolvidas quando submetidas à 1DE SDS-PAGE, impossibilitando a digestão *in gel* com tripsina. Alternativamente, testamos um protocolo de digestão com tripsina sem aplicação prévia das amostras ao 1DE SDS-PAGE, mas também não obtivemos sucesso.

Portanto, concluída a primeira fase de testes para o estabelecimento do protocolo de identificação de proteínas de superfície, tínhamos encontrado nos dois métodos testados limitações que comprometiam o estudo. A tripsinização *in vivo* estava sendo muito agressiva, causando a lise da célula e a consequente contaminação das frações proteícas citosólicas. Já a fração gerada na solubilização diferencial com Triton X-114 foi altamente hidrofóbica exigindo a utilização de detergentes para manter as proteínas em solução, tornando a amostra incompatível com a tripsinização em solução (uma vez que desnaturam a enzima) ou com a 1DE SDS-PAGE.

Assim, uma nova estratégia experimental para solucionar os problemas e limitações encontradas nas técnicas anteriores foi desenvolvida e aplicada. Para evitarmos a lise celular, realizamos a marcação da superfície celular *in vivo* com um reagente biotinilante (EZ-Link Sulfo-NHS-Biotin; Pierce) que é incapaz de atravessar a membrana plasmática. Após a marcação, as células foram lisadas e as proteínas marcadas foram capturadas por cromatografia de afinidade em matriz de agarose-avidina. Para identificarmos com sucesso amostras contendo grandes quantidades de proteínas hidrofóbicas, optamos pela utilização do 1DE SDS-PAGE e realização da digestão com tripsina *in gel*, evitando assim a trispinização em solução (contendo detergente) que anteriormente não havia funcionado. Em seguida, os peptídeos gerados foram submetidos à cromatografia líquida de fase reversa acoplada à espectrometria de massas. Este protocolo de identificação é denominado GeLC-MS/MS.

A biotinilação se mostrou-se bastante eficiente na identificação de proteínas de superfície. A ausência de proteínas ribossomais identificadas nas amostras provavelmente demonstra uma baixa contaminação com proteínas citosólicas, e indica que a utilização da biotina não ocasionou a lise celular. Além disso, foram identificadas inúmeras proteínas envolvidas em processos celulares que ocorrem na superfície celular, indicando que a marcação da superfície celular *in vivo* ocorreu eficientemente.

Dentre estas proteínas podemos destacar as envolvidas com a adesão celular. A adesão é um processo crucial para a colonização e o estabelecimento da doença em suínos infectados por *M. hyopneumoniae* e, embora seja multifatorial, as adesinas são consideradas as principais efetoras sendo, por isso, as mais estudadas (RAZIN & JACOBS, 1992). Em nossas identificações foi possível confirmar a presença na superfície celular de adesinas conhecidas, como a P76, a P97, a P102 e a P216. Também é relevante destacar que algumas proteínas envolvidas tradicionalmente em processos celulares que ocorrem no citoplasma foram encontradas expostas na superfície do *M. hyopneumoniae*. Dentre tais, estão as proteínas envolvidas com o metabolismo, como a piruvato-desidrogenase (PDH), a lactato-desidrogenase (LDH) e a gliceraldeído-3-fosfato-desidrogenase (GAPDH). Embora frequentemente localizadas no citoplasma, as mesmas também já foram descritas participando de eventos que ocorrem na superfície celular bacteriana. A PDH e LDH são consideradas imunogênicas em *M. hyopneumoniae* (FREY *et al*., 1994; PINTO *et al*., 2007), sendo que a subunidade E1 beta da PDH de *Mycoplasma pneumoniae* foi descrita como capaz de se ligar a fibronectina (DALLO *et al*., 2002). A GAPDH parece estar associada a alguns processos patogênicos na superfície do *Mycoplasma genitalium* (ALVAREZ *et al*., 2003) e do *M. pneumoniae* (DUMKE *et al.*, 2011), atuando na interação e adesão à matriz do hospedeiro.

Além destas proteínas, identificamos a presença na superfície do *M. hyopneumoniae* do fator de alongamento Tu (EF-Tu) e da chaperona molecular DnaK. A proteína EF-Tu, além de desempenhar função no processo de tradução, teve sua região carboxi-terminal recentemente descrita como exposta na superfície de *M. pneumoniae*, sendo capaz de interagir com a fibronectina (DALLO *et al*., 2002; BALASUBRAMANIAN *et al*., 2008). A proteína DnaK atua no processo de translocação de proteínas do citoplasma para a membrana (QI *et al.*, 2002), sendo a sua associação com membranas bastante conhecida (BUKAU *et al.*, 1993). Evidencias sugerem que a DnaK é secretada e/ou possui localização na superfície celular de diversas bactérias patogênicas, entre elas *Mycoplasma synoviae* (COUTO *et al*., 2012), *Bacillus anthracis* (CHITLARU *et al*., 2007) e *Mycobacterium tuberculosis* (XOLALPA *et al.*, 2007).

Além das proteínas com função conhecida, chama atenção o grande número de proteínas hipotéticas encontradas na superfície bacteriana. De fato, esta é uma característica até certo ponto esperada, uma vez que aproximadamente 36% das proteínas codificadas pelo *M. hyopneumoniae* 7448 são hipotéticas (SIQUEIRA *et al.*, 2013). Investir na identificação da função destas proteínas parece cada vez mais fundamental para melhorarmos o entendimento sobre os mecanismos de patogenicidade do *M. hyopneumoniae*.

Interessantemente, nos experimentos de GeLC-MS/MS, foi possível observar a distribuição da mesma proteína em bandas diferentes do gel, indicando a presença de variantes de diferentes tamanhos. Esse reultado corrobora com dados previamente descritos que indicam que proteínas de *M. hyopneumoniae* podem sofrer modificações proteolíticas pós-traducionais (DJORDJEVIC *et al*., 2004; PINTO *et al*., 2007). Estas modificações podem estar associadas à defesa bacteriana, auxiliando na evasão do sistema imune do hospedeiro através da variação de antígenos expostos na superfície.

Para complementar a identificação de proteínas de superfície por GeLC-MS/MS, realizamos a análise *in silico* de todas as proteínas codificadas pelo genoma de *M. hyopneumoniae,* buscando padrões e domínios que as caracterizassem como proteínas de membrana/superfície. Para tanto, utilizamos diversos programas de bioinformática de maneira combinada, identificando especialmente as proteínas transmembrana (TM) e as lipoproteínas (LPs).

Analisando comparativamente as proteínas identificadas por GeLC-MS/MS como expostas na superfície celular e as proteínas preditas através das análises *in silico*, foi possível observar que grande parte das proteínas identificadas por GeLC-MS/MS também foi predita como proteínas de superfície. Dentre as quais destacam-se as proteínas relacionadas com adesão (como a P76, a P97, a P102 e a P216), as lipoproteínas e um grande número de proteínas hipotéticas. Esta grande sobreposição dos resultados entre as duas metodologias também pode ser considerada como um indicativo de sucesso de nosso desenho experimental de marcação da superfície celular com biotina, aumentando a confiabilidade das nossas identificações.

Esta análise também mostrou que algumas proteínas identificadas por GeLC-MS/MS como estando expostas na superfície celular, não foram preditas *in silico*, podendo-se citar as proteínas PDH, LDH, GAPDH, EF-Tu e DnaK. Possivelmente, isso ocorreu devido às características do protocolo de marcação da superfície celular, que faz com que todas as proteínas que tiverem porções peptídicas expostas na superfície incorporem a biotina. Assim, nossas identificações experimentais não se restringem às proteínas que estejam associadas integralmente à membrana (TM e LPs), mas, identificando também proteínas periféricas, algumas transitórias e que estão associadas à membrana de outras formas, como por exemplo, através de interações proteína-proteína (SINGER & NICOLSON, 1972).

Ao realizarmos uma predição detalhada da topologia das proteínas identificadas por GeLC-MS/MS como expostas na superfície, foi possível observar que além das proteínas com reconhecida função no papel de adesão, outras proteínas, entre elas algumas hipotéticas, possuem topologia semelhante às adesinas, consistindo basicamente de uma hélice transmembrana e de uma grande região exposta na superfície. Estes dados sugerem que algumas destas proteínas podem estar desempenhando algum papel no processo de interação patógeno-hospedeiro, e podem ser alvos para futuros estudos.

M. hyopneumoniae 7448 coabita o trato respiratório do suíno com outras espécies de micoplasmas, como a espécie relacionada e não patogênica *Mycoplasma floculare*. Embora geneticamente estes microorganismos sejam muito semelhantes e 90% das proteínas preditas como de superfície em *M. hyopneumoniae* também são encontradas em *M. floculare* (SIQUEIRA *et al*., 2013), somente o *M. hyopneumoniae* é patogênico e causa lesões que resultam no estabelecimento da doença (KOBISCH & FRIIS, 1996). Na tentativa de estabelecer algum tipo de relação entre a composição proteica de superfície e a patogenicidade, realizamos uma busca por ortólogos em *M. floculare* das proteínas identificadas por GeLC-MS/MS como expostas na superfície celular de *M. hyopneumoniae*. Para tal, utilizando como referência a lista de regiões codificadoras de proteínas de superfície ortólogas encontradas no genoma do *M. flocculare*, *M. hyopneumoniae 7448* e *M. hyorhinis* HUB-1, previamente descrita por Siqueira e colaboradores (2013) (Apêndice 2, Additional File 12). Neste sentido, cabe ressaltar que apenas uma pequena parcela de proteínas identificadas na superfície *de M. hyopneumoniae* não foram encontradas no genoma de *M. floculare*, e podem estar associadas à patogenicidade em *M. hyopneumoniae*. Este grupo de proteínas consiste em: três hipotéticas (MHP7448_0088, MHP7448_0324 e MHP7448_0333); uma relacionada com o transporte celular (proteína periplásmica de ligação à açúcar); e uma enzima

oxidorredutase (flavina oxidorredutase dependente de NAD). Esta última aparenta ser uma das mais interessantes, uma vez que o envolvimento de oxidorredutases em processos patogênicos já é conhecido (YU & KROLL, 1999). Um estudo recente utilizando cepas mutantes de *Mycobacterium avium* subsp. *paratuberculosis* mostrou que quando o gene codificador da enzima flavina oxidorredutase foi silenciado pela adição de um transposon em sua sequência, a bactéria foi incapaz de invadir células epiteliais da mucosa intestinal de bovinos, sendo esta condição totalmente restabelecida pela adição de uma nova copia do gene (ALONSO-HEARN *et al*., 2008).

O desenvolvimento contínuo de novas estratégias de vacinação e de testes diagnósticos, associado à produção de novas drogas e antibióticos é fundamental para o controle da PES. Além disso, diversos aspectos envolvendo os mecanismos de patogenicidade do *M. hyopneumoniae*, como adesão, sinalização e modulação do sistema imune do hospedeiro, ainda não foram totalmente elucidados. Uma vez que a superfície bacteriana é a principal interface entre a bactéria e o hospedeiro, a identificação experimental por GeLC-MS/MS de proteínas de superfície de *M. hyopneumioniae,* apresentada nesta tese, permite que novos novos e promissores alvos possam ser identificados e selecionados para futuros estudos que visem melhorar o entendimento a respeito dos processos biológicos e patogênicos do *M. hyopneumoniae,* bem como para o desenvolvimento de estratégias de controle da PES.

6. PERSPECTIVAS

As principais perspectivas desta tese são:

- Utilizar os métodos proteômicos padronizados nesta tese para realizar a identificação de proteínas de superfície de *M. hyopneumoniae* linhagem J (não patogênica) e *M. flocculare*.

- Realizar a análise qualitativa e quantitativa das proteínas de superfície das linhagens 7448 e J de *M. hyopneumoniae* e de *M. flocculare* utilizando o protocolo experimental padronizado nesta tese, porém realizando as identificações em espectrômetro de massas de alta resolução (como o Orbitrap), melhorando a sensibilidade das identificações.

- Realizar a análise comparativa da composição global de proteínas de superfície da linhagem patogênica (7448) e não patogênica (J) de *M. hyopneumoniae* e da espécie relacionada e não patogênica *M. flocullare*.

- Investir na caracterização imunológica das proteínas indicadas nesta tese como potenciais alvos para o desenvolvimento de novas estratégias de vacinação, testes diagnósticos e drogas.

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8. APÊNDICES

8.1 APÊNDICE 1

Material suplementar: Survey of surface proteins from the pathogenic *Mycoplasma hyopneumoniae* strain 7448 using a biotin cell surface labeling approach.

Figure S1 - Biotin labeling and affinity capture of *M. hyopneumoniae* proteins from labeled lysed cell (LLC) and labeled intact cell (LIC) samples.

Table S1 - Proteins identified by GeLC-MS/MS in labeled intact cell (LIC) samples.

Table S2 - Different protein species identified by GeLC-MS/MS in labeled intact cell (LIC) samples.

Table S3 - Different protein species identified by GeLC-MS/MS in control samples from labeled lysed cells (LLC).

Table S4 - Lipoprotein predictions.

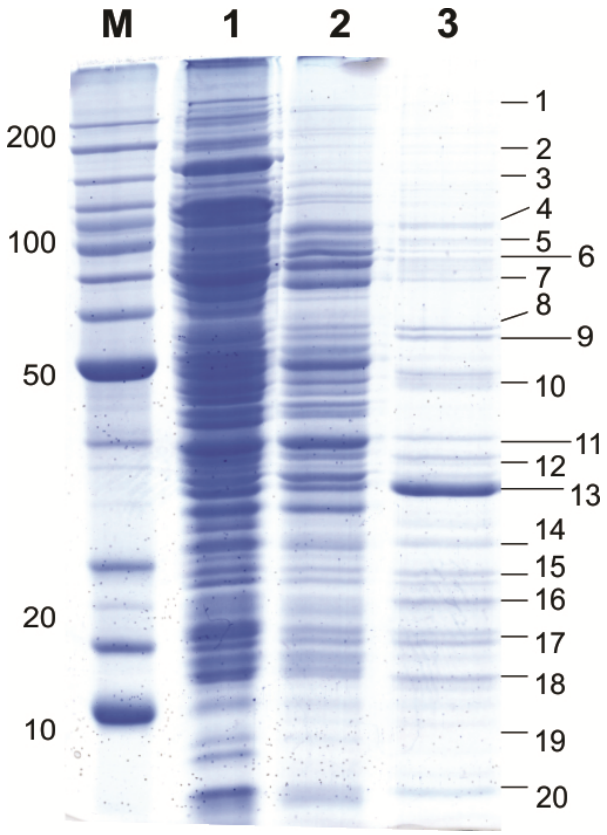

Figure S1 - Biotin labeling and affinity capture of *M. hyopneumoniae* proteins from labeled lysed cell (LLC) and labeled intact cell (LIC) samples.

SDS-PAGE 10%, and stained with Coomassie Brilliant Blue. Lane M – marker, Precision Plus prestained protein standards (Bio-Rad). Lane 1 – crude protein extracts from *M. hyopneumoniae* 7448 (15 µg). Lane 2 – avidin affinity capture of proteins from *M. hyopneumoniae* LLC samples (15 µg). Lane 3 - avidin affinity capture of proteins from *M. hyopneumoniae* LIC samples (15 µg). Bands 1 to 20 were subjected to Nano-LC/MS/MS analysis.

Table S1 - Proteins identified by GeLC-MS/MS in labeled intact cell (LIC) samples. [1]

Slice [2]	Locus Tag [3]	Protein product [4]	Protein name	Score [5]	Coverage	Mw (Da) [6]
1			No identification			
2	MHP7448_0199	YP_287596.1	Protein P102	35	1%	102268
	MHP7448_0372	YP_287766.1	Protein MHP7448_0372	27	1%	108982
3	MHP7448_0496	YP_287885.1	Putative p216 surface protein	277	15%	216487
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	105	11%	159678
	MHP7448_0663	YP_288048.1	Adhesin like-protein p146	40	9%	148795
	MHP7448_0153	YP_287550.1	Guanylate kinase	15	8%	22864
4	MHP7448_0496	YP_287885.1	Putative p216 surface protein	714	13%	216487
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	419	8%	159678
	MHP7448_0198	YP_287595.1	Protein P97	26	2%	122821
5	MHP7448_0198	YP_287595.1	Protein P97	880	23%	122821
	MHP7448_0496	YP_287885.1	Putative p216 surface protein	745	19%	216487
	MHP7448_0663	YP_288048.1	Adhesin like-protein p146	472	12%	148795
	MHP7448_0108	YP_287506.1	Protein P97	259	6%	118929
	MHP7448_0611	YP_287997.1	Hypothetical protein MHP7448_0611	259	16%	91584
	MHP7448_0621	YP_288007.1	Lipoprotein	244	9%	92215
	MHP7448_0217	YP_287614.1	Lipoprotein	126	11%	101515
	MHP7448_0138	YP_287535.1	Hypothetical protein MHP7448_0138	32	4%	113780
	MHP7448_0535	YP_287924.1	Uridylate kinase	26	8%	26359

6	MHP7448_0366	YP_287760.1	Lipoprotein	1154	40%	83939
	MHP7448_0656	YP_288041.1	Prolipoprotein p65	520	32%	71029
	MHP7448_0630	YP_288015.1	5'-nucleotidase precursor	465	22%	66008
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	412	9%	159678
	MHP7448_0480	YP_287869.1	Hypothetical protein MHP7448_0480	62	2%	83179
7	MHP7448_0656	YP_288041.1	Prolipoprotein p65	1500	50%	71029
	MHP7448_0378	YP_287772.1	Lipoprotein	597	23%	68481
	MHP7448_0198	YP_287595.1	Protein P97	561	12%	122821
	MHP7448_0367	YP_287761.1	Lipoprotein	359	17%	78357
	MHP7448_0366	YP_287760.1	Lipoprotein	347	15%	83939
	MHP7448_0067	YP_287467.1	Molecular chaperone DnaK	466	10%	65579
	MHP7448_0352	YP_287746.1	Hypothetical protein MHP7448_0352	92	2%	82877
	MHP7448_0630	YP_288015.1	5'-nucleotidase precursor	49	1%	66008
	MHP7448_0507	YP_287896.1	Dihydrolipoamide dehydrogenase	48	1%	67251
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	47	1%	159678
	MHP7448_0108	YP_287506.1	Protein P97	43	1%	118929
	MHP7448_0060	YP_287460.1	RNA polymerase sigma factor	36	1%	57497
8	MHP7448_0662	YP_288047.1	Hypothetical protein MHP7448_0662	639	16%	132382
	MHP7448_0372	YP_287766.1	Protein MHP7448_0372	629	17%	108983
	MHP7448_0604	YP_287990.1	ABC transporter xylose-binding lipoprotein	609	37%	49390
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	393	10%	159678
	MHP7448_0656	YP_288041.1	Prolipoprotein p65	239	14%	71029
	MHP7448_0353	YP_287747.1	P60-like lipoprotein	195	10%	61323

	MHP7448_0088	YP_287488.1	Hypothetical protein MHP7448_0088	192	16%	56447
	MHP7448_0324	YP_287719.1	Hypothetical protein MHP7448_0324	192	19%	49261
	MHP7448_0366	YP_287760.1	Lipoprotein	83	6%	83939
	MHP7448_0198	YP_287595.1	Protein P97	62	3%	122821
	MHP7448_0378	YP_287772.1	Lipoprotein	60	8%	68481
	MHP7448_0479	YP_287868.1	F0F1 ATP synthase subunit alpha	49	2%	57663
	MHP7448_0199	YP_287596.1	Protein P102	959	15%	102268
	MHP7448_0513	YP_287902.1	46K surface antigen precursor	811	16%	45703
	MHP7448_0523	YP_287912.1	Elongation factor Tu	254	14%	44124
	MHP7448_0604	YP_287990.1	ABC transporter xylose-binding lipoprotein	244	18%	49390
	MHP7448_0663	YP_288048.1	Adhesin like-protein p146	212	6%	148795
	MHP7448_0662	YP_288047.1	Hypothetical protein MHP7448_0662	184	4%	132382
	MHP7448_0115	YP_287513.1	Pyruvate dehydrogenase E1-alpha subunit	180	12%	42297
9	MHP7448_0497	YP_287886.1	P76 membrane protein precursor	138	2%	159678
	MHP7448_0468	YP_287858.1	Hypothetical protein MHP7448_0468	125	2%	82594
	MHP7448_0366	YP_287760.1	Lipoprotein	95	2%	83939
	MHP7448_0223	YP_287620.1	Ribonucleotide-diphosphate reductase subunit beta	83	6%	39293
	MHP7448_0234	YP_287631.1	Periplasmic sugar-binding proteins	29	3%	43883
	MHP7448_0082	YP_287482.1	NADH oxidase	29	4%	51481
	MHP7448_0225	YP_287622.1	Methylmalonate-semialdehyde dehydrogenase	29	3%	53996
	MHP7448_0088	YP_287488.1	Hypothetical protein MHP7448_0088	18	1%	56446
	MHP7448_0198	YP_287595.1	Protein P97	422	10%	122821
10	MHP7448_0368	YP_287762.1	Putative lipoprotein	374	31%	36061
	MHP7448_0663	YP_288048.1	Adhesin like-protein p146	325	6%	148795

	MHP7448_0509	YP_287898.1	Phosphotransacetylase	270	25%	35066
	MHP7448_0366	YP_287760.1	Lipoprotein	192	8%	83939
	MHP7448_0116	YP_287514.1	Pyruvate dehydrogenase	177	5%	36768
	MHP7448_0199	YP_287596.1	Protein P102	163	6%	102268
	MHP7448_0656	YP_288041.1	Prolipoprotein p65	144	14%	71029
	MHP7448_0137	YP_287534.1	L-lactate dehydrogenase	141	6%	34331
	MHP7448_0467	YP_287857.1	Hypothetical protein MHP7448_0467	48	5%	36665
	MHP7448_0223	YP_287620.1	Ribonucleotide-diphosphate reductase subunit beta	42	5%	39293
	MHP7448_0604	YP_287990.1	ABC transporter xylose-binding lipoprotein	41	8%	49390
	MHP7448_0538	YP_287927.1	Hypothetical protein MHP7448_0538	35	7%	32261
	MHP7448_0035	YP_287435.1	Glyceraldehyde 3-phosphate dehydrogenase	31	15%	37078
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	27	1%	159678
	MHP7448_0199	YP_287596.1	Protein P102	339	12%	102268
	MHP7448_0366	YP_287760.1	Lipoprotein	322	11%	83939
	MHP7448_0352	YP_287746.1	Hypothetical protein MHP7448_0352	112	4%	82877
	MHP7448_0198	YP_287595.1	Protein P97	90	2%	122821
11	MHP7448_0206	YP_287603.1	Cell division protein	88	2%	78586
	MHP7448_0507	YP_287896.1	Dihydrolipoamide dehydrogenase	80	2%	67251
	MHP7448_0372	YP_287766.1	Protein MHP7448_0372	71	2%	108983
	MHP7448_0480	YP_287869.1	Hypothetical protein MHP7448_0480	47	4%	83179
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	45	2%	159678
12	MHP7448_0199	YP_287596.1	Protein P102	50	2%	102268
13	MHP7448_0137	YP_287534.1	L-lactate dehydrogenase	101	13%	34331

	MHP7448_0198	YP_287595.1	Protein P97	78	6%	122821
	MHP7448_0064	YP_287464.1	Hypothetical protein MHP7448_0064	19	5%	171293
	MHP7448_0496	YP_287885.1	putative p216 surface protein	585	14%	216487
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	491	9%	159678
	MHP7448_0366	YP_287760.1	Lipoprotein	60	2%	83939
14	MHP7448_0373	YP_287767.1	Hypothetical protein MHP7448_0373	59	3%	113924
	MHP7448_0507	YP_287896.1	Dihydrolipoamide dehydrogenase	52	2%	67251
	MHP7448_0199	YP_287596.1	Protein P102	49	1%	102268
	MHP7448_0523	YP_287912.1	Elongation factor Tu	43	4%	44124
	MHP7448_0656	YP_288041.1	Prolipoprotein p65	29	2%	71029
	MHP7448_0198	YP_287595.1	Protein P97	883	21%	122821
	MHP7448_0663	YP_288048.1	Adhesin like-protein p146	542	10%	148795
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	343	8%	159678
	MHP7448_0611	YP_287997.1	Hypothetical protein MHP7448_0611	316	15%	91584
15	MHP7448_0496	YP_287885.1	putative p216 surface protein	225	5%	216487
	MHP7448_0217	YP_287614.1	Lipoprotein	201	6%	101515
	MHP7448_0108	YP_287506.1	Protein P97	195	12%	118929
	MHP7448_0138	YP_287535.1	Hypothetical protein MHP7448_0138	32	4%	113780
	MHP7448_0621	YP_288007.1	Lipoprotein	244	9%	92215
	MHP7448_0366	YP_287760.1	Lipoprotein	1410	34%	83939
16	MHP7448_0630	YP_288015.1	5'-nucleotidase precursor	721	29%	66008
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	446	9%	159678
	MHP7448_0114	YP_287512.1	adenine phosphoribosyltransferase	58	7%	18596

	MHP7448_0309	YP_287705.1	NADH-dependent flavin oxidoreductase	40	4%	44385
	MHP7448_0656	YP_288041.1	Prolipoprotein p65	34	2%	71029
	MHP7448_0474	YP_287864.1	Hypothetical protein MHP7448_0474	30	4%	27450
	MHP7448_0373	YP_287767.1	Hypothetical protein MHP7448_0373	24	2%	113924
	MHP7448_0611	YP_287997.1	Hypothetical protein MHP7448_0611	23	3%	91584
	MHP7448_0656	YP_288041.1	Prolipoprotein p65	1807	43%	71029
	MHP7448_0378	YP_287772.1	Lipoprotein	650	20%	68481
	MHP7448_0108	YP_287506.1	Protein P97	615	14%	118929
	MHP7448_0367	YP_287761.1	Lipoprotein	535	20%	78357
17	MHP7448_0366	YP_287760.1	Lipoprotein	429	17%	83939
	MHP7448_0199	YP_287596.1	Protein P102	135	6%	102268
	MHP7448_0067	YP_287467.1	Molecular chaperone DnaK	91	9%	65579
	MHP7448_0333	YP_287728.1	Hypothetical protein MHP7448_0333	77	2%	24082
	MHP7448_0656	YP_288041.1	Prolipoprotein p65	401	18%	71029
	MHP7448_0198	YP_287595.1	Protein P97	365	10%	122821
	MHP7448_0367	YP_287761.1	Lipoprotein	341	7%	78357
	MHP7448_0378	YP_287772.1	Lipoprotein	161	16%	68481
	MHP7448_0067	YP_287467.1	Molecular chaperone DnaK	140	6%	65579
18	MHP7448_0366	YP_287760.1	Lipoprotein	132	4%	83939
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	82	2%	159678
	MHP7448_0356	YP_287750.1	Hypothetical protein MHP7448_0356	45	2%	60852
	MHP7448_0661	YP_288046.1	Hypothetical protein MHP7448_0661	42	2%	59997
	MHP7448_0629	YP_288014.1	Hypothetical protein MHP7448_0629	39	2%	68954
	MHP7448_0355	YP_287749.1	Hypothetical protein MHP7448_0355	32	2%	64548

	MHP7448_0352	YP_287746.1	Hypothetical protein MHP7448_0352	30	2%	82877
	MHP7448_0199	YP_287596.1	Protein P102	1538	18%	102268
	MHP7448_0513	YP_287902.1	46K surface antigen precursor	371	21%	45703
	MHP7448_0662	YP_288047.1	Hypothetical protein MHP7448_0662	203	7%	132382
	MHP7448_0663	YP_288048.1	Adhesin like-protein p146	133	6%	148795
	MHP7448_0523	YP_287912.1	Elongation factor Tu	96	4%	44124
	MHP7448_0497	YP_287886.1	p76 membrane protein precursor	90	2%	159678
	MHP7448_0656	YP_288041.1	Prolipoprotein p65	61	3%	71029
19	MHP7448_0223	YP_287620.1	Ribonucleotide-diphosphate reductase subunit beta	55	6%	39293
	MHP7448_0508	YP_287897.1	Acetate kinase	54	7%	44005
	MHP7448_0366	YP_287760.1	Lipoprotein	35	6%	83939
	MHP7448_0198	YP_287595.1	Protein P97	33	1%	122821
	MHP7448_0468	YP_287858.1	Hypothetical protein MHP7448_0468	32	2%	82594
	MHP7448_0546	YP_287935.1	Potassium uptake protein	30	2%	55585
	MHP7448_0674	YP_288059.1	Hypothetical protein MHP7448_0674	27	5%	39042
	MHP7448_0490	YP_287879.1	Phosphoglycerate kinase	22	4%	44266
	MHP7448_0198	YP_287595.1	Protein P97	461	10%	122821
	MHP7448_0368	YP_287762.1	Putative lipoprotein	391	25%	36061
	MHP7448_0663	YP_288048.1	Adhesin like-protein p146	255	6%	148795
	MHP7448_0656	YP_288041.1	Prolipoprotein p65	223	12%	71029
20	MHP7448_0035	YP_287435.1	Glyceraldehyde 3-phosphate dehydrogenase	180	11%	37078
	MHP7448_0199	YP_287596.1	Protein P102	172	5%	102268
	MHP7448_0509	YP_287898.1	Phosphotransacetylase	129	14%	35066
	MHP7448_0366	YP_287760.1	Lipoprotein	110	8%	83939

MHP7448_0137	YP_ 287534.1	L-lactate dehydrogenase	109	8%	34331
MHP7448_0467	YP_287857.1	Hypothetical protein MHP7448_0467	95	5%	36665
MHP7448_0116	YP_ 287514.1	Pyruvate dehydrogenase	79	5%	36768
MHP7448_0497	YP_287886.1	p76 membrane protein precursor	53	1%	159678
MHP7448_0662	YP_288047.1	Hypothetical protein MHP7448_0662	40	1%	132382
MHP7448_0604	YP_287990.1	ABC transporter xylose-binding lipoprotein	17	8%	49390

[1] Listed proteins are those found in at least two of the three biological replicates.
[2] Gel slice number according to Supplementary Figure 1.
[3] Locus tag as defined for *M. hyopneumoniae* strain 7448 (http://www.ncbi.nlm.nih.gov/genome, NC_007332).
[4] Protein product according to NCBI database (http://www.ncbi.nlm.nih.gov).
[5] MASCOT score is -10 x log(P), where P is the probability that the observed match is a random event.
[6] Expected protein molecular weight in Daltons.

Table S2 - Different protein species identified by GeLC-MS/MS in labeled intact cell (LIC) samples.

Locus Tag [1]	Protein product [2]	Protein name	COG[3]	Surface localized[4]	TM topology[5]	Number of TM domains[5]	LP[6]	Additional criteria [7]	Ortholog MF [8]
MHP7448_0035	YP_287435.1	Glyceraldehyde 3-phosphate dehydrogenase	G	no	non-TM	0	NLP	[36, 37, 38]	yes
MHP7448_0060	YP_287460.1	RNA polymerase sigma factor	K	no	non-TM	0	NLP		yes
MHP7448_0064	YP_287464.1	Hypothetical protein MHP7448_0064	S	yes	non-TM	0	NLP		yes
MHP7448_0067	YP_287467.1	Molecular chaperone DnaK	O	no	non-TM	0	NLP	[32, 40, 41, 42]	yes
MHP7448_0082	YP_287482.1	NADH oxidase	R	no	non-TM	0	NLP		yes
MHP7448_0088	YP_287488.1	Hypothetical protein MHP7448_0088	S	no	non-TM	0	NLP		no

MHP7448_0108	YP_287506.1	Protein P97	S	yes	i12-34o	1	NLP	[8, 9, 10]	yes
MHP7448_0114	YP_287512.1	Adenine phosphoribosyltransferase	F	no	non-TM	0	NLP		yes
MHP7448_0115	YP_287513.1	Pyruvate dehydrogenase E1-alpha subunit	C	no	non-TM	0	NLP	[33, 34, 35]	yes
MHP7448_0116	YP_287514.1	Pyruvate dehydrogenase	C	no	non-TM	0	NLP	[33, 34, 35]	yes
MHP7448_0137	YP_287534.1	L-lactate dehydrogenase	C	no	non-TM	0	NLP	[39]	yes
MHP7448_0138	YP_287535.1	Hypothetical protein MHP7448_0138	S	yes	i65-87o	1	NLP		yes
MHP7448_0153	YP_287550.1	Guanylate kinase	F	no	non-TM	0	NLP		yes
MHP7448_0198	YP_287595.1	Protein P97	S	yes	i7-29o	1	NLP	[8, 9, 10]	yes

MHP7448_0199	YP_287596.1	Protein P102	S	yes	i12-34o	1	NLP	[13]	yes
MHP7448_0206	YP_287603.1	Cell division protein	O	yes	i12-34o178-200i284-306o	3	NLP		yes
MHP7448_0217	YP_287614.1	Lipoprotein	S	yes	non-TM	0	LP		yes
MHP7448_0223	YP_287620.1	Ribonucleotide-diphosphate reductase subunit beta	F	no	non-TM	0	NLP		yes
MHP7448_0225	YP_287622.1	Methylmalonate-semialdehyde dehydrogenase	C	no	non-TM	0	NLP		yes
MHP7448_0234	YP_287631.1	Periplasmic sugar-binding proteins	G	yes	i9-31o	1	NLP		no
MHP7448_0309	YP_287705.1	NADH-dependent flavin oxidoreductase	C	yes	non-TM	0	NLP	[48, 49, 50]	no
MHP7448_0324	YP_287719.1	Hypothetical protein MHP7448_0324	S	no	non-TM	0	LP		no

MHP7448_0333	YP_287728.1	Hypothetical protein MHP7448_0333	S	no	non-TM	0	NLP		no
MHP7448_0352	YP_287746.1	Hypothetical protein MHP7448_0352	S	yes	i49-71o	1	NLP	[29]	yes
MHP7448_0353	YP_287747.1	P60-like lipoprotein	R	yes	non-TM	0	LP		yes
MHP7448_0355	YP_287749.1	Hypothetical protein MHP7448_0355	S	yes	non-TM	0	NLP		yes
MHP7448_0356	YP_287750.1	Hypothetical protein MHP7448_0356	S	no	non-TM	0	LP		yes
MHP7448_0366	YP_287760.1	Lipoprotein	S	yes	non-TM	0	LP		yes
MHP7448_0367	YP_287761.1	Lipoprotein	S	yes	non-TM	0	LP		yes
MHP7448_0368	YP_287762.1	Putative lipoprotein	S	yes	non-TM	0	LP		yes

MHP7448_0372	YP_287766.1	Protein MHP7448_0372	S	yes	i9-31o	1	NLP		yes
MHP7448_0373	YP_287767.1	Hypothetical protein MHP7448_0373	S	yes	i17-39o	1	NLP	[30]	yes
MHP7448_0378	YP_287772.1	Lipoprotein	S	no	non-TM	0	LP		yes
MHP7448_0467	YP_287857.1	Hypothetical protein MHP7448_0467	S	yes	i7-29o	1	LP		yes
MHP7448_0468	YP_287858.1	Hypothetical protein MHP7448_0468	S	yes	i7-29o	1	LP		yes
MHP7448_0474	YP_287864.1	Hypothetical protein MHP7448_0474	S	no	non-TM	0	NLP		yes
MHP7448_0479	YP_287868.1	F0F1 ATP synthase subunit alpha	C	no	non-TM	0	NLP		yes
MHP7448_0480	YP_287869.1	Hypothetical protein MHP7448_0480	S	yes	o677-696i	1	NLP		yes

MHP7448_0490	YP_287879.1	Phosphoglycerate kinase	G	no	non-TM	0	NLP		yes
MHP7448_0496	YP_287885.1	Putative p216 surface protein	S	yes	i7-29o	1	NLP	[11]	yes
MHP7448_0497	YP_287886.1	p76 membrane protein precursor	S	yes	i7-29o	1	NLP		yes
MHP7448_0507	YP_287896.1	Dihydrolipoamide dehydrogenase	C	no	non-TM	0	NLP		yes
MHP7448_0508	YP_287897.1	Acetate kinase	C	no	non-TM	0	NLP		yes
MHP7448_0509	YP_287898.1	Phosphotransacetylase	C	no	non-TM	0	NLP		yes
MHP7448_0513	YP_287902.1	46K surface antigen precursor	S	yes	i9-31o	1	LP		yes
MHP7448_0523	YP_287912.1	Elongation factor Tu	J	no	non-TM	0	NLP	[18]	yes

MHP7448_0535	YP_287924.1	Uridylate kinase	F	no	non-TM	0	NLP		yes
MHP7448_0538	YP_287927.1	Hypothetical protein MHP7448_0538	S	yes	non-TM	0	NLP		yes
MHP7448_0546	YP_287935.1	Potassium uptake protein	P	yes	i21-43o83-105i139-161o213-235i256-278o348-365i386-408o454-476i	8	NLP		yes
MHP7448_0604	YP_287990.1	ABC transporter xylose-binding lipoprotein	R	yes	non-TM	0	LP	[28]	yes
MHP7448_0611	YP_287997.1	Hypothetical protein MHP7448_0611	S	yes	non-TM	0	NLP		yes
MHP7448_0621	YP_288007.1	Lipoprotein	S	no	non-TM	0	LP		yes
MHP7448_0629	YP_288014.1	Hypothetical protein MHP7448_0629	S	yes	i23-40o	1	LP		yes
MHP7448_0630	YP_288015.1	5'-nucleotidase precursor	F	yes	non-TM	0	NLP		yes

MHP7448_0656	YP_288041.1	Prolipoprotein p65	S	yes	non-TM	0	LP		yes
MHP7448_0661	YP_288046.1	Hypothetical protein MHP7448_0661	S	yes	i12-34o	1	LP		yes
MHP7448_0662	YP_288047.1	Hypothetical protein MHP7448_0662	S	yes	non-TM	0	NLP	[31, 32]	yes
MHP7448_0663	YP_288048.1	Adhesin like-protein p146	S	yes	i7-29o	1	NLP		yes
MHP7448_0674	YP_288059.1	Hypothetical protein MHP7448_0674	S	yes	non-TM	0	NLP		yes

[1] Locus tag as defined for *M. hyopneumoniae* strain 7448 (http://www.ncbi.nlm.nih.gov/genome, NC_007332).
[2] Protein product according to NCBI database (http://www.ncbi.nlm.nih.gov).
[3] COG classification (http://www.ncbi.nlm.nih.gov/COG): (C) Energy production and conversion; (F) Nucleotide transport and metabolism; (G) Carbohydrate transport and metabolism, (J) Translation; (K) Transcription; (O) Post-translational modification, protein turnover, and chaperones, (P) Inorganic ion transport and metabolism; (R) General function prediction only; (S) Function unknown.
[4] Previously predicted [23].
[5] TMHMM 2.0 Topology predictor (http://www.cbs.dtu.dk/services/TMHMM-2.0/).
[6] LIPOPREDICT (http://www.lipopredict.cdac.in/): LP = Lipoprotein; NLP = Non Lipoprotein.
[7] Published literature.
[8] Performed as previously described [26].

Table S3 - Different protein species identified by GeLC-MS/MS in control samples from labeled lysed cell (LLC).

Loccus Tag[1]	Protein product[2]	Protein name	COG[3]
MHP7448_0009	YP_287409.1	Hypothetical protein MHP7448_0009	S
MHP7448_0011	YP_287411.1	Heat shock protein	O
MHP7448_0014	YP_287414.1	Fructose-bisphosphate aldolase	G
MHP7448_0035	YP_287435.1	Glyceraldehyde 3-phosphate dehydrogenase	G
MHP7448_0056	YP_287456.1	Elongation factor Ts	J
MHP7448_0067	YP_287467.1	Molecular chaperone DnaK	O
MHP7448_0071	YP_287471.1	Nucleoid DNA-binding protein	L
MHP7448_0075	YP_287475.1	Elongation factor G	J
MHP7448_0082	YP_287482.1	NADH oxidase	R
MHP7448_0083	YP_287483.1	Thymidine phosphorylase	F
MHP7448_0084	YP_287484.1	Purine-nucleoside phosphorylase	F
MHP7448_0096	YP_287496.1	Thiol peroxidase	O
MHP7448_0098	YP_287498.1	Thioredoxin reductase	O
MHP7448_0102	YP_287502.1	Triosephosphate isomerase	G
MHP7448_0112	YP_287510.1	Hypothetical protein MHP7448_0112	S
MHP7448_0114	YP_287512.1	Adenine phosphoribosyltransferase	F
MHP7448_0115	YP_287513.1	Pyruvate dehydrogenase E1-alpha subunit	C
MHP7448_0116	YP_287514.1	Pyruvate dehydrogenase	C
MHP7448_0129	YP_287527.1	Aminopeptidase	G
MHP7448_0132	YP_287530.1	50S ribosomal protein L27	J
MHP7448_0133	YP_287531.1	Lipase-esterase	R
MHP7448_0137	YP_287534.1	L-lactate dehydrogenase	C
MHP7448_0149	YP_287546.1	Trigger factor	O

MHP7448_0168	YP_287565.1	DNA-directed RNA polymerase subunit alpha	K
MHP7448_0176	YP_287573.1	50S ribosomal protein L15	J
MHP7448_0179	YP_287576.1	50S ribosomal protein L6	J
MHP7448_0183	YP_287580.1	50S ribosomal protein L24	J
MHP7448_0198	YP_287595.1	Protein P97	S
MHP7448_0199	YP_287596.1	Protein P102	S
MHP7448_0206	YP_287603.1	Cell division protein	O
MHP7448_0207	YP_287604.1	Lysyl-tRNA synthetase	J
MHP7448_0223	YP_287620.1	Ribonucleotide-diphosphate reductase subunit beta	F
MHP7448_0225	YP_287622.1	Methylmalonate-semialdehyde dehydrogenase	C
MHP7448_0229	YP_287626.1	Myo-inositol catabolism protein	G
MHP7448_0230	YP_287627.1	Myo-inositol catabolism protein	G
MHP7448_0244	YP_287641.1	Hypothetical protein MHP7448_0244	S
MHP7448_0245	YP_287642.1	30S ribosomal protein S20	J
MHP7448_0250	YP_287647.1	Phosphopyruvate hydratase	G
MHP7448_0252	YP_287649.1	Hypothetical protein MHP7448_0252	S
MHP7448_0257	YP_287654.1	Hypothetical protein MHP7448_0257	S
MHP7448_0262	YP_287659.1	Hypoxanthine-guanine phosphoribosyltransferase	F
MHP7448_0273	YP_287670.1	Phenylalanyl-tRNA synthetase alpha chain	J
MHP7448_0279	YP_287676.1	Transcriptional regulator	K
MHP7448_0284	YP_287681.1	50S ribosomal protein L19	J
MHP7448_0296	YP_287692.1	30S ribosomal protein S6	J
MHP7448_0310	YP_287706.1	Lipoate-protein ligase A	H
MHP7448_0348	YP_287742.1	Hypothetical protein MHP7448_0348	S
MHP7448_0354	YP_287748.1	HIT-like protein	G
MHP7448_0373	YP_287767.1	Hypothetical protein MHP7448_0373	S
MHP7448_0374	YP_287768.1	PTS system, IIA component	S

MHP7448_0375	YP_287769.1	PTS system enzyme IIB component	S
MHP7448_0377	YP_287771.1	Hypothetical protein MHP7448_0377	S
MHP7448_0384	YP_287778.1	Thioredoxin	S
MHP7448_0401	YP_287793.1	Asparaginyl-tRNA synthetase	J
MHP7448_0427	YP_287818.1	Elongation factor P	J
MHP7448_0428	YP_287819.1	Transketolase	G
MHP7448_0436	YP_287827.1	L-ribulose-5-phosphate 4-epimerase	G
MHP7448_0438	YP_287829.1	3-keto-L-gulonate-6-phosphate decarboxylase	G
MHP7448_0454	YP_287845.1	Acyl carrier protein phosphodiesterase	I
MHP7448_0457	YP_287847.1	Neutrophil activating factor	P
MHP7448_0459	YP_287849.1	50S ribosomal protein L1	J
MHP7448_0460	YP_287850.1	50S ribosomal protein L11	J
MHP7448_0464	YP_287854.1	Leucyl aminopeptidase	E
MHP7448_0472	YP_287862.1	Phosphoenolpyruvate-protein phosphotransferase	G
MHP7448_0479	YP_287868.1	F0F1 ATP synthase subunit alpha	C
MHP7448_0494	YP_287883.1	Mannose-6-phosphate isomerase	G
MHP7448_0496	YP_287885.1	Putative p216 surface protein	S
MHP7448_0497	YP_287886.1	P76 membrane protein precursor	S
MHP7448_0505	YP_287894.1	Lipoprotein	S
MHP7448_0507	YP_287896.1	Dihydrolipoamide dehydrogenase	C
MHP7448_0507	YP_287896.1	Dihydrolipoamide dehydrogenase	C
MHP7448_0508	YP_287897.1	Acetate kinase	C
MHP7448_0509	YP_287898.1	Phosphotransacetylase	C
MHP7448_0513	YP_287902.1	46K surface antigen precursor	S
MHP7448_0521	YP_287910.1	Oligoendopeptidase F	E
MHP7448_0523	YP_287912.1	Elongation factor Tu	J
MHP7448_0524	YP_287913.1	Heat shock ATP-dependent protease	O

MHP7448_0529	YP_287918.1	Methionine sulfoxide reductase B	O
MHP7448_0531	YP_287920.1	Glucose-6-phosphate isomerase	G
MHP7448_0534	YP_287923.1	Ribosome recycling factor	J
MHP7448_0542	YP_287931.1	Spermidine/putrescine ABC transporter ATP-binding	E
MHP7448_0563	YP_287951.1	Hypothetical protein MHP7448_0563	S
MHP7448_0566	YP_287953.1	Hypothetical protein MHP7448_0566	S
MHP7448_0576	YP_287962.1	30S ribosomal protein S4	J
MHP7448_0587	YP_287973.1	Pullulanase	G
MHP7448_0592	YP_287978.1	ATP binding protein	L
MHP7448_0595	YP_287981.1	Phosphoglyceromutase	G
MHP7448_0604	YP_287990.1	ABC transporter xylose-binding lipoprotein	R
MHP7448_0609	YP_287995.1	Phosphocarrier protein HPr	G
MHP7448_0618	YP_288004.1	50S ribosomal protein L7/L12	J
MHP7448_0630	YP_288015.1	5'-nucleotidase precursor	F
MHP7448_0656	YP_288041.1	Prolipoprotein p65	S
MHP7448_0659	YP_288044.1	XAA-Pro aminopeptidase	E
MHP7448_0662	YP_288047.1	Hypothetical protein MHP7448_0662	S
MHP7448_0663	YP_288048.1	Adhesin like-protein p146	S
MHP7448_0668	YP_288053.1	Transcription elongation factor GreA	K

[1] Locus tag as defined for *M. hyopneumoniae* strain 7448 (http://www.ncbi.nlm.nih.gov/genome, NC_007332).
[2] Protein product according to NCBI database (http://www.ncbi.nlm.nih.gov).
[3] COG classification (http://www.ncbi.nlm.nih.gov/COG): (C) Energy production and conversion; (E) Amino Acid metabolis and transport; (F) Nucleotide transport and metabolism; (G) Carbohydrate transport and metabolism, (J) Translation; (K) Transcription; (L) Replication and repair; (O) Post-translational modification, protein turnover, and chaperones, (P) Inorganic ion transport and metabolism; (R) General function prediction only; (S) Function unknown.

Table S4 - Lipoprotein predictions. Proteins in bold were also identified in GeLC-MS/MS analysis.

			LIPOPREDICT[3]	LIPO CBU[4]		GOG[5]	
Locus tag[1]	**Protein product[2]**	**Protein name**	**Prediction**	**LipoBox Similarity**	**Cleavage site**	**COG class**	**Surface localized[6]**
MHP7448_0036	YP_287436.1	Hypothetical protein MHP7448_0036	LP	Low	19	no COG	yes
MHP7448_0112	YP_287510.1	Hypothetical protein MHP7448_0112	LP	Medium	23	no COG	yes
MHP7448_0118	YP_287516.1	Hypothetical protein MHP7448_0118	LP	Low	32	E	yes
MHP7448_0217	**YP_287614.1**	**Lipoprotein**	**LP**	**Medium**	**21**	**no COG**	**yes**
MHP7448_0299	YP_287695.1	Hypothetical protein MHP7448_0299	NLP	Low	24	no COG	yes
MHP7448_0324	**YP_287719.1**	**Hypothetical protein MHP7448_0324**	**LP**	**__**	**__**	**no COG**	**no**
MHP7448_0353	**YP_287747.1**	**P60-like lipoprotein**	**LP**	**Medium**	**30**	**no COG**	**yes**
MHP7448_0356	**YP_287750.1**	**Hypothetical protein MHP7448_0356**	**LP**	**Low**	**22**	**no COG**	**no**
MHP7448_0360	YP_287754.1	P37-like ABC transporter substrate-binding lipoprotein	LP	__	__	no COG	yes
MHP7448_0366	**YP_287760.1**	**Lipoprotein**	**LP**	**__**	**__**	**no COG**	**yes**
MHP7448_0367	**YP_287761.1**	**Lipoprotein**	**LP**	**Low**	**25**	**no COG**	**yes**
MHP7448_0368	**YP_287762.1**	**Putative lipoprotein**	**LP**	**__**	**__**	**L**	**yes**
MHP7448_0378	**YP_287772.1**	**Lipoprotein**	**LP**	**Medium**	**24**	**no COG**	**no**
MHP7448_0399	YP_287791.1	Hypothetical protein MHP7448_0399	NLP	Medium	28	no COG	yes
MHP7448_0408	YP_287800.1	Hypothetical protein MHP7448_0408	LP	Low	28	no COG	no
MHP7448_0433	YP_287824.1	Hypothetical protein MHP7448_0433	LP	Low	23	no COG	yes

MHP7448_0467	**YP_287857.1**	**Hypothetical protein MHP7448_0467**	**LP**	**Medium**	**24**	**no COG**	**yes**
MHP7448_0468	**YP_287858.1**	**Hypothetical protein MHP7448_0468**	**LP**	**Medium**	**22**	**no COG**	**yes**
MHP7448_0513	**YP_287902.1**	**46K surface antigen precursor**	**LP**	**Medium**	**31**	**G**	**yes**
MHP7448_0604	**YP_287990.1**	**ABC transporter xylose-binding lipoprotein**	**LP**	**__**	**__**	**R**	**yes**
MHP7448_0621	**YP_288007.1**	**Lipoprotein**	**LP**	**__**	**__**	**no COG**	**no**
MHP7448_0629	**YP_288014.1**	**Hypothetical protein MHP7448_0629**	**LP**	**__**	**__**	**no COG**	**yes**
MHP7448_0656	**YP_288041.1**	**Prolipoprotein p65**	**LP**	**Low**	**30**	**no COG**	**yes**
MHP7448_0660	YP_288045.1	Hypothetical protein MHP7448_0660	LP	Low	32	no COG	no
MHP7448_0661	**YP_288046.1**	**Hypothetical protein MHP7448_0661**	**LP**	**Medium**	**29**	**no COG**	**yes**

[1] Locus tag as defined for *M. hyopneumoniae* strain 7448 (http://www.ncbi.nlm.nih.gov/genome, NC_007332).
[2] Protein product according to NCBI database (http://www.ncbi.nlm.nih.gov).
[3] LIPOPREDICT (http://www.lipopredict.cdac.in/): LP = Lipoprotein; NLP = Non Lipoprotein.
[4] LIPO CBU (http://services.cbu.uib.no/tools/lipo).
[5] COG classification (http://www.ncbi.nlm.nih.gov/COG): (C) Energy production and conversion; (E) Amino acid transport and metabolism; (F) Nucleotide transport and metabolism; (G) Carbohydrate transport and metabolism, (I) Lipid metabolism; (L) DNA replication, recombination, and repair; (M) Cell envelope biogenesis, outer membrane; (O) Post-translational modification, protein turnover, and chaperones, (P) Inorganic ion transport and metabolism; (R) General function prediction only; (S) Function unknown; (U) Intracellular trafficking, secretion, and vesicular transport; (V) Defense mechanisms.
[6] Previously data [23].

8.2 APÊNDICE 2

New insights on the biology of swine respiratory tract mycoplasmas from a comparative genome analysis.

Artigo publicado em co-autoria na revista BMC Genomics, fator de impacto 4,04.

Autores: Franciele Maboni Siqueira (FMS), Claudia Elizabeth Thompson (CET), Veridiana Gomes Virginio (VGV), Taylor Gonchoroski (TG), Luciano Reolon (LR), Luiz Gonzaga Almeida (LGA), Marbella Maria da Fonsêca (MMF), Rangel de Souza (RS), Francisco Prosdocimi (FP), Irene Silveira Schrank (ISS), Henrique Bunselmeyer Ferreira (HBF), Ana Tereza Ribeiro de Vasconcelos (ATV) e Arnaldo Zaha (AZ).

Contribuição dos autores: Luciano Reolon (LR) contribuiu com este trabalho através da prospecção *in silico* e da análise comparativa das potenciais proteínas de superfície de *M. hyopneumoniae*, *M. flocculare* e *M. hyorinis*. Além disto, realizou a análise da topologia de adesinas, buscando identificar e comparar as características apresentadas pelas regiões de ligação à matriz do hospedeiro das adesinas de *M. hyopneumoniae*, *M. flocculare* e *M. hyorinis*. FMS realizou as análises relativa à organização dos genomas, as análises comparativas, participou da interpretação dos resultados e escreveu o manunscrito. CET, FP e MMF realizaram as análises de filigenética e filogenômica e a interpretação dos resultados. VGV e TG participaram das análises *in silico* de proteínas de superfície e das análises comparativas da composição de proteínas de superfície nas três espécies analisadas. LGA e RS realizaram a montagem do genoma e participaram das análises comparativas. ISS, HBF,

ATV e AZ conceberam o estudo e participaram do seu design, implementação e coordenação.

Neste artigo, mais especificamente na sessão "**Results**", subtítulo "**Repertoire of surface proteins encoded by *M. flocculare*, *M. hyopneumoniae* and *M. hyorhinis***", encontram-se descritos os seguintes resultados referentes a esta tese:

- predições *in silico* de proteínas de superfície de *M. hyopneumoniae*, *M. flocculare e M. hyorhinis*;

- classificação funcional das proteínas de superfície preditas nas três espécies;

- análise e predição de topologia e de regiões repetidas de adesinas;

- análise comparativa entre as proteínas preditas como expostas na superfície de *M. hyopneumoniae*, *M. flocculare* e *M. hyorhinis.*

O artigo está completamente reproduzido a seguir, com numeração de páginas original mantida. No final deste apêndice estão os arquivos adicionais editados e que correspondem aos resultados que são parte desta tese. A lista completa e o acesso a todos os arquivos adicionais está disponível em: http://www.biomedcentral.com/1471-2164/14/175.

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RESEARCH ARTICLE Open Access

New insights on the biology of swine respiratory tract mycoplasmas from a comparative genome analysis

Franciele Maboni Siqueira[1,4], Claudia Elizabeth Thompson[2], Veridiana Gomes Virginio[1,3], Taylor Gonchoroski[1], Luciano Reolon[1,3], Luiz Gonzaga Almeida[2], Marbella Maria da Fonsêca[2], Rangel de Souza[2], Francisco Prosdocimi[6], Irene Silveira Schrank[1,3,5], Henrique Bunselmeyer Ferreira[1,3,5], Ana Tereza Ribeiro de Vasconcelos[2*] and Arnaldo Zaha[1,3,4,5*]

Abstract

Background: *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare* and *Mycoplasma hyorhinis* live in swine respiratory tracts. *M. flocculare*, a commensal bacterium, is genetically closely related to *M. hyopneumoniae*, the causative agent of enzootic porcine pneumonia. *M. hyorhinis* is also pathogenic, causing polyserositis and arthritis. In this work, we present the genome sequences of *M. flocculare* and *M. hyopneumoniae* strain 7422, and we compare these genomes with the genomes of other *M. hyoponeumoniae* strain and to the a *M. hyorhinis* genome. These analyses were performed to identify possible characteristics that may help to explain the different behaviors of these species in swine respiratory tracts.

Results: The overall genome organization of three species was analyzed, revealing that the ORF clusters (OCs) differ considerably and that inversions and rearrangements are common. Although *M. flocculare* and *M. hyopneumoniae* display a high degree of similarity with respect to the gene content, only some genomic regions display considerable synteny. Genes encoding proteins that may be involved in host-cell adhesion in *M. hyopneumoniae* and *M. flocculare* display differences in genomic structure and organization. Some genes encoding adhesins of the P97 family are absent in *M. flocculare* and some contain sequence differences or lack of domains that are considered to be important for adhesion to host cells. The phylogenetic relationship of the three species was confirmed by a phylogenomic approach. The set of genes involved in metabolism, especially in the uptake of precursors for nucleic acids synthesis and nucleotide metabolism, display some differences in copy number and the presence/absence in the three species.

Conclusions: The comparative analyses of three mycoplasma species that inhabit the swine respiratory tract facilitated the identification of some characteristics that may be related to their different behaviors. *M. hyopneumoniae* and *M. flocculare* display many differences that may help to explain why one species is pathogenic and the other is considered to be commensal. However, it was not possible to identify specific virulence determinant factors that could explain the differences in the pathogenicity of the analyzed species. The *M. hyorhinis* genome contains differences in some components involved in metabolism and evasion of the host's immune system that may contribute to its growth aggressiveness. Several horizontal gene transfer events were identified. The phylogenomic analysis places *M. hyopneumoniae, M. flocculare* and *M. hyorhinis* in the hyopneumoniae clade.

Keywords: Mycoplasma, Comparative genomics, Adhesins, Swine respiratory tract

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Background

Mycoplasmas belong to the class Mollicutes, which is a taxon of bacteria that is characterized by the absence of a cell wall, a relatively small genome size and a strong dependence on nutrients supplied by the host environment [1]. More than 120 mycoplasma species have been described, and although they display diverse life styles, most of the species are parasitic, implying the occurrence of different mechanisms by which they interact with host cells. Several mycoplasmas associate with their host cells through adhesins, while others may also invade cells [2-8]. Among mycoplasmas, several species are responsible for human, animal and plant diseases, but some species are considered commensal organisms [1].

Mycoplasma hyopneumoniae, *Mycoplasma flocculare* and *Mycoplasma hyorhinis* are the most important species that have been identified in porcine respiratory systems [9-11]. Based on a 16S rRNA sequence comparison, *M. hyopneumoniae* and *M. flocculare* are known to be closely related [12]. *M. hyopneumoniae* is the etiological agent of porcine mycoplasmal pneumonia, while *M. hyorhinis*, which causes polyserositis and arthritis, is also frequently found in swine respiratory tracts [13]. *M. flocculare* is also widespread in swine herds, but no disease has been associated with this species [14]. *M. hyopneumoniae* can adhere to the cilia of tracheal epithelial cells and causes damage. Although *M. flocculare* can also adhere to cilia, no resulting damage has been observed, suggesting that *M. hyopneumoniae* and *M. flocculare* may possess different adhesins, facilitating the recognition of different receptor sites on the cilia [15]. Additionally, while *M. flocculare* is restricted to the swine respiratory tract, *M. hyopneumoniae* and *M. hyorhinis* can also colonize other sites, such as cardiac or joint tissues [14,16]. These bacteria can even colonize different hosts; *M. hyorhinis* has been detected in human carcinoma tissues [17,18]. The genetic maps of *M. flocculare* ATCC 27716 and *M. hyopneumoniae* strain J have been compared, revealing that at least three chromosomal inversions have occurred since the divergence of both species [19].

In recent years, the genomes of several mycoplasma species have been sequenced. The absence of several metabolic pathways, which was suggested by genetic and biochemical studies [1], has been confirmed at the genome sequence level. Among the swine-infecting mycoplasmas, the genomes of *M. hyopneumoniae* (four strains), *M. hyorhinis* (four strains) and *Mycoplasma suis* (two strains) have been sequenced, facilitating the comparison of metabolic pathways and evidencing specific mechanisms that can be utilized to survive in different host environments [20-28].

Because the genome sequences of *M. flocculare* and *M. hyopneumoniae* strain 7422 have now been completed, the current study presents a comprehensive comparison of the *M. flocculare*, *M. hyopneumoniae* and *M. hyorhinis* genomes. These three mycoplasma species can inhabit swine respiratory tracts. We have assessed the overall genome organizations including analyses of the open reading frame (ORF) clusters (OCs), inversions and rearrangements, and coding capacities, including analyses of encoded metabolic pathways and surface protein repertoires. Potential mechanisms of interaction with host cells are evidenced, and their implications on pathogenicity are discussed. Additionally, a phylogenomic approach using 32 mycoplasma genomes (including two that are reported here for the first time) was implemented to reconstruct the evolutionary history of the swine mycoplasma genomes, individual genes and/or portions of their genomes, including horizontal gene transfer analysis.

Results and discussion

General genome features of *M. flocculare* and *M. hyopneumoniae* 7422

The *M. flocculare* and *M. hyopneumoniae* 7422 genomes are composed of single, circular chromosomes of 763,948 and 899,887 bp with 28.9% and 28.4% GC contents, respectively. The *M. flocculare* genome, which by assembly remained with 13 gaps, contains 585 coding sequences (CDSs), of which 356 have known functions and 229 are annotated as hypothetical. The *M. hyopneumoniae* 7422 genome, completely closed in one contig by assembly, comprises 692 CDSs, of which 414 correspond to proteins with known functions and 278 are annotated as hypothetical. The protein-coding regions occupy approximately 87% of each *M. flocculare* and *M. hyopneumoniae* 7422 chromosome, and the average ORF length is 1,145 bp. Each genome contains one gene encoding the ribosomal RNAs (rRNAs) 16S and 23S, one gene encoding rRNA 5S and 30 genes encoding the transfer RNAs (tRNAs) representing all 20 amino acids. The general genome features of the five strains, *M. hyopneumoniae* (7422, 7448, J, 232 and 168), *M. flocculare*, and *M. hyorhinis* HUB-1, were compared in this study and are listed in Table 1.

Among the *M. flocculare* and *M. hyopneumoniae* 7422 CDSs that encode proteins with known functions, 380 and 403 CDSs, respectively, were classified into COG families comprising 18 functional categories (Table 2). A functional classification based on the KEGG [29] analysis assigned 351 and 371 CDSs from *M. flocculare* and *M. hyopneumoniae* 7422, respectively, into 15 different categories (Table 2). The performance differences produced by COG with respect to KEGG may be attributable to the presence of paralogs. As expected, the general genomic features and similarities in all of the COG and KEGG categories were strikingly similar between *M. flocculare*, *M. hyopneumoniae*, and *M. hyorhinis*, which commonly exhibited small genome sizes, high AT contents, and no two signal transduction proteins (Table 2).

Table 1 Comparison of general features of different mycoplasmas species and strains

	Organism*						
	MHP 7422	MHP 7448	MHP J	MHP 232	MHP 168	MFL	MHR HUB-1
Total length (bp)	899,887	920,079	897,405	892,758	925,576	763,948	839,615
G + C content (%)	28.4	28.5	28.5	28.6	28.4	28.9	25.8
Total no. CDSs	692	716	690	692	695	585	654
Average CDS length (bp)	1,147	1,146	1,167	1,164	1,071	1,145	1,092
Known proteins	414	418	410	304	354	356	489
Hypothetical proteins	278	298	280	388	341	229	165
No. of rRNAs	3	3	3	3	3	3	3
No. of tRNAs	30	30	30	30	30	30	30

*Abreviations: MHP = *M. hyopneumoniae*; MFL = *M. flocculare*; MHR = M. hyorhinis

To identify the genes that constitute the core and pan-genome of *M. flocculare*, *M. hyopneumoniae*, and *M. hyorhinis*, we took advantage of the bidirectional best hit (BBH) approach and plotted the data in a Venn diagram (Figure 1). We identified a considerable number of unique (i.e., organism-specific) genes in *M. hyorhinis* that may underline the phenotypic differences between this species and *M. flocculare* and *M. hyopneumoniae*. Including the repertoire of surface proteins (discussed later) and the inositol metabolism pathway, we identified 76 genes that are unique to *M. flocculare*, 69 to *M. hyopneumoniae* and 234 to *M. hyorhinis*.

When compared to other sequenced strains of *M. hyopneumoniae*, the genome of strain 7422 displays a highly similar gene composition and organization, with the exception of the localization of the integrative conjugative element (ICEH), which is positioned from 139,715 to 162,049 bp in the 7422 genome and from 518,376 to 540,705 bp in the 7448 genome. The similarity between *M. hyopneumoniae* gene repertories was 88%

Table 2 Comparison of *Mycoplasma* sp. genomes statistics using KEGG classification

Category/Organism	MHP 7422		MHR		MHP 7488		MHP J		MFL	
	Number	%	Number	%	Number	%	Number	%	Number	%
Carbohydrate metabolism	83	22.4	73	17.3	82	22.2	82	22.4	73	20.8
Energy metabolism	20	5.4	22	5.2	19	5.1	20	5.5	20	5.7
Lipid metabolism	9	2.4	11	2.6	9	2.4	9	2.5	7	2
Nucleotide metabolism	47	12.7	44	10.4	46	12.5	45	12.3	46	13.1
Amino Acid metabolism	14	3.8	15	3.6	14	3.8	14	3.8	12	3.4
Metabolism of Other Amino Acids	7	1.9	8	1.9	7	1.9	7	1.9	7	2
Glycan Biosynthesis and Metabolism	1	0.3	5	1.2	2	0.5	1	0.3	2	0.6
Metabolism of Cofactors and Vitamins	9	2.4	14	3.3	9	2.4	8	2.2	8	2.3
Metabolism of Terpenoids and Polyketides	5	1.3	1	0.2	5	1.4	5	1.4	5	1.4
Membrane Transport	36	9.7	38	9	36	9.8	36	9.8	32	9
Folding. Sorting and Degradation	10	2.7	14	3.3	10	2.7	10	2.7	9	2.6
Replication and Repair	45	12.1	44	10.4	42	11.4	42	11.5	44	12.5
Transcription	3	0.8	3	0.7	3	0.8	3	0.8	3	0.9
Translation	72	19.4	98	23.2	73	19.8	72	19.7	73	20.8
Biosynthesis of Other Secondary Metabolites	8	2.2	2	0.5	9	2.4	9	2.5	7	2
Cell Motility	0	0	0	0	0	0	0	0	0	0
Signal Transduction	0	0	0	0	0	0	0	0	0	0
TOTAL	371		392		369		366		351	

Abbreviations as in Table 1.

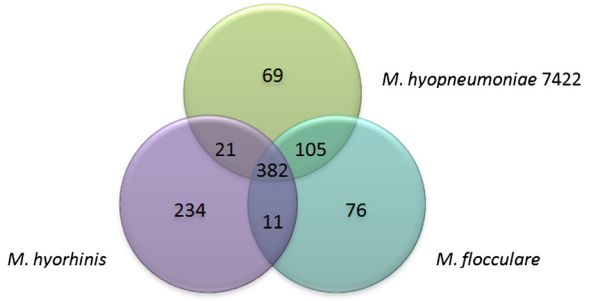

Figure 1 Venn diagram showing the distribution of *M. hyopneumoniae* 7422, *M. flocculare* and *M. hyorhinis* CDSs. Venn diagram identifying the total number of common and exclusive CDSs for each genome.

approximately. The small, but significative difference in the *M. hyopneumoniae* 7422 genome is the presence of an exclusive region of genes encoding transposases, hypothetical proteins and an ortholog of subtilisin-like serine protease (positioned from 497,277 to 510,210 bp). In comparison to 7422 genome, just one exclusive region was found in the 7448 genome, which is composed of genes encoding hypothetical proteins (positioned from 746,315 to 757,309 bp).

Comparison of OC organization in *M. flocculare, M. hyopneumoniae* and *M. hyorhinis* genomes

The gene-by-gene genome organization of *M. flocculare*, *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1 was analyzed, and the gene localization patterns were compared to detect ORFs with order conservation. The ORF cluster composition, organization and localization in the genomes were analyzed to determine the conservation level among the OC organization. Two groups of ORF clusters were created for each species, the OC group (Additional files 1 and 2) and the monocistronic gene (mC) group (Additional file 3). The general features of the OCs organization in the *M. flocculare, M. hyopneumoniae* and *M. hyorhinis* genomes are shown in Table 3 and Additional file 4.

A comparison of the OCs arrangements revealed a similar number of OCs among the *M. flocculare*, *M. hyopneumoniae* and *M. hyorhinis* genomes (Table 3). This result suggests that gene organization in *M. flocculare* and *M. hyorhinis* also occur preferably in clusters as found in *M. hyopneumoniae* [30]. Moreover, as previously described for *M. hyopneumoniae* [30], the overall ORF distribution within the OCs in *M. flocculare* and *M. hyorhinis* is highly variable with respect to the number of ORFs and the functional categories of the encoded products (Additional files 1 and 2).

An analysis of the mC group revealed a different ORF number in the *M. flocculare* genome when compared to the organization in the *M. hyopneumoniae* and *M. hyorhinis* genomes. There were 51, 34, and 30 mCs in the *M. flocculare*, *M. hyopneumoniae* and *M. hyorhinis* genomes, respectively (Additional file 3). Among all of the mCs, seventeen mCs were shared only by the *M. flocculare* and *M. hyopneumoniae* genomes. However, only the CDSs encoding an O-sialoglycoprotein endopeptidase (*gcp*) and an excinuclease ABC subunit C (*uvrC*) were found to display monocistronic organization in the three genomes (Additional file 5).

Table 3 Features of the *M. flocculare, M. hyopneumoniae* and *M. hyorhinis* OCs organization

Features	MFL*	MHP*	MHR*
Total length (base pairs)	772.687	920.079	839.615
Total No. of OCs (CDSs total)	114 (582)	117 (657)	98 (654)
Total No. of monocistronic group	51	34	34
Exclusives OCs	10	24	36

* Abbreviations as in Table 1.

A detailed analysis of the organization of each OC demonstrated a high level of conservation between the *M. flocculare* and *M. hyopneumoniae* genomes (Additional file 4). Approximately 78% and 46% of the OCs from *M. flocculare* display total or partial conserved gene distribution when compared to the OCs of *M. hyopneumoniae* and *M. hyorhinis*, respectively (Table 4; Additional file 4). Moreover, a comparative analysis of OC cluster organization among the three mycoplasma species revealed the presence of 12 OCs with complete similarity with respect to the ORF repertoires (synteny was not always detected). The majority of these OCs (seven OCs) were composed of two ORFs, with increasing numbers of up to five ORFs. These data are consistent with previous results that suggested that the majority of gene clusters in diverse organisms are formed by a string of two to four genes [30,31].

Another group of noteworthy OCs was the group classified as partially conserved among the three mycoplasma species. The number of partially conserved gene-order clusters in different genome pairs is shown in Table 4. The

Table 4 Comparison of OCs organization in *M. flocculare, M. hyopneumoniae* and *M. hyorhinis* genomes

ORF Clusters features	MFL x MHP*	MFL x MHR*	MFL x MHP x MHR*
OCs 100% conserved	44	17	12
OCs Partially conserved	44	35	33
OCs Without conservation	29	62	-

* Abbreviations as in Table 1.

M. flocculare and *M. hyopneumoniae* genomes shared 44 OCs in which the gene string was partially conserved, and 33 OCs of the 44 OCs were classified as partially conserved in all three analyzed species. It is well known that only a few operons are conserved in most bacterial genomes; the classical example of conserved organization involves the ribosomal protein operons [32]. However, a detailed analysis of the 33 partially conserved OCs revealed gene context conservation in the ribosomal operons (see $OC_{7448}28$ in Additional file 4) and in other clusters, such as clusters containing the chromosomal replication initiation protein (DnaA) (see $OC_{7448}01$ in Additional file 4) and the OC containing the cell division protein MraZ (see $OC_{7448}67$ in Additional file 4). In general, the similarity of the gene order (total or partial) among the prokaryotic genomes is maintained via the horizontal transfer of a chromosomal region. Our results suggest that individual genome pairs, such as *M. flocculare* and *M. hyopneumoniae* or *M. flocculare* and *M. hyorhinis*, share several OCs, which can partially be attributed to horizontal gene transfer.

A detailed, genomic-scale analysis of the OC organization in *M. flocculare* and *M. hyopneumoniae* demonstrated that species-specific differences are not present in genes with known function and/or related with pathogenicity (Additional file 6A-D). Apparently the 24 OCs exclusive to *M. hyopneumoniae* (not found in the *M. flocculare* genome) encode hypothetical proteins, transport-related proteins, myo-inositol utilization proteins, the integrative conjugative element (ICEH) and an additional copy of the P97 protein (Additional file 6A). In the *M. hyorhinis* genome, 35 OCs were unique to this species (Table 3; Additional file 6D), and the majority of the ORFs encode hypothetical proteins or products related to variable surface lipoproteins (*vlp* genes), which have been described as being involved in a complex system involving bacterial-host interactions [33].

Rearrangements in the *M. flocculare, M. hyopneumoniae* and *M. hyorhinis* genomes

A detailed comparative analysis of genome organization is needed to understand the evolutionary dynamics of prokaryotic genomes. Therefore, a comparative genomic analysis was performed using the *M. flocculare* contigs (MFL contigs) and *M. hyopneumoniae* genome, considering the ORF string organization and OC distribution (Additional file 7A-B). Comparisons were also performed between MFL contigs and the *M. hyorhinis* genome (Additional file 7A-B); however, in this case, both the global alignment and gene-by-gene alignment were not applicable, possibly due to the large number of transpositions and inversions that have occurred in these genomes.

In the comparison between *M. flocculare* and *M. hyopneumoniae*, 22 regions (with lengths ranging from 2 to 75 kb) were identified as being involved in inversions or rearrangements (Additional file 7B). Among these regions, only eight showed major rearrangements, although the OC organization was maintained. Notably, OCs containing several of the genes encoding pathogenicity-related proteins, such as lipoproteins and adhesins, were located within these regions. For instance, major rearrangements were observed in gene clusters encoding P97, P102 (MFL contig 13), P60, P69, P37 (MFL contig 34), P216, P76 (MFL contig 20) and the 46 K surface antigen precursor (MFL contig 4). Genes encoding transposases were found adjacent to some of the inverted segments, such as in MFL contig 23 (containing the P146 and MgPa proteins) and MFL contig 15 (containing the P97-like and P102-like adhesins), suggesting a possible role of these transposases in the rearrangements. Our findings in the comparison between the *M. flocculare* and *M. hyopneumoniae* genomes are similar to the situation found in the genomes of the two closely related species *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, whose genomes can be divided into segments with highly conserved gene organization, although the segments are arranged differently [34].

The lack of gene-order conservation beyond the operon level even between relatively closely related species has been previously described [32]. Apparently, in closely related mycoplasmas, such as *M. flocculare* and *M. hyopneumoniae*, large-scale gene-order conservation is observed, although genome collinearity is disrupted at some points. Chromosomal rearrangements are generally caused by homologous recombination between repeated sequences within the genome [19,35]. Although the number of genes involved in DNA repair and recombination in mycoplasmas is relatively small [36], the gene encoding RecA was found in all sequenced mollicute genomes. Recently, the importance of RecA in the antigenic and phase variation of the MgpB and MgpC adhesins in *M. genitalium* has been demonstrated [37].

Repertoire of surface proteins encoded by *M. flocculare, M. hyopneumoniae* and *M. hyorhinis*

A comparative *in silico* survey of the repertoire of encoded surface proteins was performed between the genome of the non-pathogenic *M. flocculare* and the genomes of two pathogenic mycoplasma species that are found in this tissue in the swine respiratory tract, *M. hyopneumoniae* (represented by the 7448 strain) and *M. hyorhinis* (represented by the HUB-1 strain). The results of this survey are summarized in Additional files 8 and 9, and the complete generated datasets are presented in Additional files 10, 11 and 12. Of the total of 585 *M. flocculare* CDSs, 277 (47.5%) were predicted to encode surface proteins; this number was similar to that of *M. hyopneumoniae* 7448 (292 out of 716; 44.4%) and higher than that of *M. hyorhinis* HUB-1 (247 out of 654, 37.7%)

(Additional file 10A-C). The proportion of CDSs encoding surface proteins in these species is considerably large considering their small genome sizes. From these surface protein sets, 28 (10.1%), 42 (14.4%), and 44 (17.8%) CDSs are unique to *M. flocculare*, *M. hyopneumoniae* and *M. hyorhinis*, respectively, with respect to the other two species (Additional file 8; Additional file 11A-C).

The repertoire of *M. flocculare* surface proteins that is not shared with the other two species consists exclusively of hypothetical proteins; although those of *M. hyopneumoniae* and *M. hyorhinis* are predominantly composed of hypothetical proteins (73.8% and 59%, respectively), they also include some proteins with assigned functions. Among these proteins with predicted functions, *M. hyopneumoniae* includes proteins involved in myo-inositol catabolism, a permease and a protein encoded by the integrative conjugative element (ICEH), and *M. hyorhinis* includes some variable antigens, secretory system components, transporters and lipoproteins.

Based on an E-value cutoff threshold of 1e-6 to define orthology, nearly 90% of the repertoire of *M. flocculare* surface proteins is shared with *M. hyopneumoniae* and/or *M. hyorhinis* (Additional file 8; Additional file 12). Searches using more stringent conditions resulted in not more than a 20% reduction in the numbers of identified orthologs (data not shown). These results are indicative of physiological similarities that would be consistent with the adaptation to the same host environment. Of the shared proteins, approximately 40% have unknown functions (hypothetical proteins), while the other 60% consist of proteins with assigned functions in at least one of the compared species. Notably, many of these shared proteins (46 proteins, marked in bold in Additional file 12) correspond to putative pathogenicity-related genes in *M. hyopneumoniae* and/or *M. hyorhinis*; these proteins include several lipoproteins and adhesins that are thought to play a role in virulence despite of the non-pathogenic nature of *M. flocculare* and the pathogenicity differences between *M. hyopneumoniae* and *M. hyorhinis*. For instance, *M. flocculare* contains orthologs for the P97 copy 2 and for the P97-like adhesins of *M. hyopneumoniae*, although it lacks an ortholog for P97 copy 1. The genomic organization of the P97 copy 2 and P97-like ortholog CDSs are similar in both species with respect to gene clustering (Figure 2); this result suggests that P97 copy 2 and P97-like are ancestral P97 paralogs and that they were present in a common ancestor to *M. flocculare* and *M. hyopneumoniae*. A second duplication event, which originated the *M. hyopneumoniae* P97 copy 1, would have occurred after the divergence of *M. hyopneumoniae* from *M. flocculare*.

Additional file 13 lists some adhesins that have been associated with pathogenicity and have been experimentally analyzed [38-43] in *M. hyopneumoniae* 232. The *M. hyopneumoniae* 7448 and *M. flocculare* genomes contain orthologs for all the adhesins with the aforementioned exception of one copy of the P97 and P102 proteins that are absent in *M. flocculare* (also shown in Figure 2). The gene organization and location was analyzed and, as described in Additional file 13, the regions containing these orthologs are involved in inversions or rearrangements (Additional file 7B) in both mycoplasma species. Specifically, three important adhesins (P216, P159 and P60) display highly conserved gene organization between *M. hyopneumoniae* 232, 7422, 7448 and 168 strains, but they display inversions and rearrangements in *M. flocculare*. The participation of *M. hyopneumoniae* adhesins in host-cell adhesion is a complex process involving specific cleavage events [44]. The set of *M. flocculare* genes that may be involved in adhesion may not be complete, which would explain the differences in host-cell adhesion with respect to *M. hyopneumoniae* [18]. These results may explain the presence of orthologs in *M. flocculare* despite its lack of pathogenic capacity.

The presence of surface virulence determinants even in the non-pathogenic *M. flocculare* and in a non-pathogenic strain of *M. hyopneumoniae* (J strain) [21,45] suggests that their roles in pathogenicity may depend on

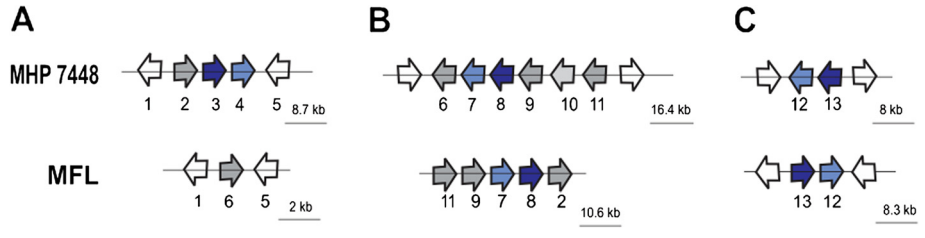

Figure 2 P97 and P102 gene organization contexts in the *M. flocculare* and *M. hyopneumoniae* 7448 genomes. (**A**) P97 copy 1 ORF cluster organization. (**B**) P97 copy 2 ORF cluster organization. **(C)** P97-like ORF cluster organization. The arrows represent the ORFs (not to scale) and indicate the transcriptional direction. The dark-blue arrows represent the P97 ORFs, and the light-blue arrows represent the P102 ORFs. The white arrows represent the ORFs that are at the limits of the OC. The numbers from one through thirteen represent the ORF name and the names of its orthologous as follows: 1- *rpsJ*; 2- MF1418 and MHP0197; 3- P97 copy 1; 4- P102 copy 1; 5- MF0249 and MHP0200; 6- MF0247 and MHP0106; 7- P102 copy 2; 8- P97 copy 2; 9- *gyrB*; 10- transposase; 11- *pfkA*; 12- P102-like; and 13- P97-like.

their expression levels and/or post-translational processing, which may vary [46]. Differences in virulence between species and strains can also be associated with the presence of variants of these proteins with or without some functional domains that are associated with features such as adhesion capacity or antigenicity, which has previously been described for several *M. hyopneumoniae* and *M. hyorhinis* virulence factors [40,41,47,48]. For instance, the *M. flocculare* P97 copy 2 and P97-like orthologs present relatively high overall identities to their *M. hyopneumoniae* counterparts (53% and 57%, respectively), but in the case of P97 copy 2, the *M. flocculare* ortholog lacks a domain (R1) regarded to be important for virulence in *M. hyopneumoniae*; instead, it contains a second R2 domain (Additional file 14). These R1 and R2 repeats are absent from the *M. flocculare* P97-like protein and its orthologs from *M. hyopneumoniae* (P97-like adhesin) and *M. hyorhinis* (P95).

Cell-surface features with implications for virulence may also reside in the 10 to 18% of the repertoires of surface proteins that are not shared between *M. flocculare*, *M. hyopneumoniae* and *M. hyorhinis*. However, because all (in the case of *M. flocculare*) or most (in the cases of *M. hyopneumoniae* and *M. hyorhinis*) of these unshared CDS products are hypothetical or conserved hypothetical proteins, their potential contributions to pathogenicity remain elusive. However, considering the nature of the unshared CDS products annotated in *M. hyopneumoniae* and *M. hyorhinis*, these 'exclusive' and unknown proteins are likely to include players of processes that are important for pathogen-host interactions, such as proteins involved in secretion, the uptake of certain molecules, conjugation and immune evasion/modulation. The variation in *M. hyorhinis* surface lipoproteins (Vlp) is considered important to protect the organism from the humoral response and may be a primary adaptive strategy for immune evasion during infection and disease [49,50]. Therefore, at least some of these proteins are expected to compose a portion of the repertoire of determinants of virulence or avirulence for each species or strain.

An additional comparison of the repertoires of surface proteins from *M. flocculare*, *M. hyopneumoniae* and *M. hyorhinis* was performed based on the COG classification of the predicted surface protein sets for each species (see Additional file 10). The produced COG functional profiles of surface proteins for the three species are summarized in Additional file 9. According to the COG, the functional surface protein profile for *M. flocculare* is similar to those for *M. hyopneumoniae* and *M. hyorhinis*; similar numbers of proteins were assigned to each category for the three species. This similarity was observed even for the U and M categories, which include secretion system components (whose repertoires are virtually equivalent for the three species; data not shown), and for the no-COG category, which included 45-53% of the proteins, most of which (82.2% for *M. flocculare*, 86.6% for *M. hyopneumoniae*, and 64.8% for *M. hyorhinis*) were represented by hypothetical proteins or in the additional category of antigen, adhesin or lipoprotein, in which proteins were included based on their prior immunological or functional characterization according to published studies. Overall, the surface protein set for *M. flocculare*, taken from the correspondent COG profile, was shown to be very similar to those of *M. hyopneumoniae* and *M. hyorhinis*. This result suggests that the species have equivalent genetic backgrounds for metabolic and growth processes. Such functional similarities may be the result of common selective pressures associated with the colonization of the same environment (i.e., the swine respiratory tract).

The L (replication, recombination and repair) and V (defense mechanisms) categories displayed differences; *M. flocculare* (and *M. hyorhinis*) contained approximately half of the number of proteins as *M. hyopneumoniae*. Protein sets assigned to the L category are heterogeneous, and the relative excess of proteins in *M. hyopneumoniae* corresponds to transposases that cannot be found in either *M. flocculare* or *M. hyorhinis*. Conversely, category V is enriched with ATP-binding cassette (ABC) transporter system proteins related to defense mechanisms, such as the *M. hyorhinis* ABC-type multidrug-like transport system ATP-binding proteins and their orthologs in *M. flocculare* and *M. hyopneumoniae*. The remaining transporters, including ABC and non-ABC transporter system components (such as those from the phosphotransferase system; PTS) appear in other COG categories, such as E, G, R or P. However, the overall number of transporters unrelated to defense mechanisms (non-V) is roughly equivalent in the three species, with 50, 49, and 47, in the *M. flocculare*, *M. hyopneumoniae* and *M. hyorhinis* surface protein sets, respectively. Although *M. hyorhinis* have 19 genes encoding transposases, and *M. hyopneumoniae* 9 genes, notably, the presence of transposases among *M. hyopneumoniae* predicted surface proteins may be an artifact due to the occurrence of helical structures in these enzymes [51]; these helices can be misidentified as transmembrane domains. However, the differential presence of at least some transporter system components is indicative of certain *M. hyopneumoniae* capabilities that are unavailable in both *M. flocculare* and *M. hyorhinis*. A larger number of transport proteins is usually related to a species' capacity to persist in different tissue environments [52], but this phenomenon does not seem to apply to *M. flocculare*, *M. hyopneumoniae* or *M. hyorhinis* because they share a large portion of the transporter repertoire. This situation is similar to that observed for secretory system components. However, according to the COG (see below), *M. hyopneumoniae* has approximately two-

fold more transporters associated with defense mechanisms than the other two species. In this aspect, *M. hyopneumoniae* is more similar to *Mycoplasma bovis* [53], which infects the respiratory tract and breast and joint tissues of bovines, than to *M. flocculare* or *M. hyorhinis*. The implications of the larger *M. hyopneumoniae* repertoire of defense mechanisms proteins (COG V) for its survival in the swine respiratory tract have not yet been investigated.

Phylogenomics and the phylogenetics of *Mycoplasmataceae*

From the entire set of 585 annotated *M. flocculare* genes, 179 gene sets were retrieved that contained at least one gene representative for each swine mycoplasma analyzed here (BLAST cut off E-10). Overall, 179 ortholog-like files representing different CDSs were concatenated, leading to an aligned file containing 104,097 amino acid residues. The neighbor-joining method (NJ) and maximum parsimony (MP) tree topologies did not differ significantly, especially when major clades were considered (Additional files 15, 16, 17; 18). There was consensus in several aspects (Figure 3). As expected, all of the *M. hyopneumoniae* strains formed a monophyletic clade. Additionally, the *M. hyopneumoniae* monophyletic clade was closely related to *M. flocculare*, with high bootstrap support. Finally, *M. hyorhinis* is basal to *Mycoplasma conjunctivae*, *M. flocculare* and *M. hyopneumoniae* in all of the phylogenomic trees.

The *Mycoplasmataceae* species were subdivided into the following clades: bovis (including *Mycoplasma agalactiae*, *M. bovis* and *Mycoplasma fermentans*), hominis (*Mycoplasma hominis* and *Mycoplasma arthritidis*), hyopneumoniae (*M. hyorhinis*, *M. conjunctivae*, *M. flocculare*, and *M. hyopneumoniae*), hemotrophic mycoplasma (*Mycoplasma suis* and *Mycoplasma haemofelis*), genitalium-pneumoniae

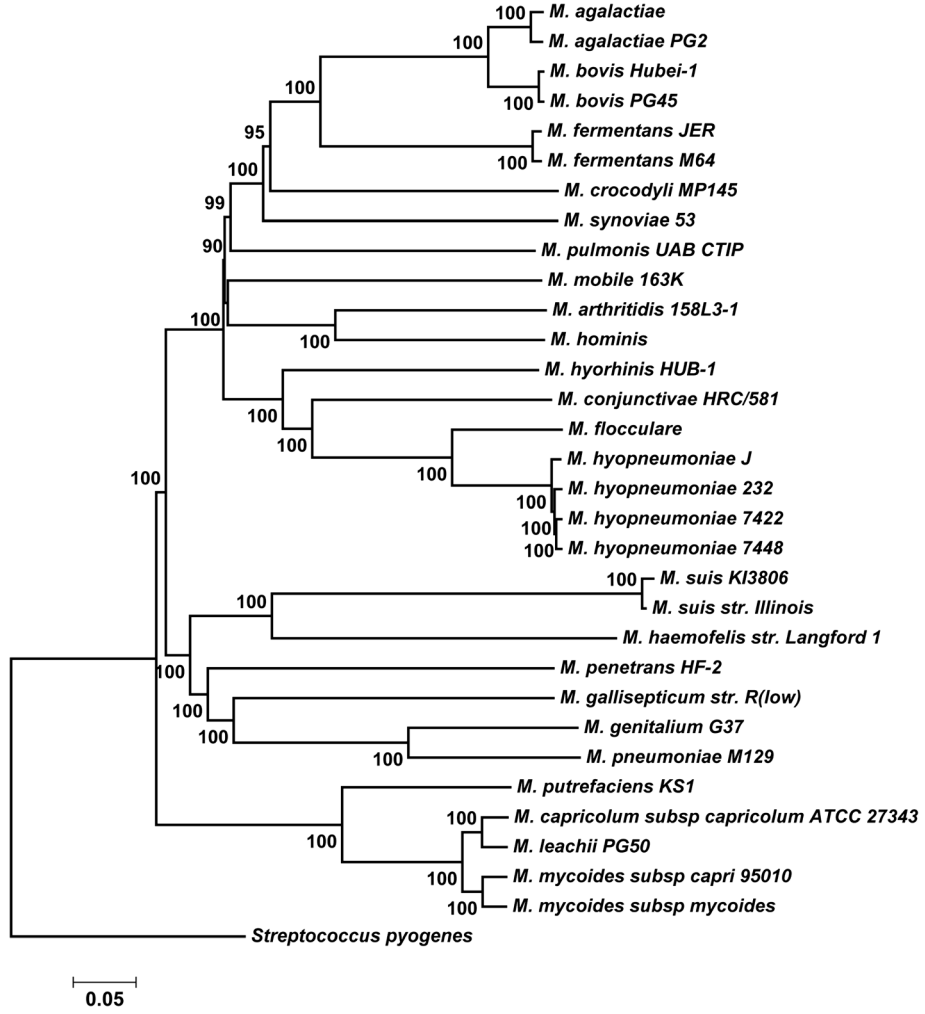

Figure 3 Evolutionary history of mycoplasmas obtained through a phylogenomic approach. The Neighbor-Joining method using the p-distance to compute the evolutionary distances and the pairwise deletion of gaps was implemented in the MEGA 5 software program. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to each branch. *Streptococcus pyogenes* was used as the outgroup.

(*Mycoplasma gallisepticum*, *Mycoplasma genitalium*, and *Mycoplasma pneumoniae*), and mycoides (*Mycoplasma putrefaciens*, *Mycoplasma capricolum*, *Mycoplasma leachii*, and *Mycoplasma mycoides*). All of these clades displayed high bootstrap values. The synoviae-pulmonis (*M. crocodyli*, *M. synoviae*, and *M. pulmonis*) group did not form a monophyletic cluster, but they are closely related to the bovis clade. *M. penetrans* HF-2 is near the genitalium-pneumoniae clade.

Our mycoplasma phylogenomic tree (Figure 3) corroborated the results that were obtained using the RNA polymerase beta subunit (rpoB), 16S-23S rRNA intergenic transcribed spacer region (ITS), and 16S rRNA genes [54,55]. *M. flocculare*, *M. hyopneumoniae* 7448, and *M. hyorhinis* HUB-1 were located in the hyopneumoniae clade.

When comparing the *M. flocculare*, *M. hyorhinis* HUB-1 and *M. hyopneumoniae* 7448 genomes, several paralog clusters were identified through the bidirectional best hit (BBH) approach, wherein a paralog cluster was defined as a gene set in which every gene is a BBH with at least one other element. Fourteen of these paralogs (DNA methylase, ATP synthase, ribulose-phosphate 3-epimerase, oligoendopeptidase F, single-strand binding protein, fructose-bisphosphate aldolase, dihydrolipoamide dehydrogenase, glucose-6-phosphate isomerase, lipoate-protein ligase, acyl-carrier-protein phosphodiesterase, lactate dehydrogenase, membrane nuclease lipoprotein, TrsE-like protein, and P97) were submitted to phylogenetic analyses to understand the evolutionary history of those paralogs.

Phylogenetic analyses of mycoplasma DNA methylases, which are enzymes that catalyze the transfer of a methyl group to DNA [56], contained ancient gene duplications in the hyopneumoniae group, leading each DNA methylase paralog to form a monophyletic group that included *M. flocculare* and *M. hyopneumoniae* (Additional file 19). Methylation, in addition to involvement in restriction systems, plays an important role in controlling gene expression, and it is one of the most significant DNA modifications [57]. The N^6-adenine methylation is involved in bacterial gene regulation and virulence [58-60]. CpG motifs in bacterial DNA may play a significant pathogenic role in inflammatory lung disease because the proinflammatory effects can be reduced by DNA methylation [61]. Microarray analyses and RT-PCR have demonstrated that the deletion of a C^5-cytosine methyltransferase in *Helicobacter pylori* strains can affect the expression of several genes related to motility, adhesion and virulence [57].

The ATP synthase phylogeny indicated that *M. flocculare*, *M. hyorhinis* HUB-1, and *M. hyopneumoniae* 7448 cluster according to the ATP synthase subunit, with the alpha subunit presenting a more complex evolutionary pattern (Additional file 20). This enzyme is required to synthesize adenosine triphosphate (ATP), providing energy to the cell. The paralogs found in mycoplasmas are related to different subunits that are required for enzymatic function [62].

Ribulose-phosphate 3-epimerase interconverts the stereoisomers ribulose-5-phosphate and xylulose-5-phosphate [63]. Its phylogeny revealed that ancient duplications occurred in the hyopneumoniae group. Other gene duplications responsible for the *M. hyorhinis* GDL-1 and *M. hyorhinis* HUB-1 split can be observed at the base of this *M. hyorhinis* clade (Additional file 21). Similarly, recent gene duplications can be observed in the *M. hyorhinis* clade in the oligoendopeptidase F (Additional file 22) and single-strand binding protein (Additional file 23) phylogenetic trees, which both contain high bootstrap support.

M. flocculare and *M. hyopneumoniae* 7448 contain two copies of fructose-bisphosphate aldolase, while *M. hyorhinis* HUB-1 contains only one copy. These duplications likely occurred prior to the diversification of the hyopneumoniae group (Additional file 24). The same process occurred during dihydrolipoamide dehydrogenase evolution, as shown in Additional file 25. *M. hyorhinis* HUB-1 contains two copies of the dimeric glycolytic enzyme glucose-6-phosphate isomerase, which catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate [64]. The lack of statistical confidence in some tree branches did not facilitate inferences regarding the evolutionary history of these copies in *M. hyorhinis* HUB-1 (Additional file 26).

Two lipoate-protein ligases are found in *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1. They are more closely related to the enzymes from other microorganisms than to each other (Additional file 27). In *M. flocculare*, only one copy was identified. The same pattern was observed in the phylogenetic tree of acyl-carrier-protein phosphodiesterase (Additional file 28), which belongs to the hydrolase family and acts on phosphoric diester bonds [65]. The topology of the lactate dehydrogenase tree showed that the two *M. hyorhinis* HUB-1 protein copies are significantly different, which led the sequences to be grouped in distant clades (Additional file 29). A recent duplication of membrane nuclease lipoprotein resulted in a *M. hyorhinis* cluster containing the two *M. hyorhinis* HUB-1 copies. A unique copy of this specific protein was found in *M. flocculare* and *M. hyopneumoniae* 7448 (Additional file 30).

Two copies of the TrsE -like protein were identified in *M. hyopneumoniae* 7448, and one copy was identified in the *M. flocculare* and *M. hyorhinis* HUB-1 genomes. Each copy is more similar to other sequences in the *M. hyopneumoniae* strain than to one another (Additional file 31). Finally, P97 is an adhesin thought to play a role in virulence. Several copies have been detected in the

hyopneumoniae group. The phylogenetic tree indicated that the copies of *M. flocculare* and *M. hyopneumoniae* 7448 are more closely related to the sequences from other species and strains than to each other (Additional file 32).

Although mycoplasmas contain reduced genomes, some paralogs are maintained in their genomes. A phylogenetic analysis was conducted to better understand the evolution of those paralogs. Gene copies are known to be preserved in a genome if the organism demands high levels of particular gene products. In other cases, positive selection can result in the diversification of the gene's function, a process called neofunctionalization [66]. Additionally, subfunctionalization can lead to the loss of function, resulting in duplicated genes whose functions differ to some degree [67]. Even highly conserved genes may have slightly or very different functions, such as glycolytic enzymes such as fructose-bisphosphate aldolase (FBA), which have been described as complex, multifunctional proteins that perform non-glycolytic functions [68].

Some mycoplasma paralog proteins, such as lipoate-protein ligase may possess different functions or differ in substrate specificity. Otherwise, essential enzymes, such as ATP synthase, may maintain multiple gene copies because they encode different subunits that are required for enzymatic function, despite the recent finds of losses of this enzyme family in the common ancestor of Mollicutes [69]. According to the standard model of phylogenomics, there is a higher similarity among orthologs than paralogs [67]. The paralog genes initially display identical sequences and functions. However, the action of selective pressures and mutations lead to divergence in regulatory and coding sequences [70].

Horizontal gene transfer

The species tree that was generated by the phylogenomic analysis was compared to the individual gene trees to investigate the occurrence of horizontal gene transfer (HGT) events. HGTs are an important source of genome innovation and evolution in prokaryotes [71], and it apparently also impacts Mycoplasmataceae evolution.

In mycoplasmas, we observed several HGT events (Additional file 33); some of the events occurred between *M. hyorhinis* HUB-1 and *M. conjunctivae* HRC. The events involving species belonging to the hyopneumoniae group occurred in ribosomal proteins, GTP-binding proteins, heat-shock proteins, DNA primase, signal recognition particle protein, ABC transporter ATP-binding proteins, phosphoesterases, cell division protein, elongation factor, fructose-biphosphate aldolase, DNA polymerase, glutamyl-tRNA synthetase, helicases, and hypothetical proteins. Regions encoding ABC transporters were likely transferred between *M. synoviae* and *M. gallisepticum* (Additional file 33), corroborating previously published results [21].

It is well-known that prokaryotes exchange genes in a sophisticated manner via lateral transfer, and bacterial phylogenies may also be viewed as a complex network of genomic exchange. However, the sequence-based methods implemented in the phylogenomic studies have yielded phylogenetic trees that are similar to rRNA trees, which were demonstrated in the current study. Consequently, lateral transfer events do not prevent the recovery of phylogenetic signals in prokaryotes, although they do add an extra source of noise [72].

Metabolism overview

Mycoplasmas contain a reduced genome; therefore, they lack many metabolic pathways, particularly biosynthetic pathways, such as those involved in cell-wall production, de novo purine biosynthesis and the biosynthesis of amino acids [73]. These organisms also lack a functional tricarboxylic acid (TCA) cycle because they are extremely fastidious in their nutritional requirements and dependent on nutrients supplied by their hosts. They produce high levels of enzymes responsible for the degradation of nucleic acids and proteins and transporters to obtain the precursors of these macromolecules. Most mycoplasma species depend on the glycolytic pathway to generate ATP. Some species may produce ATP based on the reaction involving acetyl phosphate and ADP by acetate kinase, coupled with acetyl phosphate formation from acetyl-CoA by phosphate acetyl transferase. Acetyl CoA is formed by the pyruvate dehydrogenase complex [1,74]. Some mycoplasma species such as *M. hominis* and *M. arthritidis* produce ATP through arginine degradation, using the arginine dihydrolase pathway [75]. This pathway is absent in the mycoplasma genomes analyzed in this work. All of the genes that encode enzymes of the glycolytic pathway exist in the three species; however, some differences in gene copy number were observed. Two copies of the genes encoding fructose-bisphosphate aldolase exist in *M. hyopneumoniae* and *M. flocculare*, two copies of the gene encoding d-ribulose-5-phosphate 3 epimerase are present in *M. hyopneumoniae*, and two copies of the gene encoding glucose-6-phosphate isomerase are present in *M. hyorhinis*. The possible influence of the gene copy number on the physiology of the species is unknown. However, some glycolytic enzymes have been described as virulence factors, exhibiting functions unrelated to glycolysis, such as adhesion to the host cells, may contribute to the pathogenesis of mycoplasmas infections [7,68,76-79]. The evolutionary aspects of these paralogs are discussed in the "Phylogenomics and phylogenetics of *Mycoplasmatacae*" section.

It has been shown that nuclease activities can be detected in Mollicutes and that these activities are primarily associated with the membrane and may be essential for growth and survival [80]. Genes encoding nucleases or putative

membrane-associated nucleases were found in the three analyzed *Mycoplasma* species. Two gene sets encoding membrane nucleases were observed in the three species. One set is represented by two copies of *mnu*A (MHR_0206 and MHR_0549) in the *M. hyorhinis* HUB-1 genome and a single copy in *M. hyopneumoniae* 7448 (MHP7448_0580), *M. hyopneumoniae* 7422 (MX03145) and *M. flocculare* (MF00420). Another set represented by two copies was observed in the genomes of the three species. The cell surface-exposed exonuclease (mhp379) from *M. hyopneumoniae* 232, a representative of the latter set, has been analyzed, and it has been proposed that the exonuclease activity of mhp379 may be important for importing nucleic acid precursors [81]. The presence of an extra copy of a nuclease gene in *M. hyorhinis* may represent a potential advantage of this species in acquiring nucleic acids precursors. Mycoplasmas cannot de novo synthesize purines and pyrimidines; therefore, they depend on salvage and interconversions to supply the cell with the nucleic acid precursors [82]. The three species contain a similar set of genes involved in purine (MHP7448_0084 - *M. hyopneumoniae*, MHR_0566 - *M. hyorhinis* and MF01198 - *M. flocculare*) and pyrimidine (MHP7448_0578 - *M. hyopneumoniae*, MHR_0640 - *M. hyorhinis* and MF00424 - *M. flocculare*) metabolism (See Additional file 4). However, *M. hyorhinis* contains genes encoding thymidylate synthetase (TS), allowing the conversion of dUMP to dTMP, and dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF). The presence of TS and DHFR in *M. hyorhinis* may also contribute to its ability to overgrow the other *Mycoplasma* species in the swine respiratory tract [14].

Conclusions

The comparative analyses of three mycoplasma species that inhabit the swine respiratory tract facilitated the identification of some characteristics that may promote the understanding of their different behaviors. The *M. hyopneumoniae* strain 7422 genome displays a similar organization as to the other previously described strains, but it contains rearrangements and an altered position of ICEH in the genome. The genomes of *M. hyopneumoniae* and *M. flocculare*, two closely related species, contain some blocks of synteny, but they also display many differences that may help to explain why one species is pathogenic and the other is commensal. However, it was not possible to correlate specific virulence determinant factors to the pathogenicity differences of the analyzed species. A large proportion of the repertoire of *M. flocculare* surface proteins is shared with *M. hyopneumoniae* and/or *M. hyorhinis,* which would be expected because the organisms may occupy the same niche. However, certain members of the p97 family are absent in *M. flocculare*, and some display sequence differences or lack domains that are considered to be important for host-cell adhesion. *M. hyorhinis* contains some metabolic genes that are absent in the other species, suggesting a possible advantage in the growth of this species. The differences in some components involved in evasion of the host immune system may also contribute to the aggressive growth of *M. hyorhinis*. The phylogenomic analysis confirmed previous results, placing *M. hyopneumoniae, M. flocculare* and *M. hyorhinis* in the hyopneumoniae clade. Several horizontal gene transfer events were identified, and several of them occurred between *M. hyorhinis* and *M. conjunctivae*.

Methods

Bacterial strains, culture conditions, and DNA isolation

M. hyopneumoniae strain 7422 was isolated from an infected swine in Lindóia do Sul, Santa Catarina, Brazil. *M. flocculare* (ATCC 27716) was acquired by Embrapa Suínos e Aves (Concórdia, Brazil) from the American Type Culture Collection. Both of the strains were cultivated in 5 mL of Friis medium [83] at 37°C for 48 h, and genomic DNA was extracted according to a standard protocol [84].

Genome sequencing, assembly and annotation

For each species, one 454 shotgun library was prepared with approximately 5 μg of gDNA. The library construction, titration, emulsion PCR and sequencing steps were performed explicitly according to the manufacturer's protocol. Sequencing was performed using Roche 454 GS FLX Titanium platform. *M. flocculare* was sequenced in one region of a two-region PicoTiterPlate (PTP), and *M. hyopneumoniae* 7422 was sequenced in two regions of an eight-region PTP. The contigs were assembled using the Newbler software program version 2.6 with the default parameters. The PCR assisted contig extension (PACE) strategy [85] was used for physical gap closure by the ends regions of gaps. For *M. hyopneumoniae* 7422 and *M. flocculare*, the estimated genome coverage for both genomes was 23X. The *M hyopneumoniae* 7422 genome was completely closed in one contig, and the *M. flocculare* genome retained 13 gaps.

The annotation and analysis of the sequences of both genomes were performed using the System for Automated Bacterial Integrated Annotation (SABIA) [86]. The comparative analysis was based on the Bidirectional Best Hits (BBH) [87] approach using the BLASTP (Basic Local Alignment Search Tool) [88] program to identify corresponding gene pairs recognized as the best hits in other genomes. All of the BLASTP searches were performed using the following parameters: an e-value of 10^{-5}, query coverage of 60%, and positive similarity value

of 50%. The comparative databank is available at http://www.genesul.lncc.br/comparative/.

To store and analyze the data, a databank was developed using the MySQL and Perl programming languages. This databank integrates tools and information from numerous biological databases, such as The Integrated Resource of Protein Domains and Functional Sites (Interpro) [89], Protein Subcellular Localization Prediction Tool (Psort) [90], Kyoto Encyclopedia of Genes and Genomes (KEGG) [29], Clusters of Orthologous Groups of Proteins (COG) [91], Transporter Classification Database (TCDB) [92], BLASTP of KEGG and UniProt/Swiss-Prot [93], facilitating several analyses, such as cluster with minimal genomes and clusters exclusives genes for each genome analyzed. In addition, the databank allows automatic genomic comparisons by bidirectional best hits (BBH) between five species. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession PRJNA65295 ID: 65295 for *M. flocculare* and PRJNA47327 ID: 47327 for *M. hyopneumoniae* 7422.

In silico analysis of ORF clusters (OCs)

The prediction of OCs was performed by the Artemis Release 10.5.2 software program [94] according to previously established criteria [30]. The manual examination of the possible OCs in the *M. flocculare, M. hyorhinis* and *M. hyopneumoniae* genomes was established based on the occurrence of clusters with two or more tandem genes in the same DNA strand. This procedure was performed by a systematic annotation comparison of the protein sequences encoded in all of the ORFs from the analyzed genomes. According to the complexity of the adjacent ORF rearrangements, two groups were created; the OC group was characterized by the presence of two or more ORFs in the same DNA strand until the occurrence of ORFs in the opposite strand, and the monocistronic (mC) group represented single ORFs. Differences in the annotation were evaluated by comparing the protein sequences from these three genomes using the NCBI/BLASTP program. The OC groups predicted for *M. flocculare* and *M. hyorhinis* were compared with the OC organization found in *M. hyopneumoniae* [30]. Moreover, comparative analyses were also performed between *M. hyorhinis* and *M. flocculare* to predict OC organization. The *M. flocculare* analysis was performed with each contig sequence, while the *M. hyorhinis* analysis was performed with the whole genome sequence.

Analysis of surface-protein-encoding CDSs

For the surface protein predictions, all of the translated CDS entries from the *M. flocculare* genome and from the *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1 genomes (NC_007332 and NC_014448 entries, respectively) were analyzed using the default parameters by the following software programs: SVMtm Transmembrane Domain Predictor [95], TMHMM Server v. 2.0 [96], SCAMPI [97], and PSORTb [98]. The first three programs predicted the presence of transmembrane (TM) domains, and the fourth was used for to predict subcellular localization (i.e., cytoplasmic vs. membrane). The first three independent TM predictions were merged, and the CDSs were considered as 'surface protein encoding' when they were predicted as such by at least two of the TM-predicting programs. The CDSs that were predicted to encode surface proteins by only one of the TM-predicting programs and/or by PSORTb were additionally analyzed by HMM-TM [99], and when a previously described ortholog was identified, based on the published literature. Those CDSs that were able to predict surface localization either by HMM-TM or based on the literature were also included in the list of predicted surface proteins encoded by each genome. After orthologs were identified by reciprocal pairwise comparisons between *M. flocculare*, *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1 (see below), the CDSs predicted to encode surface proteins just for one species (by the aforementioned criteria) had their corresponding orthologs (when existent) included in the list of surface-protein-encoding CDSs for the other two species. The clusters of orthologous group (COG) functional classification of *M. flocculare* predicted surface proteins using NCBI COGnitor [100], and those of *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1 predicted surface proteins obtained from NCBI.

For the ortholog and paralog identifications, bidirectional local BLAST searches [88] were performed between the *M. flocculare* predicted surface protein encoding CDSs and all of the CDSs from the other two species using the BioEdit Sequence Alignment Editor [101]. The ortholog and paralog definitions were based on the best hits from TBLASTX with the following parameters: cut-off e-value thresholds of 10^{-20}, 10^{-10} or 10^{-6}; manual inspection of query coverage, identity and similarity scores; and, if required, consideration of peculiarities of specific gene families.

Phylogenomic analyses

M. flocculare (ATCC 27716) was used as query organism. We began with all 582 genes identified in this strain and performed a BLAST search against the gene set of all other 32 organisms (Additional file 34). To select putative orthologs for all of the *M. flocculare* genes, we performed a BLAST search between this queried gene set and the individual genomes of the organisms previously cited; we retrieved the single best hit from each genome (BLAST cut-off 10). Multi-FASTA files were created containing ortholog gene sets for each query gene in the

M. flocculare strain. Only gene sets containing at least one representative in each genome were selected for further phylogenomic analysis (i.e., we only evaluated files containing 32 sequences for each analyzed genome).

Multi-FASTA putative ortholog files containing the best representative of each *M. flocculare* deduced protein sequence for all of the analyzed swine mycoplasmas were used as the input for multiple alignments using the CLUSTALw algorithm with default parameters.

The SCaFos software program [102] was used to facilitate the concatenation of the 179 alignment files. Phylogenies of 32 concatenated, deduced amino acid sequences were estimated by the NJ [103] and MP [104] methods in the Molecular Evolutionary Genetics Analysis (MEGA) program version 5.05 [105]. For the NJ method, the evolutionary distances were computed using the p-distance and the Poisson-corrected amino acids distance; both were presented in units of amino acid differences per site. The complete and pairwise deletion of gaps or missing data were implemented with the datasets containing 49,751 and 104,097 positions, respectively. The bootstrap test of the phylogeny was performed using 1,000 repetitions. The MP tree was obtained using the close-neighbor-interchange algorithm with search level 1, in which the initial trees were obtained by random addition of sequences (10 replicates). The different gap treatments were tested considering the complete deletion, partial deletion, and all sites included. The bootstrap test was implemented using 500 replicates. The TreeView software program [106] was used to visualize the resulting phylogenies.

Paralog analysis

Selected gene clusters of paralogs were subjected to a phylogenetic analysis. BLAST searches were first conducted for each gene using a 10^{-6} e-value cutoff; all of the sequences were subsequently aligned using COBALT [107]. Distance and parsimony methods in the MEGA 5 software program were applied to identify the evolutionary scenario for each paralog cluster. A bootstrap test was additionally performed with 1,000 replications.

Horizontal gene transfer

Horizontal gene transfer (HGT) was analyzed with Tree and Reticulogram reconstruction (T-Rex) [108] using the bipartition dissimilarity as the optimization criteria in the HGT detection algorithm [109]. The program infers an optimal (i.e., minimum-cost) scenario of horizontal gene transfers reconciling a given pair of species and gene trees. All of the gene trees were obtained through the distance method implemented in the MEGA 5 software program using p-distance, pairwise deletion of gaps, and bootstrap test of phylogeny with 500 replications. A bootstrap cutoff of 75% was applied to accept the HGT events. In total, 179 genes were subjected to phylogenetic and HGT analyses.

Additional files

Additional file 1: OCs organization in *M. flocculare* genome.

Additional file 2: OCs in *M. hyorhinis* genome.

Additional file 3: Monocistronic group (mC).

Additional file 4: OC organization similarities in the *M. hyorhinis* and *M. flocculare* genomes in relationship of *M. hyopneumoniae* OC organization.

Additional file 5: Monocistronic ORFs presents in the MHP, MHR and MFL genomes.

Additional file 6: A. Exclusive OCs of *M. hyopneumoniae* genome. 6B. Exclusive OCs of *M. flocculare* genome. 6C. Conserved Ocs among the three mycoplasmas species. 6D. Exclusive OCs of *M. hyorhinis* genome.

Additional file 7: A. *M. flocculare* contigs with conservation regions. B. *M. flocculare* contigs with inversion regions.

Additional file 8: Venn diagram of the predicted surface protein sets from *M. flocculare*, *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1.

Additional file 9: COG functional classifications of the predicted surface protein sets from *M. flocculare* (A), *M. hyopneumoniae* 7448 (B) and *M. hyorhinis* HUB-1 (C).

Additional file 10: A: List of predicted surface proteins of *M. flocculare* (A), *M. hyopneumoniae* 7448 (B), and *M. hyorhinis* HUB-1 (C). B: List of predicted surface proteins of *M. flocculare* (A), *M. hyopneumoniae* 7448 (B), and *M. hyorhinis* HUB-1 (C). List of predicted surface proteins of M. flocculare (A), *M. hyopneumoniae* 7448 (B), and *M. hyorhinis* HUB-1 (C).

Additional file 11: A. List of predicted surface proteins of *M. flocculare* not shared with *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB_1. B. List of predicted surface proteins of *M. hyopneumoniae* 7448 not shared with *M. flocculare* and *M. hyorhinis* HUB-1. C. List of predicted surface proteins of *M. hyorhinis* HUB-1 not shared with *M. flocculare* and *M. hyopneumoniae* 7448.

Additional file 12: List of ortholog surface protein coding CDSs between *M. flocculare*, *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1.

Additional file 13: Summary of adhesins associated to pathogenicity and Genome organization comparison.

Additional file 14: *M. flocculare*, *M. hyopneumoniae* and *M. hyorhynis* P97 and P97-like adhesin orthologs and paralogs.

Additional file 15: Evolutionary history of mycoplasmas obtained through a phylogenomic approach. The Neighbor-Joining method, using p-distance to compute the evolutionary distances and complete deletion of gaps was implemented by MEGA 5 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,500 replicates) are shown next to the branches. *S. pyogenes* was used as outgroup.

Additional file 16: Evolutionary history of mycoplasmas obtained through a phylogenomic approach. The Neighbor-Joining method, using Poisson correction to compute the evolutionary distances and complete deletion of gaps was implemented by MEGA 5 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,500 replicates) are shown next to the branches. *S. pyogenes* was used as outgroup.

Additional file 17: Evolutionary history of mycoplasmas obtained through a phylogenomic approach. The Neighbor-Joining method was the same description of the Additional file 16.

Additional file 18: Evolutionary history of mycoplasmas obtained through a phylogenomic approach. The Maximum Parsimony method using the close-neighbor-interchange algorithm, and the complete

deletion of gaps was implemented by MEGA 5 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. *S. pyogenes* was used as outgroup.

Additional file 19: Evolutionary history of DNA methylases from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method, using p-distance to compute the evolutionary distances and complete deletion of gaps was implemented by MEGA 5 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches.

Additional file 20: Evolutionary history of ATP synthases from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 21: Evolutionary history of ribulose-phosphate -3epimerases from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 22: Evolutionary history of oligoendopeptidases from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 16. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches.

Additional file 23: Evolutionary history of single-strand binding proteins from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 24: Evolutionary history of fructose-bisphosphate aldolase proteins from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 25: Evolutionary history of dihydrolipoamide dehydrogenase proteins from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 26: Evolutionary history of glucose-6-phosphate-isomerase proteins from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 27: Evolutionary history of lypoate protein ligases from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 28: Evolutionary history of acyl carrier phosphodiesterases from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 29: Evolutionary history of lactate dehydrogenases from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 30: Evolutionary history of membrane nucleases from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 31: Evolutionary history of TRSE-like proteins from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 32: Evolutionary history of P97 proteins from mycoplasmas obtained through a phylogenetic analysis. The Neighbor-Joining method was the same description of the Additional file 19.

Additional file 33: HGT events in mycoplasmas species.

Additional file 34: Bacterial strains used in the phylogenomic analyses.

Abbreviations

CDS: Coding DNA sequence; COG: Clusters of orthologous groups; HGT: Horizontal gene transfer; ORF: Open read frames; OC: ORF cluster.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

FMS performed the genomes organization analyses and comparative analyses of genomes, participated in the interpretation of the results and in the writing of the manuscript. CET, FP and MMBF carried out the phylogenomics and phylogenetics analyses and in the interpretation of the results. LGPA and RS carried out the assemblies genomes and participated in the comparative analyses of genomes. VGV, LR and TG participated in the surface proteins *in silico* analyses. ISS, HBF, ATV and AZ conceived, designed and coordinated the study, participated in the interpretation of the results and in the writing of the manuscript. All authors read and approved the final manuscript.

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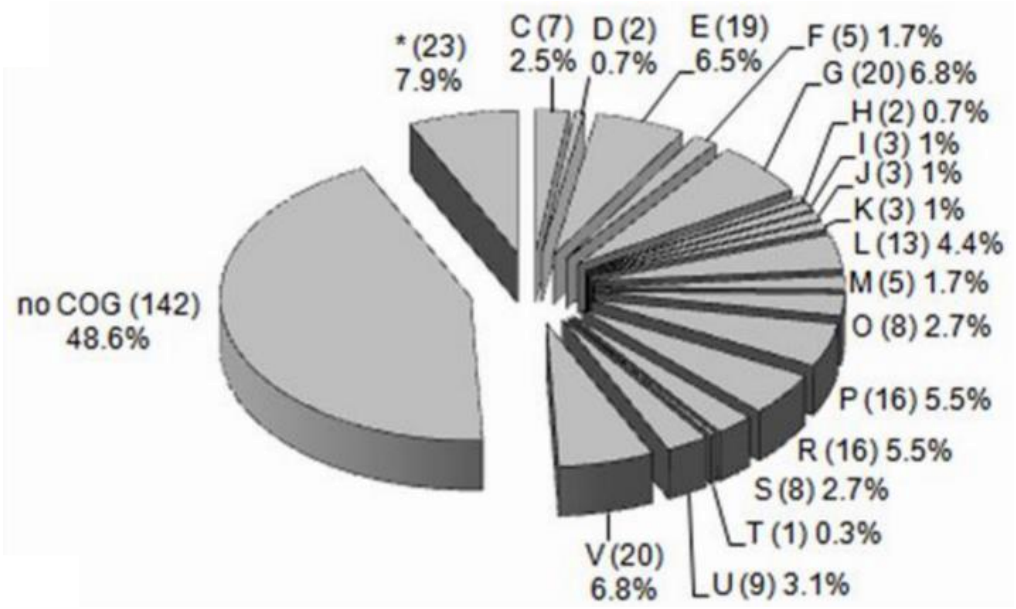
8.2.1 Material suplementar: New insights on the biology of swine respiratory tract mycoplasmas from a comparative genome analysis.

- **Additional file 9B:** COG functional classifications of the predicted surface protein sets from *M. hyopneumoniae* 7448 (B).

- **Additional file 10B:** List of predicted surface proteins of *M. hyopneumoniae* 7448 (B).

- **Additional file 11B:** List of predicted surface proteins of *M. hyopneumoniae* 7448 not shared with *M. flocculare* and *M. hyorhinis* HUB-1.

Additional file 12: List of ortholog surface protein coding CDSs between *M. flocculare, M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1.



Additional file 9B. COG functional classifications of the predicted surface protein sets from *M. hyopneumoniae* 7448. Percentages and the number (in parentheses) of proteins predicted in each functional category are indicated in the sectors of the circle. Functional categories are: (C) Energy production and conversion; (D) Cell cycle control, mitosis and meiosis; (E) Amino acid transport and metabolism; (F) Nucleotide transport and metabolism; (G) Carbohydrate transport and metabolism; (H) Coenzyme transport and metabolism; (I) Lipid transport and metabolism; (J) Translation; (K) Transcription; (L) Replication, recombination and repair; (M) Cell wall/membrane biogenesis; (O) Posttranslational modification, protein turnover, chaperones; (P) Inorganic ion transport and metabolism; (R) General function prediction only; (S) Function unknown; (T) Signal transduction mechanisms; (U) Intracellular trafficking and secretion; (V) Defense mechanisms; (no COG) Protein not related to any COG category; (*) Antigen, adhesion or lipoprotein (proteins arbitrarily classified based on their prior immunological or functional characterization according to published literature). The number of proteins in the graphic exceeds the total of predicted proteins because some of them were grouped in more than one functional category.

Additional file 10B: List of predicted surface proteins of *M. hyopneumoniae* 7448.
Inclusion criteria are described in the Methods section.

ORF ID	ORF product	COG[a]	SVMtm	TMHMM 2.0	SCAMPI	PSORTb 3.0	HMM-TM	Additional criteria[b]
MHP7448_0631	1-acyl-sn-glycerol-3-phosphate acyltransferase	I	No	No	Yes	No	Yes	PubMed link
MHP7448_0513	46K surface antigen precursor	G*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0475	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	F	No	Yes	No	No	Yes	PubMed link
MHP7448_0630	5'-nucleotidase precursor	F	No	No	No	No	n.t.	MF_00947, MHR_0049
MHP7448_0379	ABC transport ATP-binding protein	G	No	No	No	Yes	Yes	PubMed link
MHP7448_0370	ABC transport permease protein	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0362	ABC transport system permease protein p69 - like	P*	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0623	ABC transporter ATP-binding - Pr1	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0664	ABC transporter ATP-binding - Pr1	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0627	ABC transporter ATP-binding - Pr1-like	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0628	ABC transporter ATP-binding - Pr2 - like	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0665	ABC transporter ATP-binding - Pr2 - like	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0160	ABC transporter ATP-binding and permease protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0019	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No

MHP7448_0020	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0021	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0023	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0024	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0305	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0306	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0314	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0315	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0340	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0361	ABC transporter ATP-binding protein	P	No	No	No	Yes	Yes	PubMed link
MHP7448_0383	ABC transporter ATP-binding protein	V	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0452	ABC transporter ATP-binding protein	V	No	No	No	Yes	No	PubMed link
MHP7448_0469	ABC transporter ATP-binding protein	V	No	No	No	Yes	Yes	PubMed link
MHP7448_0624	ABC transporter ATP-binding protein - Pr2	CO	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0265	ABC transporter permease protein	P	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0380	ABC transporter permease protein	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0381	ABC transporter permease protein	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0371	ABC transporter permease protein	G	Yes	Yes	Yes	Yes	n.t.	No

MHP7448_0369	ABC transporter permease protein	G	No	No	No	Yes	Yes	PubMed link
MHP7448_0606	ABC transporter protein	R	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0604	ABC transporter xylose-binding lipoprotein	R	No	No	Yes	No	Yes	PubMed link
MHP7448_0663	adhesin like-protein P146	no COG*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0081	amino acid permease	E	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0113	amino acid permease	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0357	amino acid permease	E	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0049	ATP synthase subunit B	C	Yes	Yes	Yes	No	n.t.	No
MHP7448_0241	bifunctional preprotein translocase subunit SecD/SecF	U	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0038	cardiolipin synthetase	I	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0268	cation-transporting P-type ATPase	P	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0281	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	I	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0206	cell division protein	O	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0008	cell division protein ftsY	U	No	No	No	Yes	No	PubMed link
MHP7448_0393	cell division protein ftsZ	D	Yes	No	Yes	Yes	n.t.	No
MHP7448_0145	chromate transport protein	P	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0146	chromate transport protein	P	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0263	cobalt transporter ATP-binding subunit	P	No	No	No	Yes	No	PubMed link

MHP7448_0264	cobalt transporter ATP-binding subunit	P	No	No	No	Yes	No	PubMed link
MHP7448_0280	CTP synthetase	F	No	No	No	No	n.t.	MF_00072
MHP7448_0614	cytosine specific DNA methyltransferase	L	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0506	dihydrolipoamide acetyltransferase	C	Yes	No	Yes	No	n.t.	No
MHP7448_0047	F0F1 ATP synthase subunit A	C	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0053	F0F1 ATP synthase subunit beta	C	No	No	No	Yes	No	PubMed link
MHP7448_0048	F0F1 ATP synthase subunit C	C	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0655	glucose-inhibited division protein B	M	No	No	No	No	n.t.	MF_00878
MHP7448_0358	glycerol uptake facilitator protein	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0588	glycerol-3-phosphate dehydrogenase	R	No	No	No	Yes	Yes	PubMed link
MHP7448_0312	glycine cleavage system H protein	E	No	No	No	No	n.t.	MHR_0282
MHP7448_0070	GTP-binding protein EngA	R	No	No	No	No	n.t.	MF_01175
MHP7448_0156	GTP-binding protein Era	R	No	No	No	Yes	No	PubMed link
MHP7448_0073	GTP-binding protein LepA	M	No	No	No	Yes	Yes	PubMed link
MHP7448_0643	hemolysin C	R	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0136	hexosephosphate transport protein	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0405	holliday junction DNA helicase motor protein	L	No	No	No	No	n.t.	MF_00090
MHP7448_0017	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No

MHP7448_0025	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0030	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0036	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00832
MHP7448_0042	hypothetical protein	no COG	No	No	No	Yes	Yes	No
MHP7448_0044	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0046	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0064	hypothetical protein	L	No	No	No	Yes	Yes	No
MHP7448_0065	hypothetical protein	no COG	No	Yes	Yes	No	n.t.	No
MHP7448_0072	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0074	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0078	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0079	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0080	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0085	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0089	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0090	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0094	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00500
MHP7448_0100	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00488

MHP7448_0112	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0117	hypothetical protein	no COG	Yes	Yes	No	No	n.t.	No
MHP7448_0118	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0120	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0121	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0128	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00122
MHP7448_0135	hypothetical protein	S	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0138	hypothetical protein	no COG	No	Yes	Yes	No	n.t.	No
MHP7448_0148	hypothetical protein	O	No	No	No	Yes	Yes	No
MHP7448_0150	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0159	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0162	hypothetical protein	K	No	No	Yes	Yes	Yes	No
MHP7448_0197	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0200	hypothetical protein	S	No	No	No	No	n.t.	MF_00249
MHP7448_0205	hypothetical protein	D	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0211	hypothetical protein	no COG	No	Yes	Yes	No	n.t.	No
MHP7448_0216	hypothetical protein	no COG	Yes	No	Yes	Yes	n.t.	No
MHP7448_0219	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No

MHP7448_0220	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0237	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0240	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0246	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0248	hypothetical protein	R	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0278	hypothetical protein	H	No	No	No	No	n.t.	MF_00603
MHP7448_0282	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0290	hypothetical protein	no COG	No	No	Yes	No	Yes	No
MHP7448_0297	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0298	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00568
MHP7448_0299	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0300	hypothetical protein	no COG	No	No	No	Yes	Yes	No
MHP7448_0308	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00455
MHP7448_0313	hypothetical protein	no COG	Yes	No	Yes	Yes	n.t.	No
MHP7448_0317	hypothetical protein	no COG	No	No	No	No	n.t.	MF_01108
MHP7448_0318	hypothetical protein	no COG	No	No	No	Yes	Yes	No
MHP7448_0319	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0320	hypothetical protein	no COG	No	Yes	Yes	Yes	n.t.	No

MHP7448_0325	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0328	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0329	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0330	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0334	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0335	hypothetical protein	S	Yes	Yes	Yes	No	n.t.	No
MHP7448_0336	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0337	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0338	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0341	hypothetical protein	no COG	Yes	Yes	No	No	n.t.	No
MHP7448_0342	hypothetical protein	no COG	No	Yes	Yes	No	n.t.	No
MHP7448_0344	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0345	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0346	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0347	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0348	hypothetical protein	K	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0350	hypothetical protein	L	No	No	No	No	n.t.	MF_01243
MHP7448_0352	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No

MHP7448_0355	hypothetical protein	no COG	No	No	Yes	No	Yes	No
MHP7448_0363	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0364	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0373	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0377	hypothetical protein	R	No	No	No	Yes	Yes	No
MHP7448_0390	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0397	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0399	hypothetical protein	no COG	No	No	Yes	Yes	Yes	No
MHP7448_0400	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00720, MHR_0298
MHP7448_0402	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0404	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0407	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00082
MHP7448_0417	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0418	hypothetical protein	no COG	No	No	Yes	No	Yes	No
MHP7448_0429	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0432	hypothetical protein	no COG	No	Yes	No	No	Yes	No
MHP7448_0433	hypothetical protein	no COG	No	Yes	No	No	Yes	No
MHP7448_0435	hypothetical protein	R	No	No	No	No	n.t.	MF_00714

MHP7448_0441	hypothetical protein	no COG	No	Yes	No	Yes	Yes	No
MHP7448_0443	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0444	hypothetical protein	no COG	No	No	No	Yes	Yes	No
MHP7448_0446	hypothetical protein	no COG	No	Yes	No	Yes	Yes	No
MHP7448_0448	hypothetical protein	no COG	No	Yes	No	Yes	Yes	No
MHP7448_0453	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0466	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0467	hypothetical protein	no COG	No	Yes	Yes	No	n.t.	No
MHP7448_0468	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0470	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0480	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0484	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0486	hypothetical protein	no COG	No	Yes	Yes	Yes	n.t.	No
MHP7448_0488	hypothetical protein	S	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0489	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0493	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0498	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0510	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No

MHP7448_0511	hypothetical protein	no COG	No	Yes	No	Yes	Yes	No
MHP7448_0512	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0518	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0519	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0522	hypothetical protein	E	No	No	No	No	n.t.	MF_00018, MHR_0293
MHP7448_0525	hypothetical protein	no COG	No	Yes	Yes	Yes	n.t.	No
MHP7448_0530	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0536	hypothetical protein	no COG	No	Yes	Yes	No	n.t.	No
MHP7448_0537	hypothetical protein	O	No	No	No	Yes	Yes	No
MHP7448_0538	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00551, MHR_0059
MHP7448_0539	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00552, MHR_0465
MHP7448_0543	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0544	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0547	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00019
MHP7448_0555	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0556	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00306
MHP7448_0569	hypothetical protein	no COG	No	No	No	Yes	Yes	No
MHP7448_0570	hypothetical protein	O	Yes	Yes	Yes	No	n.t.	No

MHP7448_0594	hypothetical protein	S	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0596	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0599	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0602	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0610	hypothetical protein	G	No	No	No	No	n.t.	MF_00374
MHP7448_0611	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00358
MHP7448_0612	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0620	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0629	hypothetical protein	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0634	hypothetical protein	S	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0642	hypothetical protein	R	No	No	No	Yes	Yes	No
MHP7448_0646	hypothetical protein	T	No	Yes	Yes	No	n.t.	No
MHP7448_0649	hypothetical protein	J	No	No	No	Yes	Yes	No
MHP7448_0661	hypothetical protein	no COG	No	Yes	Yes	Yes	n.t.	No
MHP7448_0662	hypothetical protein	no COG	No	No	No	No	n.t.	MF_01055
MHP7448_0666	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0667	hypothetical protein	no COG	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0669	hypothetical protein	V	No	Yes	Yes	Yes	n.t.	No

MHP7448_0670	hypothetical protein	no COG	No	Yes	Yes	Yes	n.t.	No
MHP7448_0674	hypothetical protein	no COG	No	No	No	No	n.t.	MF_00996
MHP7448_0110	ISMHp1 transposase	L	Yes	Yes	Yes	No	n.t.	No
MHP7448_0218	ISMHp1 transposase	L	Yes	Yes	No	No	n.t.	No
MHP7448_0495	ISMHp1 transposase	L	Yes	Yes	Yes	No	n.t.	No
MHP7448_0636	ISMHp1 transposase	L	Yes	Yes	Yes	No	n.t.	No
MHP7448_0673	ISMHp1 transposase	L	Yes	Yes	Yes	No	n.t.	No
MHP7448_0217	lipoprotein	E*	No	No	No	No	n.t.	MF_00291
MHP7448_0366	lipoprotein	no COG*	Yes	No	Yes	No	n.t.	No
MHP7448_0367	lipoprotein	no COG*	No	No	Yes	No	Yes	PubMed link
MHP7448_0505	lipoprotein	no COG*	No	No	No	Yes	Yes	PubMed link
MHP7448_0031	lipoprotein signal peptidase	MU*	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0372	Lppt protein	no COG*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0580	membrane nuclease, lipoprotein	R*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0396	methionyl-tRNA synthetase	J	Yes	Yes	Yes	No	n.t.	No
MHP7448_0229	myo-inositol catabolism protein	E	No	No	No	Yes	Yes	PubMed link
MHP7448_0309	NADH-dependent flavin oxidoreductase	C	No	No	No	No	n.t.	MHR_0279
MHP7448_0473	NH(3)-dependent NAD+ synthetase	H	No	No	No	No	n.t.	MF_00434

MHP7448_0521	oligoendopeptidase F	E	No	No	No	No	n.t.	MF_00021, MHR_0536
MHP7448_0214	oligopeptide ABC transporter ATP-binding protein	EP	No	No	No	Yes	Yes	PubMed link
MHP7448_0215	oligopeptide ABC transporter ATP-binding protein	E	No	No	No	Yes	Yes	PubMed link
MHP7448_0501	oligopeptide ABC transporter ATP-binding protein	E	No	No	No	Yes	Yes	PubMed link
MHP7448_0502	oligopeptide ABC transporter ATP-binding protein	EP	No	No	No	Yes	Yes	PubMed link
MHP7448_0504	oligopeptide ABC transporter permease protein	EP	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0212	oligopeptide ABC transporter system permease	EP	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0213	oligopeptide transport system permease protein	EP	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0503	oligopeptide transport system permease protein	EP	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0635	O-sialoglycoprotein endopeptidase	O	No	No	No	Yes	Yes	PubMed link
MHP7448_0099	outer membrane protein - P95	no COG*	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0360	P37-like ABC transporter substrate-binding lipoprotein	no COG*	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0353	P60-like lipoprotein	no COG*	No	No	Yes	No	Yes	PubMed link
MHP7448_0497	P76 membrane protein precursor	no COG*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0234	periplasmic sugar-binding proteins	G	Yes	Yes	Yes	No	n.t.	No
MHP7448_0302	permease	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0533	phosphatidate cytidylyltransferase synthase	R	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0546	potassium uptake protein	P	Yes	Yes	Yes	Yes	n.t.	No

MHP7448_0175	preprotein translocase subunit SecY	U	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0097	prolipoprotein diacylglyceryl transferase	M	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0656	prolipoprotein p65	no COG*	No	No	Yes	Yes	No	PubMed link
MHP7448_0107	protein P102	no COG*	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0199	protein P102	no COG*	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0271	protein P102-like	no COG*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0108	protein P97	no COG*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0198	protein P97	no COG*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0272	protein P97-like	no COG*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0376	PTS system ascorbate-specific transport subunit IIC	S	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0552	PTS system ascorbate-specific transport subunit IIC	S	No	Yes	Yes	Yes	n.t.	No
MHP7448_0492	PTS system fructose-specific transporter subunit IIABC	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0591	PTS system glucose-specific enzyme IIB component	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0550	PTS system mannitol-specific transporter subunit IIBC	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0574	PTS system N-acetylglucosamine-specific II ABC component	G	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0040	PTS system, lichenan-specific IIA component	M	No	No	Yes	No	Yes	PubMed link
MHP7448_0414	putative ICEF-II	no COG	Yes	Yes	Yes	No	n.t.	No
MHP7448_0423	putative ICEF-IIA	U	No	Yes	Yes	Yes	n.t.	No

MHP7448_0676	putative inner membrane protein translocase component YidC	U	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0368	putative lipoprotein	L*	No	No	Yes	No	Yes	PubMed link
MHP7448_0487	putative MG2+ transport protein	P	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0496	putative P216 surface protein	no COG*	Yes	Yes	Yes	No	n.t.	No
MHP7448_0233	putative ribose ABC transporter	G	No	Yes	Yes	Yes	n.t.	No
MHP7448_0605	putative sugar ABC transporter ATP-binding protein	R	No	No	No	Yes	Yes	PubMed link
MHP7448_0677	ribonuclease P protein component	J	No	Yes	No	Yes	Yes	No
MHP7448_0221	ribonucleotide-diphosphate reductase subunit beta	F	No	Yes	Yes	No	n.t.	No
MHP7448_0231	ribose ABC transport ATP-binding protein	G	No	No	No	Yes	No	PubMed link
MHP7448_0654	ribose-phosphate pyrophosphokinase	FE	No	No	No	No	n.t.	MF_00882
MHP7448_0026	signal peptidase I	OU	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0057	signal recognition particle protein	U	No	No	No	Yes	No	PubMed link
MHP7448_0542	spermidine/putrescine ABC transporter ATP-binding	E	No	No	No	Yes	Yes	PubMed link
MHP7448_0540	spermidine/putrescine ABC transporter permease protein	E	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0541	spermidine/putrescine ABC transporter permease protein	E	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0332	subtilisin-like serine protease	O	Yes	Yes	Yes	No	n.t.	No
MHP7448_0607	sugar ABC transporter permease protein	R	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0637	transcription antitermination protein NusG	K	No	No	No	No	n.t.	MF_00929

MHP7448_0236	transposase	L	Yes	Yes	Yes	No	n.t.	No
MHP7448_0323	transposase	L	Yes	Yes	Yes	No	n.t.	No
MHP7448_0253	triacylglycerol lipase	R	No	No	No	Yes	No	PubMed link
MHP7448_0247	TRSE-like protein	R	Yes	Yes	Yes	Yes	n.t.	No
MHP7448_0416	TRSE-like protein	U	No	Yes	Yes	Yes	n.t.	No
MHP7448_0130	uracil-DNA glycosylase	L	No	No	No	No	n.t.	MF_00126
MHP7448_0514	xylose ABC transporter ATP-binding protein	G	No	No	No	Yes	Yes	PubMed link
MHP7448_0515	xylose ABC transporter permease protein	G	Yes	Yes	Yes	Yes	n.t.	No

n.t.: not tested.

[a] COG functional classes: (C) Energy production and conversion; (D) Cell cycle control, mitosis and meiosis; (E) Amino acid transport and metabolism; (F) Nucleotide transport and metabolism; (G) Carbohydrate transport and metabolism; (H) Coenzyme transport and metabolism; (I) Lipid transport and metabolism; (J) Translation; (K) Transcription; (L) Replication, recombination and repair; (M) Cell wall/membrane biogenesis; (O) Posttranslational modification, protein turnover, chaperones; (P) Inorganic ion transport and metabolism; (R) General function prediction only; (S) Function unknown; (T) Signal transduction mechanisms; (U) Intracellular trafficking and secretion; (V) Defense mechanisms; (no COG) Protein not related to any COG category; (*) Antigen, adhesin or lipoprotein (proteins arbitrarily classified based on their prior immunological or functional characterization according to published literature).

[b] Published literature (PubMed link) or predicted surface protein ortholog in one or in the two other analyzed species (CDS id.).

Additional file 11B. List of predicted surface proteins of *M. hyopneumoniae* 7448 not shared with *M. flocculare* and *M. hyorhinis* HUB-1.

ORF	GI	YP	ORF product	COG[a]
MHP7448_0017	72080359	287417.1	Hypothetical protein	no COG
MHP7448_0085	72080427	287485.1	Hypothetical protein	no COG
MHP7448_0117	72080457	287515.1	Hypothetical protein	no COG
MHP7448_0118	72080458	287516.1	Hypothetical protein	no COG
MHP7448_0120	72080460	287518.1	Hypothetical protein	no COG
MHP7448_0121	72080461	287519.1	Hypothetical protein	no COG
MHP7448_0150	72080489	287547.1	Hypothetical protein	no COG
MHP7448_0219	72080558	287616.1	Hypothetical protein	no COG
MHP7448_0220	72080559	287617.1	Hypothetical protein	no COG
MHP7448_0290	72080629	287687.1	Hypothetical protein	no COG
MHP7448_0320	72080657	287715.1	Hypothetical protein	no COG
MHP7448_0328	72080665	287723.1	Hypothetical protein	no COG
MHP7448_0329	72080666	287724.1	Hypothetical protein	no COG
MHP7448_0330	72080667	287725.1	Hypothetical protein	no COG
MHP7448_0334	72080671	287729.1	Hypothetical protein	no COG
MHP7448_0337	72080674	287732.1	Hypothetical protein	no COG
MHP7448_0338	72080675	287733.1	Hypothetical protein	no COG
MHP7448_0344	72080680	287738.1	Hypothetical protein	no COG
MHP7448_0345	72080681	287739.1	Hypothetical protein	no COG
MHP7448_0347	72080683	287741.1	Hypothetical protein	no COG
MHP7448_0418	72080751	287809.1	Hypothetical protein	no COG
MHP7448_0432	72080765	287823.1	Hypothetical protein	no COG
MHP7448_0441	72080774	287832.1	Hypothetical protein	no COG
MHP7448_0446	72080778	287836.1	Hypothetical protein	no COG
MHP7448_0448	72080780	287838.1	Hypothetical protein	no COG
MHP7448_0493	72080824	287882.1	Hypothetical protein	no COG
MHP7448_0543	72080874	287932.1	Hypothetical protein	no COG
MHP7448_0569	72080897	287955.1	Hypothetical protein	no COG
MHP7448_0596	72080924	287982.1	Hypothetical protein	no COG
MHP7448_0599	72080927	287985.1	Hypothetical protein	no COG

MHP7448_0666	72080993	288051.1	Hypothetical protein	no COG
MHP7448_0110	72080450	287508.1	Ismhp1 transposase	L
MHP7448_0218	72080557	287615.1	Ismhp1 transposase	L
MHP7448_0495	72080826	287884.1	Ismhp1 transposase	L
MHP7448_0636	72080963	288021.1	Ismhp1 transposase	L
MHP7448_0673	72081000	288058.1	Ismhp1 transposase	L
MHP7448_0229	72080568	287626.1	Myo-inositol catabolism protein	E
MHP7448_0234	72080573	287631.1	Periplasmic sugar-binding proteins	G
MHP7448_0302	72080640	287698.1	Permease	G
MHP7448_0423	72080756	287814.1	Putative ICEF-IIA	U
MHP7448_0236	72080575	287633.1	Transposase	L
MHP7448_0323	72080660	287718.1	Transposase	L

[a]COG functional classes: (E) Amino acid transport and metabolism; (G) Carbohydrate transport and metabolism; (L) Replication, recombination and repair; (U) Intracellular trafficking and secretion; (no COG) Protein not related to any COG category.

Additional file 12. List of ortholog surface protein coding ORFs between *M. flocculare*, *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1.

Ortholog and paralog definition criteria are described in the Material and Methods section.

ORF product[a,d]	ORFs			Identity / Similarity (%)		
	M. flocculare	*M. hyopneumoniae* 7448	*M. hyorhinis* HUB-1	Mf X Mhp	Mf X Mhr	Mhr X Mhp
conserved hypothetical protein	MF_00009	MHP7448_0397	MHR_0367	77 / 85	48 / 67	46 / 67
methionyl-tRNA synthetase	MF_00011	MHP7448_0396	MHR_0334	75 / 88	49 / 70	49 / 68
conserved hypothetical protein	MF_01158	MHP7448_0065	n.f.	60 / 74	–	–
		n.f.	MHR_0293	–	30 / 56	–
prolipoprotein P65	MF_00871	MHP7448_0656		52 / 69	30 / 52	31 / 50
conserved hypothetical protein	MF_00018	MHP7448_0522		61 / 78	23 / 45	23 / 46
101 kDa protein[c]	MF_00019	MHP7448_0547	n.f.	71 / 83	–	–
		n.f.	MHR_0249	–	21 / 41	–
101 kDa protein[c]	MF_00579	n.f.		–	22 / 41	–
		MHP7448_0547	n.f.	68 / 83	–	–
		n.f.	MHR_0043	–	22 / 42	–
		n.f.	MHR_0225	–	22 / 39	–
oligoendopeptidase F	MF_00021	MHP7448_0521	MHR_0356	82 / 93	52 / 73	52 / 72
		n.f.	MHR_0363	–	52 / 72	–
hypothetical protein	MF_00023	MHP7448_0512	MHR_0617	52 / 70	52 / 74	46 / 66
		MHP7448_0240	n.f.	45 / 64	–	–
		MHP7448_0519	n.f.	39 / 59	–	–
conserved hypothetical protein	MF_00025	MHP7448_0511	MHR_0616	51 / 76	46 / 64	58 / 73
hypothetical protein	n.f.	MHP7448_0237	MHR_0167	–	–	23 / 44
conserved hypothetical protein	MF_00026		MHR_0614	52 / 70	45 / 64	41 / 63

		MHP7448_0510	n.f.	39 / 56	–	–
		MHP7448_0518	n.f.	37 / 58	–	–
hypothetical protein	n.f.		MHR_0619	–	–	28 / 48
xylose ABC transporter permease protein	MF_00033	MHP7448_0515	MHR_0164	94 / 97	80 / 90	82 / 91
xylose ABC transporter ATP-binding protein	MF_00035	MHP7448_0514	MHR_0163	93 / 96	87 / 94	86 / 95
		MHP7448_0231	n.f.	36 / 59	–	–
46K surface antigen precursor	MF_00037	MHP7448_0513	MHR_0162	89 / 95	65 / 76	65 / 76
dihydrolipoamide acetyltransferase	MF_00043		MHR_0515	86 / 92	62 / 75	61 / 75
lipoprotein	MF_00046	MHP7448_0505	MHR_0639	72 / 85	37 / 55	38 / 56
oligopeptide ABC transporter permease protein	MF_00047	MHP7448_0504	MHR_0638	80 / 90	59 / 76	58 / 77
oligopeptide transport system permease protein	MF_00051	MHP7448_0503	MHR_0637	79 / 92	61 / 76	57 / 75
oligopeptide ABC transporter ATP binding protein	MF_00061	MHP7448_0502	MHR_0636	82 / 90	72 / 87	73 / 88
oligopeptide ABC transporter ATP-binding protein	MF_00063	MHP7448_0501	MHR_0635	73 / 84	45 / 66	43 / 65
hypothetical protein	MF_00069	MHP7448_0300	n.f.	98 / 99	–	–
putative ICEF-II[b]	MF_00071	MHP7448_0414	n.f.	39 / 55	–	–
		n.f.	MHR_0483	–	26 / 46	–
putative ICEF-II[c]	MF_00683	MHP7448_0246		66 / 82	29 / 46	27 / 46
		MHP7448_0414	n.f.	27 / 45	–	–
CTP synthase	MF_00072	MHP7448_0280	n.f.	88 / 95	–	–
		n.f.	MHR_0273	–	60 / 77	–
CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	MF_00073	MHP7448_0281	n.f.	66 / 80	–	–
hypothetical protein	MF_00075	MHP7448_0282	MHR_0286	71 / 84	31 / 48	28 / 51
conserved hypothetical protein	MF_00082	MHP7448_0407	n.f.	70 / 83	–	–
		n.f.	MHR_0260	–	39 / 56	–
holliday junction DNA helicase RuvA	MF_00090	MHP7448_0405	n.f.	81 / 92	–	–

		n.f.	MHR_0264	–	57 / 75	–
hypothetical protein	MF_00092	MHP7448_0404	n.f.	65 / 80	–	–
hypothetical protein	MF_00095	MHP7448_0402	MHR_0268	68 / 84	28 / 46	27 / 46
ribonucleoside-diphosphate reductase beta chain	MF_00115	MHP7448_0223	MHR_0662	95 / 98	84 / 92	83 / 93
hypothetical protein	MF_00122	MHP7448_0128	n.f.	76 / 87	–	–
uracil-dna glycosylase	MF_00126	MHP7448_0130	n.f.	74 / 89	–	–
		n.f.	MHR_0207	–	60 / 78	–
lipase-esterase	MF_00131	n.f.	MHR_0284	–	42 / 63	
ABC transporter, permease protein[b]	MF_00132	MHP7448_0135	MHR_0643	88 / 93	41 / 62	43 / 63
hexosephosphate transport protein	MF_00133	MHP7448_0136	MHR_0432	87 / 94	51 / 67	53 / 67
conserved hypothetical protein	MF_00138	MHP7448_0138	MHR_0285	74 / 87	29 / 47	31 / 48
chromate transport protein	MF_00162	MHP7448_0145	MHR_0326	78 / 90	46 / 68	44 / 65
chromate transport protein	MF_00164	MHP7448_0146	MHR_0327	75 / 88	40 / 62	42 / 63
hypothetical 33 kDa chaperonin[c]	MF_00170	MHP7448_0148	MHR_0610	74 / 88	41 / 62	41 / 62
GTP-binding protein	MF_00188	MHP7448_0156	MHR_0313	82 / 94	49 / 69	48 / 69
ABC transporter ATP-binding and permease protein	MF_00190	MHP7448_0160	MHR_0310	92 / 96	45 / 70	46 / 70
transcription termination factor[c]	MF_00194	MHP7448_0162	MHR_0308	84 / 92	45 / 68	46 / 68
preprotein translocase SecY subunit	MF_00215	MHP7448_0175	MHR_0383	87 / 94	65 / 80	64 / 80
26.3 kDa protein in cilium adhesin operon	MF_00249	MHP7448_0200	n.f.	74 / 87	–	–
		n.f.	MHR_0291	–	52 / 74	–
tRNA(Ile)-lysidine synthase[c]	MF_00253	MHP7448_0205	MHR_0533	76 / 90	44 / 68	42 / 68
cell division protein	MF_00257	MHP7448_0206	MHR_0532	88 / 94	63 / 76	61 / 75
PTS system, fructose-specific IIABC component	MF_00267	MHP7448_0492	MHR_0231	30 / 50	55 / 73	30 / 49
conserved hypothetical protein	MF_00272	MHP7448_0211	n.f.	58 / 72	–	–
oligopeptide ABC transporter system permease	MF_00274	MHP7448_0212	MHR_0362	89 / 95	71 / 80	69 / 80

oligopeptide transport system permease protein	MF_00275	MHP7448_0213	MHR_0361	96 / 99	71 / 87	71 / 87
oligopeptide transport system permease protein	MF_00279	MHP7448_0214	n.f.	89 / 96	–	–
		n.f.	MHR_0360	–	54 / 67	–
oligopeptide transport system permease protein	MF_00281	MHP7448_0214		93 / 98	80 / 90	69 / 80
oligopeptide ABC transporter ATP-binding protein	MF_00285	MHP7448_0215	MHR_0359	84 / 93	54 / 72	56 / 73
conserved hypothetical protein	MF_00289	MHP7448_0216	MHR_0358	67 / 81	32 / 51	31 / 49
lipoprotein	MF_00291	MHP7448_0217	n.f.	79 / 91	–	–
		n.f.	MHR_0357	–	47 / 67	–
mannitol-1-phosphate 5-dehydrogenase	MF_00292	n.f.	MHR_0170	–	35 / 60	–
PTS system, mannitol-specific IIBC component	MF_00293	MHP7448_0550	n.f.	79 / 90	–	–
conserved hypothetical protein	MF_00297	n.f.	MHR_0613	–	41 / 66	–
PTS system enzyme IIC component	MF_00300	MHP7448_0552	MHR_0459	98 / 99	25 / 45	24 / 44
transport protein sgaT	MF_00747	MHP7448_0376		85 / 92	54 / 73	56 / 74
hypothetical protein	MF_00303	MHP7448_0555	n.f.	68 / 79	–	–
conserved hypothetical protein	MF_00306	MHP7448_0556	n.f.	70 / 88	–	–
ABC transporter permease protein	MF_00324	MHP7448_0453	MHR_0318	70 / 83	35 / 56	35 / 55
conserved hypothetical protein	MF_00350	MHP7448_0620	MHR_0660	74 / 85	65 / 78	63 / 75
hypothetical protein	MF_00357	MHP7448_0612		71 / 82	25 / 49	24 / 50
lipoprotein[c]	MF_00358	MHP7448_0611	n.f.	75 / 87	–	–
		n.f.	MHR_0659	–	27 / 44	–
	MF_00345	n.f.		–	69 / 82	–
		n.f.		–	69 / 82	–
PTS system, glucose-specific IIABC component	MF_00374	MHP7448_0610	n.f.	58 / 75	–	–
sugar ABC transporter permease protein	MF_00383	MHP7448_0607	MHR_0075	91 / 96	50 / 67	49 / 67
ABC transporter protein	MF_00385	MHP7448_0606	MHR_0074	75 / 88	35 / 58	36 / 58
sugar ABC transporter ATP-binding protein	MF_00386	MHP7448_0605	MHR_0073	85 / 95	57 / 76	58 / 76

ABC transporter xylose-binding lipoprotein	MF_00388	MHP7448_0604	MHR_0076	71 / 82	41 / 57	40 / 56
		n.f.	MHR_0487	–	25 / 42	–
conserved hypothetical protein	MF_00390	MHP7448_0602	MHR_0647	61 / 81	31 / 57	29 / 56
conserved hypothetical protein	MF_00396	MHP7448_0594	MHR_0600	91 / 97	76 / 86	77 / 88
membrane nuclease, lipoprotein	MF_00420	MHP7448_0580	MHR_0549	67 / 80	30 / 49	30 / 48
		n.f.	MHR_0206	–	31 / 52	–
PTS system, N-acetylglucosamine-specific II ABC component	MF_00429	MHP7448_0574	n.f.	94 / 96	–	–
NH(3)-dependent NAD+ synthetase	MF_00434	MHP7448_0473	n.f.	80 / 87	–	–
		n.f.	MHR_0509	–	61 / 75	–
5'-methylthioadenosine nucleosidase S-adenosyl homocysteine nucleosidase	MF_00437	MHP7448_0475	MHR_0295	67 / 82	39 / 54	38 / 56
ATP synthase beta chain	MF_00449	MHP7448_0053	MHR_0653	36 / 62	58 / 79	38 / 61
	MF_01135		MHR_0223	92 / 95	79 / 89	78 / 89
conserved hypothetical protein	MF_00452	MHP7448_0480	MHR_0655	71 / 83	21 / 42	24 / 43
hypothetical protein	MF_00455	MHP7448_0308	n.f.	25 / 41	–	–
		n.f.	MHR_0330	–	21 / 43	–
putative protein P97	MF_00472	MHP7448_0198	n.f.	48 / 62	–	–
		n.f.	MHR_0344	–	22 / 37	–
hypothetical protein	MF_00488	MHP7448_0100	n.f.	64 / 78	–	–
putative LppT protein	MF_00739	MHP7448_0372	n.f.	56 / 71	–	–
		n.f.	MHR_0440	–	23 / 41	–
putative adhesin like-protein P146	MF_01050	MHP7448_0663		42 / 60	21 / 39	25 / 47
putative P216 surface protein	MF_00848	MHP7448_0496		55 / 70	20 / 38	21 / 39
protein P97-like	MF_00620	MHP7448_0272		57 / 74	22 / 39	21 / 38
putative protein P97 (p97 paralog)	MF_00472	MHP7448_0108		53 / 68	21 / 40	23 / 43
outer membrane protein - P95	MF_00492	MHP7448_0099		58 / 73	21 / 39	24 / 40

	n.f.		MHR_0652	–	–	25 / 42
prolipoprotein diacylglyceryl transferase	MF_00497	MHP7448_0097	MHR_0442	82 / 90	64 / 78	60 / 75
conserved hypothetical protein	MF_00500	MHP7448_0094	n.f.	54 / 69	–	–
		n.f.	MHR_0546	–	29 / 53	–
excinuclease ABC subunit A	MF_00504	n.f.	MHR_0443	–	61 / 79	–
conserved hypothetical protein	MF_00526	MHP7448_0525	MHR_0562	75 / 84	36 / 58	37 / 58
phosphatidate cytidylyltransferase synthase	MF_00538	MHP7448_0533	MHR_0594	63 / 77	37 / 52	34 / 50
hypothetical protein	MF_00544	MHP7448_0536	MHR_0597	73 / 86	23 / 42	23 / 40
conserved hypothetical protein	MF_00551	MHP7448_0538	MHR_0059	69 / 82	34 / 54	33 / 50
conserved hypothetical protein	MF_00552	MHP7448_0539	MHR_0465	79 / 89	49 / 69	49 / 69
spermidine/putrescine ABC transporter permease protein	MF_00553	MHP7448_0540	MHR_0466	87 / 94	67 / 84	67 / 82
spermidine/putrescine ABC transporter permease protein	MF_00556	MHP7448_0541	MHR_0467	85 / 92	64 / 81	65 / 83
spermidine/putrescine ABC transporter permease protein	MF_00559	MHP7448_0542	MHR_0468	87 / 92	72 / 84	70 / 83
potassium uptake protein	MF_00566	MHP7448_0546	MHR_0650	85 / 93	45 / 63	45 / 63
conserved hypothetical protein	MF_00568	MHP7448_0298	n.f.	54 / 71	–	–
hypothetical protein	MF_01448	MHP7448_0297	n.f.	46 / 61	–	–
conserved hypothetical protein	MF_00577		n.f.	45 / 59	–	–
hypothetical protein	MF_00571		n.f.	44 / 58	–	–
conserved hypothetical protein	MF_00574	MHP7448_0299	n.f.	55 / 77	–	–
amino acid permeases	MF_00592	MHP7448_0113	MHR_0529	85 / 92	49 / 70	49 / 71
lipoprotein[c]	MF_01320	MHP7448_0468	MHR_0021	64 / 79	28 / 46	26 / 45
		n.f.	MHR_0026	–	23 / 43	–
lipoprotein, putative[b]	MF_00595	MHP7448_0112		60 / 75	22 / 41	26 / 42
hypothetical protein	n.f.		MHR_0250	–	–	26 / 44

transcriptional regulator	MF_00598	n.f.	MHR_0574	–	28 / 49	–
conserved hypothetical protein	MF_00603	MHP7448_0278	n.f.	66 / 82	–	–
		n.f.	MHR_0539	–	37 / 56	–
cation-transporting P-type ATPase	MF_00636	MHP7448_0268	MHR_0257	72 / 86	45 / 65	45 / 65
ABC transporter permease protein	MF_00640	MHP7448_0265	MHR_0110	88 / 94	63 / 80	62 / 80
ABC transporter ATP-binding protein	MF_00644	MHP7448_0264	MHR_0109	85 / 94	68 / 80	69 / 84
ABC transporter ATP-binding protein	MF_00645	MHP7448_0263	MHR_0108	87 / 94	68 / 82	69 / 83
ribosomal RNA small subunit methyltransferase I[c]	MF_00655	n.f.	MHR_0106	–	53 / 74	–
DNA polymerase III subunit delta	MF_00657	n.f.	MHR_0105	–	34 / 52	–
triacylglycerol lipase	MF_00668	MHP7448_0253	n.f.	62 / 80	–	–
hypothetical protein	MF_00676	MHP7448_0248	MHR_0481	61 / 74	21 / 45	21 / 45
		MHP7448_0417	n.f.	24 / 48	–	–
		n.f.	MHR_0551	–	23 / 39	–
TRSE-like protein	MF_00678	MHP7448_0247	MHR_0482	89 / 93	27 / 48	27 / 50
		MHP7448_0416	n.f.	44 / 67	–	–
protein-export membrane protein	MF_00687	MHP7448_0241	MHR_0143	85 / 93	60 / 77	61 / 78
hypothetical protein	MF_00704		MHR_0503	71 / 84	43 / 65	43 / 62
conserved hypothetical protein	MF_00707	n.f.	MHR_0354	–	45 / 60	–
conserved hypothetical protein	MF_00708	MHP7448_0433	n.f.	45 / 57	–	–
conserved hypothetical protein	MF_00714	MHP7448_0435	n.f.	69 / 83	–	–
		n.f.	MHR_0453	–	42 / 61	–
lipoprotein	MF_00724	MHP7448_0367	MHR_0062	64 / 80	35 / 54	35 / 55
		MHP7448_0366	n.f.	26 / 46	–	–
		n.f.	MHR_0061	–	31 / 52	–
putative lipoprotein	MF_00726	MHP7448_0368	MHR_0063	58 / 76	40 / 61	35 / 57
ABC transporter permease protein	MF_00727	MHP7448_0369	MHR_0064	79 / 89	47 / 61	47 / 61

		n.f.	MHR_0194	–	40 / 57	–
ABC transport permease protein	MF_00736	MHP7448_0370	MHR_0065	84 / 91	60 / 77	60 / 77
		n.f.	MHR_0196	–	26 / 47	–
ABC transporter permease protein	MF_00737	MHP7448_0371	MHR_0066	80 / 91	67 / 83	60 / 79
		n.f.	MHR_0195	–	27 / 46	–
conserved hypothetical protein	MF_00741	MHP7448_0373	n.f.	55 / 73	–	–
transport protein sgaT	MF_00747	MHP7448_0376	MHR_0459	85 / 92	54 / 73	56 / 74
hypothetical protein	MF_00750	MHP7448_0377	MHR_0460	90 / 96	81 / 91	79 / 91
lipoprotein	MF_00751	n.f.	MHR_0276	–	33 / 54	–
ABC transport ATP-binding protein	MF_00752	MHP7448_0379	MHR_0123	72 / 84	47 / 61	44 / 59
ABC transporter permease protein	MF_00753	MHP7448_0380	MHR_0122	77 / 88	45 / 64	45 / 62
ABC transporter permease protein	MF_00762	MHP7448_0381	MHR_0121	72 / 87	43 / 66	42 / 64
ABC transporter ATP-binding protein	MF_00767	MHP7448_0383	n.f.	77 / 88	–	–
hypothetical protein	MF_00789	MHP7448_0348	MHR_0314	70 / 82	25 / 48	25 / 49
conserved hypothetical protein	MF_00813	MHP7448_0319	n.f.	52 / 71	–	–
conserved hypothetical protein	MF_00815	MHP7448_0318	n.f.	47 / 68	–	–
lipoprotein signal peptidase	MF_00818	MHP7448_0031	MHR_0133	75 / 87	50 / 65	51 / 69
hypothetical protein	MF_00832	MHP7448_0036	n.f.	50 / 65	–	–
		n.f.	MHR_0298	–	24 / 43	–
hypothetical protein	MF_00720	MHP7448_0400		75 / 88	29 / 46	31 / 48
hypothetical protein	MF_00834	MHP7448_0444	n.f.	28 / 47	–	–
P76 membrane protein precursor	MF_00844	MHP7448_0497	n.f.	38 / 55	–	–
P3^{c}	MF_00856	MHP7448_0489	MHR_0435	52 / 64	21 / 38	24 / 41
conserved hypothetical protein	MF_00857	MHP7448_0488	MHR_0434	84 / 92	46 / 73	46 / 68
putative MG2+ transport protein	MF_00861	MHP7448_0487	MHR_0433	82 / 92	60 / 78	60 / 79
hypothetical protein	MF_00863	MHP7448_0486	n.f.	68 / 80	–	–

hypothetical protein	MF_00866	MHP7448_0484	MHR_0658	66 / 77	21 / 44	22 / 41
glucose-inhibited division protein B	MF_00878	MHP7448_0655	n.f.	73 / 86	–	–
		n.f.	MHR_0118	–	50 / 68	–
ribose-phosphate pyrophosphokinase	MF_00882	MHP7448_0654	n.f.	90 / 95	–	–
		n.f.	MHR_0117	–	68 / 84	–
conserved hypothetical protein	MF_00895	MHP7448_0649	n.f.	67 / 82	–	–
conserved hypothetical protein	MF_00905	MHP7448_0646	MHR_0233	88 / 95	37 / 55	41 / 60
conserved hypothetical protein	MF_00909	n.f.		–	48 / 67	–
hemolysin C	MF_00917	MHP7448_0643	MHR_0236	88 / 96	49 / 69	48 / 68
conserved hypothetical protein	MF_00920	MHP7448_0642	n.f.	77 / 90	–	–
transcription antitermination protein	MF_00929	MHP7448_0637	n.f.	88 / 94	–	–
		n.f.	MHR_0243	–	47 / 66	–
O-sialoglycoprotein endopeptidase	MF_00934	MHP7448_0635	n.f.	78 / 90	–	–
conserved hypothetical protein	MF_00936	MHP7448_0634	n.f.	70 / 84	–	–
segregation and condensation protein A[c]	MF_00943	n.f.	MHR_0417	–	53 / 77	–
1-acyl-sn-glycerol-3-phosphate acyltransferase	MF_00945	MHP7448_0631	MHR_0416	68 / 84	42 / 62	40 / 61
5'-nucleotidase precursor	MF_00947	MHP7448_0630	MHR_0049	73 / 86	73 / 86	69 / 82
conserved hypothetical protein	MF_00949	MHP7448_0629	MHR_0042	97 / 98	67 / 79	62 / 73
putative ABC transporter ATP-binding - Pr2-like protein	MF_00951	MHP7448_0628	MHR_0150	96 / 98	26 / 47	26 / 48
		MHP7448_0624	n.f.	50 / 71	–	–
cell division protein ftsY	MF_00971	MHP7448_0008	MHR_0084	88 / 93	61 / 76	63 / 78
putative inner membrane protein translocase component YidC / membrane protein oxaA[c]	MF_00993	MHP7448_0676	MHR_0679	83 / 92	52 / 69	54 / 70
hypothetical protein	MF_00996	MHP7448_0674	n.f.	63 / 80	–	–
conserved hypothetical protein	MF_01005	MHP7448_0670	MHR_0011	84 / 93	48 / 68	48 / 70
conserved hypothetical protein	MF_01010	MHP7448_0669	MHR_0012	75 / 87	37 / 60	39 / 61

hypothetical protein	MF_01015	MHP7448_0667	MHR_0014	66 / 83	28 / 49	26 / 46
putative ABC transporter ATP-binding - Pr2-like protein	MF_01042	MHP7448_0665	MHR_0149	71 / 85	26 / 46	26 / 46
ABC transporter ATP-binding - Pr1	MF_01044	MHP7448_0664		77 / 89	28 / 51	29 / 51
ABC transporter ATP-binding - Pr1- like protein	MF_00952	MHP7448_0627		97 / 98	28 / 50	30 / 49
		MHP7448_0623	n.f.	50 / 67	–	–
		n.f.	MHR_0651	–	42 / 63	–
conserved hypothetical protein	MF_01055	MHP7448_0662	n.f.	36 / 54	–	–
		n.f.	MHR_0439	–	22 / 42	–
P102-like protein	MF_00623	MHP7448_0271		60 / 75	22 / 38	22 / 38
protein P102	MF_00475	MHP7448_0199		45 / 63	24 / 39	22 / 39
		MHP7448_0107	n.f.	43 / 61	–	–
conserved hypothetical protein	MF_01064	MHP7448_0661	n.f.	54 / 70	–	–
		n.f.	MHR_0538	–	24 / 44	–
hypothetical protein	MF_01068	MHP7448_0313	n.f.	93 / 94	–	–
		MHP7448_0025	n.f.	31 / 57	–	–
ABC transporter ATP-binding protein	MF_01070	MHP7448_0019	n.f.	41 / 58	–	–
		MHP7448_0020	n.f.	37 / 54	–	–
		MHP7448_0021	n.f.	40 / 60	–	–
		MHP7448_0023	n.f.	37 / 55	–	–
		MHP7448_0024	n.f.	42 / 60	–	–
		MHP7448_0306	n.f.	57 / 73	–	–
		MHP7448_0340	n.f.	34 / 53	–	–
		MHP7448_0314	MHR_0319	98 / 99	30 / 56	31 / 58
ABC transporter ATP-binding protein	MF_00322	MHP7448_0452		85 / 91	63 / 78	65 / 79
ABC transporter ATP-binding protein	MF_01072	MHP7448_0315		98 / 99	30 / 57	30 / 57
		MHP7448_0305	n.f.	61 / 77	–	–

conserved hypothetical protein	MF_01086	MHP7448_0570	n.f.	100 / 100	–	–
cell division protein ftsZ	MF_01095	MHP7448_0393	MHR_0353	74 / 86	27 / 53	26 / 52
hypothetical protein	MF_01105	MHP7448_0390	MHR_0294	66 / 80	26 / 43	26 / 49
conserved hypothetical protein	MF_01108	MHP7448_0317	n.f.	46 / 65	–	–
		n.f.	MHR_0174	–	34 / 53	–
cardiolipin synthetase	MF_01110	MHP7448_0038	MHR_0209	76 / 88	45 / 66	45 / 64
PTS system, lichenan-specific IIA component	MF_01115	MHP7448_0040	MHR_0211	72 / 86	49 / 66	52 / 69
ATP synthase A chain	MF_01122	MHP7448_0047	MHR_0217	84 / 91	52 / 70	51 / 72
ATP synthase subunit B	MF_01126	MHP7448_0049	MHR_0219	72 / 83	32 / 60	32 / 59
ATP synthase delta chain	MF_01129	n.f.	MHR_0220	–	28 / 58	–
signal recognition particle protein	MF_01141	MHP7448_0057	MHR_0411	86 / 94	61 / 78	61 / 79
Putative ATP-binding helicase protein/Glycosyltransferase/DNA2-like helicase[c]	MF_01154	MHP7448_0064	MHR_0601	70 / 84	24 / 45	25 / 44
		n.f.	MHR_0269	–	24 / 44	–
		n.f.	MHR_0667	–	25 / 44	–
conserved hypothetical protein	MF_01163	MHP7448_0065	n.f.	52 / 72	–	–
GTP-binding protein EngA	MF_01175	MHP7448_0070	n.f.	80 / 90	–	–
		n.f.	MHR_0068	–	63 / 79	–
GTP-binding protein LepA	MF_01176	MHP7448_0073	MHR_0070	92 / 97	77 / 87	80 / 88
hypothetical protein	MF_01177	MHP7448_0074	MHR_0093	70 / 82	25 / 45	26 / 47
conserved hypothetical protein	MF_01186	MHP7448_0079	MHR_0017	85 / 90	53 / 70	52 / 70
amino acid permease	MF_01193	MHP7448_0081	MHR_0146	81 / 91	52 / 71	54 / 74
conserved hypothetical protein	MF_01206	MHP7448_0364	MHR_0051	77 / 89	32 / 49	28 / 50
putative ABC transport system permease protein p69-like protein	MF_01208	MHP7448_0362	MHR_0623	74 / 87	42 / 60	41 / 61
ABC transporter ATP-binding protein	MF_01216	MHP7448_0361	MHR_0624	75 / 85	40 / 62	44 / 65
P37-like ABC transporter substrate-binding lipoprotein	MF_01218	MHP7448_0360	MHR_0625	75 / 87	38 / 58	39 / 57

glycerol uptake facilitator protein	MF_01224	MHP7448_0358	n.f.	70 / 83	–	–
amino acid permease	MF_01226	MHP7448_0357	MHR_0586	71 / 85	46 / 65	44 / 62
hypothetical protein	MF_01232	MHP7448_0355	n.f.	62 / 79	–	–
P60-like lipoprotein	MF_01236	MHP7448_0353	MHR_0627	73 / 84	42 / 63	42 / 62
hypothetical protein	MF_01239	MHP7448_0352	MHR_0628	78 / 88	38 / 62	40 / 62
conserved hypothetical protein	MF_01243	MHP7448_0350	n.f.	63 / 80	–	–
		n.f.	MHR_0632	–	32 / 51	–
conserved hypothetical protein	MF_01251	MHP7448_0089	n.f.	52 / 66	–	–
		MHP7448_0325	n.f.	51 / 65	–	–
		MHP7448_0332	MHR_0137	33 / 51	93 / 96	33 / 51
conserved hypothetical protein	MF_01252	n.f.	MHR_0138	–	93 / 95	–
		n.f.	MHR_0136	–	52 / 76	–
conserved hypothetical protein	MF_01254	n.f.	MHR_0139	–	94 / 96	–
conserved hypothetical protein	MF_01255	n.f.	MHR_0140	–	96 / 97	–
conserved hypothetical protein	MF_01264	MHP7448_0090	MHR_0598	91 / 97	55 / 76	54 / 77
GTP-binding protein	MF_01266	n.f.	MHR_0548	–	51 / 69	–
hypothetical protein	MF_01288	MHP7448_0443	MHR_0298	48 / 64	23 / 37	23 / 42
conserved hypothetical protein	MF_01290		n.f.	53 / 70	–	–
		n.f.	MHR_0298	–	26 / 44	–
leucyl aminopeptidase	MF_01294	n.f.	MHR_0030	–	53 / 71	–
hypothetical protein	n.f.	MHP7448_0466	MHR_0028	–	–	30 / 48
hypothetical protein	MF_01303		n.f.	47 / 61	–	–
lipoprotein[c]	MF_01315	MHP7448_0467	MHR_0022	66 / 80	27 / 50	29 / 53
		n.f.	MHR_0027	–	25 / 44	–
putative ABC transporter ATP-binding protein	MF_01322	MHP7448_0469	MHR_0020	90 / 96	59 / 76	58 / 77
		n.f.	MHR_0025	–	44 / 69	–

conserved hypothetical protein	MF_01324	MHP7448_0470	MHR_0019	82 / 91	44 / 67	43 / 67
		n.f.	MHR_0024	–	37 / 62	–
PTS system glucose-specific enzyme IIB component	MF_01330	MHP7448_0591	MHR_0602	81 / 89	47 / 67	45 / 64
conserved hypothetical protein	MF_01338	MHP7448_0026	n.f.	74 / 84	–	–
DUTPase[c]	MF_01350	n.f.	MHR_0590	–	45 / 62	–
conserved hypothetical protein	MF_01353	MHP7448_0544	n.f.	95 / 96	–	–
conserved hypothetical protein	MF_01354	MHP7448_0537	n.f.	78 / 91	–	–
hypothetical protein	n.f.		MHR_0558	–	–	39 / 64
conserved hypothetical protein	MF_01356	MHP7448_0530	n.f.	40 / 63	–	–
conserved hypothetical protein	MF_01277	n.f.	MHR_0152	–	20 / 37	–
		n.f.		–	25 / 43	–
hypothetical protein	MF_01358	n.f.	MHR_0153	–	37 / 58	–
hypothetical protein	MF_01359	MHP7448_0080	MHR_0016	81 / 90	47 / 72	46 / 69
conserved hypothetical protein	MF_01360	MHP7448_0399	n.f.	59 / 77	–	–
hypothetical protein	MF_01373	MHP7448_0159	n.f.	85 / 95	–	–
hypothetical protein	MF_01378	MHP7448_0046	n.f.	52 / 68	–	–
hypothetical protein	MF_01380	MHP7448_0072	n.f.	73 / 92	–	–
hypothetical protein	MF_01382	MHP7448_0042	n.f.	69 / 83	–	–
hypothetical protein	MF_01408	MHP7448_0044	MHR_0214	84 / 92	29 / 57	36 / 66
hypothetical protein	MF_01418	MHP7448_0197	MHR_0634	75 / 90	39 / 61	40 / 63
F0F1 ATP synthase subunit C	MF_01464	MHP7448_0048	MHR_0218	77 / 89	44 / 65	46 / 68
hypothetical protein	n.f.	MHP7448_0078	MHR_0018	–	–	57 / 77
hypothetical protein	n.f.	MHP7448_0363	MHR_0050	–	–	29 / 53
NADH-dependent flavin oxidoreductase	n.f.	MHP7448_0309	MHR_0279	–	–	49 / 67
glycine cleavage system H protein	n.f.	MHP7448_0312	MHR_0282	–	–	51 / 77

variant surface antigen D[b]	n.f.	MHP7448_0662	MHR_0337	–	–	22/ 43
variant surface antigen G[b]	n.f.		MHR_0343	–	–	30 / 51
variant surface antigen A[b]	n.f.		MHR_0344	–	–	24 / 41
myo-inositol catabolism protein	n.f.	MHP7448_0226	MHR_0511	–	–	20 / 39
LemA-family protein[b]	n.f.	MHP7448_0335	MHR_0589	–	–	36 / 55

Mf, *M. flocculare*; Mhp, *M. hyopneumoniae* 7448; Mhr, *M. hyorhinis* HUB-1; n.f.: ortholog not found.

[a] ORF product name according to the *M. flocculare* genome annotation; in cases of *M. flocculare* hypothetical or conserved hypothetical ORFs with annotated orthologs in *M. hyopneumoniae* or *M. hyorhinis*, the annotation of *M. hyopneumoniae* and/or *M. hyorhinis* was used; in cases of absence of *M. flocculare* orthologs, the annotation of *M. hyopneumoniae* and/or *M. hyorhinis* was also used.

[b] Hypothetical protein in *M. flocculare*, but annotated as such for *M. hyopneumoniae* and/or *M. hyorhinis* orthologs

[c] Conserved hypothetical protein in *M. flocculare*, but annotated as such for *M. hyopneumoniae* and/or *M. hyorhinis* orthologs

[d] ORF products previously associated to virulence in *M. hyopneumoniae* (Ferreira & Castro, 2007) or *M. hyorhinis* (Yogev, 1995) are in bold.

8.3 APÊNDICE 3

Padronização dos cultivos de *M. hyopneumoniae*

Neste apêndice encontram-se descritos os resultados relacionados:

- à padronização das culturas em grande escala de *M. hyopneumoniae* 7448;

- ao desenvolvimento de um método para o acesso às curvas de crescimento e à análise do crescimento bacteriano;

- à analise qualitativamente e quantitativamente da influencia da concentração de soro suíno no perfil global de proteínas produzidas pelo *M. hyopneumoniae*, bem como a avaliação dos efeitos da substituição total do soro suíno por bovino neste perfil protéico.

Estes resultados estão organizados na forma do manuscrito científico "Insights in pathogenic *Mycoplasma hyopneumoniae* culture", a ser submetido na revista XXX, onde constam dados referentes a estudos desenvolvidos por Luciano A. Reolon (LAR) e Bianca G. Bittencourt (BGB), coordenados e orientados por Marilene H. Vainstein (MHV) e Irene S. Schrank (ISS).

Insights in pathogenic *Mycoplasma hyopneumoniae* culture.

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Abstract

Mycoplasma hyopneumoniae is the agent of enzootic pneumonia, a widespread chronicle disease that occurs in pigs herds throughout the world. As other mycoplasmas, *M. hyopneumoniae* is a fastidious microorganism that requires a complex culture media based in serum, peptone, beef hearth infusion and yeast extract to supply its growth. Here, we evaluate the *M. hyopneumoniae* growth in media with different swine serum concentrations, as well as in a medium with total replacement of swine for bovine serum. The growth and protein production curves analysis showed that the replacement of swine by bovine serum is able to support *M. hyopneumoniae* growth. In mass spectrometry analysis, the pattern of protein expression revealed absence of significant qualitative difference between the evaluated media. Some surface and/or cytoadhesion related proteins were overexpressed in medium with bovine serum, suggesting a non-specific host response of antigenic related proteins to swine serum. Furthermore, since *M. hyopneumoniae* standard growth curves based in time of culture are limited, mainly due the serum batches and strains variability, we propose an OD_{560} measure based method to rapid access of growth curve. This method was able to determine an estimated cell number and protein production in the evaluated culture conditions.

Keywords

Mycoplasma hyopneumoniae; porcine enzootic pneumonia; modified Friis medium; grown curves pattern proteins MS/MS analysis.

1. Introduction

Mycoplasma hyopneumoniae is the etiological agent of Enzootic Pneumonia (EP). This chronical disease, characterized by high morbidity and low mortality, is responsible for the infection of approximately 200 million pigs every year, causing hundreds of millions of dollars of loss for swine farmers worldwide. *M. hyopneumoniae* attaches to the cilia of the tracheal epithelial cells, causing a reduction in ciliary action (DeBey and Ross, 1994; Zielinski and Ross, 1993), predisposing the swine to secondary pathogens such as *Pasteurella multocida* (Amass *et al.,* 1994) and porcine reproductive and respiratory syndrome virus (PRRSV) (Thacker *et al.,* 1999).

Mycoplasmas are fastidious microorganisms and the majority of the species has a slow growth in available culture media. Furthermore, there are descriptions of *in vitro* cultivation of only few *Mycoplasma* species (Razin, 1998). As other mycoplasmas, *M. hyopneumoniae* required a complex medium based in serum, peptone, beef heart infusion and yeast extract to supply its growth (Friis, 1975, Razin, 1998). Serum is one of the most important supplements in mycoplasma culture media, since it provides nutrients, fatty acids and cholesterol, an essential membrane component (Slutzky *et al.,* 1976). Swine serum has been used as the preferentially serum in most of the Friis-like media. A related batch-to-batch variation of *M. hyopneumoniae* growth in Friis medium (Miles, 1992), linked with recently observations that swine serum component is highly variable in support growth of recent isolates (Madsen *et al.,* 2007), decreases the reproducibility and consensus in standard *M. hyopneumoniae* growth curves.

The availability of reports on growth parameters of *M. hyopneumoniae* strain 7448 are few and commonly uses laborious methods, as color-changing unit (CCU) assay (Stemke and Robertson, 1982), ATP measurements (Stemke and Robertson, 1990; Calus *et. al*., 2010) and flow citometry analysis (Assunção *et al*., 2004). The development of a

rapid, simple and reproducible method for analyzing and monitoring the growth of *M. hyopneumoniae* is essential for experiments that require standardized bacterial cultures such as those involving proteomics surface, RNA extraction and biochemical experiments.

As the serum is one of the main compounds of the *M. hyopneumoniae* culture medium, we evaluate the impact of changes in the medium swine sera concentration and in the total replacement of swine by bovine serum in *M. hyopneumoniae* growth and total protein production. For this, we propose a simple and reliable method for growth analysis, based in 560 nm optical density (OD_{560}) measurements, to access a growth curve and correlating with the estimate number of cells and protein production.

2. Materials and Methods

2.1 Bacterial strain and Modified Friis (MF) medium

Mycoplasma hyopneumoniae strain 7448 was isolated from infected swine from Lindóia do Sul (Santa Catarina, Brazil) (Vasconcelos *et. al*., 2005) by CNPSA, EMBRAPA (Concórdia, Santa Catarina, Brazil).

Three Modified Friis medium were used as describe in Supplementary Table 1. Briefly, MFP (Modified Friis with increase of double amounts of Phenol red) is the basic modified Friis Medium (Friis, 1975), containing 25% (v/v) swine pathogen-free (SPF) serum (CNPSA, EMBRAPA, Concórdia, Santa Catarina, Brazil) and double amounts of phenol red. MFPL (MFP and Low concentration of serum) has a reduction to 5% of swine serum. In MFPB (MFP and Bovine serum) the swine serum was totality replaced by 25% of bovine serum (Gibco).

2.2 Growth assays

To evaluate the growth of *M. hyopneumoniae* in liquid culture medium, 500 µL of bacterial suspension was inoculated into 4.5 ml of culture medium (Supplementary Table 1) in a 15 ml polypropylene tube (Greiner-Bio One). The culture was incubated at 37ºC with slow agitation in roller for different periods of time. The optical density at 560 nm (OD_{560}) was measured in an UV/Visible spectrophotometer (GE Healthcare). To assess the colony forming units (CFU) of *M. hyopneumoniae,* serial dilutions were performed from liquid inoculation and plated in MFP solid medium. The plates were then incubated at 37°C in microaerophilic conditions for 14 days and the CFU was determined. Five independent experiments were performed for the liquid and solid media experiments for each medium evaluated.

2.3 Total protein extraction

For ESI-Q-ToF MS/MS identification, protein extraction was performed as previously described (Pinto *et al.,* 2007) with few modifications. Cells from four days culture were harvested by centrifugation at 5000 x g for 15 min and washed three times with PBS pH 7.4. Cell suspensions were then lysed by sonication at 25 Hz on ice bath with five 30 s cycles with a 1 min interval between pulses.

For protein quantification in different times and culture media, a hot SDS bacterial lysis was performed. *M. hyopneumoniae* cell pellets were suspended of in 1% (w/v) hot SDS and incubated for 3 min at 95 ºC and submitted to centrifugation (10.000 g – 10 min.). The supernantant were stored (-80ºC) for further analysis.

Proteins quantifications were performed using Quant-iT™ Protein Assay Kit (Invitrogen) with fluorescence emission analyzed in a Qubit™ Fluorometer (Invitrogen) and BCA Protein Assay Kit (Pierce).

2.4 Mass spectrometry and data analysis

Liquid chromatography (LC) separation coupled with tandem mass spectrometry (MS/MS) was performed to identify the expression proteins profiles in three different culture media. MS/MS analyses were performed in an electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) Q-ToF Micro mass spectrometer (Micromass) coupled to a capillary liquid chromatography system (Waters Corporation). For ESI-Q-TOF MS/MS identification, samples of 100 µg of proteins were directly digested with trypsin (Promega) and desalted using an OASIS® HLB Cartridge column (Waters Corporation). The resulting peptide mixture was separated in a Nanoease C18 (75 µm ID) capillary column by elution with 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.

Data analysis and protein identification was performed using MASCOT software (Matrix Science) and all tandem mass spectra were searched against the *in silico* digested *M. hyopneumoniae* strain 7448 protein database, available from Uniprote-MS (CBiot, UFRGS, Brazil). The following search parameters were used: trypsin was used as the cutting enzyme, the mass tolerance for monoisotopic peptide window was set to ±0.2 Da, the MS⁄MS tolerance window was set to ±0.2 Da, one missed cleavage was allowed, and carbamidomethylation of cysteine and oxidation of methionine were chosen as variable modifications.

For each protein match identified by MASCOT, a corresponding exponentially modified protein abundance index (emPAI) (Ishihama *et al.,* 2005) was calculated. To compare the protein abundance in MFP, MFPL and MFPB, twofold or more difference between the normalized emPAIs values were used to consider a protein as differentially expressed (Ishihama *et al.,* 2005; Pinto *et al.*, 2009). Three technical replicates

identification were performed on each three independent protein extraction samples for MFP, MFPL and MFPB, totalizing nine protein profiles to each culture media. Proteins identified at least in six of nine identification and with significant score threshold ($p<0.05$) had their emPAI values used to quantitative (means of normalized protein emPAIs) and qualitative protein profile comparison between evaluated media.

3. Results and Discussion

3.1 OD changes during M. hyopneumoniae *growth*

Colony-forming units (CFU) and color-changing units (CCU) assays are not friendly to be used routinely, since these techniques requires long periods of incubation and have some drawbacks, as underestimation of number of live cells due to the limited accuracy and clump formation. In addition, the high variability of *M. hyopneumoniae* growth cultures difficult the use of a standard growth curves based in cultures time and make necessary a new CCU or CFU assay for each new serum batch, bacterial isolation and others culture changes. For this reason, an indirect analysis, based in OD changes during *M. hyopneumoniae* growth was performed.

The color change from red to yellow can be accessed by OD_{560} measurement. As cells develop and metabolize the culture components, there is an intense acidification of the media, which leads to a red to yellow color shift and consequent reduction of OD_{560}. We hypothesized that following the OD_{560} during the *M. hyopneumoniae* in MFP or derived media could represent a strategy for indirect cell quantification and curve growth access. However, to improve the sensitivity of medium color change analysis, a modified Phenol red Friis Medium (MFP) was proposed by adding double amount of the pH sensitive color indicator phenol red (Friis, 1975). This modification increased the OD_{560}

value approximated two fold in fresh media, when compared with traditionally culture medium described by Friis (1975), yielding a more reliable measure determination.

The growth rate of *M. hyopneumoniae* was then evaluated on MFP containing alterations in the sera. Figure 1a represents the growth response determined by OD_{560} *versus* time for three modified Friis media: MFP (25% swine serum), MFPL (5% swine serum) and MFPB (25% bovine serum). The higher decrease in OD_{560} values found in MFP and MFPB reflect a color shift due to acidification of the medium by cell metabolism and suggest a better growth in this medium when compared with MFPL. The lower decrease in MFPL OD_{560} values, when plateau was reached just after six days of growth, occurs probably due to the low and late growth in response to the nutrients limitations in low serum concentration. In all evaluated media, after reaching the OD_{560} peak, the values remained stable suggesting entry into the stationary phase.

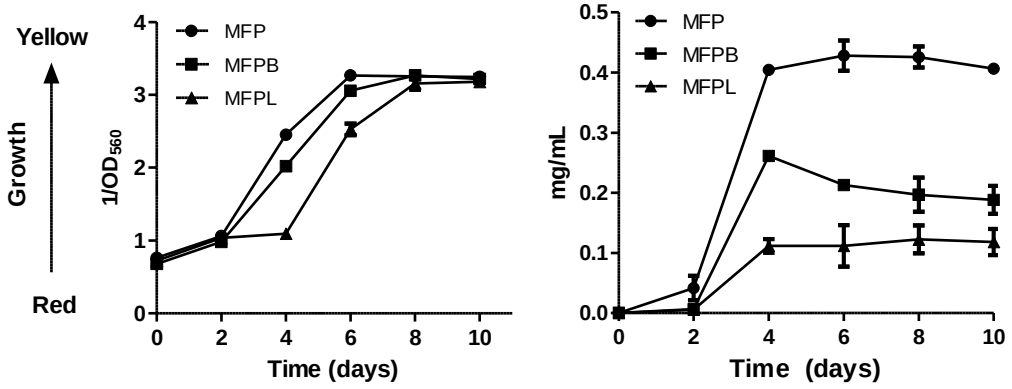

Figure 1: Indirect growth analysis of *M. hyopneumoniae* strain 7448 in MFP, MFPL and MFPB culture media.

(a) OD_{560} changes during *M. hyopneumoniae* strain 7448 growth in MFP, MFPL and MFPB liquid medium. $1/OD_{560}$ is an extinction of red measure. (b) Total protein production. Data points represent means of five biological replicates measurements (± standard deviation). MFP - Modified Friis; MFPL - Modified Friis Low serum; MFPB - Modified Friis Bovine serum.

In research laboratory routine, cultures in liquid medium from two and four days are commonly employed in several assays, including RNA extraction (two days) (Siqueira *et. al.*, 2011), as well DNA and protein extraction (four days) (Pinto *et al.*, 2007). Therefore, a colony-forming units (CFU) assay was performed at these days and reveals that, at the second day of growth, approximated 10^6 CFU.ml^{-1} in all three culture media were observed. However, as expected, at the fourth day of culture a difference between MFP (2 x 10^8 CFU.ml^{-1}), MFPB (5 x 10^7 CFU.ml^{-1}) and MFPL (8 x 10^6 CFU.ml^{-1}) could be detected.

Changes in cell mass content can be also used for bacterial growth evaluation. A direct chemical measurement of some cell compound, as total proteins, is an easy and very useful technique to access a growth curve (Bratbak and Dundas, 1984). In the first two days of growth, protein production was very similar among the three media evaluated (Figure 1b). However, analysis of third day cultures revealed a significant difference among the media evaluated. *M. hyopneumoniae* growing on MFP medium showed better rates of total protein production. Furthermore a plateau on the growth curves was observed after four days, suggesting that the bacterial culture has entered into a stationary phase. This result is in agreement with previous report where the same growth profile was observed in experiments using CCU (Stemke and Robertson, 1982) and ATP production measurements (Calus *et. al.*, 2010; Stemke and Robertson, 1990).

In order to attain a systematic visualization of protein production in different media and culture time, a SDS-PAGE analysis was performed. The results demonstrated that *M. hyopneumoniae* growing on three different media has a similar profile of protein expression (also confirmed by LC-MS/MS analysis) (Figure 2).

Altogether, the data presented here allowed establishing a fast and reliable way to access an estimate the cell number of *M. hyopneumoniae* strain 7448 cultures by OD_{560}

measurements. Therefore, its possible to establish that, for example, a four day culture in MFP with an OD_{560} of 0.4 has approximated 10^8 CFU.ml^{-1} an produces 0.4 mg.ml^{-1} (note that the protein concentration refers to hot SDS 1% extraction method being possible be optimized according to the interest).

2.5 Comparative shotgun proteomics

Elucidation of mechanisms that leads to modifications on protein expression profile could help in the developments of better media formulations. In the present case, characterized by the reduction or total exchange of the main constituent (serum), evaluation of the protein expression profile is highly required. Therefore, a qualitative and quantitative comparative proteomic analysis based on LC-MS/MS protein expression profiles of *M. hyopneumoniae* strain *7448* in MFP, MFPL and MFPB media was performed. Three independent biological replicates were analyzed by three technical replicates by LC-MS/MS, resulting in nine protein profiles referent to each medium evaluated.

We identify a total of 67, 56 and 58 proteins in *M. hyopneumoniae* strain 7448 growth in MFP, MFPL and MFPB respectively (Supplementary Table 1). From this, 41 proteins involved in a broad range of cellular processes (Figure 3), and identified at last in six from nine replicates in three media, were chosen to perform an average of the relative abundance using their emPAI values (Table 1). These averages were used in the following comparative analysis.

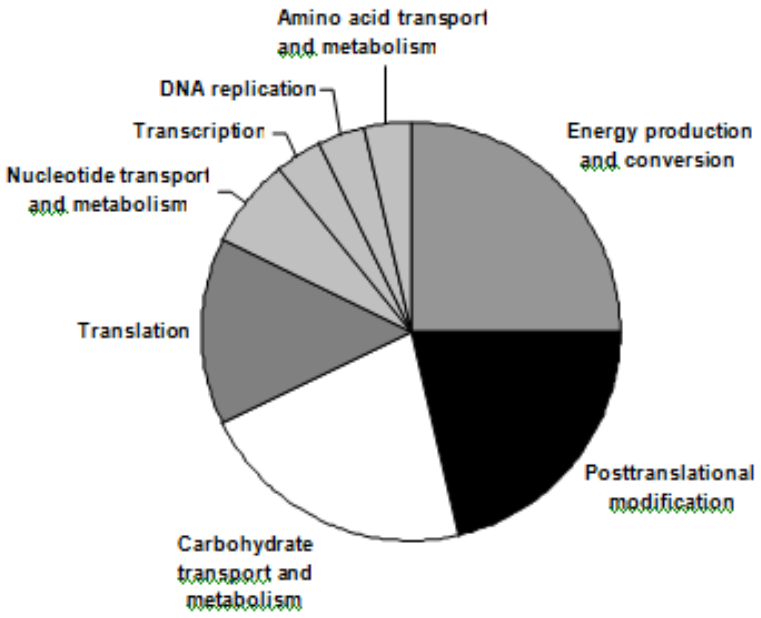

Figure 3: Diagram of COG functional categories distribution of identified proteins.

Quantitative differences identified between culture medium (twofold differences) among the calculated emPAIs were considered differentially expressed (Ishihama *et al.*, 2005; Pinto *et. al*., 2009). Interesting, some overexpressed surface and/or cytoadhesion related proteins (P46, P97, P146 and 46K surface antigen precursor) (Ferreira and Castro, 2007) were identified in *M. hyopneumoniae* grown in MFPB (Table 1). In addition, a previously described pathogenic strain protein MHP7448_0662 (Pinto *et. al*., 2009), similar to P-102 like proteins, was also overexpressed in MFPB. These results suggest a non specific host response of antigenic related proteins to swine serum, indicating that low amounts of swine serum or total replacement for bovine serum can be performed with no decrease and/or suppression of these proteins. Bivariant plots (Figure 4) show the similar protein amounts and the small quantitative difference between MFP, MFPL and MFPB.

Table 1: The emPAI value comparison and COG functional classification of identified proteins from *M. hyopneumoniae* growing on MFP, MFPL and MFPB media.

			emPAI [4]		
Accession [1]	**Protein description [2]**	**COG [3]**	**MFP**	**MFPL**	**MFPB**
YP_287409.1	Hypothetical protein MHP7448_0009	S	0,006	0,004	0,006
YP_287414.1	Fructose-bisphosphate aldolase	G	0,009	0,008	0,01
YP_287435.1	Glyceraldehyde 3-phosphate dehydrogenase	G	0,016	0,02	0,027
YP_287456.1	Elongation factor Ts	J	0,006	0,008	0,015
YP_287467.1	Molecular chaperone DnaK	O	0,032	0,049	0,036
YP_287471.1	Nucleoid DNA-binding protein	L	0,019	0,022	0,024
YP_287475.1	Elongation factor G	J	0,01	0,006	0,009
YP_287482.1	NADH oxidase	R	0,063	0,06	0,045
YP_287496.1	Thiol peroxidase	O	0,046	0,032	0,04
YP_287498.1	Thioredoxin reductase	O	0,012	0,01	0,012
YP_287512.1	Adenine phosphoribosyltransferase	F	0,014	0,022	0,024
YP_287513.1	Pyruvate dehydrogenase E1-alpha subunit	C	0,047	0,048	0,047
YP_287514.1	Pyruvate dehydrogenase	C	0,085	0,084	0,095
YP_287527.1	Aminopeptidase	G	0,024	0,017	0,022
YP_287534.1	L-lactate dehydrogenase	C	0,087	0,061	0,049
YP_287546.1	Trigger factor	O	0,01	0,024	0,012
YP_287595.1	Protein P97	S	0,01	0,018	0,031
YP_287596.1	Protein P102	S	0,002	0,005	0,009
YP_287620.1	Ribonucleotide-diphosphate reductase	F	0,004	0,006	0,007
YP_287622.1	Methylmalonate-semialdehyde dehydrogenase	C	0,018	0,02	0,011
YP_287647.1	Phosphopyruvate hydratase	G	0,006	0,01	0,009
YP_287649.1	Hypothetical protein MHP7448_0252	S	0,011	0,011	0,015
YP_287654.1	Hypothetical protein MHP7448_0257	S	0,036	0,027	0,021
YP_287769.1	PTS system enzyme IIB component	G	0,076	0,054	0,061
YP_287778.1	Thioredoxin	O	0,067	0,078	0,065
YP_287883.1	Mannose-6-phosphate isomerase	G	0,005	0,006	0,007

YP_287885.1	Putative p216 surface protein	S	0,009	0,012	0,015
YP_287886.1	P76 membrane protein precursor	S	0,01	0,009	0,019
YP_287897.1	Acetate kinase	C	0,005	0,008	0,011
YP_287898.1	Phosphotransacetylase	C	0,021	0,014	0,015
YP_287902.1	46K surface antigen precursor	S	0,013	0,013	0,034
YP_287912.1	Elongation factor Tu	J	0,031	0,05	0,035
YP_287918.1	Methionine sulfoxide reductase B	O	0,021	0,016	0,02
YP_287957.1	Dihydrolipoamide dehydrogenase	C	0,007	0,014	0,008
YP_287990.1	ABC transporter xylose-binding lipoprotein	R	0,009	0,014	0,017
YP_288004.1	50S ribosomal protein L7/L12	J	0,059	0,037	0,033
YP_288041.1	Prolipoprotein p65	S	0,012	0,016	0,013
YP_288044.1	XAA-Pro aminopeptidase	E	0,007	0,008	0,011
YP_288047.1	Hypothetical protein MHP7448_0662	S	0,003	0,006	0,007
YP_288048.1	Adhesin like-protein P146	S	0,004	0,005	0,01
YP_288053.1	Transcription elongation factor GreA	K	0,01	0,012	0,005

[1] 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] COG database functional classes: (J) Translation, ribosomal structure and biogenesis, (K) Transcription, (L) DNA replication, recombination and repair, (O) Posttranslational modification, protein turnover, chaperones, (C) Energy production and conversion, (G) Carbohydrate transport and metabolism, (E) Amino acid transport and metabolism, (F) Nucleotide transport and metabolism, (R) General function prediction only, and (S) Function unknown.

[4] 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).

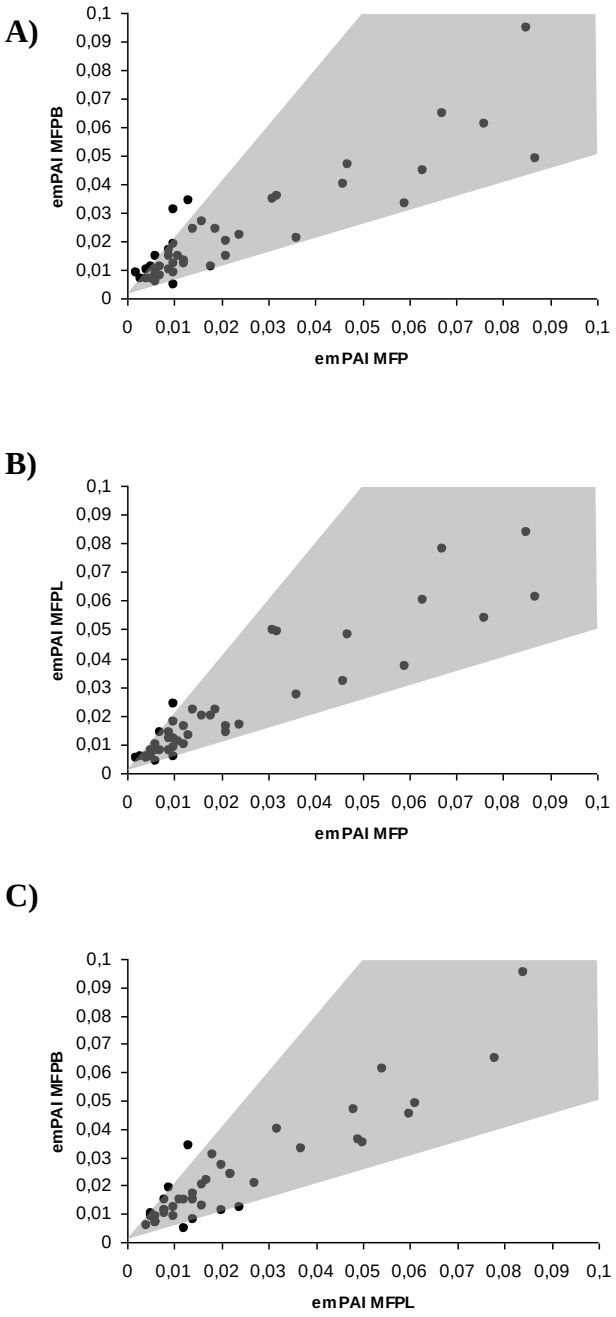

Figure 4: Comparative analyses of *M. hyopneumoniae* strain 7448 protein profile in different culture media based on emPAI relative abundance.

Bivariants plots of *M. hyopneumoniae* strain 7448 proteins expressed in MFP, MFPL or MFPB medium based on LC-MS/MS emPAI relative abundance. (a) MFPB against MFP; (b) MFP versus MFPL; and (c) MFPL versus MFPB. Proteins outside the gray area (V-shaped shaded) were assumed to be overexpressed in the culture medium whose emPAI values are assigned to the proximal axis. Two fold or higher differences between the emPAI values for a given protein were considered as overexpressed.

Few qualitative differences (presence or absence) in protein expression between the three different culture medium analyzed were identified and it is probably related with the low abundance of these proteins (Supplementary Table 1). A remarkable aspect of this analysis refers to raised levels of proteins associated with the inositol pathway in *M. hyopneumoniae* cultivated in MFP compared to other conditions. This may indicate a specific response to host serum since *M. hyopneumoniae* is the only mycoplasma so far sequenced that has all genes involved in the inositol metabolism pathway.

4. Conclusions

The results presented in this work suggest that is possible a total replacement of the swine for the bovine serum without significant changes in total protein expression profile. The reduction of swine serum concentrations is also viable, however the lower rates of growth indicates that higher amounts of culture are required to achieve the same cell population of the traditional Friis medium. Furthermore, a fast and reliable method to access the *M. hyopneumoniae* strain 7448 growth curve using OD_{560} measurements was proposed and was able to easily access an estimate bacterial cell number in laboratory routine.

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SUPPLEMENTARY DATA

Supplementary Table 1: Modified Friis media.

	MFP[a]	**MFPL[b]**	**MFPB[c]**
Hank's balanced salt solution A[d]	1,5%	1,5%	1,5%
Hank's balanced salt solution B[e]	1,5%	1,5%	1,5%
Yeast extract[f]	3,4% (v/v)	3,4% (v/v)	3,4% (v/v)
PPLO broth base[g]	0,4% (w/v)	0,4% (w/v)	0,4% (w/v)
BHI[h]	0,3% (w/v)	0,3% (w/v)	0,3% (w/v)
Phenol Red 0,25% (w/v)[i]	1% (v/v)	1% (v/v)	1% (v/v)
Thallium acetate	0,01% (w/v)	0,01% (w/v)	0,01% (w/v)
Swine serum[j]	25% (v/v)	5% (v/v)	-
Foetal bovine serum[l]	-	-	25% (v/v)
Ultrapure water	up to 1 liter	up to 1 liter	up to 1 liter

[a] Modified Friis double amounts of Phenol red.

[b] Modified Friis double amounts of Phenol red and Low amounts of swine serum.

[c] Modified Friis double amounts of Phenol red and total replace of swine serum to Bovine serum.

[d] 6% (w/v) NaCl; 0,8% (w/v) KCl; 0,2% (w/v) $MgSO_4$; 0,2% (w/v) $MgCl_2$; 0,28% (w/v) $CaCl_2$.

[e] 0,3% (w/v) Na_2HPO_4; 0,12% (w/v) KH_2PO_4.

[f] Yeast extract from *Saccharomyces cerevisiae* type 2 (Sigma Aldrich).

[g] Base for the isolation and cultivation of Mycoplasma (Difco).

[h] Brain hearth broth (Merck).

[i] Solution of phenol red (Acros, Organics) at 0,2% pH 7.0.

[j] Swine serum form SPF swine (CNPSA, EMBRAPA, Concórdia, Santa Catarina, Brazil).

[k] Bovine serum Gibco.

Supplementary Table 1: Biological and technical replicates emPAI value of LC-MS/MS identified proteins in *M. hyopneumoniae* strain 7448 growth in MFP.

		MFP[1]								
		emPAI[2]								
		1ª Biological replicate			2ª Biological replicate			3ª Biological replicate		
Protein[3]	EmPAI Average[4]	Technical replicate 1	Technical replicate 2	Technical replicate 3	Technical replicate 1	Technical replicate 2	Technical replicate 3	Technical replicate 1	Technical replicate 2	Technical replicate 3
pyruvate dehydrogenase	0,085	0,125	0,099	0,101	0,066	0,068	0,052	0,088	0,069	0,095
pyruvate dehydrogenase E1-alpha subunit	0,047	0,068	0,061	0,071	0,040	0,055	0,029	0,034	0,032	0,034
p76 membrane protein precursor	0,010	0,011	0,016	0,011	0,008	0,008	0,009	0,011	0,008	0,007
putative p216 surface protein	0,009	0,011	0,006	0,008	0,008	0,011	0,011	0,007	0,008	0,008
molecular chaperone DnaK	0,032	0,033	0,038	0,035	0,023	0,031	0,030	0,036	0,033	0,029
L-lactate dehydrogenase	0,087	0,058	0,062	0,063	0,111	0,088	0,081	0,097	0,104	0,122
protein P97	0,010	0,011	0,013	0,013	0,008	0,010	0,012	0,006	0,014	0,007
NADH oxidase	0,063	0,040	0,053	0,060	0,071	0,073	0,053	0,103	0,059	0,052
aminopeptidase	0,024	0,027	0,024	0,019	0,021	0,022	0,031	0,020	0,012	0,037
phosphotransacetylase	0,021	0,016	0,021	0,028	0,018	0,025	0,035	0,015	0,014	0,019
dihydrolipoamide dehydrogenase	0,007	0,011	0,010	0,013	0,004	0,004	0,004	0,007	0,007	0,006
adhesin like-protein P146	0,004	0,005	0,007	0,004	0,004	0,002	0,002	0,001	0,003	0,005

elongation factor Tu	0,031	0,023	0,016	0,021	0,033	0,034	0,034	0,032	0,045	0,040
elongation factor G	0,010	0,010	0,011	0,006	0,012	0,010	0,011	0,007	0,012	0,008
prolipoprotein p65	0,012	0,016	0,015	0,015	0,011	0,016	0,012	0,011	0,003	0,005
46K surface antigen precursor	0,013	0,017	0,007	0,016	0,018	0,006	0,014	0,011	0,010	0,014
glyceraldehyde 3-phosphate dehydrogenase	0,016	0,015	0,020	0,020	0,012	0,013	0,018	0,014	0,020	0,011
methylmalonate-semialdehyde dehydrogenase	0,018	0,018	0,009	0,013	0,018	0,015	0,032	0,014	0,013	0,026
thiol peroxidase	0,046	0,034	0,031	0,032	0,040	0,028	0,058	0,070	0,065	0,056
trigger factor	0,010	0,007	0,006	0,010	0,009	0,016	0,013	0,010	0,014	0,008
thioredoxin	0,067	0,055	0,050	0,051	0,067	0,069	0,065	0,078	0,071	0,096
adenine phosphoribosyltransferase	0,014	0,034	0,019	0,009	0,008	0,008	0,011	0,013	0,012	0,011
PTS system enzyme IIB component	0,076	0,018	0,067	0,068	0,059	0,096	0,087	0,105	0,096	0,084
50S ribosomal protein L7/L12	0,059	0,032	0,074	0,075	0,093	0,067	0,037	0,019	0,070	0,061
hypothetical protein MHP7448_0009	0,005	0,005	0,004	0,004	0,004	0,006	0,002	0,007	0,012	0,005
thioredoxin reductase	0,012	0,010	0,010	0,005	0,019	0,014	0,012	0,007	0,014	0,019
hypothetical protein MHP7448_0662	0,003	0,003	0,002	0,004	0,003	0,002	0,003	0,006	0,005	0,003
hypothetical protein MHP7448_0257	0,021	0,020	0,018	0,018	0,016	0,016	0,023	0,028	0,025	0,022
protein P102	0,002	0,002	0,001	0,003	0,002	0,001	0,001	0,002		0,002
acetate kinase	0,005	0,009	0,004	0,004	0,007	0,003	0,005	0,006		0,005
ABC transporter xylose-binding lipoprotein	0,009	0,014	0,010	0,015	0,009	0,006	0,009		0,005	0,004

fructose-bisphosphate aldolase	0,009	0,012	0,011	0,005	0,005	0,009	0,014		0,008	0,007
XAA-Pro aminopeptidase	0,007	0,004	0,004	0,004	0,008	0,008	0,011		0,012	
hypothetical protein MHP7448_0252	0,011	0,018	0,007	0,007	0,014	0,014	0,009			0,009
methionine sulfoxide reductase B	0,021	0,011	0,022	0,022	0,019	0,047	0,012	0,015		
transcription elongation factor	0,010	0,010	0,005			0,008	0,012	0,014	0,013	0,011
nucleoid DNA-binding protein	0,019				0,013	0,014	0,02	0,024	0,022	0,019
ribonucleotide-diphosphate reductase subunit beta	0,004	0,004	0,004	0,004	0,003	0,003	0,005			
elongation factor Ts	0,006	0,005	0,005	0,005	0,004				0,007	0,012
mannose-6-phosphate isomerase	0,006	0,005				0,004	0,006	0,007	0,006	0,005
myo-inositol catabolism protein	0,005	0,005	0,005	0,005	0,004	0,005	0,007			
phosphopyruvate hydratase	0,006	0,004	0,007	0,007		0,003		0,005	0,01	
phosphocarrier protein HPr	0,022	0,019		0,018			0,022	0,027	0,025	
lipoprotein	0,005	0,009	0,006	0,008		0,002	0,002			
3-keto-L-gulonate-6-phosphate decarboxylase	0,006	0,007	0,006	0,006	0,005	0,006				
50S ribosomal protein L27	0,021	0,020	0,018	0,019			0,024		0,026	
triosephosphate isomerase	0,007	0,006	0,006	0,006		0,011	0,007			
dihydrolipoamide dehydrogenase	0,003	0,003	0,003		0,003	0,003	0,003			
heat shock protein	0,007	0,006	0,005	0,012		0,005	0,005			
leucyl aminopeptidase	0,005		0,003			0,003	0,003	0,01		
oligoendopeptidase F	0,003		0,002	0,002	0,004		0,003			

lipoate-protein ligase A	0,007	0,009	0,004	0,004			0,011			
ribosome recycling factor	0,009	0,009		0,008	0,007		0,011			
hypoxanthine-guanine phosphoribosyltransferase	0,010			0,008		0,007	0,011	0,013		
transketolase	0,004				0,002	0,002			0,007	0,003
myo-inositol catabolism protein	0,003	0,002	0,002		0,004					0,002
asparaginyl-tRNA synthetase	0,003	0,003	0,003	0,003		0,003				
phenylalanyl-tRNA synthetase alpha chain	0,006	0,006	0,006		0,005					
30S ribosomal protein S6	0,005	0,006	0,005	0,005						
hypothetical protein MHP7448_0373	0,002	0,003	0,001	0,002						
L-ribulose-5-phosphate 4-epimerase	0,006	0,006	0,006	0,006						
thymidine phosphorylase	0,003	0,004	0,003	0,003						
neutrophil activating factor	0,009		0,010		0,008	0,009				
lipase-esterase	0,008							0,008	0,008	0,007
purine-nucleoside phosphorylase	0,007	0,007	0,006				0,008			
elongation factor P	0,008				0,007	0,007				0,01
cell division protein	0,002	0,002	0,002							
5'-nucleotidase precursor	0,002		0,002	0,002						
spermidine/putrescine ABC transporter ATP-binding	0,003	0,003	0,003							
HIT-like protein	0,014	0,014	0,013							

acyl carrier protein phosphodiesterase	0,007		0,007		0,006					
hypothetical protein MHP7448_0112	0,004			0,003				0,005		
heat shock ATP-dependent protease	0,001				0,001	0,001				
ATP binding protein	0,001				0,001	0,001				
phosphoglyceromutase	0,004						0,004	0,004		
phosphoenolpyruvate-protein phosphotransferase	0,003	0,003								
pullulanase	0,002	0,002								
hypothetical protein MHP7448_0563	0,003	0,003								
hypothetical protein MHP7448_0377	0,004	0,004								
PTS system, IIA component	0,010	0,010								
F0F1 ATP synthase subunit alpha	0,003	0,003								
hypothetical protein MHP7448_0566	0,002		0,002							
50S ribosomal protein L24	0,014		0,014							
hypothetical protein MHP7448_0348	0,005		0,005							
lysyl-tRNA synthetase	0,003			0,003						
dihydrolipoamide acetyltransferase	0,009				0,009					
glucose-6-phosphate isomerase	0,006				0,006					
30S ribosomal protein S4	0,006				0,006					
50S ribosomal protein L6	0,007				0,007					
50S ribosomal protein L11	0,008				0,008					

DNA-directed RNA polymerase subunit alpha	0,004				0,004					
hypothetical protein MHP7448_0244	0,008				0,008					
transcriptional regulator	0,005					0,005				

[1] MFP (Modified Friis with increase of double amounts of Phenol red), containing 25% (v/v) swine serum.

[2] 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).

[3] Protein identification according to NCBI database (http://www.ncbi.nlm.nih.gov).

[4] Protein emPAI average from biological and technical replicates. All positive identifications were used to calculate the average.

Supplementary Table 1: Biological and technical replicates emPAI value of LC-MS/MS identified proteins in *M. hyopneumoniae* strain 7448 growth in MFPL.

	MFPL[1]								
	emPAI[2]								
	1ª Biological replicate			2ª Biological replicate			3ª Biological replicate		
Protein[3]	emPAI Average [4]	Technical replicate 1	Technical replicate 2	Technical replicate 3	Technical replicate 1	Technical replicate 2	Technical replicate 3	Technical replicate 1	Technical replicate 2	Technical replicate 3
pyruvate dehydrogenase E1-alpha subunit	0,048	0,084	0,061	0,070	0,023	0,038	0,042	0,033	0,035	0,042
pyruvate dehydrogenase	0,084	0,120	0,088	0,102	0,053	0,044	0,108	0,079	0,098	0,061
L-lactate dehydrogenase	0,061	0,093	0,096	0,075	0,038	0,026	0,068	0,034	0,068	0,055
molecular chaperone DnaK	0,049	0,049	0,049	0,042	0,041	0,045	0,051	0,063	0,050	0,054
elongation factor Tu	0,050	0,034	0,048	0,044	0,078	0,044	0,061	0,045	0,049	0,047
NADH oxidase	0,060	0,046	0,097	0,065	0,055	0,070	0,050	0,050	0,054	0,052
putative p216 surface protein	0,012	0,011	0,018	0,020	0,010	0,007	0,005	0,009	0,011	0,013
prolipoprotein p65	0,016	0,032	0,033	0,032	0,013	0,012	0,005	0,005	0,003	0,005
aminopeptidase	0,017	0,028	0,024	0,028	0,005	0,015	0,007	0,016	0,011	0,017
adhesin like-protein P146	0,005	0,009	0,004	0,002	0,005	0,006	0,004	0,005	0,004	0,005
thiol peroxidase	0,032	0,067	0,034	0,066	0,012	0,015	0,017	0,023	0,011	0,039

protein P97	0,018	0,011	0,020	0,011	0,015	0,022	0,021	0,020	0,023	0,021
thioredoxin	0,078	0,106	0,092	0,106	0,067	0,085	0,056	0,061	0,065	0,064
p76 membrane protein precursor	0,009	0,008	0,011	0,013	0,005	0,007	0,010	0,009	0,008	0,008
trigger factor	0,024	0,013	0,011	0,021	0,042	0,024	0,019	0,027	0,029	0,034
glyceraldehyde 3-phosphate dehydrogenase	0,020	0,019	0,036	0,009	0,034	0,016	0,017	0,017	0,012	0,018
dihydrolipoamide dehydrogenase	0,014	0,015	0,013	0,015	0,010	0,017	0,014	0,012	0,013	0,016
46K surface antigen precursor	0,013	0,007	0,028	0,015	0,010	0,006	0,006	0,009	0,014	0,019
phosphopyruvate hydratase	0,010	0,014	0,019	0,007	0,005	0,011	0,006	0,012	0,009	0,008
elongation factor G	0,006	0,009	0,004	0,004	0,008	0,007	0,004	0,005	0,006	0,008
protein P102	0,005	0,003	0,003	0,003	0,005	0,006	0,003	0,008	0,004	0,010
acetate kinase	0,008	0,008	0,007	0,008	0,005	0,007	0,007	0,014	0,010	0,010
PTS system enzyme IIB component	0,054	0,035	0,030	0,035	0,090	0,028	0,129	0,046	0,049	0,048
hypothetical protein MHP7448_0009	0,004	0,004	0,004	0,004	0,003	0,003	0,004	0,005	0,006	0,005
oligoendopeptidase F	0,008	0,005	0,008	0,009	0,006	0,007	0,008	0,008	0,012	0,005
transketolase	0,006	0,005	0,009		0,006	0,008	0,005	0,006	0,006	0,003
ABC transporter xylose-binding lipoprotein	0,014	0,007	0,026	0,030	0,009	0,017		0,008	0,009	0,004
thioredoxin reductase	0,010	0,020	0,009	0,010	0,013	0,008	0,009	0,006	0,006	
methionine sulfoxide reductase B	0,016	0,020	0,018	0,020	0,013	0,016	0,018	0,012		0,012
methylmalonate-semialdehyde dehydrogenase	0,020			0,006	0,012	0,028	0,025	0,025	0,022	0,021

hypothetical protein MHP7448_0257	0,027			0,038	0,024	0,030	0,034	0,021	0,023	0,022
phosphotransacetylase	0,014	0,009	0,018			0,016	0,008	0,005	0,027	
dihydrolipoamide acetyltransferase	0,019				0,014	0,017	0,03	0,019	0,013	0,02
adenine phosphoribosyltransferase	0,022				0,012	0,015	0,017	0,023	0,04	0,024
nucleoid DNA-binding protein	0,022				0,021	0,026	0,029	0,019	0,02	0,019
mannose-6-phosphate isomerase	0,006		0,008		0,006	0,008		0,005	0,006	0,005
hypoxanthine-guanine phosphoribosyltransferase	0,025				0,023	0,029	0,016	0,034	0,036	0,01
DNA-directed RNA polymerase subunit alpha	0,008				0,006	0,007		0,011	0,006	0,012
hypothetical protein MHP7448_0252	0,011	0,015		0,015				0,009	0,009	0,009
50S ribosomal protein L7/L12	0,037	0,027			0,039		0,024	0,059		0,036
ribonucleotide-diphosphate reductase subunit beta	0,006			0,008	0,007			0,005	0,005	0,005
elongation factor Ts	0,008		0,009		0,006			0,006	0,006	0,013
hypothetical protein MHP7448_0662	0,006				0,006	0,007		0,005	0,005	0,005
heat shock ATP-dependent protease	0,002				0,002	0,002	0,003	0,002		0,002
XAA-Pro aminopeptidase	0,008		0,016			0,007		0,005	0,005	0,005
fructose-bisphosphate aldolase	0,008	0,011			0,007	0,009		0,006		
phosphocarrier protein HPr	0,024					0,029		0,021	0,022	0,022
hypothetical protein MHP7448_0373	0,003	0,003		0,003		0,002				0,002
50S ribosomal protein L6	0,013					0,014	0,016		0,011	0,01

asparaginyl-tRNA synthetase	0,006	0,006	0,005	0,006						
3-keto-L-gulonate-6-phosphate decarboxylase	0,024	0,030	0,011	0,030						
myo-inositol catabolism protein	0,005				0,003			0,005	0,006	
30S ribosomal protein S8	0,014				0,015			0,013	0,014	
50S ribosomal protein L27	0,026				0,024	0,031				0,023
lysyl-tRNA synthetase	0,004				0,004	0,005		0,003		
30S ribosomal protein S4	0,009				0,009			0,008	0,009	
ribosome recycling factor	0,013				0,011		0,016		0,011	
50S ribosomal protein L15	0,013					0,016		0,012		0,012
purine-nucleoside phosphorylase	0,008							0,008	0,008	
phosphoglycerate kinase	0,006		0,006			0,006				
phenylalanyl-tRNA synthetase alpha chain	0,007		0,011							0,002
6-phosphofructokinase	0,009		0,008	0,009						
transcription elongation factor	0,022				0,026		0,017			
glucose-6-phosphate isomerase	0,006				0,005	0,006				
cell division protein	0,003				0,003					0,002
lipase-esterase	0,008					0,009		0,006		
hypothetical protein MHP7448_0675	0,003						0,003			0,002
50S ribosomal protein L19	0,015							0,014	0,015	
amino acid permease	0,006	0,006								

hypothetical protein MHP7448_0483	0,016			0,016						
myo-inositol 2-dehydrogenase	0,006				0,006					
recombination protein RecR	0,010				0,010					
50S ribosomal protein L21	0,020				0,020					
Lppt protein	0,002				0,002					
triosephosphate isomerase	0,008				0,008					
transcriptional regulator	0,009				0,009					
30S ribosomal protein S20	0,029					0,029				
30S ribosomal protein S9	0,019					0,019				
XAA-Pro aminopeptidase	0,007					0,007				
50S ribosomal protein L6	0,016						0,016			
50S ribosomal protein L11	0,012								0,012	
periplasmic sugar-binding proteins	0,005								0,005	
elongation factor P	0,010								0,01	
acyl carrier protein phosphodiesterase	0,009									0,009
leucyl aminopeptidase	0,007	0,007								

[1] MFPL (Modified Friis with increase of double amounts of Phenol red), containing 5% (v/v) swine serum.

[2] 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).

[3] Protein identification according to NCBI database (http://www.ncbi.nlm.nih.gov).

[4] Protein emPAI average from biological and technical replicates. All positive identifications were used to calculate the average.

Supplementary Table 1: Biological and technical replicates emPAI value of LC-MS/MS identified proteins in *M. hyopneumoniae* strain 7448 growth in MFPB.

		MFPB[1]								
		emPAI[2]								
		1ª Biological replicate			2ª Biological replicate			3ª Biological replicate		
Protein[3]	emPAI Average[4]	Technical replicate 1	Technical replicate 2	Technical replicate 3	Technical replicate 1	Technical replicate 2	Technical replicate 3	Technical replicate 1	Technical replicate 2	Technical replicate 3
protein P97	0,031	0,015	0,010	0,014	0,050	0,040	0,036	0,037	0,039	0,042
p76 membrane protein precursor	0,019	0,016	0,015	0,017	0,026	0,029	0,016	0,016	0,022	0,016
putative p216 surface protein	0,015	0,011	0,008	0,01	0,015	0,011	0,017	0,024	0,022	0,021
NADH oxidase	0,045	0,048	0,073	0,047	0,072	0,017	0,014	0,046	0,048	0,038
46K surface antigen precursor	0,034	0,033	0,019	0,004	0,031	0,069	0,034	0,037	0,031	0,045
adhesin like-protein P146	0,010	0,009	0,005	0,007	0,009	0,015	0,004	0,016	0,016	0,010
L-lactate dehydrogenase	0,049	0,038	0,043	0,037	0,056	0,078	0,021	0,065	0,055	0,052
pyruvate dehydrogenase	0,095	0,150	0,121	0,147	0,039	0,090	0,073	0,072	0,062	0,097
elongation factor Tu	0,035	0,015	0,009	0,027	0,031	0,044	0,058	0,047	0,032	0,055
molecular chaperone DnaK	0,036	0,040	0,037	0,044	0,032	0,029	0,030	0,029	0,053	0,034
glyceraldehyde 3-phosphate dehydrogenase	0,027	0,034	0,031	0,042	0,018	0,025	0,031	0,029	0,022	0,013
ABC transporter xylose-binding lipoprotein	0,017	0,018	0,017	0,018	0,028	0,018	0,022	0,010	0,010	0,010

thiol peroxidase	0,040	0,011	0,023	0,024	0,037	0,024	0,100	0,047	0,070	0,028
hypothetical protein MHP7448_0662	0,007	0,006	0,003	0,003	0,010	0,013	0,011	0,007	0,007	0,007
dihydrolipoamide dehydrogenase	0,008	0,010	0,012	0,013	0,005	0,008	0,006	0,011	0,007	0,004
thioredoxin	0,065	0,066	0,061	0,065	0,099	0,036	0,029	0,075	0,077	0,074
prolipoprotein p65	0,013	0,013	0,012	0,009	0,009	0,012	0,005	0,014	0,023	0,022
pyruvate dehydrogenase E1-alpha subunit	0,047	0,081	0,075	0,070	0,024	0,021	0,027	0,041	0,042	0,040
thioredoxin reductase	0,012	0,013	0,012	0,012	0,010	0,013	0,011	0,007	0,015	0,014
protein P102	0,009	0,004	0,004	0,006	0,003	0,013	0,014	0,012	0,010	0,012
elongation factor G	0,009	0,008	0,013	0,014	0,004	0,005	0,004	0,013	0,013	0,006
lipoprotein	0,006	0,005	0,005	0,007	0,004	0,005	0,013	0,006	0,009	0,003
hypothetical protein MHP7448_0009	0,006	0,006	0,005	0,002	0,004	0,012	0,010	0,003	0,007	0,003
aminopeptidase	0,022	0,017	0,022	0,017	0,026		0,019	0,027	0,020	0,027
trigger factor	0,012	0,013	0,012	0,008	0,020	0,009	0,007	0,015	0,005	0,005
phosphopyruvate hydratase	0,009	0,004	0,008	0,004	0,007	0,009		0,015	0,016	0,010
PTS system enzyme IIB component	0,061	0,022		0,021	0,075	0,045	0,149	0,057	0,059	0,056
hypothetical protein MHP7448_0373	0,004		0,002	0,002	0,006	0,008	0,006	0,002	0,002	0,002
fructose-bisphosphate aldolase	0,010	0,014	0,006	0,007	0,010	0,015	0,012	0,008	0,008	
methionine sulfoxide reductase B	0,020	0,013	0,012	0,012	0,019	0,059		0,014	0,015	0,014
50S ribosomal protein L7/L12	0,033		0,035		0,057	0,034	0,028	0,043	0,019	0,018
phosphotransacetylase	0,015	0,013	0,012	0,019	0,019	0,012	0,010	0,022		

hypothetical protein MHP7448_0257	0,036	0,024	0,052	0,055			0,039	0,027	0,028	0,026
3-keto-L-gulonate-6-phosphate decarboxylase	0,011	0,008	0,008	0,008				0,009	0,022	0,009
adenine phosphoribosyltransferase	0,024				0,017	0,024	0,019	0,028	0,029	0,028
methylmalonate-semialdehyde dehydrogenase	0,011	0,017	0,016	0,017		0,008	0,006		0,004	
XAA-Pro aminopeptidase	0,011	0,011	0,005	0,011			0,019	0,013	0,006	
acetate kinase	0,011	0,015	0,014	0,01			0,017	0,006	0,006	
ribosome recycling factor	0,012	0,011	0,01	0,011				0,012	0,013	0,012
ribonucleotide-diphosphate reductase subunit beta	0,007	0,005	0,005	0,005	0,008	0,011		0,006		
50S ribosomal protein L27	0,031	0,024	0,022	0,024	0,036	0,050				
pyruvate kinase	0,008	0,004	0,004	0,004	0,012	0,017				
phosphoglycerate kinase	0,006	0,004	0,004	0,004	0,007	0,009				
hypothetical protein MHP7448_0377	0,007	0,011	0,005	0,005				0,006		0,006
purine-nucleoside phosphorylase	0,011	0,008				0,017	0,014	0,009		0,009
nucleoid DNA-binding protein	0,024		0,019		0,031			0,024	0,023	
hypothetical protein MHP7448_0252	0,015	0,021	0,019	0,009				0,011		
5'-nucleotidase precursor	0,004	0,003	0,003	0,003		0,007				
transketolase	0,004	0,003	0,003					0,004	0,004	
phosphocarrier protein HPr	0,023	0,023	0,022	0,021						0,025
spermidine/putrescine ABC transporter ATP-binding	0,004	0,004	0,004	0,004					0,004	

mannose-6-phosphate isomerase	0,007	0,005					0,01		0,007	0,006
lipoate-protein ligase A	0,007	0,005	0,01	0,005						
cell division protein	0,003			0,002			0,004		0,003	
DNA-directed RNA polymerase subunit alpha	0,009	0,006	0,011		0,009					
oligoendopeptidase F	0,007					0,007		0,007	0,006	
triosephosphate isomerase	0,010		0,007	0,007		0,016				
transcription elongation factor	0,012	0,012	0,011							0,013
lipase-esterase	0,007	0,007		0,007						
phosphoenolpyruvate-protein phosphotransferase	0,003	0,003		0,003						
dihydrolipoamide acetyltransferase	0,010	0,006		0,013						
elongation factor Ts	0,015							0,007	0,023	
hypoxanthine-guanine phosphoribosyltransferase	0,012			0,011						0,012
leucyl aminopeptidase	0,004	0,004								
hexosephosphate transport protein	0,004	0,004								
F0F1 ATP synthase subunit alpha	0,004	0,004								
phenylalanyl-tRNA synthetase alpha chain	0,007		0,007							
myo-inositol catabolism protein	0,006		0,006							
hypothetical protein MHP7448_0477	0,006		0,006							
uracil phosphoribosyltransferase	0,009		0,009							

transcription elongation factor	0,011		0,011							
ATP binding protein	0,002			0,002						
ascorbate-specific PTS system enzyme IIC	0,003			0,003						
heat shock protein	0,007			0,007						
30S ribosomal protein S8	0,022				0,022					
triosephosphate isomerase	0,016					0,016				
30S ribosomal protein S5	0,018					0,018				
hypothetical protein MHP7448_0483	0,017						0,017			
L-ribulose-5-phosphate 4-epimerase	0,013						0,013			
50S ribosomal protein L6	0,018						0,018			
hypothetical protein MHP7448_0159	0,018									0,018
6-phosphofructokinase	0,006									0,006

[1] MFPB (Modified Friis with increase of double amounts of Phenol red), containing 25% (v/v) bovine serum.

[2] 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).

[3] Protein identification according to NCBI database (http://www.ncbi.nlm.nih.gov).

[4] Protein emPAI average from biological and technical replicates. All positive identifications were used to calculate the average.

9. CURRICULUM VITAE

REOLON, LA.

1. DADOS PESSOAIS

Nome:

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Local e data de nascimento:

16/02/1985 - Ronda Alta, Rio Grande do Sul, Brasil

Endereço profissional:

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2. FORMAÇÃO:

Formação acadêmica/titulação

2010 Doutorado em andamento em Biologia Celular e Molecular (Conceito CAPES 6).

Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Título: indefinido,

Orientador: Henrique Bunselmeyer Ferreira.

Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

Grande área: Ciências Biológicas / Área: Genética / Subárea: Genética Molecular e de Microorganismos.

2008 – 2010 Mestrado em Biologia Celular e Molecular (Conceito CAPES 6).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.
Título: Análise de proteínas que ligam ao DNA de *Mycoplasma hyopneumoniae* 7448,
Ano de Obtenção: 2010.
Orientador: Irene Silveira Schrank.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.
Palavras-chave: Mycoplasma hyopneumoniae; Promotores; genômica; proteômica; RNAP.
Grande área: Ciências Biológicas / Área: Genética / Subárea: Genética Molecular e de Microorganismos.

2008 – 2010 Especialização em Formação de Especialistas em Acupuntura.
Colégio Brasileiro de Estudos Sistêmicos, CBES, Brasil.
Título: Acupuntura e infertilidade: uma visão atual..
Orientador: Deise Mernak.

2004 – 2007 Graduação em Biomedicina.
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.
Título: Caracterização Funcional de Promotores de *Mycoplasma hyopneumoniae*.
Orientador: Irene Silveira Schrank.

Formação complementar

2014 - Integração Curricular e Educação Interprofissional. (Carga horária: 20h).
Laureate International Universities.
2013 - Curso de Formação de Cipistas. (Carga horária: 20h).
Centro Universitário Ritter dos Reis, UniRITTER, Brasil.
2013 - Professores Laureate no Século XXI. (Carga horária: 20h).
Laureate International Universities.
2013 - Aprendizado Cooperativo. (Carga horária: 20h).
Laureate International Universities.

2013 - Academia de Lideranças - Planejamento Estratégico. (Carga horária: 4h).
Business School São Paulo Ltda.
2013 - Academia de Lideranças - Módulo Gestão Financeira. (Carga horária: 4h).
Business School São Paulo Ltda.
2013 - Academia de Lideranças - Módulo Gestão de Pessoas. (Carga horária: 4h).
Business School São Paulo Ltda.
2013 - Academia de Lideranças - Módulo Resultados. (Carga horária: 4h).
Business School São Paulo Ltda.
2013 - Academia de Lideranças - Módulo Comunicação. (Carga horária: 4h).
Business School São Paulo Ltda.
2013 - Capacitação docente - Módulo Estrutura Humana. (Carga horária: 11h).
Centro Universitário Ritter dos Reis, UniRITTER, Brasil.
2013 - Capacitação docente - Aprendizagem por Competências. (Carga horária: 4h).
Centro Universitário Ritter dos Reis, UniRITTER, Brasil.
2013 - Capacitação docente - Módulo Simulação Clínica. (Carga horária: 16h).
Centro Universitário Ritter dos Reis, UniRITTER, Brasil.
2013 - Capacitação docente - Módulo Processos Biológicos. (Carga horária: 16h).
Centro Universitário Ritter dos Reis, UniRITTER, Brasil.
2012 - Curso de Formação de Cipistas. (Carga horária: 20h).
Centro Universitário Ritter dos Reis, UniRITTER, Brasil.
2007 - Extensão universitária em Introdução à Biologia Molecular Computacional. (Carga horária: 35h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

3. ESTÁGIOS:

Bolsas de Iniciação Científica

2006 – 2008 Laboratório de Microorganismos Diazotróficos Centro de Biotecnologia/UFRGS.
Projeto: Caracterização funcional de promotores de *Mycoplasma hyopneumoniae.*
Bolsista de Iniciação Científica – PIBIC/CNPq, Carga horária: 20h.
Orientadora: Irene Silveira Schrank.

2005 – 2006 Laboratório de Lectinas Bioquímica/UFRGS.
Projeto: Purificação da Lectina de Mikania Laevigata por Cromatografia em Coluna de Estroma-Acrilamida.
Bolsista de Iniciação Científica - FAPERGS, Carga horária: 20h.
Orientadora: Magdolna Maria Vozari Hampe.

Estágio curricular supervisionado

2008 Laboratório e Banco de Sangue Marques Pereira.
Estágio Supervisionado em Análises Clínicas.
Carga-horária: 500h

2008 Laboratório de Microorganismos Diazotróficos Centro de Biotecnologia/UFRGS.
Estágio Supervisionado em Pesquisa – Biologia Molecular.
Carga-horária: 500h

4. EXPERIÊNCIA PROFISSIONAL OU DIDÁTICA ANTERIOR

Centro Universitário Ritter dos Reis, UniRITTER, Brasil.

2012 – Atual Vínculo: Professor, Enquadramento Funcional: Tempo Integral, Carga horária: 40

Atividades

03/2012 – Atual Direção e administração, Faculdade de Ciências da Saúde/UniRitter, Curso de Graduação em Biomedicina.
Cargo ou função: Coordenador de Curso.

03/2012 – Atual Ensino, Faculdade de Saúde UniRitter, Nível: Graduação.
Disciplinas ministradas (Biomedicina):
Práticas em Biomedicina.
Bioética e Legislação.
Tecnologia Genética e Diagnóstico Molecular.
Módulo Processos Biológicos - Biologia Celular, Biologia Molecular, Bioquímica e Genética.

Disciplinas ministradas (Enfermagem, Farmácia, Fisioterapia, Nutrição e Medicina Veterinária):

Biologia Celular.

Módulo Processos Biológicos - Biologia Celular, Biologia Molecular, Bioquímica e Genética.

2013 - 2014 Direção e administração, Faculdade de Ciências da Saúde/UniRitter**.**

Cargo ou função: Coordenação do Módulo Processos Biológicos - Biologia Celular, Biologia Molecular, Bioquímica e Genética.

07/2012 - 06/2013 Direção e administração, Faculdade de Ciências da Saúde/UniRitter.

Cargo ou função: Coordenador do Curso de Graduação em Farmácia.

09/2011 - 03/2012 Conselhos, Comissões e Consultoria, UniRitter.

Cargo ou função: Consultor área biomédica.

5. ARTIGOS COMPLETOS PUBLICADOS

- REOLON, L. A.; MARTELLO C.L.; SCHRANK I.S., & FERREIRA H.B. Survey of surface proteins from the pathogenic Mycoplasma hyopneumoniae strain 7448 using a biotin cell surface labeling approach. PLoS. One., 9(11): e112596, 2014.

- MARTELLO, C.; LEAL, F.; VIRGINIO, V.; REOLON, L.; SCHRANK, I.; ZAHA, A. & FERREIRA, H. Orthologous surface proteins from Mycoplasma hyopneumoniae and Mycoplasma flocculare: in silico comparison and heterologous expression of differential extracellular domains. BMC Proceedings, v. 8, p. P157, 2014.

- SIQUEIRA, F. M.; THOMPSON C.E.; VIRGINIO V.G.; GONCHOROSKI T.; REOLON L.; ALMEIDA L.G.; DA FONSECA M.M.; DE S.R.; PROSDOCIMI F.; SCHRANK I.S.; FERREIRA H.B.; DE VASCONCELOS A.T., & ZAHA A. New insights on the biology of swine respiratory tract mycoplasmas from a comparative genome analysis. BMC. Genomics, 14: 175, 2013.

- MOITINHO-SILVA, L.; HEINECK B.L.; REOLON L.A.; PAES J.A.; KLEIN C.S.; REBELATTO R.; SCHRANK I.S.; ZAHA A., & FERREIRA H.B. Mycoplasma hyopneumoniae type I signal peptidase: expression and evaluation of its diagnostic potential. Vet. Microbiol., 154(3-4): 282-291, 2012.

- SANT'ANNA, F. H.; ALMEIDA L.G.; CECAGNO R.; REOLON L.A.; SIQUEIRA F.M.; MACHADO M.R.; VASCONCELOS A.T., & SCHRANK I.S. Genomic insights into the versatility of the plant growth-promoting bacterium Azospirillum amazonense. BMC. Genomics, 12: 409, 2011.

6. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

Resumos publicados em anais de congressos

- REOLON, L. A.; WEBER, S. S.; LOPES, B. M. T.; BITTENCOURT, B. G. F.; SILVA, S. C.; VAINSTEIN, M. H.; SCHRANK, I. S. In vivo promoter activities in Mycoplasma hyopneumoniae: evaluation of a reporter system. In: X Congresso Argentino de Microbiologia, 2007, Córdoba. Revista Argentina de Microbiologia. Buenos Aires: Associacion Argentina de Microbiologia. v. 39. p. 47-47.

- LOPES, B. M. T.; REOLON, L. A.; BITTENCOURT, B. G. F.; VAINSTEIN, M. H.; SCHRANK, I. S.; SILVA, S. C. Mycoplasma hyopneumoniae: transformation with a new replicative vector. In: Congresso Argentino de Microbiologia, 2007, Cordoba. Revista Argentina de Microbiologia. Buenos Aires: Associação argentina de microbiologia. v. 39. p. 47-47.

- REOLON, L. A.; WEBER, S. S.; SILVA, S. C.; SCHRANK, I. S. Atividade in vivo de promotores de Mycoplasma hyopneumoniae: avaliação de um sistema reportes. In: XIX Salão de Iniciação Científica e XVI Feira de Iniciação Científica da UFRGS, 2007, Porto Alegre. Livro de Resumos - XIX Salão de Iniciação Científica e XVI Feira de Iniciação Científica da UFRGS. Porto Alegre: UFRGS, 2007. p. 456-457.

- REOLON, L. A.; WEBER, S. S.; SILVA, S. C.; SCHRANK, I. S. Caracterização funcional de promotores de Mycoplasma hyopneumoniae. In: XVIII SALÃO DE INICIAÇÃO CIENTÍFICA; XV FEIRA DE INICIAÇÃO CIENTÍFICA, 2006, Porto Alegre. Livro de Resumos - XVIII SALÃO DE INICIAÇÃO CIENTÍFICA; XV FEIRA DE INICIAÇÃO CIENTÍFICA. Porto Alegre: UFRGS, 2006.

- REOLON, L. A.; Hampe M. M. V.; Zanetti G. D.; Dresch R. R. . Purificação da Lectina de Mikania Laevigata por Cromatografia em Coluna de Estroma-Acrilamida. In: XVII Salão de Iniciação Científica UFRGS, 2005, Porto Alegre. Anais do Salão de Iniciação Científica 2005 - UFRGS, 2005.

Apresentações de Trabalho

- REOLON, L. A.; WEBER, S. S.; SCHRANK, I. S. Atividade in vivo de promotores de Mycoplasma hyopneumoniae: avaliação de um sistema reporter. 2007. (Apresentação de Trabalho/Outra).

- REOLON, L. A.; WEBER, S. S.; SILVA, S. C.; SCHRANK, I. S. Caracterização Funcional de Promotores de Mycoplasma hyopneumoniae. 2006. (Apresentação de Trabalho/Outra).

- REOLON, L. A.; Zanetti G. D.; Dresch R. R.; Hampe M. M. V. Purificação da lectina de Mikania laevigata por cromatografia em coluna de estroma acrilamida. 2005. (Apresentação de Trabalho/Outra).