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

Identificação de determinantes de patogenicidade de *Mycoplasma hyopneumoniae*

Tese de Doutorado

Jéssica Andrade Paes Vieira

Porto Alegre, dezembro de 2018

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS, como requisito parcial para a obtenção do grau de Doutor em Biologia Celular e Molecular

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ÍNDICE

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

µg – microgramas
µl - microlitros
A – adenina
ABPA – Associação Brasileira de Proteína Animal
CDS – sequência de DNA codificadora, do inglês *coding DNA sequence*
DAMPs - padrões moleculares associados ao dano, do inglês *damage-associated molecular patterns*
DNA – ácido desoxirribonucleico, do inglês *deoxyribonucleic acid*
ELISA – ensaio de imunoabsorção ligado à enzima, do inglês, *enzyme-linked immunosorbent assay*
emPAI – do inglês *exponentially modified protein abundance index*
G – guanina
G + C – guanina e citosina
GO – ontologia gênica, do inglês *gene ontology*
IBGE – Instituto Brasileiro de Geografia e Estatística
IL - interleucina
kb – quilobase(s)
LC-MS/MS – cromatografia líquida acoplada à espectrometria de massas em tandem, do inglês *liquid chromatography tandem-mass spectrometry*
mg – miligramas
ml – mililitros
NSAF – do inglês *normalized spectral abundance factor*
ORF – fase aberta de leitura, do inglês *open reading frame*
PCR – reação em cadeia da polimerase, do inglês, *polymerase chain reaction*
PES – pneumonia enzoótica suína
PRDC - complexo de doenças respiratórias suínas
PRRSV - vírus da síndrome suína reprodutiva e respiratória
PS – soro suíno, do inglês *porcine serum*
RNA – ácido ribonucleico, do inglês *ribonucleic acid*
ROS – espécies reativas de oxigênio, do inglês *reactive oxygen species*
rRNA – ácido ribonucleico ribossômico, do inglês *ribosomal ribonucleic acid*
SIV - vírus da influenza suíno
sRNA – pequeno RNA, do inglês, *small* RNA
TNF – fator de necrose tumoral, do inglês tumor necrosis factor
U – uracila
USDA – Departamento de Agricultura dos Estados Unidos, do inglês *United States Department of Agriculture*
UT – unidade transcricional

LISTA DE FIGURAS E TABELAS

Introdução

Seção 4.1

Seção 4.2

Seção 4.3

RESUMO

Mycoplasma hyopneumoniae e *Mycoplasma flocculare* coabitam o trato respiratório suíno, são geneticamente similares e compartilham a maioria dos genes conhecidos como codificadores de fatores de virulência. Entretanto, *M. hyopneumoniae* é patogênico, sendo o agente etiológico da pneumonia enzoótica suína (PES), enquanto *M. flocculare* é comensal. Neste contexto, o objetivo deste estudo foi analisar comparativamente os proteomas e secretomas de *M. hyopneumoniae* e *M. flocculare*, a fim de identificar diferenças relacionadas a determinação de patogenicidade de *M. hyopneumoniae*. Para isso, uma abordagem de fracionamento celular combinada a espectrometria de massas (LC-MS/MS) foi utilizada para analisar comparativamente os proteomas intracelular, de superfície e os secretomas de *M. hyopneumoniae* e *M. flocculare*. Mais de 50% das proteínas preditas nos proteomas dos micoplasmas analisados foram identificadas, incluindo diversas proteínas diferencialmente representadas entre *M. hyopneumoniae* e *M. flocculare*. Entre estas proteínas, podem-se destacar aquelas relacionadas a processos biológicos envolvidos na interação micoplasmas-hospedeiro, que incluem adesão, processamento proteolítico, proteção contra estresses, e vias do metabolismo energético e de síntese proteica, as quais foram consideradas potenciais determinantes de patogenicidade de *M. hyopneumoniae*. Além disso, a análise comparativa dos secretomas de *M. hyopneumoniae* e *M. flocculare* demonstrou que os repertórios de proteínas secretadas por estas micoplasmas são diferenciais, sendo que diversos potenciais determinantes de patogenicidade foram detectados como secretados por *M. hyopneumoniae*. Os proteomas das linhagens *M. hyopneumoniae* 7448 (patogênica) e *M. hyopneumoniae* J (não-patogênica) também foram comparativamente analisados em condições de estresse oxidativo e térmico. Interessantemente, diversos potenciais determinantes de patogenicidade foram detectados em maior abundância em *M. hyopneumoniae* 7448 em situações de estresse, sugerindo que o estresse pode atuar como gatilho para expressão destas proteínas nesta micoplasma. Em conclusão, as análises proteômicas comparativas realizadas permitiram a identificação de mais de uma centena de proteínas diferencialmente representadas entre *M. hyopneumoniae* e *M. flocculare*, muitas das quais são potenciais determinantes de patogenicidade de *M. hyopneumoniae*.

Palavras-chave: Proteômica comparativa; *Mycoplasma hyopneumoniae*; *Mycoplasma flocculare*; determinantes de patogenicidade

ABSTRACT

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* coinhabit the porcine respiratory tract, are genetically similar and share most of the known virulence factor coding genes. However, *M. hyopneumoniae* is pathogenic, being the etiological agent of porcine enzootic pneumonia (PEP) while *M. flocculare* is commensal. In this context, the aim of this study was comparatively analyze the proteomes and secretomes of *M. hyopneumoniae* and *M. flocculare*, in order to identify differences related to *M. hyopneumoniae* pathogenicity determination. For that, a cell fractioning approach combined to mass spectrometry was used to analyze the intracellular and surface proteomes, and the secretomes of *M. hyopneumoniae* and *M. flocculare.* Over 50% of proteins predicted in the analyzed mycoplasmas proteomes were identified, including many differentially represented proteins between *M. hyopneumoniae* and *M. flocculare*. Among these proteins, were of particular interest those related to biological processes involved in mycoplasma-host relationship, which include adhesion, post-translation proteolytical processing, stress protection, and energetic metabolism and protein synthesis pathways, which were considered potential pathogenicity determinants of *M. hyopneumoniae*. Moreover, the comparative analyses of *M. hyopneumoniae* and *M. flocculare* secretomes demonstrated that the secreted protein repertoires of these mycoplasmas are differential, once several potential pathogenicity determinants were detected as secreted by *M. hyopneumoniae*. The proteomes of the strains *M. hyopneumoniae* 7448 (pathogenic) and *M. hyopneumoniae* J (non-pathogenic) were also comparatively analyzed in oxidative and heat stress conditions. Interestingly, several potential pathogenicity determinants were detected in higher abundance in *M. hyopneumoniae* 7448 in stress conditions, suggesting that the stress may act as a trigger for the expression of these proteins. Overall, the performed proteomics comparative analyses allowed the identification of more than one hundred differentially represented proteins between *M. hyopneumoniae* and *M. flocculare*, many of which are potential pathogenicity determinants.

Key-words: Comparative proteomics; *Mycoplasma hyopneumoniae*; *Mycoplasma flocculare*; pathogenicity determinants.

1. INTRODUÇÃO

1.1 A suinocultura no mundo e no Brasil

A carne suína ocupa o segundo lugar no ranking das carnes mais produzidas e consumidas no mundo, alcançando mais de 110 milhões de toneladas em 2016 (USDA, 2016). Entre os anos de 2005 e 2015, a produção mundial de carne suína cresceu, em média, 1,6% ao ano, percentual superior ao verificado, no mesmo período, para a carne bovina (0,4% ao ano). Entretanto, a quantidade relativa de carne suína transacionada internacionalmente é menor que as das demais carnes, o que pode ser explicado por questões de segurança alimentar e por restrições de natureza religiosa. Neste contexto, os cinco países maiores produtores de carne suína, como China, União Europeia, EUA, Rússia e Brasil, também são os cinco maiores consumidores, sendo responsáveis por mais de 80% da produção e consumo mundial dessa carne.

Em 2015, o Brasil foi o quarto maior produtor e exportador mundial de carne suína. Produziu pouco mais de 3,5 milhões de toneladas, representando cerca de 3% do total mundial (USDA, 2016). Em relação às exportações, o Brasil respondeu, no mesmo ano, por quase 9% do total mundial em volume. Considerando a produção de carnes de frango, bovina e pescados, a carne suína está em terceiro lugar no Brasil. Além disso, entre 2005 e 2015, a produção brasileira de carne suína cresceu 3,2% ao ano.

A concentração regional da produção de carne suína está na Região Sul, responsável, em 2015, por 67% dos abates com algum tipo de fiscalização (federal, estadual ou municipal). As Regiões Sudeste e Centro-Oeste foram responsáveis por 18% e 14% da produção de carne suína, respectivamente, enquanto as regiões Norte e Nordeste produziram apenas 1%. Dentre os Estados, destacaram-se os de Santa Catarina, com 27% do total, do Rio Grande do Sul e do Paraná, com 20% cada (IBGE, 2016).

Em relação ao consumo, a carne suína é a terceira mais consumida no país, sendo o mercado interno o principal destino da produção nacional (85% em 2015). Entre 2005 e 2015, o consumo nacional de carne suína aumentou cerca de 30%. A elevação do consumo de carne suína foi provocada não só pelo aumento do poder de compra das camadas mais pobres da população no período, que aumentou o consumo geral de carnes, mas, sobretudo, pela redução do preço relativo da carne suína diante da carne bovina e da carne de frango no Brasil (Ipeadata, 2016).

Nos últimos anos, o Brasil tem exportado aproximadamente 15% da sua produção de carne suína, sendo 80% dos cortes destinados para mais de 70 países, incluindo Rússia

(45%) e Hong Kong (23%) (ABPA, 2016). As exportações não são maiores em razão, sobretudo, de barreiras sanitárias de alguns países que são grandes importadores como a China e os Estados Unidos. Atualmente, negociações comerciais e avanços no reconhecimento internacional da sanidade da suinocultura brasileira têm auxiliado na reversão parcial de algumas das barreiras impostas a exportação de carne suína. Como exemplo, Estados Unidos e Japão permitem a importação de carne suína oriunda de Santa Carina, que é o único estado livre de febre aftosa sem vacinação. Neste contexto, o aumento das exigências sanitárias e do bem-estar dos animais nas granjas, atendendo não só à demanda dos consumidores, mas também às mudanças nas legislações nacionais e internacionais é importante para melhorar os índices de exportação da carne suína brasileira.

1.2 Doenças respiratórias suínas e pneumonia enzoótica suína

Doenças de trato respiratório suíno são responsáveis por significantes perdas econômicas para suinocultura. Entre estas doenças, a pneumonia se destaca por ser uma das doenças respiratórias mais frequentes em granjas de suínos, cuja prevalência varia de 19% a 79% (Fablet *et al.*, 2012). A bactéria *Mycoplasma hyopneumoniae* é o patógeno primário associado com a pneumonia enzoótica suína (PES). Os principais sinais clínicos da PES são tosse seca e não-produtiva, redução da taxa de crescimento e aumento de taxa de conversão alimentar, sendo caracterizada como uma doença que causa alta morbidade, mas baixa mortalidade. Lesões pulmonares características da infecção por *M. hyopneumoniae* consistem em manchas roxas ou cinzas na área de consolidação pulmonar (Garcia-Morante *et al.*, 2016). Além disso, ocorre infiltração de células inflamatórias nos alvéolos e brônquios e acumulação de linfócitos nos vasos sanguíneos das vias aéreas de suínos afetados pela PES.

Suínos infectados por *M. hyopneumoniae* podem ser coinfectados com outros patógenos respiratórios, incluindo as bactérias *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica* e *Pasteurella multocida*, e os vírus da síndrome suína reprodutiva e respiratória (PRRSV), o circovírus suíno tipo 2 e o vírus da influenza suíno (SIV). Esta infecção polimicrobiana é clinicamente conhecida como complexo de doenças respiratórias suínas (PRDC). Assim como a PES, a PRDC é uma das doenças que causam mais prejuízos econômicos aos suinocultores, com perdas estimadas em mais de 600 milhões de dólares por ano (Holtkamp, 2014).

Diferentes estudos têm correlacionado a prevalência da infecção por *M. hyopneumoniae* com o aumento da severidade de doenças respiratórias, como a PRDC (Sibila *et al.*, 2008; Li *et al.*, 2015; Chae, 2016). Além disso, sabe-se que infecções por *M. hyopneumoniae* podem predispor os suínos a novas infecções. Neste contexto, estratégias de monitoramento, diagnóstico e prevenção da doença são importantes para combater a PES e a PRDC.

O monitoramento do status de saúde dos suínos reprodutores é importante para evitar o contágio dos demais suínos da granja. Este monitoramento é baseado principalmente nos sinais clínicos da PES. Entretanto, alguns animais podem possuir lesões pulmonares, mas sem apresentar os sinais clínicos, ou o animal pode apresentar os sinais clínicos da doença, mas estar infectado com outro patógeno. Neste contexto, o diagnóstico clínico da PES deve ser confirmado por exames laboratoriais.

O isolamento de *M. hyopneumoniae* a partir de suínos potencialmente infectados é o método diagnóstico considerado padrão-ouro. Entretanto, este método requer a utilização de meio de cultura especializado, é demorado e dispendioso, além de haver a possibilidade de contaminação por outras bactérias. Por outro lado, um estudo recente demonstrou uma nova formulação de meio de cultura mais adequado para o crescimento de *M. hyopneumoniae*, evitando contaminações por outros micoplasmas, como *Mycoplasma hyorhinis* (Cook *et al.*, 2016). Além do isolamento bacteriano, o método diagnóstico da PES mais comum é baseado na detecção de anticorpos anti-*M. hyopneumoniae*, como o ELISA. Entretanto, este teste não diferencia a presença de anticorpos derivados da mãe, ou anticorpos induzidos pela infecção, e/ou por vacinação (Thacker e Minion, 2010). Além do ELISA, testes moleculares como a PCR têm sido utilizados como uma alternativa para o diagnóstico de *M. hyopneumoniae*. Atualmente, novas estratégias de monitoramento/diagnóstico da PES em suínos têm sido propostas, baseadas na presença de sinais clínicos e na combinação de dois testes diagnósticos, como ELISA e PCR. Estas estratégias são focadas principalmente para suínos recém adquiridos, que constituem o maior risco de introdução da PES em granjas de suínos.

Além do monitoramento e diagnóstico da PES, estratégias de prevenção e controle são cruciais para manter os rebanhos suínos livres de *M. hyopneumoniae*. Entre estas estratégias estão o uso de antimicrobianos e vacinação. *M. hyopneumoniae* é resistente a antimicrobianos relacionados à polimerização de parede celular, como os beta-lactâmicos, sendo sensível tetraciclinas, macrolídeos, fluoroquinolonas, aminoglicosídeos e aminociclitois. A eficiência destes antimicrobianos tem sido

demonstrada em diversos estudos, sendo observado que suínos tratados têm a performance melhorada, além das lesões pulmonares e sinais clínicos diminuídos. Entretanto, estudos de campo têm demonstrado que os sinais clínicos da PES e a proliferação bacteriana reaparecem após o fim do tratamento. Neste contexto, estratégias de prevenção, como a vacinação, podem ser mais efetivas para o controle da PES.

A vacinação contra a PES é amplamente utilizada como uma estratégia de controle e prevenção da doença. Mundialmente, é estimado que 70% dos rebanhos suínos são vacinados contra *M. hyopneumoniae* (Martelli *et al.*, 2014). Atualmente, diferentes estratégias de vacinação têm sido adotadas, dependendo do tipo de rebanho, sistema de produção e condições de manejo (Maes *et al.*, 2018). A vacinação reduz os sinais clínicos e as lesões pulmonares causadas pela doença e aumenta a performance dos suínos, mas não previne a colonização bacteriana e não reduz os riscos de transmissão da doença (Villarreal *et al.*, 2011). As vacinas comerciais são chamadas de bacterinas e consistem em células bacterianas inativadas e adjuvantes, e são administradas por via intramuscular. Devido ao baixo grau de proteção conferido pelo uso das bacterinas, esforços constantes têm sido dirigidos para o desenvolvimento de novas formulações vacinais. Vários estudos têm avaliado diferentes proteínas recombinantes de *M. hyopneumoniae* e diferentes formulações e vias de imunização.

1.3 *Mycoplasma hyopneumoniae* e *Mycoplasma flocculare*

M. hyopneumoniae e *Mycoplasma flocculare* são espécies bacterianas que compartilham o mesmo habitat, o trato respiratório suíno, e possivelmente o mesmo nicho. *M. hyopneumoniae* é uma espécie patogênica, sendo agente etiológico da PES, enquanto *M. flocculare* é uma espécie comensal.

1.3.1 Taxonomia e evolução

M. hyopneumoniae e *M. flocculare* pertencem à classe Mollicutes, à ordem Mycoplasmatales e à família Mycoplasmataceae. A classe Mollicutes é composta por bactérias que apresentam em comum células de pequeno tamanho, ausência de parede celular, genoma de tamanho reduzido, baixo conteúdo de G + C, e vias metabólicas simplificadas (Sirand-Pugnet *et al.*, 2007). Os molicutes se reúnem em um ramo único da árvore filogenética do Domínio Bacteria e se derivaram de um ancestral comum do Filo Firmicutes, com baixo conteúdo de G + C, há 605 milhões de anos atrás (Razin *et al.*, 1998). Reconstruções evolutivas mostram que os molicutes se dividiram em dois ramos

principais: o ramo APP, que contém os gêneros *Acholeplasma*, *Anaeroplasma*, *Asteroleplasma* e o filo Phytoplasma; e o ramo SEM, que inclui os gêneros *Spiroplasma*, *Mesoplasma*, *Ureaplasma* e *Mycoplasma*.

Eventos evolutivos ocorridos no ramo SEM resultaram em mudanças dramáticas, como a necessidade de esteróis, que são componentes importantes da membrana plasmática, e a conversão do códon de terminação UGA para a codificação de triptofano (Sirand-Pugnet *et al.*, 2007). Devido ao baixo conteúdo de G + C dos molicutes, o aminoácido triptofano é mais frequentemente codificado pelo códon UGA do que pelo códon UGG nas espécies do ramo SEM (*Spiroplasma* spp., *Mesoplasma* spp., *Ureaplasma* spp. e *Mycoplasma* spp.).

A simplicidade biológica das espécies da classe Mollicutes sugeria que as bactérias pertencentes a este grupo eram organismos primitivos e, a partir deles, bactérias mais complexas teriam emergido durante a evolução. No entanto, um estudo baseado em análises de rRNA demonstrou que os molicutes apresentam características evolutivas diferentes de quase todas as bactérias, sendo considerados organismos taquitélicos (Woese *et al.*, 1984). Por isso, os molicutes não devem ser considerados como bactérias primitivas, mas sim organismos que evoluem mais rapidamente.

Análises baseadas na sequência nucleotídica de rRNA 16S demonstraram que *M. hyopneumoniae* e *M. flocculare* são filogeneticamente relacionados (Stemke *et al.*, 1992). Recentemente, análises filogenômicas demonstraram que *M. hyopneumoniae* e *M. flocculare* apresentam um ancestral comum mais recente e formam um grupo monofilético (Figura 1) (Siqueira *et al.*, 2013). Além disso, *M. hyopneumoniae* e *M. flocculare* formam um clado chamado hyopneumoniae dentro da família Mycoplasmataceae, juntamente com *Mycoplasma hyorhinis* e *Mycoplasma conjuctivae*. Sabe-se que a herança vertical de genes através de relações filogenéticas e a aquisição de novos genes relacionados à adaptação a habitats e modos de vida são forças complementares que modelam os genomas bacterianos (Tamames *et al.*, 2016). Como estas bactérias coabitam o trato respiratório suíno, as forças da herança vertical de genes e do ambiente podem explicar a relação filogenética próxima entre *M. hyopneumoniae* e *M. flocculare*, embora não explique os modos de vida distintos destas bactérias.

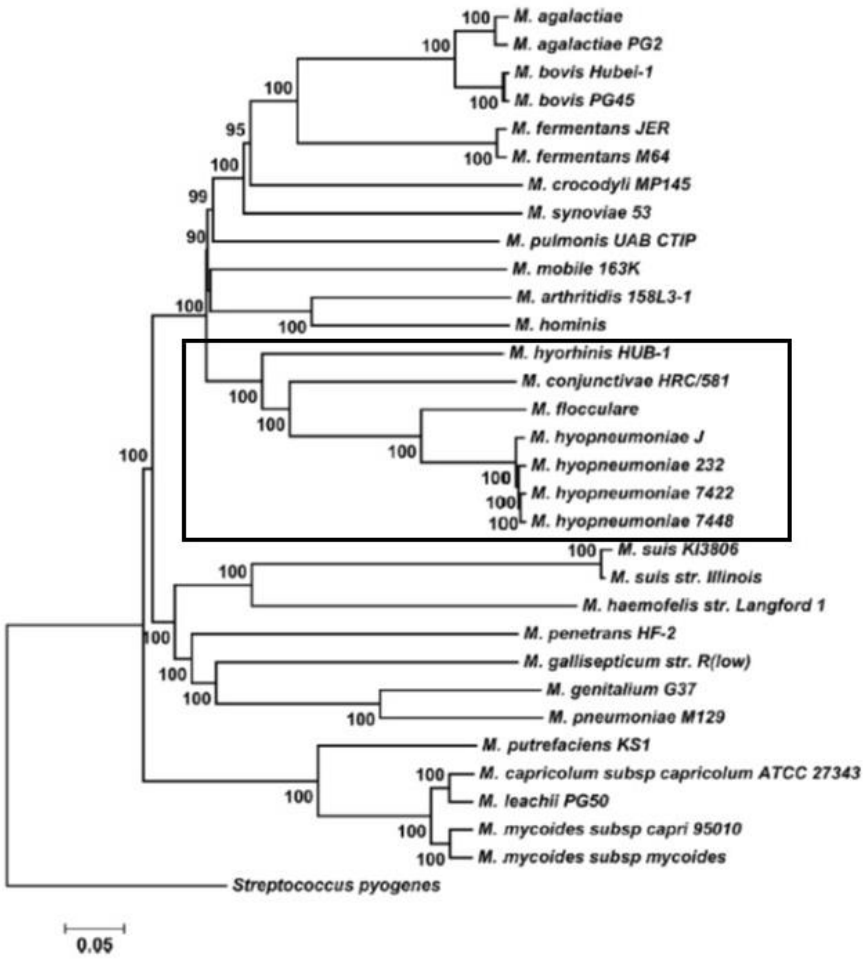

Figura 1: História evolutiva de micoplasmas obtida através de abordagens filogenômicas. O clado hyopneumoniae está destacado e contém *M. hyopneumoniae*, *M. flocculare*, *M. hyorhinis* e *M. conjunctivae*. Figura adaptada de Siqueira *et al.* (2013).

1.3.2 Estudos comparativos

Os genomas de *M. hyopneumoniae* e *M. flocculare* são compostos por um único cromossomo circular contendo aproximadamente 900 kb e 760 kb, respectivamente, e aproximadamente 28% de conteúdo de G + C (Vasconcelos 2005; Siqueira 2013). Análises genômicas comparativas entre *M. hyopneumoniae* e *M. flocculare* demonstraram a alta similaridade genética destas bactérias, que compartilham mais de 70% de seus genes. A análise dos transcritomas de *M. hyopneumoniae* e *M. flocculare* demonstrou que mais de 90% dos genes preditos são transcritos (Siqueira *et al.*, 2014).

Aproximadamente 18% das ORFs *M. hyopneumoniae* e *M. flocculare* estão organizadas em unidades transcricionais (UT), sendo que a distribuição de 78% destas UTs é total ou parcialmente conservada (Siqueira *et al.*, 2013). Apesar do alto grau de conservação, a análise comparativa revelou a presença de regiões contendo rearranjos ou

inversões gênicas em algumas UTs compartilhadas por *M. hyopneumoniae* e *M. flocculare*. Análises transcriptômicas posteriores comprovaram a coexpressão de genes organizados em UTs para *M. hyopneumoniae* e *M. flocculare* (Siqueira *et al.*, 2014).

Apesar da natureza comensal, o genoma de *M. flocculare* apresenta ortólogos para 90% dos genes preditos como codificadores de proteínas de superfície de *M. hyopneumoniae*, incluindo a maioria dos genes preditos como fatores de virulência (Ferreira e Castro, 2007; Siqueira *et al.*, 2013). Dentre os genes compartilhados, 85% apresentam domínios diferenciais, que incluem inserções/deleções de porções gênicas ou regiões diferenciais com menos de 30% de identidade de sequência entre o par de ortólogos (Leal *et al.*, 2016). Interessantemente, os 10% de genes preditos como codificadores de proteínas de superfície não compartilhados entre *M. hyopneumoniae* e *M. flocculare* correspondem a genes codificadores de proteínas hipotéticas.

Considerando potenciais fatores de virulência, o genoma de *M. flocculare* contém ortólogos para quase todas as adesinas de *M. hyopneumoniae*, com exceção de uma cópia das adesinas P97 e P102 (Siqueira *et al.*, 2013). Entretanto, as regiões gênicas contendo os ortólogos de adesinas apresentam inversões e rearranjos. Por exemplo, os genes codificadores das adesinas P216, P159 e P60 de *M. hyopneumoniae* são altamente conservados quando comparados entre linhagens patogênicas da mesma espécie, mas quando comparados com *M. flocculare* estes ortólogos apresentam rearranjos gênicos. Além disso, o gene codificador da adesina P97 (cópia 2) de *M. hyopneumoniae* apresenta um domínio relacionado a virulência (R1) (Deutscher *et al.*, 2010), que está ausente no ortólogo de *M. flocculare* (Siqueira *et al*., 2013). Análises transcriptômicas quantitativas demonstraram que apesar de que todos os genes codificadores de adesinas de *M. hyopneumoniae* e *M. flocculare* sejam transcritos, há diferenças nos níveis de expressão destes genes (Siqueira *et al.*, 2014). Curiosamente, os transcritos correspondentes as adesinas P97-like, P102-like, P97-cópia 2, P102-cópia 2, P95 e P60 foram encontrados em maior abundância das amostras de *M. flocculare* do que em *M. hyopneumoniae*.

A alta similaridade de composição e organização genômicas entre *M. hyopneumoniae* e *M. flocculare* também se reflete nas capacidades metabólicas destas espécies (Siqueira *et al.*, 2013). Análises *in silico* baseadas em predições de categorias funcionais (COG e KEGG) demonstraram grandes semelhanças no número de genes envolvidos em rotas metabólicas distintas. Além disso, foi demonstrado que os genes com maior nível de expressão para ambas micoplasmas são aqueles relacionados ao metabolismo basal (Siqueira *et al.*, 2014). Recentemente, análises metabolômicas

comparativas demonstraram que *M. hyopneumoniae* e *M. flocculare* apresentam capacidades metabólicas similares (Ferrarini *et al.*, 2016). Entretanto, a presença exclusiva de genes relacionados ao catabolismo de mio-inositol e do gene *glpO* (codificador da enzima glicerol-3-fosfato oxidase) em *M. hyopneumoniae* podem estar relacionadas com a patogenicidade desta bactéria.

A análise dos genomas de *M. hyopneumoniae* e *M. flocculare* permitiu a identificação de pequenos RNAs não-codificantes (sRNA), e a expressão de vários destes transcritos foi validada experimentalmente (Siqueira *et al.*, 2016). Interessantemente, dois sRNAs de *M. hyopneumoniae* foram diferencialmente expressos em condições de estresse oxidativo e/ou térmico, sugerindo que estes transcritos podem desempenhar um papel de RNA regulatórios em resposta estresse.

Em geral, os estudos comparativos realizados entre *M. hyopneumoniae* e *M. flocculare* demonstraram grandes semelhanças entre estas duas espécies bacterianas. Diferenças pontuais a nível de organização genômica, transcrição e metabolismo foram encontradas e podem ser correlacionadas com a patogenicidade de *M. hyopneumoniae* e ausência de patogenicidade de *M. flocculare*. No entanto, o compartilhamento da maioria dos potenciais fatores de virulência preditos entre *M. hyopneumoniae* e *M. flocculare* sugere que as naturezas patogênica e comensal destas espécies, respectivamente, podem ser explicadas a nível proteico, incluindo variações no nível de expressão de proteínas e presença de modificações pós-traducionais diferenciais.

Até o momento, apenas um estudo proteômico comparativo havia sido realizado entre linhagens patogênicas (7448 e 7422) e uma não-patogênica (J) de *M. hyopneumoniae* (Pinto *et al.*, 2009). A abundância diferencial de proteínas relacionadas a patogenicidade, como adesinas e proteínas de resposta ao estresse, foi observada, uma vez que estas proteínas foram mais abundantes nas amostras de linhagens patogênicas do que na linhagem não-patogênica. Além disso, este estudo evidenciou o processamento proteolítico diferencial entre linhagens de *M. hyopneumoniae* para adesina P97, uma vez que isoformas adicionais desta proteína foram exclusivamente detectadas nas amostras de *M. hyopneumoniae* 7448 e 7422.

1.4 Fatores de virulência de *M. hyopneumoniae*

M. hyopneumoniae depende essencialmente da adesão ao epitélio ciliar respiratório suíno para efetivamente colonizá-lo e estabelecer a infecção pulmonar. Este

micoplasma não possui organela de atracamento e, portanto, suas adesinas estão possivelmente distribuídas por toda a sua superfície (Hovind-Hougen e Friis, 1991; Reolon *et al.*, 2014; Raymond *et al.*, 2015). No processo de adesão de *M. hyopneumoniae* ao epitélio respiratório suíno, algumas adesinas são capazes de interagir com diversas proteínas do hospedeiro, como a heparina, a fibronectina e o plasminogênio (Jarocki *et al.*, 2015; Raymond *et al.*, 2015). Ao aderir no tecido epitelial, *M. hyopneumoniae* pode interferir na ação de receptores de membrana e nos mecanismos de transporte de íons e nutrientes das suas células-alvo. O bloqueio de canais de K^+ está entre os mecanismos de transporte de íons mais afetados. O bloqueio destes canais nas células ciliadas dos brônquios é a principal causa da ciliostase observada na PES (Deutscher *et al.*, 2010; Raymond *et al.*, 2015).

Muitas das proteínas de superfície, inclusive adesinas, de *M. hyopneumoniae* e de outros micoplasmas são antigênicas e podem estar envolvidas em outros mecanismos importantes para sobrevivência e patogenicidade, como os de mimetismo molecular e de plasticidade fenotípica (Li *et al.*, 2011). O mimetismo molecular de proteínas de superfície bacterianas, com o compartilhamento de epítopos entre elas e proteínas dos respectivos hospedeiros, atua possivelmente na evasão da resposta imune e/ou na indução da produção de autoanticorpos, a qual é observada durante a infecção por algumas espécies de micoplasmas (Zeng *et al.*, 2012). A plasticidade fenotípica, por sua vez, definida como a capacidade de um dado genótipo produzir diferentes fenótipos em resposta a diferentes variações ambientais (Forsman, 2014), também já foi descrita para proteínas de superfície de micoplasmas, muitas das quais são antigênicas. Nos casos descritos em *Mycoplasma genitalium*, *Mycoplasma hyorhinis* e *Mycoplasma hominis*, a plasticidade fenotípica é proporcionada por mecanismos de *DNA slippage*, rearranjo de DNA por recombinases sítio-específicas ou recombinação recíproca (Ma *et al.*, 2014; Chae, 2016). Em *M. hyopneumoniae*, variações no número de repetições em tandem de aminoácidos (VNTRs) em proteínas de superfície antigênicas já foram demonstradas entre isolados (De Castro *et al.*, 2006), o que também evidencia possíveis mecanismos de plasticidade fenotípica nesta espécie. Variação adicional no repertório de proteínas apresentadas na superfície de *M. hyopneumoniae* pode advir também de processamento proteolítico pós-traducional, já evidenciado para algumas adesinas e outras proteínas da bactéria (Pinto *et al.*, 2007; Seymour *et al.*, 2010; Bogema *et al.*, 2012; Deutscher *et al.*, 2012; Raymond *et al.*, 2015; Tacchi *et al.*, 2016b; Berry *et al.*, 2017). Assim, diferentes porções/domínios de uma dada proteína, podem estar diferencialmente representados na

superfície bacteriana (Tacchi *et al.*, 2014; Raymond *et al.*, 2015). As variações assim proporcionadas em proteínas de superfície antigênicas podem desempenhar papéis importantes, por exemplo, na evasão da resposta imune do hospedeiro (Wood *et al.*, 2013).

Além da adesão e ações dependentes de proteínas de superfície, processos intracelulares responsáveis pela sobrevivência da bactéria e por sua capacidade de colonização de um ambiente (hospedeiro) são essenciais para o desenvolvimento da infecção. Em *M. hyopneumoniae*, já foi evidenciado que uma peroxirredoxina é capaz de proteger o DNA da bactéria contra danos causados por espécies reativas de oxigênio (ROS), que são geradas tanto endogenamente como pelas células do hospedeiro (Machado *et al.*, 2009). Recentemente, o papel de aminopeptidases na maturação de proteínas de *M. hyopneumoniae* foi demonstrado, em que foram observados eventos de processamento endoproteolítico de 164 proteínas de *M. hyopneumoniae*, com destaque para a excisão de metioninas (Berry *et al.*, 2017). O metabolismo do mio-inositol também é um processo celular importante para *M. hyopneumoniae*, uma vez que as enzimas relacionadas a este metabolismo foram encontradas apenas nesta micoplasma (Siqueira *et al.*, 2013; Ferrarini *et al.*, 2016). O mio-inositol pode ser utilizado como uma fonte de carbono alternativa, que pode ser encontrada na composição de surfactantes pulmonares, como um subproduto da degradação de fosfatidilinositol (Veldhuizen 1998). A capacidade de *M. hyopneumoniae* absorver mio-inositol a partir de meios de cultura foi demonstrada para uma linhagem patogênica e para outra não-patogênica, mas não houve diferença significativa entre os níveis de absorção deste metabólito entre as linhagens testadas (Galvao Ferrarini *et al.*, 2018).

Produtos proteicos de secreção estão entre os principais determinantes de patogenicidade bacterianos, uma vez que constituem a linha de frente nas interações entre as bactérias patogênicas e suas espécies hospedeiras (Wooldridge, 2009; Peña e Arechaga, 2013). Aproximadamente 30% das proteínas bacterianas são destinadas para a membrana celular ou para o meio extracelular, sendo que a maioria destas proteínas atua na estruturação celular, na absorção de nutrientes, na excreção, na sinalização e em mecanismos de defesa (Driessen e Nouwen, 2008). Graças ao conjunto de funções vitais que desempenha, o secretoma constitui um reservatório de fatores de virulência para bactérias patogênicas, incluindo, por exemplo, proteínas de adesão, toxinas, superantígenos e proteínas relacionadas com a evasão da resposta imune (Kusch e Engelmann, 2014). Nos genomas de *M. hyopneumoniae* e *M. flocculare*, os genes

codificadores da maior parte das proteínas componentes típicas da via Sec-dependente já foram identificadas (Vasconcelos *et al.*, 2005; Siqueira *et al.*, 2013). A presença e funcionalidade deste mecanismo convencional de secreção são adicionalmente evidenciadas pela presença de peptídeo-sinal putativo em um número considerável de proteínas codificadas pelos genomas destas bactérias. A ocorrência de outras vias alternativas de secreção em micoplasmas, incluindo vias para a secreção de proteínas através de vesículas, ainda necessita ser investigada. A secreção de proteínas através de vesículas extracelulares já foi demonstrada na micoplasma *Acholeplasma laidlawii*, um patógeno de plantas (Chernov *et al.*, 2014), em que foi evidenciada a presença de várias proteínas potencialmente envolvidas com patogenicidade e proteção a estresses ambientais.

Outro aspecto importante de interações entre *M. hyopneumoniae* com o hospedeiro diz respeito à capacidade da bactéria de induzir apoptose em células suínas. Alguns estudos demonstraram que uma fração de proteínas da superfície de *M. hyopneumoniae* é capaz de induzir apoptose em macrófagos alveolares e células sanguíneas mononucleares de suínos (Bai *et al.*, 2013; Bai *et al.*, 2015). Este mecanismo mediado por proteínas de superfície induz, nas células suínas, a produção de espécies reativas de oxigênio (ROS) e de óxido nítrico e a decorrente morte celular por apoptose. Mais recentemente, nosso grupo de pesquisa demonstrou que uma sinal-peptidase I putativa de *M. hyopneumoniae* é capaz de induzir morte celular por apoptose em células epiteliais de suínos *in vitro* (Paes *et al.*, 2017). A citotoxicidade de *M. hyopneumoniae* para as células hospedeiras sugere uma maior participação da bactéria na indução das lesões pulmonares, características da PES, as quais eram anteriormente associadas apenas ou predominantemente à resposta imune do hospedeiro.

2. JUSTIFICATIVAS

As atividades relacionadas à suinocultura ocupam lugar de destaque na matriz produtiva do agronegócio brasileiro, possuindo grande importância no âmbito econômico e social, através da produção, do consumo e da exportação da carne suína e da geração de empregos. Entretanto, a competitividade da suinocultura brasileira tem sido comprometida devido a questões sanitárias (ABIPECS, 2013), especialmente pela falta de controle das infecções respiratórias que acometem os rebanhos. Dentre as infecções respiratórias, a PES se destaca por contribuir diretamente para a perda de produtividade e predispõe os suínos a diversas coinfecções (Fablet *et al.*, 2012). *M. hyopneumoniae*, agente etiológico da PES, é uma bactéria endêmica nos rebanhos suínos brasileiros e o seu controle é prejudicado, entre outros fatores, pela falta de métodos diagnósticos mais práticos (Simionatto *et al.*, 2013).

Além de *M. hyopneumoniae*, *M. flocculare* é outra espécie frequentemente identificada colonizando o trato respiratório suíno (Kobisch e Friis, 1996). Ambas as espécies são capazes de se aderir ao epitélio ciliar respiratório, mas com diferentes consequências para o hospedeiro. *M. hyopneumoniae* causa ciliostase, destruição dos cílios e morte de células epiteliais (Debey e Ross, 1994; Siqueira *et al.*, 2013) e provoca uma intensa resposta inflamatória (Rodríguez *et al.*, 2004), características da PES. *M. flocculare*, por outro lado, não provoca danos evidentes ao epitélio respiratório suíno e é, por isso, considerada uma bactéria comensal para suínos (Young *et al.*, 2000). Estudos genômicos e transcritômicos comparativos demonstraram grande similaridade entre *M. hyopneumoniae* e *M. flocculare*, como o compartilhamento da maioria dos potenciais fatores de virulência conhecidos, e não conseguiram evidenciar diferenças suficientes para explicar a diferença de patogenicidade entre estas duas espécies (Siqueira *et al.*, 2013; Siqueira *et al.*, 2014). São, portanto, necessários mais estudos comparativos entre estas espécies, principalmente a respeito dos repertórios de proteínas apresentados por cada uma delas ao hospedeiro suíno, a fim de identificar diferenças entre *M. hyopneumoniae* e *M. flocculare* que possam estar associadas a determinação do quadro clínico da PES.

Os conhecimentos atuais relacionados aos determinantes do quadro clínico da PES são escassos, especialmente em nível molecular. Estes determinantes incluem os fatores de virulência e mecanismos de patogenicidade da bactéria, bem como o tráfego de proteínas entre o patógeno e o hospedeiro (Peña e Arechaga, 2013). Até o momento,

apenas alguns poucos fatores de virulência de *M. hyopneumoniae*, como adesinas e algumas enzimas foram estudados individualmente (Chen *et al.*, 2006; Machado *et al.*, 2009; Deutscher *et al.*, 2010; Bogema *et al.*, 2012; Moitinho-Silva *et al.*, 2012; Moitinho-Silva *et al.*, 2013) e alguns esforços de nosso grupo foram direcionados à análise e identificação de outros fatores de virulência da bactéria em maior escala, a partir de estudos proteômicos. Tais estudos foram realizados para comparação de extratos celulares totais de linhagens patogênicas e não patogênicas de *M. hyopneumoniae* (Pinto *et al.*, 2007; Pinto *et al.*, 2009) ou para a análise da composição do proteoma de superfície da linhagem patogênica 7448 desta espécie (Reolon *et al.*, 2014). No entanto, análises proteômicas comparativas entre os repertórios proteicos de *M. hyopneumoniae* e *M. flocculare*, utilizando equipamentos de espectrometria de massas de alta resolução e sensibilidade, ainda não haviam sido realizadas. A comparação entre os repertórios proteicos de duas espécies/linhagens aparentadas filogeneticamente, mas com níveis de virulência diferentes, como *M. hyopneumoniae* e *M. flocculare*, é importante para evidenciar diferenças quali/quantitativas potencialmente relacionadas a determinação de patogenicidade. A análise proteômica de frações proteicas citoplasmáticas e de superfície de *M. hyopneumoniae* e *M. flocculare* é importante para melhorar o número de identificações de proteínas potencialmente relacionadas à patogenicidade e poderá revelar diferenças quali/quantitativas entre repertórios de proteínas de *M. hyopneumoniae* e *M. flocculare* que contribuirão para a identificação de potenciais determinantes de patogenicidade de *M. hyopneumoniae*.

Além da análise dos proteomas de *M. hyopneumoniae* e *M. flocculare*, a identificação dos repertórios de proteínas secretadas por estas micoplasmas é importante para identificação de novos fatores de virulência de *M. hyopneumoniae* e/ou proteínas relevantes para determinação da PES. Entretanto, o estudo dos secretomas de micoplasmas tem sido dificultado devido à necessidade destas bactérias se multiplicarem em meios de cultura muito ricos, incluindo altas concentrações de soro suíno na sua composição (Friis, 1975). Apesar disso, recentes aprimoramentos em técnicas para depleção de proteínas abundantes em extratos proteicos (Fonslow *et al.*, 2011), bem como a evolução em termos de sensibilidade e resolução dos espectrômetros de massas (Eliuk e Makarov, 2015), passaram a viabilizar a identificação dos repertórios de proteínas secretadas por micoplasmas (Rebollo Couto *et al.*, 2012; Chernov *et al.*, 2014). Estes avanços permitirão a análise comparativa dos secretomas de *M. hyopneumoniae* e *M. flocculare*, bem como a identificação de potenciais determinantes de patogenicidade.

Considerando as relações entre *M. hyopneumoniae* e o hospedeiro suíno, é sabido que estresses oxidativo e térmico ocorrem quando da colonização do hospedeiro e no desencadeamento e progresso da PES (Madsen *et al.*, 2006; Schafer *et al.*, 2007). Neste contexto, análises proteômicas comparativas entre linhagens patogênica e não patogênica de *M. hyopneumoniae* cultivadas em condições de estresse serão importantes para identificação de proteínas relacionadas a resposta a estresse e patogenicidade.

A análise comparativa entre os diferentes repertórios proteicos de *M. hyopneumoniae* e *M. flocculare* é uma abordagem relevante para a identificação de potenciais determinantes de patogenicidade de *M. hyopneumoniae*. A identificação de proteínas envolvidas com a determinação de patogenicidade permitirá a seleção de potenciais alvos para o desenvolvimento de novas estratégias de diagnóstico, prevenção e tratamento da PES.

3. OBJETIVOS

O objetivo geral deste estudo é a identificação de determinantes de patogenicidade de *M. hyopneumoniae* a partir de análises proteômicas comparativas abrangentes entre linhagens patogênicas e não patogênicas de *M. hyopneumoniae*, e entre *M. hyopneumoniae* e *M. flocculare*.

Objetivos específicos:

a) Comparar os repertórios de proteínas citoplasmáticas e de superfície entre linhagem patogênica e linhagem não patogênica de *M. hyopneumoniae* e entre *M. hyopneumoniae* e *M. flocculare*.

b) Comparar os repertórios de proteínas secretadas por *M. hyopneumoniae* e *M. flocculare*.

c). Comparar os repertórios proteicos entre linhagem patogênica e linhagem não patogênica de *M. hyopneumoniae* em condições de estresse oxidativo e térmico.

4. MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÃO

Na seção 4.1, materiais e métodos, resultados e discussão referentes à análise proteômica comparativa de *M. hyopneumoniae* 7448 e J e *M. flocculare* estão apresentados na forma de artigo já publicado.

Na seção 4.2, materiais e métodos, resultados e discussão referentes à análise comparativa dos repertórios de proteínas secretadas de *M. hyopneumoniae* e *M. flocculare* estão apresentados na forma de um segundo artigo já publicado.

Na seção 4.3, materiais e métodos, resultados e discussão referentes à análise comparativa dos proteomas de duas linhagens de *M. hyopneumoniae* (7448 e J) em condições de estresse oxidativo e térmico estão apresentados na forma de manuscrito já submetido.

4.1 Análise comparativa dos proteomas citoplasmáticos e de superfície de *M. hyopneumoniae* e *M. flocculare*

Referência do artigo original publicado

Jéssica Andrade Paes, Lais Del Prá Netto Machado, Fernanda Munhoz dos Anjos Leal, Sofia Nóbrega de Moraes, Hercules Moura, John R. Barr, Henrique Bunselmeyer Ferreira *Comparative proteomics of two* Mycoplasma hyopneumoniae *strains and* Mycoplasma flocculare *identified potential porcine enzootic pneumonia determinants*. *Virulence*, 9(1):1230-1246, 2018.

Contribuição dos autores:
JAP e LDPNM compartilham a primeira autoria. JAP: delineamento experimental, execução da análise do proteoma citoplasmático de *M. hyopneumoniae* e *M. flocculare*, redação do manuscrito; LDPNM: delineamento experimental, execução da análise do proteoma de superfície de *M. hyopneumoniae* e *M. flocculare*, redação do manuscrito; FMAL: auxílio nas análises de LC-MS/MS, discussão dos resultados; SNM: auxílio na preparação de amostras para LC-MS/MS; HM e JRB: auxílio nas análises de LC-MS/MS, discussão dos resultados, revisão do manuscrito; HBF: delineamento experimental, análise e discussão de resultados e revisão do manuscrito.

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Comparative proteomics of two *Mycoplasma hyopneumoniae* strains and *Mycoplasma flocculare* identified potential porcine enzootic pneumonia determinants

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ABSTRACT

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* are genetically similar bacteria, which coinhabit the porcine respiratory tract. These mycoplasmas share most of the known virulence factors, but, while *M. hyopneumoniae* causes porcine enzootic pneumonia (PEP), *M. flocculare* is a commensal species. To identify potential PEP determinants and provide novel insights on mycoplasma-host interactions, the whole cell proteomes of two *M. hyopneumoniae* strains, one pathogenic (7448) and other non-pathogenic (J), and *M. flocculare* were compared. A cell fractioning approach combined with mass spectrometry (LC-MS/MS) proteomics was used to analyze cytoplasmic and surface-enriched protein fractions. Average detection of ~ 50% of the predicted proteomes of *M. hyopneumoniae* 7448 and J, and *M. flocculare* was achieved. Many of the identified proteins were differentially represented in *M. hyopneumoniae* 7448 in comparison to *M. hyopneumoniae* J and *M. flocculare*, including potential PEP determinants, such as adhesins, proteases, and redox-balancing proteins, among others. The LC-MS/MS data also provided experimental validation for several genes previously regarded as hypothetical for all analyzed mycoplasmas, including some coding for proteins bearing virulence-related functional domains. The comprehensive proteome profiling of two *M. hyopneumoniae* strains and *M. flocculare* provided tens of novel candidates to PEP determinants or virulence factors, beyond those classically described.

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Comparative proteomics; swine respiratory pathogens; mycoplasma; subcellular fractions; virulence factors; bacterium-host interactions

Introduction

The identification and characterization of virulence factors is of upmost relevance to discover new targets for the development of diagnostic methods, therapeutic drugs, and vaccines [1]. However, the multifactorial nature of pathogenicity poses difficulties to identify disease-related proteins and mechanisms in pathogenic species. Comparisons between virulent and avirulent strains of a pathogenic species and/or two closely-related species that coinhabit the same host species, being one pathogenic and the other a commensal, are expected to provide valuable information on determinants of pathogenic/commensal ways of life.

Among the mycoplasmas that coinhabit the swine respiratory tract, there are two interesting species for comparative studies: the pathogenic *Mycoplasma hyopneumoniae* and the commensal *Mycoplasma flocculare* [2]. *M. hyopneumoniae* adheres to the host respiratory epithelium and causes the porcine enzootic pneumonia (PEP). *M. flocculare* also adheres to porcine respiratory epithelium and can be isolated from normal and pneumonic lungs. This species is usually regarded as non-pathogenic [3,4], although it is considered by some authors an opportunistic pneumonic pathogen in coinfections with *M. hyopneumoniae* [5]. Despite the pathogenic nature of *M. hyopneumoniae*, there are some strains that vary in their virulence levels, or even are avirulent, such as *M. hyopneumoniae* J, which has reduced adhesion capacity to porcine cilia [6]. Comparisons between the genomes of *M. hyopneumoniae* pathogenic and non-pathogenic strains (7448 and J, respectively) revealed no extensive genomic differences [7]. Moreover, previous comparative phylogenetic and phylogenomic studies provided evidences of

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Supplementary data for this article can be accessed here.

the close relationship of *M. hyopneumoniae* and *M. flocculare* [7–9], which share most of the known virulence-related genes [10]. The differences between *M. hyopneumoniae* and *M. flocculare* include the absence, in *M. flocculare*, of the *glpO* gene, related to *M. hyopneumoniae* hydrogen peroxide generation and cytotoxicity [11,12], and differential domains between orthologs from the P97 family of adhesins and from other surface proteins [13]. However, 90% of *M. flocculare* predicted surface proteins are shared with *M. hyopneumoniae* [9], and the observed genomic differences between *M. hyopneumoniae* strains, and between *M. hyopneumoniae* and *M. flocculare* so far do not fully explain their differential phenotypes of virulence/pathogenicity.

Differential expression of ortholog genes may also contribute to differences in pathogenicity or virulence level between *M. hyopneumoniae* strains or between *M. hyopneumoniae* and *M. flocculare*. However, previous comparative transcriptomic studies between *M. hyopneumoniae* and *M. flocculare* [14] failed to find differences in the relative transcription levels for most genes. On the other hand, pioneer proteomic studies, have provided evidences of differential protein abundance and post-translational processing between *M. hyopneumoniae* pathogenic (7448 and 7422) and non-pathogenic (J) strains [15]. Moreover, a recent comparative proteomics study between *M. hyopneumoniae* and *M. flocculare* secreted proteins revealed several virulence-related differences between these mycoplasma species [16]. This study showed that the *M. hyopneumoniae* secretome included several virulence-related proteins, like adhesins, transporters, nucleases and uncharacterized proteins bearing virulence-related functional domains, not found in the *M. flocculare*, secretome. Overall, these previous studies indicate the necessity of further and more comprehensive comparative proteomic studies, to deeply investigate possible pathogenicity or virulence-related differences at the protein level.

Here, the whole cell proteomes of *M. hyopneumoniae* strains 7448 (pathogenic) and J (non-pathogenic), and *M. flocculare* were compared by a mass spectrometry (MS)-based approach to identify differences in protein abundance associated with pathogenicity or virulence. Mycoplasma cells were fractioned into cytoplasmic- and surface-enriched protein fractions and their protein contents were analyzed by high-resolution and high-sensitivity MS. Several significant differences among *M. hyopneumoniae* strains and *M. flocculare* proteomes were depicted, and their biological significance for mycoplasma-host interactions, for virulence and PEP determination are discussed.

Results

MS-based identification of proteins from M. hyopneumoniae 7448 and J, and M. flocculare

LC-MS/MS analyzes of proteins from soluble and insoluble fractions identified overall totals of 344 out of 695 (~ 50%), 343 out of 672 (~ 51%), and 315 out of 581 (~ 54%) protein species from *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare*, respectively. Detailed peptide and protein identification data are presented in Supplementary Tables 1 and 2, respectively. Around 70% (242) of the detected proteins were shared between *M. hyopneumoniae* 7448, J, and *M. flocculare* (Figure 1(A), Supplementary Table 3A). The average peptide coverage for both *M. hyopneumoniae* and *M. flocculare* identified proteins was ~ 40% in soluble fraction and ~ 20% in insoluble fraction. The calculated zero false discovery rates (FDR) for the proteins and peptides of all samples validated all MS/MS results.

Considering the detected proteins in soluble fractions, 287, 239, and 286 proteins were identified in *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* samples, respectively. Most (188) of the identified proteins in this fraction type (considering ortholog ones) were shared between the two *M. hyopneumoniae* strains, and *M. flocculare* (Figure 1(B), Supplementary Table 3B). Considering the number of detected proteins in insoluble fractions, 267 proteins were identified for *M. hyopneumoniae* 7448, 304 proteins, for *M. hyopneumoniae* J, and 179 proteins, for *M. flocculare*. Most (135) proteins identified in these fractions are shared among all analyzed mycoplasmas (Figure 1(C), Supplementary Table 3C).

Overall, these preliminary comparisons indicated qualitative differences among the proteomes of *M. hyopneumoniae* 7448 and J, and *M. flocculare*. Some of these differences between pathogenic and non-pathogenic mycoplasmas may be associated with pathogenicity.

Enrichment of surface-related proteins in insoluble extracts of M. hyopneumoniae 7448 and J, and M. flocculare

The identified protein repertoires from soluble and insoluble protein extracts were compared to confirm the enrichment of the insoluble fraction with surface proteins. *In silico* subcellular localization predictions for proteins identified in insoluble and soluble extracts are detailed in Supplementary Table 4. Higher numbers of proteins predicted as surface proteins were identified in the insoluble fractions of *M. hyopneumoniae* strains 7448 (111;

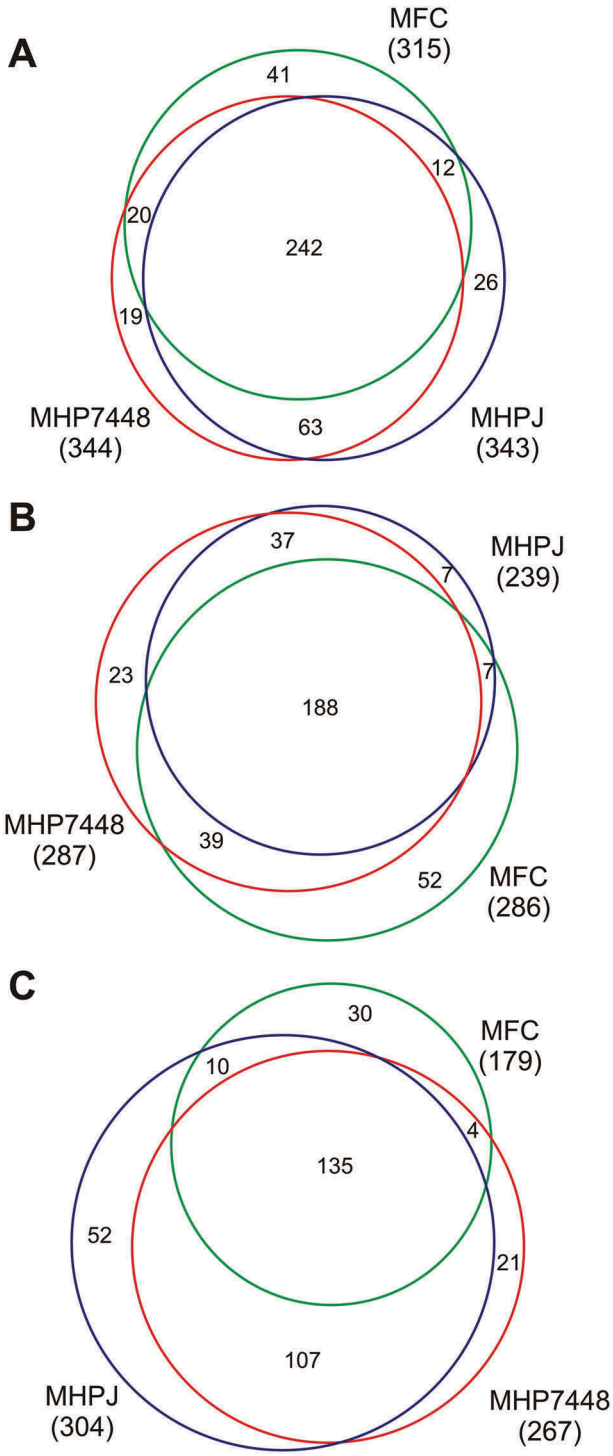

Figure 1. Overview of proteins identified in *M. hyopneumoniae* 7448 (MHP7448) and J (MHPJ), and *M. flocculare* (MFC) samples. (A) Total proteins. (B) Proteins detected in soluble fractions. (C) Proteins detected in insoluble fractions. Overall numbers of proteins identified for each sample between parentheses. Numbers of proteins exclusively detected in each sample or shared between them are indicated within the Venn diagrams.

41.6%) and J (116; 38.2%), and for *M. flocculare* (84; 46.9%) in comparison to those identified in the corresponding soluble extracts (~ 25%). Conversely, most proteins identified in the soluble fractions of *M. hyopneumoniae* 7448 (168; 58.5%), *M. hyopneumoniae* J (144; 60.3%) and *M. flocculare* (161; 56.3%) were predicted as cytoplasmic proteins. Overall, these preliminary results showed qualitative

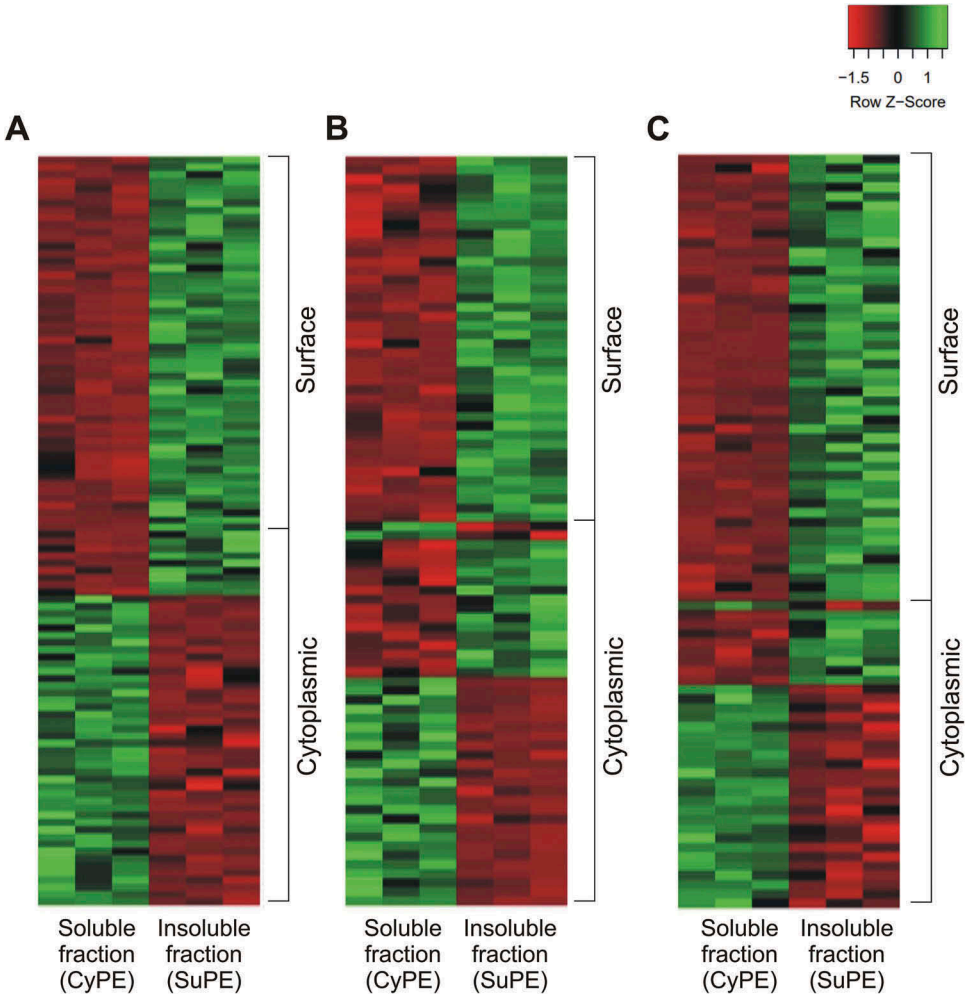

Figure 2. Heatmaps showing the enrichment of surface-related proteins in insoluble extracts of (A) *M. hyopneumoniae* 7448, (B) *M. hyopneumoniae* J, and (C) *M. flocculare*. In each heat map, all shared proteins showing statistically significant abundances ($p < 0.05$) between soluble (CyPE) and insoluble (SuPE) fractions are represented (red, low abundance; green, high abundance). Distribution of surface and cytoplasmic-predicted proteins are indicated on the right. NSAF values, converted in Z-scores, were used to quantify relative differences in protein abundance, and the *t*-test was applied to determine statistically significant differences between shared proteins.

differences between extracts that demonstrate the enrichment of surface proteins in insoluble extracts of *M. hyopneumoniae* (7448 and J) and *M. flocculare*.

Additionally, to verify the quantitative enrichment of surface proteins in insoluble protein extracts, the differential abundance of shared proteins between soluble and insoluble fractions were assessed using NSAF values. For *M. hyopneumoniae* 7448, 125 out of 210 proteins (59%) were differentially abundant between these fractions, while 93 out of 200 (46%) and 89 out of 150 (59%) were differentially abundant for *M. hyopneumoniae* J, and for *M. flocculare*, respectively (Supplementary Table 4). Among overrepresented proteins, there was an evident enrichment of surface-predicted proteins in insoluble fractions for all analyzed mycoplasmas, while cytoplasmic proteins were more abundant in soluble fractions (Figure 2).

Overall, the repertoires of differential proteins demonstrated the clear enrichments of surface proteins and cytoplasmic proteins in the analyzed insoluble and soluble fractions, respectively. Therefore, from now on these mycoplasma soluble and insoluble fractions will be treated as cytoplasmic-enriched protein extracts (CyPE) and surface-enriched protein extracts (SuPE), respectively.

Differences between the whole-cell protein contents of M. hyopneumoniae 7448 and J, and M. flocculare

The whole-cell proteome (including proteins detected in both CyPE and SuPE) of *M. hyopneumoniae* 7448 was

qualitatively and quantitatively analyzed and compared to those from *M. hyopneumoniae* J and *M. flocculare*. Qualitative comparisons were based on presence/absence of detected ortholog proteins, while quantitative comparisons were performed between ortholog proteins shared between *M. hyopneumoniae* 7448 and J, and between *M. hyopneumoniae* 7448 and *M. flocculare*.

In the whole-cell proteomes, 39 and 82 proteins were exclusively detected in *M. hyopneumoniae* 7448 samples in comparison to *M. hyopneumoniae* J and *M. flocculare*, respectively (see Figure 1). Separately analyzing the CyPEs, 62 and 60 proteins were found exclusively in *M. hyopneumoniae* 7448 samples in comparison to *M. hyopneumoniae* J and *M. flocculare*, respectively. On the other hand, 25 and 128 proteins were *M. hyopneumoniae* 7448-exclusive in comparison to *M. hyopneumoniae* J and *M. flocculare*, respectively, considering only SuPE samples.

Quantitative analyzes were performed with shared proteins between samples based on emPAI values (Supplementary Table 5). *M. hyopneumoniae* 7448 and J strains shared 305 proteins, while *M. hyopneumoniae* 7448 and *M. flocculare* shared 262. Among the proteins shared between *M. hyopneumoniae* 7448 and J strains, 78 proteins were differentially abundant. In comparison to *M. hyopneumoniae* J, 25 CyPE proteins and 18 SuPE proteins were overrepresented in *M. hyopneumoniae* 7448 samples. The differences in abundance ranged from 1.5 to 7.3-fold. Only some SuPE proteins (35 proteins) were underrepresented in *M. hyopneumoniae* 7448 samples in comparison to the J strain.

Quantitative comparisons between *M. hyopneumoniae* 7448 to *M. flocculare* found 79 proteins differentially abundant. Twenty CyPE and 44 SuPE proteins were overrepresented in *M. hyopneumoniae* 7448 samples, with differences in abundance ranging from 1.7 to 63-fold. Nine CyPE and 6 SuPE proteins were underrepresented in *M. hyopneumoniae* 7448 samples in this comparison. Among these differentially abundant proteins from *M. hyopneumoniae* 7448 and *M. flocculare*, only 9 presented significant abundance differences in both CyPE and SuPE. Of these proteins seven were overrepresented in both protein extracts of *M. hyopneumoniae* 7448. The other 2 represent cases of differential enrichment between the subcellular fractions in these two species. An aminopeptidase was overrepresented in the CyPE and underrepresented in the SuPE, in *M. hyopneumoniae* 7448, and vice-versa, in *M. flocculare*. Conversely, an uncharacterized protein (MHP7448_0356), was underrepresented in the CyPE and overrepresented in the SuPE, in *M. hyopneumoniae* 7448, and vice-versa, in *M. flocculare*.

Overall, these results showed important qualitative and quantitative differences between *M. hyopneumoniae* 7448 and J strains, between *M. hyopneumoniae* 7448 and *M. flocculare* regarding whole-cell proteomes. These differences can be associated with the differential pathogenic and non-pathogenic natures of these mycoplasmas and may point out some potential PEP determinants as described in the next sections.

Potential PEP determinants differentially represented in M. hyopneumoniae 7448

Differential proteins between *M. hyopneumoniae* 7448 and its non-pathogenic counterparts were assumed to be potential PEP determinants. This assumption was validated by the fact that, among these differential proteins, there were many virulence-related proteins previously described in the literature, like adhesins, proteases, redox balancing protein, and membrane transporters. The observed qualitative differences are graphically represented in Figure 3, and quantitative differences are presented in Table 1.

Qualitative comparisons revealed that most of the differential proteins were detected in both CyPE and SuPE samples for all analyzed mycoplasmas (see Supplementary Table 2). However, some proteins were exclusively detected in only one subcellular fraction, as follows. Methionine aminopeptidase was exclusively found in the CyPE from *M. hyopneumoniae* 7448 (MHP7448_0173), *M. hyopneumoniae* J (MHJ_0169) and, *M. flocculare* (MFC_0210). The XAA-PRO aminopeptidase was exclusively detected in the CyPE in *M. flocculare*, while in both *M. hyopneumoniae* strains it was detected in both CyPE and SuPE. The neutrophil activating factor, which is involved in oxidative stress, was exclusively detected in *M. hyopneumoniae* 7448 CyPE samples (MHP7448_0457). Most of the detected membrane transporters protein species were found only in SuPE or in both CyPE and SuPE samples from all analyzed mycoplasmas. As expected, those membrane transporters shared between CyPE and SuPE samples were mostly enriched in the SuPE samples (see Supplementary Table 4).

Considering the proteins detected in both CyPE and SuPE, several quantitative differences were observed involving the *M. hyopneumoniae* 7448 adhesin repertoire in comparison to those of the non-pathogenic counterparts. In comparison to *M. hyopneumoniae* J, the P97-like (MHP7448_0272) and MgpA-like (MHP7448_0005) adhesins were overrepresented in *M. hyopneumoniae* 7448 CyPE. On the other hand, the P95 (MHP7448_0099) and P102-copy 1 (MHP7448_0199) adhesins, were underrepresented in *M. hyopneumoniae* 7448 SuPE. Comparisons between *M. hyopneumoniae* 7448 and *M. flocculare* adhesins, showed that the P65 adhesin (MHP7448_0656) was overrepresented in *M. hyopneumoniae* 7448 in both

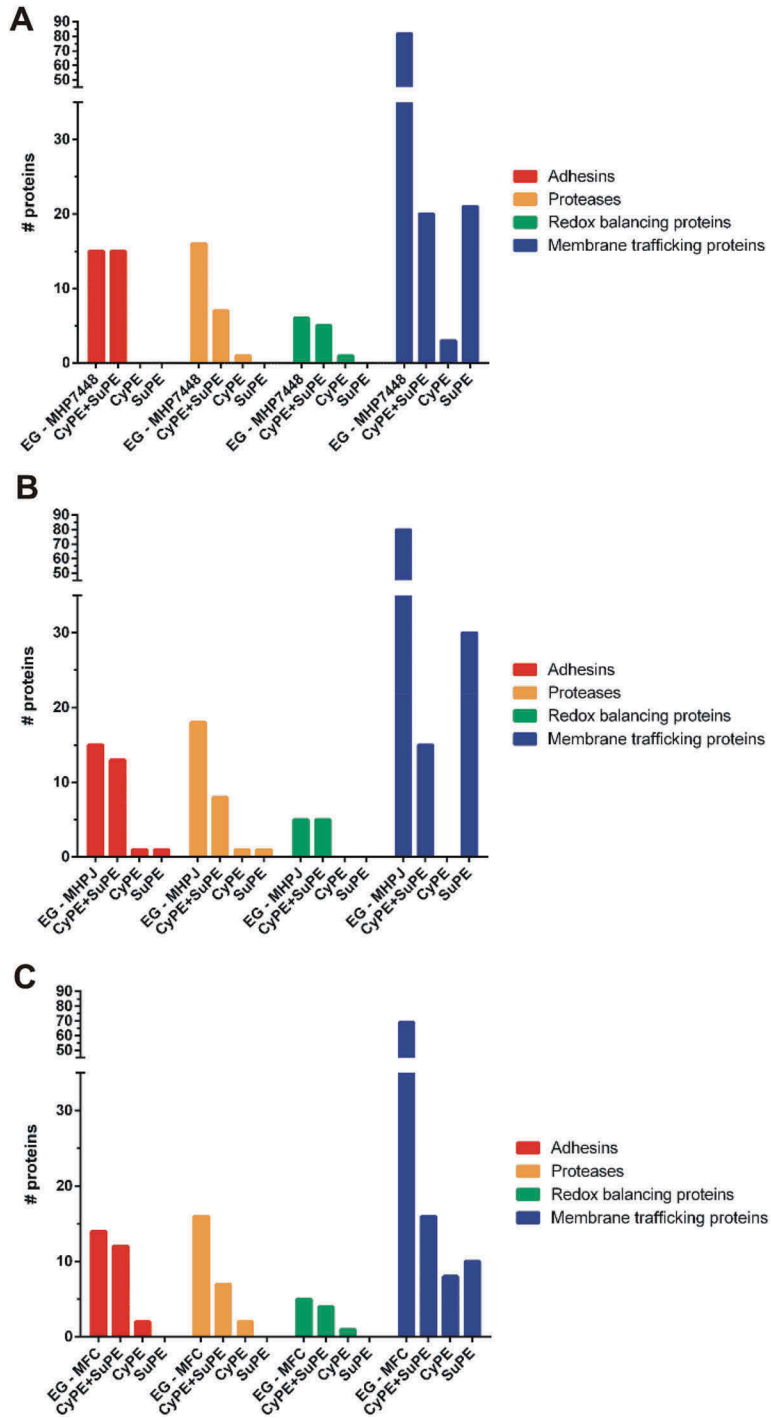

Figure 3. Qualitative differences in potential PEP determinants detected in CyPE and/or SuPE of (A) *M. hyopneumoniae* 7448, (B) *M. hyopneumoniae* J, and (C) *M. flocculare*.

Bar colors represent the different classes of potential PEP determinant, as indicated. CyPE+SuPE, proteins detected in both CyPE and SuPE samples; CyPE, proteins detected only in CyPE samples; SuPE, proteins detected only in SuPE samples. A bar representing the overall number of genes encoding each class of PEP determinants in the corresponding mycoplasma genome was included for reference (EG).

CyPE and SuPE. Interestingly, in *M. flocculare*, the P95 (MFC_00492) and P60-like (MFC_01236) adhesins, in CyPE, and the P97 copy-2 adhesin (MFC_00472), in SuPE, were overrepresented in comparison to *M. hyopneumoniae* 7448.

Proteases are often involved in the virulence of several pathogens, including pathogenic mycoplasmas. In the protease repertoires identified in *M. hyopneumoniae* strains and *M. flocculare* samples, several quantitative differences were observed, which are suggestive of differential

Table 1. Potential virulence-related proteins overrepresented ($p < 0.05$ and FC > 1.5) in *M. hyopneumoniae* 7448 samples. Association with virulence was based on the cited references.

		Fold-changes[1]				
		CyPE[2]		SuPE[2]		
NCBI accession number	Protein name	MHP7448/ MHPJ	MHP7448/ MFC	MHP7448/ MHPJ	MHP7448/ MFC	Reference
MHP7448_0210	ABC transporter ATP-binding protein	2.93	3.13	-	-	[34]
MHP7448_0314	ABC transporter ATP-binding protein	-	2.67	2.35	2.42	[34]
MHP7448_0315	ABC transporter ATP-binding protein	-	-	1.84	-	[34]
MHP7448_0452	ABC transporter ATP-binding protein	-	-	-	3.04	[34]
MHP7448_0129	Aminopeptidase	-	12.75	-	-	[10]
MHP7448_0051	ATP synthase subunit alpha	2.96	-	-	2.28	[70]
MHP7448_0101	ATP-dependent protease binding protein	-	-	-	4.77	[10]
MHP7448_0068	Chaperone protein DnaJ	-	-	-	5.20	[10]
MHP7448_0507	Dihydrolipoyl dehydrogenase	-	-	-	2.61	[41]
MHP7448_0075	Elongation factor G	-	-	-	3.34	[71]
MHP7448_0056	Elongation factor Ts	-	-	-	10.00	[71]
MHP7448_0523	Elongation factor Tu	-	-	-	2.61	[72]
MHP7448_0263	Energy-coupling factor transporter ATP-binding protein EcfA1	-	5.76			[73]
MHP7448_0464	Leucyl aminopeptidase	-	-	2.09	3.95	[10]
MHP7448_0133	Lipase-esterase	-	13.40	-	-	[74]
MHP7448_0137	L-lactate dehydrogenase	-	62.92	-	-	[36]
MHP7448_0524	Lon protease (ATP-dependent protease La)	-	-	-	3.17	[10]
MHP7448_0173	Methionine aminopeptidase	-	3.86	-	-	[10]
MHP7448_0082	NADH oxidase	-	3.90	-	2.48	[75]
MHP7448_0521	Oligoendopeptidase F	-	-	1.59	3.74	[27]
MHP7448_0501	Oligopeptide ABC transporter ATP-binding protein	-	-	-	4.32	[34]
MHP7448_0360	p37-like ABC transporter substrate-binding lipoprotein	-	-	4.24	-	[34]
MHP7448_0272	p97-like protein	2.50	-	-	-	[10]
MHP7448_0161	Phosphopentomutase	3.28	-	-	-	[42]
MHP7448_0656	Prolipoprotein p65	-	4.67	-	2.67	[10]
MHP7448_0376	PTS system ascorbate-specific transporter subunit IIC	-	-	1.58	-	[76]
MHP7448_0375	PTS system enzyme IIB component	3.61	-			[76]
MHP7448_0005	Putative MgpA-like protein	2.84	-	-	-	[10]
MHP7448_0116	Pyruvate dehydrogenase	-	16.00	-	4.35	[41]
MHP7448_0115	Pyruvate dehydrogenase E1-alpha subunit	-	2.61	-	12.91	[41]
MHP7448_0037	Ribonuclease R	3.60	3.03	-	-	[10]
MHP7448_0096	Thiol peroxidase	5.63	-	-	-	[10]
MHP7448_0384	Thioredoxin	-	-	1.55	-	[10]
MHP7448_0098	Thioredoxin reductase	-	-	-	2.81	[10]

[1] Fold changes were based on emPAI quantitative values of MHP7448 divided by those of MHPJ or MFC.
[2] Dashes means that the determined *M. hyopneumoniae* 7448 protein were not differentially abundant in CyPE and/or SuPE and, in comparison to *M. hyopneumoniae* J and/or *M. flocculare*

mechanisms for regulation of protein abundance and subcellular localization. Comparing proteases found in both *M. hyopneumoniae* 7448 and J SuPE, the oligoendopeptidase F and leucyl aminopeptidase were overrepresented in the pathogenic mycoplasma (MHP7448_0521, and MHP7448_0464, respectively). In comparison to *M. flocculare*, 6 proteases were overrepresented in *M. hyopneumoniae* 7448 samples, as follows. An aminopeptidase (MHP7448_0129) and the methionine aminopeptidase (MHP7448_0173) were more abundant in *M. hyopneumoniae* 7448 CyPE. The oligoendopeptidase F (MHP7448_0521), the leucyl aminopeptidase (MHP7448_0464), the ATP-dependent protease binding protein (MHP7448_0101), and the lon protease (MHP7448_0524) were more abundant in *M. hyopneumoniae* 7448 SuPE. Interestingly, the MHP7448_0129 aminopeptidase, overrepresented in *M. hyopneumoniae* 7448 CyPE, was differentially enriched in *M. flocculare*, being ~ 15 times more abundant in SuPE.

Redox balancing proteins can be also associated with virulence of several pathogens, including mycoplasmas, and some of them were differentially represented in the performed proteomic analyzes. *M. hyopneumoniae* neutrophil activating factor (MHP7448_0457) was detected only in *M. hyopneumoniae* 7448 CyPE. Regarding proteins differentially abundant, a thiol peroxidase (MHP7448_0096) and a thioredoxin (MHP7448_0384) (detected in CyPE and SuPE, respectively) were overrepresented in *M. hyopneumoniae* 7448 in comparison to *M. hyopneumoniae* J. In comparison to *M. flocculare*, a NADH oxidase (MHP7448_0082) and a thioredoxin reductase (MHP7448_0098) were overrepresented in the pathogenic mycoplasma.

Membrane transport proteins, such as ABC transporters, permeases and PTS system proteins, correspond to ~ 12% of the proteins encoded by *M. hyopneumoniae* and *M. flocculare* genomes. Around 47% (25) of membrane transporters species detected by LC-MS/MS were

shared among the three analyzed proteomes. Among these membrane transporters, 6 (3 from CyPE and 3 from SuPE) and 3 (from SuPE) proteins were overrepresented and underrepresented, respectively, in *M. hyopneumoniae* 7448 in comparison to *M. hyopneumoniae* J. In comparison to *M. flocculare*, 7 (3 from CyPE and 4 from SuPE) and 1 (from CyPE) membrane transporters were overrepresented and underrepresented, respectively, in *M. hyopneumoniae* 7448.

Besides the canonical virulence-related proteins described above, *M. hyopneumoniae* 7448 also presents several potential virulence-related enzymes that were differentially represented in comparison to *M. hyopneumoniae* J, and *M. flocculare*. In comparison to *M. hyopneum oniae* J, a phosphopentomutase (MHP7448_0161), and a ribonuclease (MHP7448_0037) were 3–4 times more abundant in *M. hyopneumoniae* 7448 CyPE. Comparing to *M. flocculare*, a lipase-esterase (MHP7448_0133), a ribonuclease (MHP7448_0037), two glycolytic enzymes, namely lactate dehydrogenase (LDH, MHP7448_0137), and pyruvate dehydrogenase (represented by three of its four subunits: MHP7448_0116, MHP7448_0115, and MHP7448_0507), a chaperone DnaJ (MHP7448_0068), and three translation elongation factors (MHP7448_0075, MHP7448_0056 and MHP7448_0523) were from 2.6 to ~ 63 times more abundant in *M. hyopneumoniae* 7448.

Overall, these results showed important qualitative and quantitative differences in virulence-related proteins that might be PEP determinants. Importantly, along with these previously described virulence-related proteins, at least 47 other proteins were overrepresented in *M. hyopneumoniae* 7448 proteome in comparison to the samples of non-pathogenic mycoplasmas. The potential of these proteins as PEP determinants deserves further investigation.

Differences between the protein repertoires of M. hyopneumoniae J and M. flocculare

The whole cell proteomes of *M. hyopneumoniae* J and *M. flocculare* were also qualitative and quantitatively analyzed and compared between each other. Among the proteins detected in *M. hyopneumoniae* J and *M. flocculare* samples, 26 and 41 proteins were exclusively detected, respectively (see Figure 1). Among the proteins shared between *M. hyopneumoniae* J and *M. flocculare*, 21 and 68 proteins from CyPE and SuPE, respectively, were differentially abundant (Supplementary Table 5C). The differences in abundance of both CyPE and SuPE proteins ranged from ~ 1.6 to ~ 19-times fold. Regarding CyPE differentially abundant proteins, 4 and 17 were overrepresented in *M. hyopneumoniae* J and *M. flocculare*, respectively. On the other hand, 63 and 5 SuPE proteins were overrepresented in *M. hyopneumoniae* J and *M. flocculare*, respectively. All 4 *M. hyopneumoniae* J overrepresented CyPE proteins were also overrepresented in SuPE samples. Interestingly, two *M. hyopneumoniae* J proteins, an ABC transporter (MHJ_0450) and an arginine-tRNA ligase (MHJ_0012), were differentially enriched, once they were underrepresented in CyPE and overrepresented in SuPE.

Overall, the comparisons between the proteomes of *M. hyopneumoniae* J and *M. flocculare* did not provide evidence of common features that could be clearly associated with the lack of virulence of these related bacteria. However, the observed qualitative and quantitative differences point out to physiological differences between them that deserve further investigation.

Functional enrichment analyzes of the whole cell protein sets of M. hyopneumoniae 7448 and J, and M. flocculare

GO functional enrichment analyzes were performed for the whole cell protein sets of all mycoplasma samples to provide clues on functional differences between strains and species. Totals of 292 *M. hyopneumoniae* 7448 proteins (Supplementary Table 6A), 292 *M. hyopneumoniae* J proteins (Supplementary Table 6B), and 286 *M. flocculare* proteins (Supplementary Table 6C) were categorized according to GO terms into “biological process” (BP), “molecular function” (MF), and “cellular component”(CC) categories. No annotations were retrieved for 52, 51, and 29 proteins of *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare*, respectively. Several functional BP, CC, and MF subcategories were commonly overrepresented in all mycoplasma samples. On the other hand, some functional subcategories were exclusively found as overrepresented in each of the analyzed samples, as follows. “Cellular macromolecule metabolic process”, “phosphorus metabolic process” and “ribose phosphate metabolic process” (BP subcategories); and “nucleic acid binding”, “oxidoreductase activity”, and “translation factor activity, RNA binding” (MF subcategories) were enriched only in *M. hyopneumoniae* 7448. For *M. hyopneumoniae* J. only the MF subcategories “hydrolase activity”, “nucleoside-triphosphatase activity”, and “pyrophosphatase activity” (MF) were exclusively enriched. Finally, some subcategories involved in nucleotide metabolism, as “pyridine nucleotide metabolic process”, “nucleobase-containing compound biosynthetic process”, “aromatic compound biosynthetic process” (BP subcategories), and other nucleotide-metabolism related MF subcategories were exclusively enriched in *M. flocculare*.

The performed GO functional analyzes showed that *M. hyopneumoniae* 7448 and J strains and *M. flocculare* present overall metabolic similarities, as expected. However, some interesting differences were highlighted among them, pointing out specific functional distinctions with possible impact for their proliferation, and survival capacities in the natural host.

Uncharacterized proteins detected in the M. hyopneumoniae 7448 and J, and M. flocculare proteomes

Our proteomic data provided experimental validation for 70, 73 and 69 genes previously regarded as hypothetical for *M. hyopneumoniae* 7448 and J, and *M. flocculare*, respectively, establishing interesting subsets of mycoplasma uncharacterized proteins (Supplementary Table 3), which deserve further attention. The Venn diagram in Figure 4(A), summarizes the exclusive and shared repertoires of detected *M. hyopneumoniae* 7448 and J, and *M. flocculare* uncharacterized proteins. Cell fraction analyzes revealed that ~ 44% of the uncharacterized protein species were found in both CyPE and SuPE (Figure 4(B)). A large number of uncharacterized protein species were exclusively detected in SuPE samples from *M. hyopneumoniae* 7448 (25, 35%) and J (39, 53%). For *M. flocculare*, only 24% of uncharacterized proteins were detected in SuPE.

In silico functional predictions were performed in order to provide clues on the functional roles of the detected uncharacterized proteins. A total of 41 different domains from the Pfam database were found distributed among 52 out of the total of 109 different uncharacterized proteins species detected in the analyzed samples (Supplementary Table 7). The "N-6 DNA methylase" domain was exclusively found in the *M. hyopneumoniae* 7448 set of uncharacterized proteins, while the DUF1410, 'DUF4231' and "tRNA synthetases class II" domains were exclusively found in the *M. hyopneumoniae* J set. Finally, domains related to replication initiation, peptidase and recombinase functions were found exclusively in the *M. flocculare* set.

Among the uncharacterized proteins shared between *M. hyopneumoniae* 7448 and J, and/or between *M. hyopneumoniae* 7448 and *M. flocculare*, 14 virulence-related domains were identified, including domains of potential peptidases, lipases, nucleases, permeases, thioredoxins and chaperones, were found distributed among 18 protein species. Among these proteins, only 4 were overrepresented in *M. hyopneumoniae* 7448, namely MHP7448_0431, bearing a "phosphatidylethanolamine-binding" domain; MHP7448_0064, bearing a "AAA domain"; MHP7448_0522, bearing a "GDSL-like lipase/acylhydrolase" domain; and MHP7448_0148, bearing a 'Hsp33' domain. While MHP7448_0431 and MHP7448_0064 were overrepresented in comparison to *M. hyopneumoniae* J, MHP7448_0431, MHP7448_0522 and MHP7448_0148 were overrepresented in comparison to *M. flocculare*.

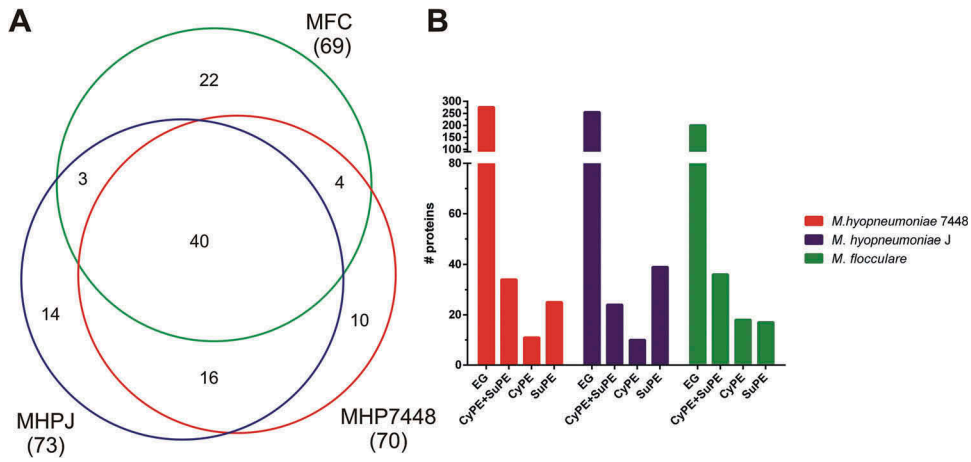

Figure 4. Overview of the uncharacterized proteins identified in *M. hyopneumoniae* 7448 and J, and *M. flocculare* samples. (A) Venn diagram of uncharacterized proteins detected in *M. hyopneumoniae* 7448 (MHP7448) and J (MHPJ), and *M. flocculare* (MFC) samples. Overall numbers of proteins identified for each sample between parentheses. The numbers of uncharacterized proteins exclusively detected in each sample or shared between them are indicated within the Venn diagram. (B) Distribution of uncharacterized proteins detected in CyPE and/or SuPE samples. Bar colors represent the different analyzed mycoplasma samples. CyPE+SuPE, proteins detected in both CyPE and SuPE samples; CyPE, proteins detected only in CyPE samples; SuPE, proteins detected only in SuPE samples. A bar representing the overall number of genes encoding uncharacterized proteins in the corresponding mycoplasma genome was included for reference (EG).

Discussion

Bacterial pathogenicity and virulence are multifactorial features that can be better assessed in comparative studies at the protein level, as protein abundance is the result of transcriptional regulation, post-translational processing and/or protein degradation. In this study, we compared the protein repertoires of cytoplasmic and surface-enriched protein fractions, comprehending the whole-cell proteomes, from the pathogenic and non-pathogenic *M. hyopneumoniae* strains (7448 and J, respectively), and *M. flocculare*, a non-pathogenic related species. For the first time, subcellular fractions of *M. hyopneumoniae* and *M. flocculare* were comparatively assessed using high-sensitivity high-resolution mass spectrometry. Qualitative and quantitative differences between the pathogenic *M. hyopneumoniae* 7448 and its non-pathogenic were found, involving potential PEP determinants, such as adhesins, proteases, and proteins related to redox balancing or membrane trafficking.

Cell fractioning procedures are useful to reduce proteome complexity, allowing the enrichment of low-abundance proteins. They improve the efficiency of MS-based protein identification and allow the association of different sets of proteins to specific cell compartments [17]. The carried out fractioning approach allowed to generate soluble fractions, enriched with cytoplasmic proteins (CyPE), and insoluble fractions, enriched with surface proteins (SuPE). For SuPE preparation, protein solubilization was carried out using the RapiGest SF surfactant, instead of the usual Triton X-114 or SDS solubilization protocols [18]. This surfactant allowed efficient protein solubilization in a one-step procedure and improved MS-protein identification. *In silico* subcellular localization prediction associated with the quantitative proteomics of CyPE and SuPE, confirmed their enrichment with cytoplasmic and surface proteins, respectively, for all analyzed mycoplasma samples. A previous *M. hyopneumoniae* 7448 surface protein survey carried out by our group identified only 34 surface-predicted proteins detected using a biotin cell surface labeling approach [19]. Our fractionation/solubilization approach, in turn, allowed the identification of 111 surface-predicted proteins in the *M. hyopneumoniae* 7448 SuPE (38% of the predicted surfaceome).

The cell fractioning approach combined with a high-resolution and sensitivity LC-MS/MS provided a high proteome coverage for all three mycoplasmas analyzed. The LC-MS/MS approach sensitivity was evidenced by comparing our data to those published by Pinto *et al.* (2009). In comparison to the former data, proteome coverage was improved 28% (from 22% to 50%) for *M. hyopneumoniae* 7448, and 27% (from 24% to 51%) for *M. hyopneumoniae* J. The remaining ~ 50% of predicted proteins not covered by our proteomic data may not have been detected due to their low abundance or lack of expression in culture conditions.

M. hyopneumoniae 7448 shared ~ 70% of the detected proteins with *M. hyopneumoniae* J and *M. flocculare*. Despite these high similarities between the sets of proteins detected for *M. hyopneumoniae* strains and *M. flocculare*, many qualitative and quantitative differences were detected, several of them likely associated with pathogenicity/PEP determination. Within the sets of proteins differentially represented in *M. hyopneumoniae* 7448 in comparison to the non-pathogenic samples, there are representatives of several classes of proteins and/or functions that may be potential PEP determinants, such as adhesins, proteases, oxidative stress-related proteins, and membrane transporters, among others.

Genomic comparative analyzes demonstrated that the sets of adhesin-encoding genes from *M. hyopneumoniae* 7448 and J, and *M. flocculare* are quite similar, containing few qualitative differences between the adhesin repertoires of *M. hyopneumoniae* and *M. flocculare* [9]. The only differences are the absence of *M. flocculare* orthologs for one P97 paralog (P97 copy-1, MHP7448_0198), and one P102 paralog (P102 copy-1, MHP7448_0199), and some rearrangements in *M. hyopneumoniae* genomic regions containing adhesin genes in comparison to *M. flocculare*. Despite these differences, the overall high qualitative similarity between the *M. hyopneumoniae* and *M. flocculare* adhesin sets was confirmed at proteomic level by the data described here. However, our data also pointed out some interesting quantitative differences, as three adhesins (P97-like, MgpA-like and P65) were more abundant in *M. hyopneumoniae* 7448 than in the non-pathogenic mycoplasmas, which may be associated with the higher adherence capacity of pathogenic *M. hyopneumoniae*. Conversely, four adhesins were more abundant in the non-pathogenic mycoplasmas. P95 and P97, for example, were more abundant in *M. flocculare* than in *M. hyopneumoniae* 7448. However, the *M. flocculare* orthologs are quite divergent (only ~ 55% of sequence identity to the *M. hyopneumoniae* orthologs), which may imply different adhesion properties. Moreover, *M. flocculare* has only one copy of P97 (MFC_00472), while *M. hyopneumoniae* has two, and, in this case, the overrepresentation of the single *M. flocculare* P97 may be resultant of a compensating mechanism.

Additionally, we observed higher peptide coverages in CyPE in comparison to those in SuPE, for *M. hyopneumoniae* and *M. flocculare* proteins, including adhesins. This suggests that these proteins are more fragmented in the cell surface than in in the cytoplasm, when they are expected to be mostly unprocessed. Previous studies have showed that adhesins are targets of post-translational proteolytic events [20–24] which can be differential between *M. hyopneumoniae* strains [15]. Along with differential adhesin abundance, the possibly differential adhesin post-translational proteolytic processing likely impact on bacterial pathogenicity and deserve further investigation.

As mediators of post-translational proteolytic events and other important cell processes, proteases play an important role to shape the *M. hyopneumoniae* proteome. Most of the proteases found in the whole cell proteomes of *M. hyopneumoniae* strains and *M. flocculare* were detected in both CyPE and SuPE. Interestingly, most of the overrepresented proteases of *M. hyopneumoniae* 7448 in comparison to *M. hyopneumoniae* J and *M. flocculare* were detected in SuPE. These differences in protease abundance between subcellular fractions and between pathogenic and non-pathogenic mycoplasmas could be resultant of differential enzyme activity or regulation for their targeting to preferential substrates in cell surface. With that, specific proteolytic activities could be targeted, for example, to the processing of surface adhesins.

Some of the proteases overrepresented in *M. hyopneumoniae* had their activities experimentally assessed [25–27]. Interestingly, *M. hyopneumoniae* leucyl aminopeptidase has been associated with plasminogen, heparin and foreign DNA binding and is localized on mycoplasma cell surface [26], which corroborated its higher abundance in *M. hyopneumoniae* 7448 SuPE. Moreover, oligoendopeptidase F and XAA-PRO aminopeptidase were previously associated with host kallikrein-kinin system, participating in inflammatory processes [27]. Overall, overrepresentation of proteases in the surface of *M. hyopneumoniae* 7448, along with previous functional studies, indicate the involvement of these enzymes with important pathogenicity-related mechanisms from adhesion to host immunomodulation.

Endogenous production of hydrogen peroxide through glycerol metabolism is essential for cytotoxicity of pathogenic mycoplasmas, as *Mycoplasma pneumoniae* and *Mycoplasma mycoides* subsp. *mycoides* [28,29]. In line with that, it was recently demonstrated that pathogenic strains of *M. hyopneumoniae* were able to produce hydrogen peroxide from glycerol metabolism, but that the non-pathogenic strain J and *M. flocculare* were not [11]. *M. hyopneumoniae* uptakes and metabolizes glycerol, while *M. flocculare* does not, failing to produce cytotoxic levels of hydrogen peroxide, which can be explained by the absence, in the *M. flocculare* genome, of the *glpO* gene, related to glycerol metabolism and hydrogen peroxide production [12].

Among proteins involved with oxidoreduction processes, a neutrophil activating factor was exclusively detected in *M. hyopneumoniae* 7448 CyPE. In *Helicobacter pylori*, this protein was previously related to neutrophil activation by the production of reactive oxygen species (ROS) [30]. Moreover, several redox balancing proteins were more abundant in *M. hyopneumoniae* 7448 than in *M. hyopneumoniae* J and *M. flocculare*. These results agreed with the functional enrichment analyzes, which demonstrated that the "oxidoreductase activity" subcategory, including all detected proteins related to redox balancing, was exclusively enriched in *M. hyopneumoniae* 7448. These differentially abundant proteins can be considered potential PEP determinants, due to their importance for bacterial survival in the context of endogenous (mycoplasma) and exogenous (host) ROS production [31–33]. For *M. flocculare*, its inability to produce endogenous hydrogen peroxide may be associated with its commensal nature, being less harmful to the host.

Membrane transporters have been described as virulence-related proteins, as they may be associated with multidrug resistance, metal ions uptake, and cell attachment [34], which are important for bacterial survival, and host colonization. *M. hyopneumoniae* and *M. flocculare* genomes have ~ 80 membrane transporters coding genes each, including genes coding for ABC transporters, permeases and PTS systems. In the LC-MS/MS analyzes, ~ 68% of the sets of membrane transporters species identified in *M. hyopneumoniae* and *M. flocculare* predicted proteomes were detected, with a partial (~ 50%) overlapping. Moreover, abundance differences were found between membrane transporters orthologs shared by *M. hyopneumoniae* 7448 and *M. hyopneumoniae* J, or by *M. hyopneumoniae* 7448 and *M. flocculare*. Overall, these evident qualitative and quantitative differences among the sets of membrane transporters of *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, and *M. flocculare* are suggestive of substantial differences in transporting activities/capabilities and may also contribute to their differential virulence/pathogenicity.

Many proteins not classically related to virulence were also differential represented between *M. hyopneumoniae* 7448 and its non-pathogenic counterparts analyzed here. Functional enrichment analyzes showed some important metabolic subcategories specifically

enriched in the *M. hyopneumoniae* 7448 whole cell proteome. The "phosphorous metabolic process" subcategory, which includes several glycolytic enzymes and kinases, and the "RNA binding" subcategory, which includes ribosomal proteins, translational elongation factors and aminoacyl tRNA ligases, were exclusively enriched in this pathogenic mycoplasma. In agreement to the functional enrichment analyzes, several proteins with canonical functions in metabolic pathways were overrepresented in *M. hyopneumoniae* 7448 protein repertoire, including the glycolytic enzymes LDH and pyruvate dehydrogenase, the pentose pathway enzyme phosphopentomutase, and translation-related proteins. Overall, the exclusive enrichment of all these metabolic functions suggests a higher metabolic capacity for the pathogenic *M. hyopneumoniae* strain, which may favor its proliferation and survival, contributing to the colonization, and infection of the porcine respiratory tract.

Glycolytic enzymes and other differential *M. hyopneumoniae* 7448 proteins not usually regarded as virulence factors, such as proteins involved in pentose phosphate pathway, DNA replication, and translation may have also alternative (moonlighting) functions of relevance for pathogenicity [35–37]. For instance, LDH is highly immunogenic and may have an immunomodulatory role [38,39], while pyruvate dehydrogenase and phosphopentomutase are proteins that play roles in adherence to the host extracellular matrix and DNA repair, respectively [40–42].

Around 37% of the sequenced genomes of *M. hyopneumoniae* strains and *M. flocculare* codes for hypothetical proteins. For pathogenic species, such set of hypothetical proteins is of particular interest, once it represents a potential reservoir of unknown virulence factors. For *M. pneumoniae*, many novel virulence factors were predicted upon *in silico* analyzes of hypothetical proteins [43]. In our study, several *M. hyopneumoniae* and *M. flocculare* coding DNA sequences (CDSs) whose putative products have been annotated as "hypothetical proteins" had their proteins products experimentally detected by LC-MS/MS. This allowed to confirm these CDSs as functional genes, and to change the status of their products to that of "uncharacterized proteins". Among the detected uncharacterized proteins, several functional domains were predicted, including virulence-related ones, and most of them were conserved among the orthologs. More importantly, abundance differences between *M. hyopneumoniae* 7448 and its assessed non-pathogenic counterparts were observed for some of the uncharacterized proteins bearing functional domains, including virulence-related ones. Future analyzes of these and other uncharacterized proteins along with the characterization of their functional domains will be important steps towards the elucidation of their functions in *M. hyopneumoniae* biology and their possible roles as novel virulence factors.

Conclusion

Our results provided a comprehensive profiling of the whole cell proteomes of two *M. hyopneumoniae* strains and *M. flocculare*, and an extended list of tens of candidates to pathogenicity determinants, beyond those classically described. Several protein classes with potential virulence-related functions were identified as overrepresented in the *M. hyopneumoniae* 7448 pathogenic strain, including adhesins, proteases, oxidative stress proteins, membrane transporters, and proteins with moonlighting functions, along with many so far uncharacterized proteins. Based on our proteomics results, the pathogenic nature of *M. hyopneumoniae* may be explained, at least in part, by the overrepresentation of several virulence-related proteins. These overrepresented proteins are involved in a wide range of biological processes, including adhesin processing and cell adhesion regulation, detoxification, overall metabolism regulation, and host-pathogen cell trafficking, among others. Although no specific commensalism determinants were found, the underrepresentation of several virulence-related proteins encoded by the non-pathogenic mycoplasmas may be a key point to explain their commensal natures.

Several of the identified proteins in *M. hyopneumoniae* strains and *M. flocculare* repertoires deserve future studies to elucidate mechanisms related to pathogenicity or commensalism, respectively. Of particular interest will be proteins with unknown function or with possible moonlighting functions overrepresented in the pathogenic *M. hyopneumoniae* 7448 strain. Moreover, the identification and characterization of *M. hyopneumoniae* virulence factors is of upmost relevance to discover new targets for the development of novel diagnostic methods, therapeutic drugs, and preventive vaccines against PEP.

Materials and methods

Bacterial growth conditions

M. hyopneumoniae pathogenic strain 7448 was isolated from an infected swine from Lindóia do Sul (SC, Brazil) [7]. *M. hyopneumoniae* non-pathogenic strain J (ATCC 25,934), and the non-pathogenic *M. flocculare* (ATCC 27,716) were acquired from American Type Culture Collection by the Empresa Brasileira de Pesquisa

Agropecuária-Centro Nacional de Pesquisa de Suínos e Aves (EMBRAPA-CNPSA, Concórdia, SC, Brazil). For soluble and insoluble protein extracts, respectively, all bacteria were cultivated in 50 mL and 100 mL of Friis medium [44] for 48 h [45], at 37°C. Cultures were carried out independently in triplicates (biological replicates), and immediately used for protein extraction.

Protein extraction and sample preparation for mass spectrometry

For protein extraction, cultured mycoplasma cells were pelleted by centrifugation (3500 x *g*, 15 min, 4°C), and washed three-times with PBS (pH 7.4). Cells were resuspended and lysed by sonication at 25 Hz in an ice bath by five 30 s cycles with 1 min intervals between pulses. The lysates were centrifuged at 10,000 x *g*, for 20 min, at 4°C and the supernatant (soluble fraction) was recovered for proteomics analyzes. The pellet (insoluble fraction) was resuspended in RapiGest SF Surfactant (Waters Corporation, Number 186,001,861). Soluble and insoluble protein extracts were quantified using the microBCA Protein Assay Kit (Thermo Fischer Scientific, Number 23,235) using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific). Three protein extracts were independently produced to provide the three biological replicates for each sample.

Samples containing 100 µg and 50 µg of proteins from the soluble and insoluble fractions, respectively, were treated for MS analyzes. For soluble fraction analyzes, proteins were precipitated with TCA 20%-acetone, incubated for 16 h at 4°C, and further centrifuged at 20,000 × g for 10 min. Protein pellets were dried and then solubilized with 8 M urea. Next, proteins were reduced with 2 µg of DTT (Bio-Rad, Number 161–0611) at 37°C for 1 h, and alkylated with 10 µg of iodoacetamide (Bio-Rad, Number 163–2109) in the dark, at room temperature. Protein samples were diluted to a final 1 M urea concentration, and further digested with 1 µg of trypsin (Promega, Number V5280). For insoluble fraction analysis, samples resuspended in RapiGest SF were reduced with DTT (Bio-Rad) at 60°C for 30 min to a final concentration of 5 mM and alkylated with iodoacetamide (Bio-Rad) 15 mM (final concentration) at room temperature for 30 min in the dark. Proteins were then digested overnight with 0.5 µg of trypsin (Promega) at 37°C, and RapiGest SF was removed as recommended by the manufacturer (Waters). Resulting soluble and insoluble fractions peptides were desalted in HLB cartridges (Waters, Number 186,000,383), and eluted with 50% acetonitrile/0.1% TFA. Peptides were then lyophilized using a Concentrator Plus (Eppendorf), prior to MS analyzes.

Mass spectrometry analyzes

Processed peptide samples were analyzed for protein identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described [16,46]. Briefly, each peptide sample was reconstituted using 0.1% formic acid in water, loaded onto a nanoAcquity HPLC system (Waters Corporation, MA, USA). A two-step LC was performed, using first a trap column PepMap 100 C18 LC column (300 µm x 5 mm) (Thermo Fischer Scientific, IL, USA), at a flow rate of 5 µl/min, and then an Easy-Spray Column PepMap RSLC C18 (75 µm x 15 cm) analytical column (Thermo Fischer Scientific). For the gradient elution, the mobile phase solvents consisted of 0.1% formic acid in water (solvent A), and 0.1% formic acid in acetonitrile (Burdick and Jackson) (solvent B). The gradient flow was set at 0.3 µl/min. The elution profile consisted of a hold at 5% solvent B for 5 min, followed by a ramp up to 35% solvent B over 25 min; a ramp up to 95% solvent B in 5 min; and a hold at 95% for 5 min, prior to a return to 5% solvent B in 5 min, and re-equilibration at 5% solvent B for 20 min. After LC, the peptides were introduced into a MS/MS Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer (Thermo Fischer Scientific). A 2.0 kV voltage was applied to the nano-LC column. The mass spectrometer was programmed to perform data-dependent acquisition by scanning the mass-to-charge (m/z) range from 400 to 1600, at a nominal resolution setting of 60,000 for parent ion acquisition. For the MS/MS analyzes, the mass spectrometer was programmed to select the top 15 most intense ions with two or more charges. Each biological replicate was independently analyzed by LC-MS/MS two times (technical replicates).

LC-MS/MS data analyzes

The MS/MS raw data were processed using msConvert version 3 (ProteoWizard) [47], and the peak lists were exported in the Mascot Generic Format (.mgf). MS/MS processed data were analyzed using Mascot Search Engine version 2.3.02 (Matrix Science, MA, USA) against local databases available for *M. hyopneumoniae* 7448 and J strains, and *M. flocculare*. These local databases were derived from the fully sequenced genomes from *M. hyopneumoniae* 7448 (920,079 bp), *M. hyopneumoniae* J (897,405 bp), and *M. flocculare* (763,948 bp), and included all deduced amino acid sequences (695, 672, and 581,

respectively) from the corresponding genomes annotation (Siqueira et al 2013; Vasconcelos et al 2005), available at NCBI (https://www.ncbi.nlm.nih.gov/protein/) and Uniprot (http://www.uniprot.org/). The MASCOT search parameters for protein identification included a fragment ion mass tolerance of 0.5 Da, peptide ion tolerance of 7 ppm, and three missed cleavages of trypsin. Carbamidomethylation of cysteine was specified as a fixed modification, whereas the oxidation of methionine, acetylation of lysine and N-terminal ends of proteins, and phosphorylation of tyrosine and serine/threonine were specified as variable modifications [48].

Scaffold software version 4.8.1 (Proteome Software Inc., OR, USA) was used to validate the peptide and protein identifications. The peptide identifications were accepted if they could be established at greater than 99.0% probability as assigned by the Peptide Prophet algorithm [49]. The protein identifications were accepted if they could be established at greater than 95% probability as assigned by the Protein Prophet algorithm [50]; were based on at least 2 identified peptides; and were detected in at least two out of three replicates (both biological and technical).

Identification of ortholog proteins among M. hyopneumoniae 7448 and J, and M. flocculare

In order to allow comparisons among protein repertoires from *M. hyopneumoniae* 7448 and J, and *M. flocculare*, ortholog sequences were determined using OrthoFinder [51]. Orthologs were then established based on the resulting bidirectional best hits, using as parameters identity ≥ 40% and a cutoff value of $1e^{-6}$.

In silico subcellular localization predictions

Proteins identified in soluble and insoluble fractions of *M. hyopneumoniae* 7448 and J strains, and *M. flocculare* were analyzed *in silico* to predict their subcellular localization, being classified as surface or cytoplasmic proteins. Membrane proteins were initially predicted based on positive predictions as lipoproteins, using LipoP 1.0 [52], and PRED-LIPO [53]. Non-lipoproteins were then analyzed for transmembrane domain prediction using TMHMM v.2.0 [54], Phobius [55], HMMTOP [56], CW-PRED [57], and HMM-TM [58]. Non-transmembrane proteins were further analyzed for subcellular localization using PSORTb v. 3.0.2 [59], iLoc-Gpos [60], and CELLO v.2.5 [61].

Proteins not predicted as membrane proteins were then classified as secreted or cytoplasmic. Secreted proteins were predicted based on the presence of signal peptide or on non-classical secretion prediction. Signal peptide predictions were made using SignalP 4.1 [62], Phobius [55], and PrediSi [63]. Non-classical secretion was predicted using SecretomeP 1.0 [64]. Remaining proteins, not classified as membrane or secreted proteins, were considered cytoplasmic proteins. For any given prediction, coincidence in all or at least most of the used predictors was required for validation.

Quantitative and qualitative comparisons between LC-MS/MS data of insoluble and soluble protein extracts from M. hyopneumoniae 7448 and J, and M. flocculare

To confirm the enrichment of surface proteins in the insoluble fractions, the LC-MS/MS datasets of proteins identified in the *M. hyopneumoniae* 7448 and J, and *M. flocculare* insoluble fractions were compared to those of the corresponding soluble fractions. For that, differentially represented proteins, exclusively detected or more abundant in the insoluble protein fraction in comparison to the soluble extract of the same species or strain, were analyzed based on subcellular localization predictions. Protein abundance was measured based on normalized spectral abundance factor (NSAF) values [65] and quantitative differences between proteins detected in both insoluble and soluble protein fractions were statistically analyzed in Scaffold software using the Student's *t*-test, with the Benjamini-Hochberg FDR multiple-testing correction. A p-value < 0.05 was considered statistically significant. Proteins with differential abundances between surface-enriched and soluble protein extracts were represented in heat-maps using the Heatmapper web server (http://www.heatmapper.ca) using the Z-score calculation of NSAF values.

Comparative quantitative analyzes of proteins shared between M. hyopneumoniae 7448, J, and M. flocculare

For quantitative comparisons between ortholog proteins shared between (i) *M. hyopneumoniae* 7448 and *M. hyopneumoniae* J; (ii) *M. hyopneumoniae* 7448 and *M. flocculare*; and (iii) *M. hyopneumoniae* J and *M. flocculare*, the analyzes were based on the exponentially modified protein abundance index (emPAI) values [66]. EmPAI values were calculated for each protein in the Scaffold software, not using the normalization option, to allow intraprotein (between ortholog proteins), and intersample (*M. hyopneumoniae* 7448 vs. *M. hyopneumoniae* J; *M. hyopneumoniae* 7448 vs. *M. flocculare*; or *M. hyopneumoniae* J vs. *M. flocculare*) comparisons. The emPAI values were statistically compared using Student's *t*-test using Prism

GraphPad Software version 6 (GraphPad Software, Inc, CA, USA). Fold-changes (FC) were calculated for each pair of ortholog proteins. Proteins with a p-value < 0.05 and a FC > 1.5 were considered differentially abundant by both statistical and FC parameters.

In silico functional analyzes

In silico functional analyzes of *M. hyopneumoniae* and *M. flocculare* proteins identified by LC-MS/MS were based on gene ontology (GO). Mycoplasma identified proteins were submitted to hierarchical GO overrepresentation tests using the Cytoscape 2.6.3 26 plugin BiNGO 2.3 [67]. Custom *M. hyopneumoniae* 7448 and J GO annotation files were acquired from Uniprot (http://www.uniprot.org/). *M. flocculare* GO annotations were acquired using BLAST2GO version 3.0 [68]. For that, online BlastP searches were performed against the NCBInr database and GO mapping, and annotation were performed based on BlastP results (E-value ≤ 1.0×10^{-3}). The ontology files were retrieved from the GO database (http://www.geneontology.org/). Both annotation and ontology files were edited in-house as BiNGO input files. The hypergeometric overrepresentation tests were performed at a 0.05 level of significance, with the Benjamini-Hochberg FDR multiple-testing correction. Uncharacterized proteins were further analyzed in order to predict functional domains using the Pfam software version 29.0 (http://pfam.xfam.org/) [69].

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Disclosure statement

No potential conflict of interest was reported by the authors.

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4.2 Análise comparativa dos secretomas de *M. hyopneumoniae* e *M. flocculare*

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Secretomes of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* reveal differences associated to pathogenesis

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ABSTRACT

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* cohabit the porcine respiratory tract. However, *M. hyopneumoniae* causes the porcine enzootic pneumonia, while *M. flocculare* is a commensal bacterium. Comparative analyses demonstrated high similarity between these species, which includes the sharing of all predicted virulence factors. Nevertheless, studies related to soluble secretomes of mycoplasmas were little known, although they are important for bacterial-host interactions. The aim of this study was to perform a comparative analysis between the soluble secreted proteins repertoires of the pathogenic *Mycoplasma hyopneumoniae* and its closely related commensal *Mycoplasma flocculare*. For that, bacteria were cultured in medium with reduced serum concentration and secreted proteins were identified by a LC-MS/MS proteomics approach. Altogether, 62 and 26 proteins were identified as secreted by *M. hyopneumoniae* and *M. flocculare*, respectively, being just seven proteins shared between these bacteria. In *M. hyopneumoniae* secretome, 15 proteins described as virulence factors were found; while four putative virulence factors were identified in *M. flocculare* secretome. For the first time, clear differences related to virulence were found between these species, helping to elucidate the pathogenic nature of *M. hyopneumoniae* to swine hosts.

Biological significance: For the first time, the secretomes of two porcine respiratory mycoplasmas, namely the pathogenic *M. hyopneumoniae* and the commensal *M. flocculare* were compared. The presented results revealed previously unknown differences between these two genetically related species, some of which are associated to the *M. hyopneumoniae* ability to cause porcine enzootic pneumonia.

1. Introduction

Mycoplasma hyopneumoniae and *Mycoplasma flocculare* are two bacterial species often identified in the porcine respiratory tract [1]. *M. hyopneumoniae* adheres to porcine respiratory epithelium causing damage to ciliated cells of trachea, bronchi and bronchioles, being the etiological agent of porcine enzootic pneumonia (PEP) [2,3]. *M. flocculare*, on the other hand, despite also being able to adhere and colonize the porcine respiratory epithelium, causes no damage, sharing a commensal relationship with the porcine host [3].

Comparative analyses of the *M. hyopneumoniae* and *M. flocculare* small genomes demonstrated that these species are genetically similar, sharing approximately 78% of its coding DNA sequences (CDS) and >90% of their predicted surface proteins [4]. Moreover, all the *M. hyopneumoniae* virulence factors described so far are also found in *M. flocculare*, and comparative transcriptomic studies between *M. hyopneumoniae* and *M. flocculare* found few differences between the transcription levels of orthologues genes coding for virulence-related genes [5]. These results suggest that major differences between *M. hyopneumoniae* and *M. flocculare* could be determined at post-transcriptional level, including qualitative and quantitative differences in protein repertoires expressed by these bacteria.

Pioneering *M. hyopneumoniae* proteomics studies have been carried out by our group, assessing both intracellular [6,7] and surface proteins [8]. The comparative proteomic analysis between *M. hyopneumoniae* pathogenic and non-pathogenic strains [7] showed that pathogenic strains present constitutive expression of adhesins, which prompt the bacterial cells for adhesion and infection prior to host contact, and proteolytic cleavage of adhesins prior to their secretion to the cell surface, diversifying the bacterial surface protein repertoire (the 'unsoluble secretome', [9]). *M. hyopneumoniae* soluble secreted proteins (the 'soluble secretome', [9]), however, have not been studied so far, due to limitations in the bacterial culture system. The relatively low yields of secreted proteins due to bacterial slow growth [10] and requirements

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of relatively high serum concentrations in the culture medium [11] have hampered the use of proteomic methods to study the mycoplasma secretomes.

The repertoire of bacterial secreted proteins include cell-signaling mediators, defense mechanism effectors and virulence factors, among others [12]. Recently, soluble proteins secreted by bacterial pathogens have been associated with bacterial-host interactions that are relevant for pathogenicity [13–15]. Therefore, the characterization of the soluble secretome of pathogenic mycoplasmas may help elucidate some of the still obscure mechanisms of bacterial-host interactions relevant to pathogenicity. In the case of *M. hyopneumoniae*, a comparative analysis of its soluble secretome with that of its close relative *M. flocculare*, which is nonpathogenic and also has swine as hosts, is expected to reveal determinant traits of both *M. hyopneumoniae* pathogenicity and *M. flocculare* commensal nature.

In this study, we established *M. hyopneumoniae* and *M. flocculare* culture conditions in a medium with low porcine serum (PS) concentration, which allowed the identification of soluble secreted proteins in culture supernatants by mass spectrometry (MS). The comparative analysis of the secretomes of *M. hyopneumoniae* and *M. flocculare* uncovered, for the first time, clear differences between these two species that can be associated with pathogenicity and commensalism, respectively.

2. Methods

2.1. Bacterial strains and culture

M. hyopneumoniae strain 7448 was originally isolated from pigs naturally infected in Lindóia do Sul (Santa Catarina, Brazil) and its pathogenicity was experimentally demonstrated [16]. *M. flocculare* ATCC 27716 was originally acquired by Empresa Brasileira de Pesquisa Agropecuária-Centro Nacional de Pesquisa de Suínos e Aves (EMBRAPA-CNPSA, Concórdia, Santa Catarina, Brazil) and kindly donated to our research group. *M. hyopneumoniae* and *M. flocculare* standard cultures were carried out in Friis medium containing 25% of PS, as previously described [11]. The same medium and conditions were used in the standardization of cultures with reduced serum concentrations (from 15% to 1% of PS). Bacterial growth in each culture condition was followed for 72 h with the optical density of cells measured at 600 nm (OD_{600}) in a Biomate 3 spectrophotometer (Thermo Scientific, IL, USA) every 24 h. Cultures were carried out in 3 ml replicas and, for each growth curve time point, cells from one replica were pelleted by centrifugation (4000 $\times g$, 15 min), washed three times with PBS pH 7.4, for removal of medium components, and suspended in 0.5 ml of PBS prior to OD measurement. Growth curve analyses were performed independently twice for each culture condition and the average results for each time point were compared between different culture conditions with statistical analyzes performed using One-way ANOVA (GraphPad Prism 6). The Tukey's post-test was used for multiple comparisons. Differences were considered significant when *P*-values ≤0.05.

2.2. Enrichment of proteins from culture supernatants of M. hyopneumoniae and M. flocculare and sample preparation for mass spectrometry

For the enrichment of *M. hyopneumoniae* and *M. flocculare* soluble secreted proteins from culture supernatants, bacteria were cultured in 5 ml batches of Friis medium containing 5% of PS for 48 h at 37 °C. For selective removal of serum albumin, bacterial cells were then pelleted by centrifugation at 3500 $\times g$ for 15 min and culture supernatants were collected, filtered (0.22 μm), and dialyzed against sodium acetate 5 mM pH 5.0 [17] using the SnakeSkin® Dialysis Tubing (Thermo Scientific, IL, USA), with molecular weight cut-off of 3.5 kDa. Culture supernatant proteins were recovered by centrifugation (20,000 $\times g$ for 1 h), suspended in 0.5 ml of PBS (pH 7.4) and quantified by microBCA Protein Assay Kit (Thermo Scientific, IL, USA) using NanoDrop 2000 spectrophotometer (Thermo Scientific, IL, USA). Samples obtained from Friis medium containing 5% of PS, submitted to the same procedures described above, were used as negative control. All cultures were performed independently three times, for the production of three biological replicates for each of them. Protein profiles of all samples were assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the presence of bacterial secretion products in *M. hyopneumoniae* test samples was confirmed by immunoblot, using a pool of 10 sera from pigs experimentally infected with *M. hyopneumoniae* 7448 [18].

Samples containing 100 μg of supernatant proteins were treated for mass spectrometry analysis. Briefly, proteins were precipitated with TCA 20%-acetone, incubated for 16 h at 4 °C and further centrifuged at 20,000 $\times g$ for 10 min. Protein pellets were dried and then solubilized with 8 M urea. Next, proteins were reduced with 2 μg of DTT (Bio-Rad, CA, USA) at 37 °C for 1 h and alkylated with 10 μg of iodoacetamide (Bio-Rad, CA, USA) in the dark at room temperature. Protein samples were diluted to a final 1 M urea concentration and further digested with 1 μg of trypsin (Promega, WI, USA). Resulting peptides were desalted in HLB cartridges (Waters Corporation, MA, USA) and eluted with 50% acetonitrile/0.1% TFA. Peptides were lyophilized using a Concentrator Plus (Eppendorf, Germany).

2.3. Mass spectrometry for the identification of M. hyopneumoniae and M. flocculare soluble secreted proteins

Processed samples from bacterial soluble secreted proteins were analyzed for protein identification using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach. Peptides from each sample were reconstituted using 0.1% formic acid (Thermo Scientific, IL, USA) in water (Burdick and Jackson, MI, USA), loaded onto a nanoAcquity UPLC system (Waters Corporation, MA, USA) and separated using a gradient elution as described before [19]. Briefly, the mobile phase solvents consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (Burdick and Jackson, MI, USA)(solvent B). The gradient flow was set at 300 nl/min. The profile consisted of a hold at 5% solvent B for 5 min, followed by a ramp up to 35% solvent B over 25 min, then a ramp up to 95% solvent B in 5 min, a hold at 95% for 5 min before returning to 5% solvent B in 5 min and re-equilibration at 5% solvent B for 20 min. After chromatography, the peptides were introduced into an Orbitrap Elite tandem mass spectrometer (Thermo Scientific, IL, USA). A 2.0 kV voltage was applied to the nano-LC column. The mass spectrometer was programmed to perform data-dependent acquisition by scanning the mass range from mass-to-charge (m/z) 400 to 1600 at a nominal resolution setting of 60,000 for parent ion acquisition. For the MS/MS analysis, the mass spectrometer was programmed to select the top 15 most intense ions with two or more charges. Each biological replicate was independently analyzed by LC-MS/MS three times (technical replicates).

2.4. LC-MS/MS data analysis

The MS/MS raw data were processed using msConvert (ProteoWizard, version 3) [20], and the peak lists were exported in the Mascot Generic Format (.mgf). The MS/MS data were analyzed using Mascot Search Engine (Matrix Science, version 2.3.02) against locals *M. hyopneumoniae* and *M. flocculare* databases (695 and 581 sequences, respectively) containing the deduced amino acid sequences from the genomes annotation available at NCBI (https://www.ncbi.nlm.nih.gov/protein/?term=mycoplasma+hyopneumoniae+7448, for *M. hyopneumoniae*; and https://www.ncbi.nlm.nih.gov/protein/?term=mycoplasma+flocculare+27716, for *M. flocculare*) [4,16]. Data from proteins of negative control (medium) samples were analyzed against both locals *M. hyopneumoniae* and *M. flocculare* databases, to exclude false-positive protein identification, and against *Sus scrofa* and *Saccharomyces cerevisiae* databases (http://www.uniprot.org), to assess medium protein content. The search parameters included a fragment

ion mass tolerance of 1 Da, peptide ion tolerance of 10 ppm and three missed cleavages of trypsin. Carbamidomethylation of cysteine was specified as a fixed modification, whereas the oxidation of methionine, acetylation of lysine and N-terminal ends of proteins and phosphorylation of tyrosine and serine/threonine were specified as a variable modification. These modifications were chosen due the high content of adenine and thymine in mycoplasmas genome, which correlates with the probability of lysine acetylation and serine phosphorylation [21,22].

Scaffold (Proteome Software Inc., version 4.4.1) [23] was used to validate the peptide and protein identifications. The peptide identifications were accepted if they could be established at >96.0% probability as assigned by the Peptide Prophet algorithm [24]. The protein identifications were accepted if they could be established at >99% probability as assigned by the Protein Prophet algorithm [25]. In case only a single peptide was identified for a protein, the corresponding MS/MS spectrum was manually validated and was accepted only if it showed at least five consecutive *b*- or *y*-type fragment ions [26]. Proteins identified were accepted if they could be identified in at least two of three replicates. The normalized spectral abundance factor (NSAF) [27] was calculated for each protein in Scaffold software.

2.5. In silico comparative and functional analyses

Correlations between *M. hyopneumoniae* and *M. flocculare* transcriptional data and secreted protein abundance were assessed by comparing gene expression data (RPKM values), available in Siqueira et al. [5], with corresponding proteomic quantitative data (NSAF values). Statistical analyses were carried out using the Spearman correlation test (GraphPad Prism 6).

In silico functional analyses of *M. hyopneumoniae* and *M. flocculare* proteins identified by LC-MS/MS were based on predictions of subcellular protein localization and secretion type, and COG and domain functional annotations. Subcellular protein localization of was predicted using PSORTb version 3.0.2 (http://www.psort.org/psortb/) [28], including membrane topology prediction using TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and Phobius (http://phobius.sbc.su.se/) [29]. When the results of PSORTb were "unknown", the subcellular location was defined according to transmembrane domains and extracellular location prediction in TMHMM and Phobius software. The signal-peptide presence was predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) [30], PrediSi (http://www.predisi.de/) and Phobius (http://phobius.sbc.su.se/). Not signal-peptide triggered protein secretion was predicted by the use of SecretomeP 2.0 Server (http://www.cbs.dtu.dk/services/SecretomeP/) [31]. The secretion type of each protein identified was predicted as "classical" if signal-peptide was identified in at least two software, and "non-classical" if prediction of SecretomeP was positive and one or none signal-peptide was identified. If no signal-peptide was predicted and the SecretomeP analysis was negative, the protein was assigned to the "undefined" secretion type.

COG functional annotation for the proteins identified was acquired using the EggNOG database version 4.5 (http://eggnogdb.embl.de/#/app/seqscan) [32]. Proteins with function unknown were analyzed in order to predict functional domains using the InterPro version 56.0 (https://www.ebi.ac.uk/interpro/) [33] and Pfam version 29.0 (http://pfam.xfam.org/) [34]. The conservation of these proteins along the taxonomic classification was determined using Protein Blast (BLASTP) and considering >40% of identity for ortholog identification.

3. Results

3.1. M. hyopneumoniae *and* M. flocculare *growth in medium with reduced concentrations of PS*

To establish *M. hyopneumoniae* and *M. flocculare* culture conditions compatible with downstream LC-MS/MS analysis of soluble secreted proteins, bacteria were cultivated in modified Friis medium, with decreasing PS concentrations (15%, 10%, 5% and 1%). The obtained results (Fig. 1) showed that the highest OD values were achieved in control cultures, supplemented with 25% of PS, which is the traditional concentration of PS used in Friis medium. For *M. hyopneumoniae*, concentrations of 15% or 10% allowed a growth just slightly reduced ($P < 0.0001$) in comparison to that of bacteria in control Friis medium, with 25% of PS. For *M. flocculare*, this reduction was not observed. Although *M. hyopneumoniae* and *M. flocculare* proliferated better in Friis medium with 10–25% PS, the resulting high contents of serum proteins in culture medium would probably hamper the identification of bacterial proteins in culture supernatants using MS approaches. Five percent was the lowest tested serum concentration in which *M. hyopneumoniae* and *M. flocculare* growth was above the minimum assumed as necessary for yielding secretion products in detectable amounts for LC-MS/MS, as there was virtually no bacterial growth in a 1% concentration of PS. In cultures with 5% serum, both species presented growth curves without statistical differences between corresponding OD_{600} values ($P > 0.05$), with peaks at 48 h. Significant reduction in the OD_{600} values was observed at 72 h in all test conditions for both species, corresponding to increasing bacterial cell death in the 48 h–72 h interval ($P < 0.0001$). Based on these results, both *M. hyopneumoniae* and *M. flocculare* were cultured for 48 h in Friis medium containing 5% of PS in the experiments for purification and identification of secreted proteins in culture supernatants.

3.2. Recovery of soluble secreted proteins from M. hyopneumoniae *and* M. flocculare *culture supernatants*

Soluble proteins secreted by *M. hyopneumoniae* and *M. flocculare* were enriched from culture supernatants by selective precipitation. The presence of albumin, the main serum component, in the samples was drastically reduced in the selective precipitation step, which was demonstrated comparing supernatant samples prior and after processing (Fig. 2). Similar SDS-PAGE protein profiles were observed for all control (medium) and test (*M. hyopneumoniae* or *M. flocculare*) samples and replicas, showing that, despite albumin depletion, several other medium proteins were still present. The presence of bacterial secreted

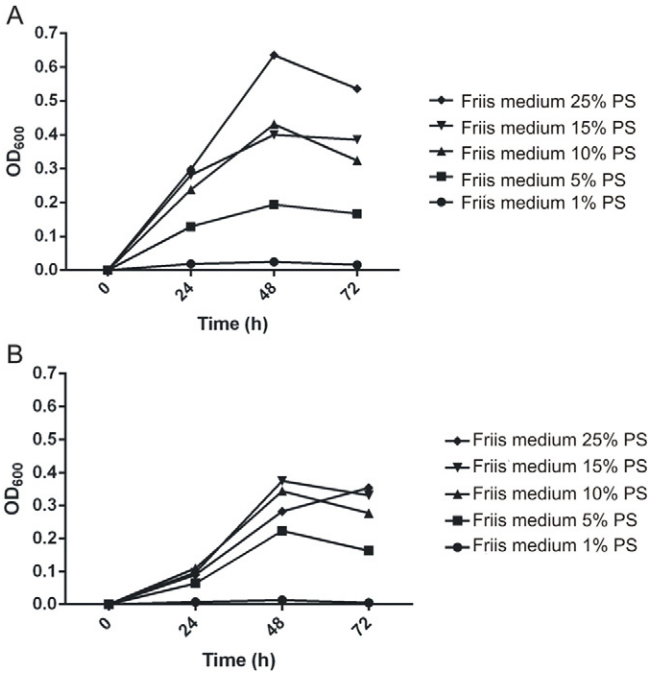

Fig. 1. Growth curves of *M. hyopneumoniae* (A) and *M. flocculare* (B) in Friis medium with different concentrations of PS. Each growth curve point is an average of OD_{600} values of replicas. Standard deviations were not shown due to the high homogeneity of replicas.

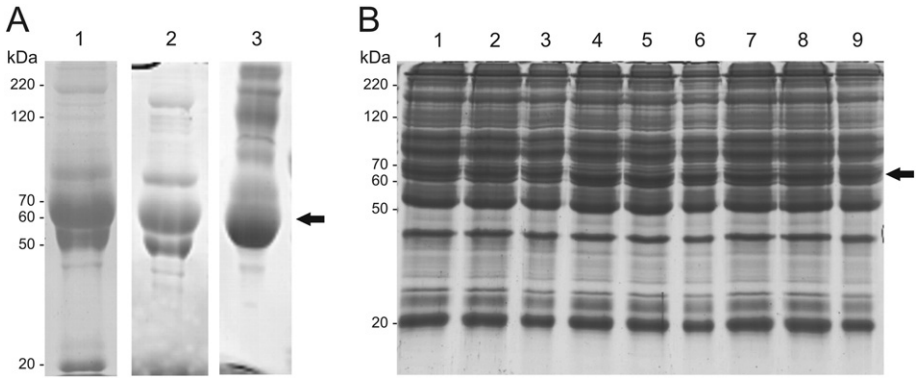

Fig. 2. Electrophoretic protein profiles of *M. hyopneumoniae* and *M. flocculare* culture supernatants. In **(A)**, protein profiles of negative control (Friis medium with 5% PS) (lane 1); and of *M. hyopneumoniae* and *M. flocculare* culture supernatants (lanes 2 and 3, respectively) prior to treatment for selective albumin removal. In **(B)**, protein profiles of the three biological replicates of negative control (Friis medium with 5% PS) (lane 1–3); and of *M. hyopneumoniae* and *M. flocculare* culture supernatants (lanes 4–6 and 7–9, respectively) after treatment for selective albumin removal. Molecular weight markers are indicated (kDa), along with the expected migration for the porcine albumin band (arrow).

proteins in *M. hyopneumoniae* test samples was confirmed by immunoblot using an antiserum pool from *M. hyopneumoniae*-infected pigs (Fig. S1). Overall protein yields in the order of 5.0 mg were obtained from 5 ml of culture for the test samples and for the medium negative control sample as well.

3.3. MS-based identification of proteins secreted by M. hyopneumoniae and M. flocculare in vitro

LC-MS/MS analyses of proteins purified from bacterial culture supernatants identified 62 *M. hyopneumoniae* and 26 *M. flocculare* proteins (Tables 1 and 2, respectively). Detailed peptide and protein identification data are presented in Tables S1 and S2, respectively. Reproducibility among replicates was assured considering as valid only proteins identified in at least two out of three biological replicates. MS data from the three technical replicates for each validated protein were condensed as an average of spectral counts for quantification purposes.

Only seven ortholog proteins were shared between *M. hyopneumoniae* (indexed as MHP) and *M. flocculare* (indexed as MFC) samples, including P102 adhesin (MHP7448_0199/MFC_00475), *N*-acetylglucosamine-6-phosphate deacetylase (MHP7448_0572/MFC_00431), an ABC transporter ATP-binding protein (MHP7448_0315/MFC_01072), a putative type II DNA modification enzyme (MHP7448_0291/MFC_00461), and three proteins with function unknown (MHP7448_0147/MFC_00169; MHP7448_0480/MFC_00452; and MHP7448_0547/MFC_00019). An additional 55 proteins were exclusively detected in the *M. hyopneumoniae* culture supernatant extract, including seven adhesins, namely P60-like lipoprotein (MHP7448_0353), prolipoprotein p65 (MHP7448_0656), protein P102-copy 2 (MHP7448_0107), protein P97-copy 1 (MHP7448_0198), protein P97-copy 2 (MHP7448_0108), Lppt protein (MHP7448_0372), and putative MgpA-like protein (MHP7448_0005); and an additional six proteins previously described as *M. hyopneumoniae* putative virulence factors [35], including a lipoprotein (MHP7448_0367), a DNA adenine methylase (MHP7448_0622), and a membrane nuclease (MnuA - MHP7448_0580). In the *M. flocculare* repertoire of exclusive secreted proteins, only two proteins associated with virulence (by orthology to *M. hyopneumoniae* putative virulence factor) were found, namely the P216 (MFC_00848) adhesins, and the DnaJ chaperone (MFC_01169). Overall, 15 virulence-related proteins were found in the *M. hyopneumoniae* secreted protein repertoire, while only four were found for *M. flocculare*, with just two of them (P102 adhesin and a type II DNA modification enzyme) shared with *M. hyopneumoniae*. Moreover, 39% (24 out of 62) and 42% (11 out of 26) of the proteins identified as secreted by *M. hyopneumoniae* and *M. flocculare*, respectively, are proteins with function unknown. *M. hyopneumoniae* secreted proteins repertoire showed a higher peptide abundance than in *M. flocculare* repertoire, indicating a higher peptide complexity and representation in *M. hyopneumoniae* repertoire. Therefore, the *M. hyopneumoniae* set of identified soluble secreted proteins was more complex, 2.3 times larger (62 vs. 26 proteins) and included 3.75 times more (15 vs. 4) virulence-related proteins than the *M. flocculare* corresponding set.

Quantitative differences were also found between and within the *M. hyopneumoniae* and *M. flocculare* sets of identified secreted proteins. NSAF values for *M. hyopneumoniae* proteins were between 0.328 and 0.0056, with a median of 0.0187, and for *M. flocculare* proteins were between 0.021 and 0.00073, with a median of 0.0032, suggesting an overall abundance of secreted proteins were considerably larger in *M. hyopneumoniae*. Within the *M. hyopneumoniae* samples, the 50S and 30S ribosomal proteins (MHP7448_0131 and MHP7448_0177, respectively), a protein with function unknown (MHP7448_0412), the adhesion P97-copy 1 (MHP7448_0198), and a DNA methylase (MHP7448_0154), were the most abundant. For *M. flocculare*, the proteins with function unknown MFC_0544 and MFC_0247 were the most abundant.

In the negative control (proteins purified from Friis medium with 5% PS), no *M. hyopneumoniae* or *M. flocculare* proteins were identified. Proteins from culture medium (either from PS or from yeast extract), on the other hand, were similarly identified in all test and control samples, totalizing 119 PS and 14 yeast proteins (data not shown).

Qualitative and quantitative differences between the *M. hyopneumoniae* and *M. flocculare* identified repertoires of soluble secreted proteins could be, at least in part, determined by differences in the expression levels of the corresponding genes. In order to investigate expression levels, gene RPKM values [5] and NSAF values of the corresponding proteins were assessed (Table S3). These comparisons demonstrated that virtually all genes encoding the detected secreted proteins (97% for *M. hyopneumoniae* and 100% for *M. flocculare*) are transcribed. However, the comparison between RPKM data and NSAF showed no correlation ($P = 0.5703$) between gene transcriptional levels and protein abundance in the secretome. Therefore, protein abundance in the secretome is likely defined, for both, *M. hyopneumoniae* and *M. flocculare*, at a post-transcriptional level.

3.4. Comparative functional analyses of the M. hyopneumoniae and M. flocculare repertoires of in vitro secreted proteins

Proteins identified within the sets of *M. hyopneumoniae* and *M. flocculare in vitro* secretion proteins were classified according to predicted secretion pathways and COG functional terms. Regarding secretion pathways, the *M. hyopneumoniae* and *M. flocculare* identified proteins were initially classified according to predictions of transmembrane helices and subcellular location (Fig. 3A), and type of secretion (Fig. 3B). Additional data on these predictions are available at Tables 1, 2 and S4. For *M. hyopneumoniae*, 26 proteins (42%) were described as located in

Table 1
Identification and functional predictions of proteins secreted by *M. hyopneumoniae in vitro*.

NCBI accesion numbers	Protein name	Subcellular location predicted	Type of secretion predicted	Functional classification (EggNOG)[a]
MHP7448_0177	30S ribosomal protein S5	Cytoplasmic	Undefined	J
MHP7448_0131	50S ribosomal protein L21	Extracellular	Undefined	J
MHP7448_0024	ABC transporter ATP-binding protein	Cytoplasmic membrane	Non-classical	V, P
MHP7448_0021	ABC transporter ATP-binding protein	Cytoplasmic membrane	Non-classical	V, P
MHP7448_0305	ABC transporter ATP-binding protein	Cytoplasmic membrane	Non-classical	V, S
MHP7448_0315	ABC transporter ATP-binding protein	Cytoplasmic membrane	Non-classical	V, P
MHP7448_0340	ABC transporter ATP-binding protein	Cytoplasmic membrane	Non-classical	V, P, E
MHP7448_0383	ABC transporter ATP-binding protein	Cytoplasmic membrane	Non-classical	V, P
MHP7448_0369	ABC transporter permeasse protein	Cytoplasmic membrane	Non-classical	G, E
MHP7448_0604	ABC transporter xylose-binding lipoprotein	Extracellular	Classical	S
MHP7448_0268	Cation-transporting P-type ATPase	Cytoplasmic membrane	Non-classical	P
MHP7448_0584	Chromosome partition protein Smc	Cytoplasmic	Undefined	D, L, S
MHP7448_0280	CTP synthase	Cytoplasmic	Undefined	F, J, S, E
MHP7448_0622	DNA adenine methylase	Extracellular	Undefined	L, V
MHP7448_0109	DNA gyrase subunit B	Cytoplasmic	Undefined	L
MHP7448_0154	DNA methylase	Extracellular	Undefined	L, J, S, H
MHP7448_0256	DNA polymerase III gamma and tau subunit	Cytoplasmic	Undefined	L
MHP7448_0091	Excinuclease ABC subunit A	Extracellular	Undefined	L
MHP7448_0359	Glycerolkinase	Cytoplasmic	Undefined	C, G
MHP7448_0010	Heat-inducible transcription repressor HrcA	Cytoplasmic	Undefined	K
MHP7448_0367	Lipoprotein	Extracellular	Classical	S, G
MHP7448_0372	Lppt protein	Cytoplasmic membrane	Classical	–
MHP7448_0580	Membrane nuclease, lipoprotein	Extracellular	Classical	S
MHP7448_0572	*N*-acetylglucosamine-6-phosphate deacetylase	Extracellular	Undefined	G, F, Q, S
MHP7448_0353	P60-like lipoprotein	Extracellular	Classical	S
MHP7448_0656	Prolipoprotein p65	Cytoplasmic membrane	Classical	S, E
MHP7448_0199	Protein P102-copy 1	Cytoplasmic membrane	Classical	S
MHP7448_0107	Protein P102-copy 2	Cytoplasmic membrane	Classical	S
MHP7448_0198	Protein P97-copy 1	Extracellular	Non-classical	S
MHP7448_0108	Protein P97-copy 2	Extracellular	Non-classical	S
MHP7448_0039	Protein RecA	Cytoplasmic	Non-classical	L, S, T
MHP7448_0492	PTS system, fructose-specific IIABC component	Cytoplasmic membrane	Undefined	G, T
MHP7448_0005	Putative MgpA-like protein	Cytoplasmic	Undefined	S, J, T
MHP7448_0291	Putative type II DNA modification enzyme: methyltransferase	Extracellular	Non-classical	V, L
MHP7448_0126	Pyruvate kinase	Cytoplasmic	Undefined	G, P
MHP7448_0231	Ribose ABC transport ATP-binding protein	Cytoplasmic membrane	Undefined	P
MHP7448_0083	Thymidine phosphorylase	Cytoplasmic	Undefined	F, E
MHP7448_0584	Translation initiation factor IF-2	Cytoplasmic	Undefined	J, T, M
MHP7448_0147	Uncharacterized protein	Cytoplasmic	Undefined	S, L
MHP7448_0308	Uncharacterized protein	Extracellular	Classical	–
MHP7448_0355	Uncharacterized protein	Extracellular	Non-classical	S
MHP7448_0412	Uncharacterized protein	Extracellular	Undefined	S
MHP7448_0417	Uncharacterized protein	Cytoplasmic membrane	Non-classical	S
MHP7448_0435	Uncharacterized protein	Cytoplasmic	Non-classical	S, H
MHP7448_0547	Uncharacterized protein	Cytoplasmic membrane	Classical	S
MHP7448_0025	Uncharacterized protein	Cytoplasmic membrane	Non-classical	–
MHP7448_0117	Uncharacterized protein	Cytoplasmic membrane	Non-classical	–
MHP7448_0138	Uncharacterized protein	Cytoplasmic membrane	Non-classical	S
MHP7448_0328	Uncharacterized protein	Cytoplasmic membrane	Non-classical	–
MHP7448_0334	Uncharacterized protein	Cytoplasmic	Non-classical	S
MHP7448_0341	Uncharacterized protein	Cytoplasmicmembrane	Non-classical	–
MHP7448_0407	Uncharacterized protein	Cytoplasmic	Undefined	S
MHP7448_0419	Uncharacterized protein	Extracellular	Non-classical	–
MHP7448_0444	Uncharacterized protein	Extracellular	Non-classical	–
MHP7448_0480	Uncharacterized protein	Cytoplasmic membrane	Classical	S
MHP7448_0634	Uncharacterized protein	Cytoplasmic membrane	Non-classical	G, S
MHP7448_0660	Uncharacterized protein	Extracellular	Non-classical	S
MHP7448_0661	Uncharacterized protein	Cytoplasmic membrane	Classical	S
MHP7448_0662	Uncharacterized protein	Cytoplasmic membrane	Non-classical	S
MHP7448_0716	Uncharacterized protein	Cytoplasmic	Undefined	–
MHP7448_0061	Uncharacterized protein	Cytoplasmic	Undefined	S
MHP7448_0646	Uncharacterized protein	Cytoplasmic membrane	Non-classical	T, J, S

[a] Functional categories are: *(C) energy production and conversion;(D) cell cycle control and mitosis; (E) amino acid metabolism and transport; (F) nucleotide metabolism and transport; (G) carbohydrate metabolism and transport;(H) coenzyme metabolism; (J) translation; (K) transcription; (L) replication and repair; (M) cell wall/membrane/envelope biogenesis; (NC) not in COG; (O) post-translational modification, protein turnover, chaperone functions;(P) inorganic ion transport and metabolism; (Q) secondary metabolites biosynthesis; (S) function unknown; (T) signal transduction; (V) defense mechanisms.*

the cytoplasmic membrane, 18 proteins (29%) as cytoplasmic, and 18 (29%) as extracellular proteins. Regarding secretion pathways, 12 proteins (19%) were predicted to be secreted by the classical pathway, while 27 proteins (44%) were predicted as non-classically secreted, and 23 proteins (37%) were assigned to an undefined type of secretion. For *M. flocculare*, 9 proteins (34.5%) were predicted as localized in cytoplasmic membrane, 8 (31%) as cytoplasmic and 9 (34.5%) as extracellular ones. In this secretome, classical secretion was predicted for 8 proteins (31%), non-classical secretion was predicted for 9 proteins (34.5%) and 9 proteins (34.5%) were assigned to an undefined secretion type.

Table 2
Identification and functional predictions of secreted proteins of *M. flocculare in vitro*.

NCBI accession numbers	Protein name	Subcellular location predicted	Type of secretion	Functional classification (EggNOG)[a]
MFC_00225	30S ribosomal protein S3	Cytoplasmic	Undefined	J
MFC_00233	50S ribosomal protein L22	Cytoplasmic	Non-classical	J
MFC_01072	ABC transporter ATP-binding protein	Cytoplasmic membrane	Non-classical	V, P
MFC_00093	ATP-dependent helicase PcrA	Cytoplasmic	Undefined	L
MFC_01169	Chaperone protein DnaJ	Cytoplasmic	Non-classical	O
MFC_00829	Glyceraldehyde-3-phosphate dehydrogenase	Cytoplasmic	Undefined	G
MFC_00431	*N*-acetylglucosamine-6-phosphate deacetylase	Extracellular	Undefined	G, F, S, Q
MFC_00616	Phenylalanyl-tRNAsynthetase beta chain	Cytoplasmic	Undefined	J
MFC_00475	Protein P102	Cytoplasmic membrane	Classical	S
MFC_00293	PTS system, mannitol-specific IIBC component	Cytoplasmic membrane	Non-classical	G
MFC_00345	Putative lipoprotein	Extracellular	Non-classical	S
MFC_00848	Putative P216 surfaceprotein	Extracellular	Classical	S
MFC_00980	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA	Extracellular	Undefined	D, J, S, C, O
MFC_00461	Type II DNA modification enzyme	Extracellular	Non-classical	V, L
MFC_00544	Uncharacterized protein	Cytoplasmic membrane	Classical	S
MFC_00018	Uncharacterized protein	Cytoplasmic membrane	Classical	S, E
MFC_00488	Uncharacterized protein	Cytoplasmic membrane	Non-classical	S
MFC_01074	Uncharacterized protein	Extracellular	Non-classical	V, L
MFC_00075	Uncharacterized protein	Cytoplasmic membrane	Classical	S
MFC_00247	Uncharacterized protein	Extracellular	Undefined	S
MFC_00169	Uncharacterized protein	Cytoplasmic	Undefined	S, L
MFC_00452	Uncharacterized protein	Cytoplasmic membrane	Classical	S
MFC_00023	Uncharacterized protein	Cytoplasmic membrane	Non-classical	S
MFC_00019	Uncharacterized protein	Extracellular	Classical	S
MFC_00065	Uncharacterized protein	Extracellular	Classical	–
MFC_01003	Valine-tRNA synthetase	Cytoplasmic	Undefined	J

[a] Functional categories are: *(C) energy production and conversion; (D) cell cycle control and mitosis; (E) amino acid metabolism and transport; (F) nucleotide metabolism and transport; (G) carbohydrate metabolism and transport; (H) coenzyme metabolism; (J) translation;(K) transcription; (L) replication and repair; (M) cell wall/membrane/envelope biogenesis; (NC) not in COG; (O) post-translational modification, protein turnover, chaperone functions;(P) inorganic ion transport and metabolism; (Q) secondary metabolites biosynthesis; (S) function unknown; (T) signal transduction; (V) defense mechanisms.*

The proteins identified as secreted by *M. hyopneumoniae* and *M. flocculare* were categorized according to COG [36,37], and the functional prediction are summarized in Fig. 3C and D, respectively. For both *M. hyopneumoniae* and *M. flocculare*, the identified secreted proteins were mostly related to the "function unknown" (S; 34% and 37%, respectively). Metabolism-related categories were also well represented for both species, comprehending 29% of *M. hyopneumoniae* identified proteins (divided in P, G, E, F, Q, C, and H categories), and 21%of *M. flocculare* identified proteins (divided in P, G, E, F, Q, and C categories). Four functional categories related to "signal transduction mechanisms" (five proteins), "transcription" (one protein), "coenzyme metabolism" (two proteins) and "membrane biogenesis" (one protein) were found only in *M. hyopneumoniae*, while the "post-translational modification, protein turnover and chaperones" (one protein) category was found only in *M. flocculare*.

Among the identified proteins secreted by *M. hyopneumoniae* and *M. flocculare*, 24 and 11, respectively, were proteins with function unknown. Functional domain predictions were then performed for these proteins to provide clues on their functions (Table S5). For *M. hyopneumoniae*, 12 proteins had functional domains identified (one for each protein), including a putative peptidase DUF31 domain (in MHP7448_0138), an YqaJ-like viral recombinase domain (in MHP7448_0147), and an extracellular matrix-binding protein domain (MHP7448_0308). For *M. flocculare*, 5 proteins had functional domains identified (one for each protein), including a SGNH hydrolase-type esterase domain (in MFC_00018), a type III restriction enzyme domain (in MFC_01074), and an YqaJ-like viral recombinase domain (in MFC_00169, orthologue of MHP7448_0138, mentioned above).

The conservation of all secreted proteins with function unknown was also assessed by searching putative orthologues in public databases (Table S5). All 24 *M. hyopneumoniae* secreted proteins with function unknown are conserved among Mollicutes, while 19 out 24 (79%) of them had orthologues found only within the *Mycoplasma* genus. For *M. flocculare*, 7 out 11 (63%) of all secreted proteins with function unknown have orthologues within the Mycoplasmataceae family, while 36% of them had orthologues found only among species of the *Mycoplasma* genus. MFC_00065 protein was found as exclusive for *M. flocculare*, with no known orthologue so far.

4. Discussion

The detailed analysis of the proteomic composition of secretomes is critical for understanding important biological processes, including cell communication and pathogenicity [38]. However, the proteomic profiling of secretomes from cells cultivated in culture medium supplemented with serum remains extremely challenging, due to the relative underrepresentation of secreted proteins in comparison to the complex background of over represented serum proteins [38]. *M. hyopneumoniae* and *M. flocculare* are fastidious to growth in vitro, and their cultivation requires the use of the so called Friis medium [11], which contains a high concentration (25%) of PS.

The use of serum-free medium removes the serum interference and facilitates the identification of secreted protein, but complete serum deprivation is known to disturb cell metabolism and proliferation, affecting protein expression and secretion [39]. In the conditions established here, Friis medium PS content was reduced to a minimum of 5% and this was compatible with *M. hyopneumoniae* and *M. flocculare* growth. Although bacterial growth was somewhat reduced in lower serum conditions, growth curve profiles were maintained, with growth peak at 48 h, time point in which supernatant samples were collected.

The use of medium with 5% PS, followed by a step for selective removal of serum albumin, reduced the dynamic range of the culture supernatants to values in which the abundance of medium proteins did not interfered significantly with bacterial secretion products. It cannot be assumed that the protein profiles secreted by these bacteria have not changed with the use of the adapted culture medium, but it can be assumed that both *M. hyopneumoniae* and *M. flocculare* have preserved their ability to secrete protein products in these conditions,

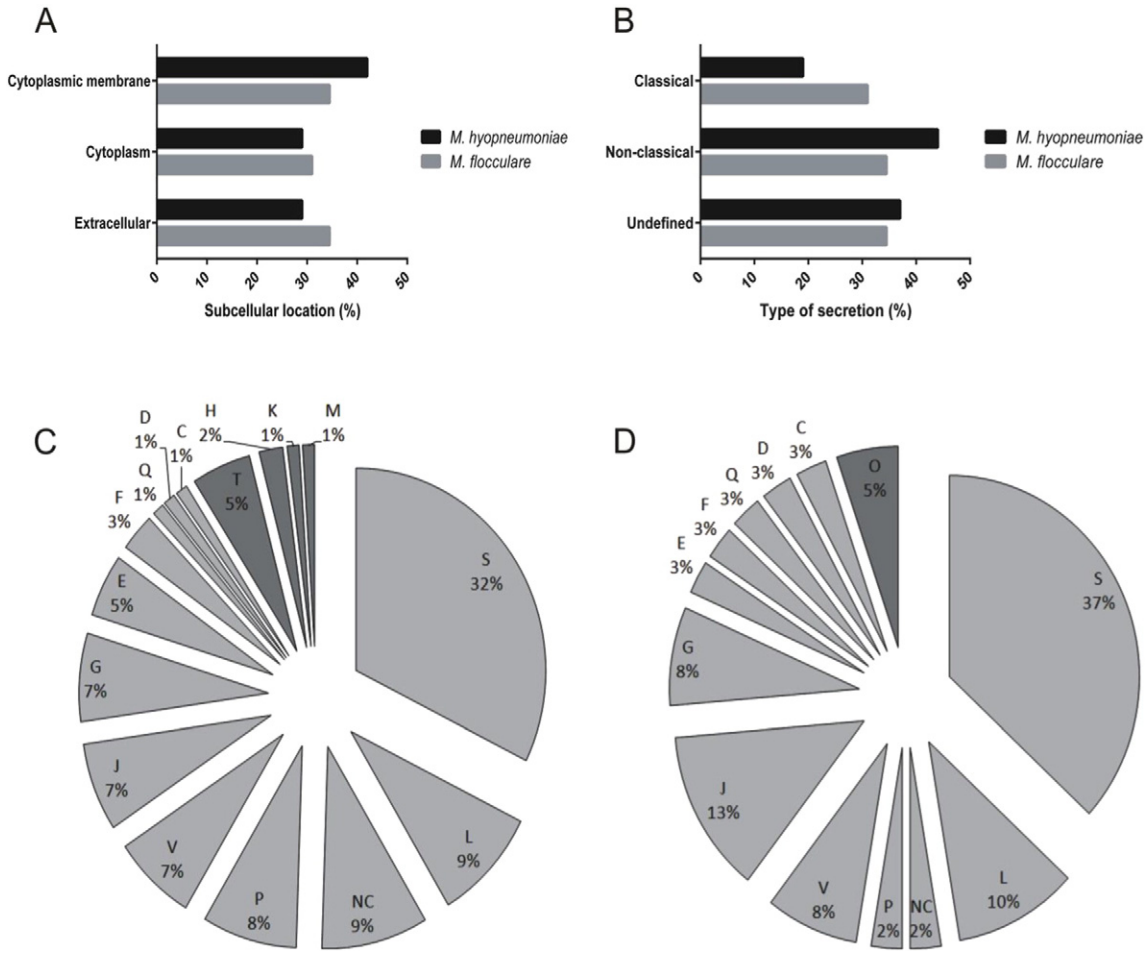

Fig. 3. In silico functional predictions for *M. hyopneumoniae* and *M. flocculare* in vitro secreted proteins identified by LC-MS/MS. Predictions of subcellular location (A), and type of secretion (B); bars represent the percentage of proteins assigned to a given category relative to the total of proteins identified in the sample. COG functional distribution *M. hyopneumoniae* (C) and *M. flocculare* (D) secreted proteins; categories highlighted in dark grey were exclusive for *M. hyopneumoniae* or *M. flocculare*, and percentages of proteins assigned to a given category relative to the total of proteins identified in the sample are indicated; some of the proteins were assigned to more than one functional category. Functional categories are: *(C) energy production and conversion;(D) cell cycle control and mitosis;(E) amino acid metabolism and transport; (F) nucleotide metabolism and transport; (G) carbohydrate metabolism and transport; (H) coenzyme metabolism; (J) translation; (K) transcription; (L) replication and repair; (M) cell wall/membrane/envelope biogenesis; (NC) not in COG; (O) post-translational modification, protein turnover, chaperone functions; (P) inorganic ion transport and metabolism; (Q) secondary metabolites biosynthesis; (S) function unknown; (T) signal transduction; (V) defense mechanisms.*

which was confirmed by the downstream LC-MS/MS analysis. Additionally, these proteomic results can, at least in part, represent the secretion profiles of *M. hyopneumoniae* and *M. flocculare* in the natural host environment, once both bacteria were cultivated in contact with porcine components, derived from PS.

The possible contamination with intracellular proteins in supernatant samples cannot be formally excluded, but its degree can be assumed to be low, as "contaminant" cells would be only the few ones that would surpass the centrifugation and filtration steps in sample processing. Indeed, two thirds of the detected proteins were typical secreted ones, and those considered as typical cytoplasmic ones, like ribosomal proteins and glycolytic enzymes, have been already described as secreted by non-classical pathways [40,41]. Also in line with that is the fact that, from the >40 ribosomal proteins encoded by *M. hyopneumoniae* and *M. flocculare* genomes, only two were detected for each species, suggesting specificity in their assumed secretion.

The standardized experimental conditions allowed to generate *M. hyopneumoniae* and *M. flocculare* supernatant samples from which productive LC-MS/MS analyses could be achieved. With that, the soluble secreted protein repertoires of these two closely related mycoplasma species, which thrive in the swine respiratory tract, could be comparatively assessed for the first time. This provided evidence of significant virulence-related differences between *M. hyopneumoniae* and *M. flocculare*, not observed to the same extant at the genomic or transcriptomic levels [4,5]. No correlation between previously assessed gene transcriptional levels and protein abundance in the *M. hyopneumoniae* and *M. flocculare* secretomes. This lack of correlation has been described [42] and is expected, as abundance of any given protein in the secretome depends not only of the transcription of its corresponding gene, but also on mRNA stability and translation efficiency, protein stability (turnover rate), and secretion efficiency.

Our data indicate that *M. hyopneumoniae* secretes more soluble proteins than *M. flocculare*, both in qualitative and quantitative terms. The *M. hyopneumoniae* soluble secretome was more complex, with 62 proteins discovered versus the 26 protein species detected for *M. flocculare*. Moreover, the NSAF value ranges are suggestive of higher abundances for *M. hyopneumoniae* secreted proteins than for *M. flocculare* ones. Qualitative and quantitative differences between *M. hyopneumoniae* and *M. flocculare* soluble secretomes could be related to differences between the secretion mechanisms of each species. *M. hyopneumoniae* classical Sec-system has less annotated protein components (6 out of the 11 expected for a complete Sec-system, [43]), than *M. flocculare* (with 8 out of 11). As both systems are functional, since signal peptide-containing proteins were found in the secretomes of both species, these differences, along with quantitative differences, suggest a less conserved and possibly more selective and efficient Sec-system for *M.*

hyopneumoniae. However, this system apparently is not the favored pathway for *M. hyopneumoniae*, as most of its identified secreted proteins were predicted as secreted by non-classical pathways or as localized in cytoplasmic membrane. These data are suggestive of alternative and preferential pathways for protein secretion in *M. hyopneumoniae*, including one mediated by extracellular vesicles, which would explain the presence of cytoplasmic membrane proteins in the bacterial supernatants [44]. Extracellular vesicle-mediated protein secretion was recently demonstrated for *Acholeplasma laidlawii* [45], but the data described here for *M. hyopneumoniae* and *M. flocculare* provided the first evidence of such secretion pathway within the genus *Mycoplasma*.

It has been described that some *M. hyopneumoniae* surface proteins [46–48] undergo atypical proteolytic processing (not related to signal peptide cleavage), resulting in differential representation of certain protein fragments in the cell surface. However, this issue has never been addressed for secretion products and our bottom-up proteomic approach did not allow to define whether the identified proteins are proteolytically processed prior, during or after their secretion. Future top-down proteomic analyses [49,50] would be necessary in order to specifically address this question.

Regarding proteins with known functions, a higher number of putative virulence factors [35] were found among proteins secreted by *M. hyopneumoniae*, including adhesins, methylases, nucleases, and antigenic lipoproteins, in comparison to that of *M. flocculare* [4], including just two adhesins. This is interesting, as *M. hyopneumoniae* shares its entire repertoire of known virulence factors with *M. flocculare* at genomic level [4,5,51], and all these genes coding for virulence-related proteins are transcribed by both *M. hyopneumoniae* and *M. flocculare* [5].

COG functional analyses showed that most proteins secreted by *M. hyopneumoniae* and *M. flocculare* were assigned to the "function unknown" category, composed mostly of proteins with function unknown, with a few annotated ones. As functional domain predictions for *M. hyopneumoniae* proteins with function unknown revealed virulence-related domains (e.g. peptidase and extracellular matrix binding domains), the presence of such proteins in the secretome of a disease-causing species (but not in its non-pathogenic close relative) reinforces their association with pathogenicity.

COG categorization of proteins with known functions was predominantly assigned to intracellular metabolic functions (e.g. carbohydrate, amino acid and nucleotide metabolism). The presence of these typical intracellular proteins in an ectopic compartment, as the soluble secretome, is suggestive of alternative, non-canonical functions, known as moonlighting functions [41,52]. Surface or extracellular ectopic localization have been described for several of these classic cytoplasmic proteins found in the *M. hyopneumoniae* and *M. flocculare*, including ribosomal proteins, aminoacyl tRNA synthetases, glycolytic enzymes, chaperones, transcription and translation factors, DNA polymerase, DNA gyrase and DNA helicase [41], but the function(s) performed by these proteins outside the cell still remains to be elucidated.

5. Conclusions

Clear qualitative and quantitative differences were demonstrated between both *M. hyopneumoniae* and *M. flocculare* soluble secreted protein repertoires. These differences pointed out a new direction for studies aiming the elucidation of the mechanisms underlining the infectious nature of *M. hyopneumoniae* and the commensal nature of *M. flocculare* in the porcine respiratory tract. Among the differentially represented secretion products, there were several *M. hyopneumoniae* virulence-related proteins and other potential relevant proteins with possible moonlighting functions, with transcribed orthologs in the *M. flocculare* genome, but not found in its secretome.

Several of the identified proteins secreted by *M. hyopneumoniae* and *M. flocculare* deserve future studies in order to provide clues on mechanisms related to pathogenicity or commensalism, respectively. Of particular interest will be virulence-related proteins with function unknown and moonlighting functions. Moreover, comparative studies on the secretion pathways of these two closely related species are needed, especially for the description of alternative pathways, including for the confirmation of secretion by extracellular vesicles.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2016.12.002.

Conflict of interest statement

The authors declare no conflict of interest.

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4.3 Análise comparativa dos proteomas de *M. hyopneumoniae* 7448 e J em condições de estresse oxidativo e choque térmico

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Differential responses to stress of two Mycoplasma hyopneumoniae *strains.*

Contribuição dos autores:

JAP e FMAL compartilham a primeira autoria. JAP: delineamento experimental, execução da análise dos proteomas de *M. hyopneumoniae* 7448 e J em estresse térmico, redação do manuscrito; FMAL: delineamento experimental, execução da análise dos proteomas de *M. hyopneumoniae* 7448 e J em estresse oxidativo, redação do manuscrito; HM e JRB: auxílio nas análises de LC-MS/MS, discussão dos resultados, revisão do manuscrito; HBF: delineamento experimental, análise e discussão de resultados e revisão do manuscrito.

Differential responses to stress of two *Mycoplasma hyopneumoniae* strains

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Running title: *Mycoplasma hyopneumoniae* stress response

Abstract

Mycoplasma hyopneumoniae is a respiratory pathogen, causing the porcine enzootic pneumonia. To survive in the porcine respiratory tract, *M. hyopneumoniae* must cope with both oxidative and heat stress imposed by the host. To get insights into *M. hyopneumoniae* stress responses and pathogenicity mechanisms, the protein profiles of two *M. hyopneumoniae* strains, the pathogenic 7448 strain and the non-pathogenic strain J, were surveyed under oxidative (OS) or heat (HS) stress. *M. hyopneumoniae* strains were submitted to OS (0.5% hydrogen peroxide) or HS (temperature shifts to 42°C) conditions and protein profiling was carried out by LC-MS/MS. Qualitative and quantitative differences involving from 40 to 60 *M. hyopneumoniae* proteins were observed for both strains when comparing bacteria exposed to OS or HS to non-treated controls. However, no differences in abundance were found in the proteins classically related to stress responses, as peroxidases and chaperones, suggesting that these proteins would be constitutively expressed by both strains in the tested conditions. Interestingly, under stress conditions, more virulence-related proteins were detected in *M. hyopneumoniae* 7448 differentially represented proteins than in *M. hyopneumoniae* J, suggesting that stress may trigger a differential response of the corresponding genes, shared by both strains.

Biological significance: *M. hyopneumoniae* is a respiratory pathogen of swine, causing the porcine enzootic pneumonia. To survive in the swine respiratory tract, *M. hyopneumoniae* must cope with both oxidative and heat stress imposed by the host, and, to get insights into the bacterial stress responses and pathogenicity mechanisms, the protein profiles of the pathogenic 7448 strain and the non-pathogenic strain J were surveyed under oxidative or heat stress. The performed comparative proteomics analyses provided evidence of differential stress responses between the 7448 and J strains, involving tens of proteins, including some known virulence factors. The described results suggest that stress conditions trigger the expression of potential virulence factors in the pathogenic *M. hyopneumoniae* 7448, but not in the non-pathogenic *M. hyopneumoniae* J.

Keywords: *Mycoplasma hyopneumoniae*; oxidative stress; heat stress; LC-MS/MS; virulence

1. Introduction

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia (PEP), a chronic respiratory disease in pigs [1]. PEP is highly prevalent causing significant economic losses in pig industry [2]. *M. hyopneumoniae* adheres to the porcine respiratory epithelium, causing ciliostasis, loss of cilial function and epithelial cell death. As a result, infected pigs become more susceptible to infections with other respiratory pathogens.

M. hyopneumoniae infection results in acute inflammation of the respiratory tract with infiltration of neutrophils, mononuclear and inflammatory cells [3]. Some adhesins from *M. hyopneumoniae* interact with host extracellular matrix components which stimulate macrophage signaling and result in production of reactive oxygen species (ROS) and pyrogenic cytokine release [4, 5]. *M. hyopneumoniae* seems to induce an environment of oxidative and heat stress in the porcine host and the pathogen has developed mechanisms to resist these stress conditions.

Little is known about the *M. hyopneumoniae* responses to oxidative and heat stress conditions. Transcriptional studies have demonstrated no differences in expression of genes related to oxidative stress protection [6, 7]. It was demonstrated that *M. hyopneumoniae* responds to temperature shifts increasing the transcription of genes encoding heat shock proteins, as DnaK and DnaJ [8], and some sRNAs [9]. However, unknown post-transcriptional processes may be regulating proteins in order to protect *M. hyopneumoniae* from oxidative stress and/or heat stress that may have a role in *M. hyopneumoniae* pathogenesis.

Despite the pathogenic nature of *M. hyopneumoniae*, there are some strains with different virulence levels, or are even avirulent, such as *M. hyopneumoniae* J. Comparisons between the genomes of *M. hyopneumoniae* pathogenic and non-pathogenic strains (7448 and J, respectively) showed no extensive genomic differences [10]. On the other hand, proteomic studies have provided evidences of differential protein abundance between *M. hyopneumoniae* pathogenic and non-pathogenic strains [11, 12], and their possible involvement in the pathogenicity. Considering these previous results, comparative proteomics analyses between *M. hyopneumoniae* pathogenic and non-pathogenic strains under stress conditions are needed to assess potential stress response mechanisms and to investigate possible pathogenicity factors related to stress responses.

In the present study, the cellular proteomes of *M. hyopneumoniae* strains 7448 (pathogenic) and J (non -pathogenic) were compared with and without exposure to

oxidative and heat stresses. An MS-based proteomic approach was used in order to identify proteins differentially represented in response to stress, and to compare the stress responses between pathogenic and non-pathogenic *M. hyopneumoniae* strains. The use of high resolution MS instruments allowed the identification of several differences among *M. hyopneumoniae* strains stress responses, some of them related to pathogenicity mechanisms.

2. Materials and Methods

2.1 Bacterial growth and stress conditions

M. hyopneumoniae 7448 was isolated from an infected swine from Lindóia do Sul (SC, Brazil) and had its pathogenicity experimentally confirmed [10]. The *M. hyopneumoniae* non-pathogenic strain J (ATCC 25934) has reduced adhesion capacity to porcine cilia [13] and was acquired from American Type Culture Collection by the Empresa Brasileira de Pesquisa Agropecuária-Centro Nacional de Pesquisa de Suínos e Aves (EMBRAPA-CNPSA, Concórdia, SC, Brazil). *M. hyopneumoniae* 7448 and J control cultures were grown with standard conditions in 50 mL of Friis medium [14] for 48 h, at 37°C, as previously described [15]. Bacterial cultures under oxidative stress (OS) and heat stress (HS) conditions were carried out as described in [7] and [8], respectively. All control and stressed mycoplasma cultures were performed in three independent replicates (biological replicates).

2.2 Protein extraction, SDS-PAGE analysis and sample preparation for mass spectrometry

After culture, mycoplasma cells were pelleted by centrifugation (3500 x g, 15 min, 4°C), and washed three-times with PBS (pH 7.4) for protein extracts preparation. Cells were then resuspended in PBS and lysed by sonication at 25 Hz in an ice bath, with five 30 s cycles and 1 min interval between pulses. The lysates were centrifuged at 10,000 x g, for 20 min, at 4°C for debris removal, and the resulting soluble protein extracts were quantified using the microBCA Protein Assay Kit (Thermo Fischer Scientific, IL, USA) using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, IL, USA).

Samples containing 10 µg of protein extracts were qualitatively evaluated by SDS-PAGE 12%, stained with Coomassie Brilliant Blue.

Samples containing 100 µg of the protein extracts were treated for MS analysis as previously described [12]. Briefly, proteins were precipitated with TCA 20%-acetone, incubated for 16 h at 4°C, and further centrifuged at 20,000 × g for 10 min. Protein pellets were dried and then solubilized with 8 M urea. Next, proteins were reduced with 2 µg of DTT (Bio-Rad, CA, USA) at 37°C for 1 h, and alkylated with 10 µg of iodoacetamide (Bio-Rad, CA, USA) in the dark, at room temperature. Protein samples were diluted to a final 1 M urea concentration, and further digested with 1 µg of trypsin (Promega, WI, USA). Resulting peptides were desalted in HLB cartridges (Waters Corporation, MA, USA), and eluted with 50% acetonitrile/0.1% TFA. Peptides were then lyophilized using a Concentrator Plus (Eppendorf, Germany), prior to MS analyses.

2.3 Mass spectrometry analyses

Processed peptide samples were analyzed for protein identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described [16]. Briefly, each peptide sample was reconstituted using 0.1% formic acid in water, loaded onto a nanoAcquity HPLC system (Waters Corporation, MA, USA). A two-step LC was performed, using first a trap column PepMap 100 C18 LC column (300 µm x 5 mm) (Thermo Fischer Scientific, IL, USA), at a flow rate of 5 µl/min, and then an Easy-Spray Column PepMap RSLC C18 (75 µm x 15 cm) analytical column (Thermo Fischer Scientific, IL, USA). For the gradient elution, the mobile phase solvents consisted of 0.1% formic acid in water (solvent A), and 0.1% formic acid in acetonitrile (Burdick and Jackson, MI, USA) (solvent B). The gradient flow was set at 0.3 µl/min. The elution profile consisted of a hold at 5% solvent B for 5 min, followed by a ramp up to 35% solvent B over 25 min; a ramp up to 95% solvent B in 5 min; and a hold at 95% for 5 min, prior to a return to 5% solvent B in 5 min, and re-equilibration at 5% solvent B for 20 min. After LC, the peptides were introduced into an Orbitrap Elite Hybrid Ion-trap-orbitrap tandem mass spectrometer (Thermo Fischer Scientific, IL, USA) for high resolution and MS/MS analysis. A 2.0 kV voltage was applied to the nano-LC column. The mass spectrometer was programmed to perform data-dependent acquisition by scanning the mass-to-charge (m/z) range from 400 to 1600, at a nominal resolution setting of 60,000 for parent ion acquisition. For the MS/MS analyses, the mass spectrometer was

programmed to select the top 15 most intense ions with two or more charges. Each biological replicate was independently analyzed by LC-MS/MS three times (technical replicates).

2.4 LC-MS/MS data analysis

MS data were processed using the Mascot Software (Matrix Science, UK). Raw MS data files were processed using Mascot Distiller. The database search was performed against *M. hyopneumoniae* 7448 and J local databases containing the deduced amino acid sequences (with 695 and 672 amino acid sequences, respectively) from the genomes annotation [10, 17] available at NCBI (https://www.ncbi.nlm.nih.gov/protein/). The search parameters included a fragment ion mass tolerance of 0.6 Da, peptide ion tolerance of 10 ppm, and three missed cleavages of trypsin. Carbamidomethylation of cysteine was specified as a fixed modification, whereas the oxidation of methionine, acetylation of lysine and N-terminal ends of proteins, phosphorylation of tyrosine and phosphorylation of serine/threonine were specified as variable modifications. These modifications were chosen due to the high content of adenine and thymine in mycoplasmas genome, which correlates with high probabilities of lysine acetylation and serine phosphorylation [18].

Scaffold version 4.8.1 (Proteome Software Inc., OR, USA) was used to validate the peptide and protein identifications. The Scaffold bundled version of X!Tandem was used to increase protein identification confidence. The peptide identifications were accepted if they could be established at > 99.0% probability as assigned by the Peptide Prophet algorithm [19]. The protein identifications were accepted if they could be established at > 95% probability as assigned by the Protein Prophet algorithm [20]; were based on at least 2 identified peptides; and were detected in at least two out of three replicates (both biological and technical). The normalized spectral abundance factor (NSAF) was used to quantify relative differences in protein abundance between control and stress samples. Quantitative differences between of *M. hyopneumoniae* 7448 and J ortholog proteins were analyzed based on exponentially modified protein abundance index (emPAI) values [21], without using the normalization option, to allow comparisons between ortholog proteins, and between strains (*M. hyopneumoniae* 7448 vs *M. hyopneumoniae* J [22]. Quantitative differences were statistically analyzed in Scaffold software using Student's t-test, with values of $p < 0.05$ considered statistically significant.

Functional analyses were performed based on gene ontology (GO) categorization. For that, GO annotations for proteins differentially represented between control and stress samples were retrieved from Uniprot database (http://www.uniprot.org/).

3. Results

3.1 SDS-PAGE profiling of *M. hyopneumoniae* 7448 and J protein extracts from stress conditions

The *M. hyopneumoniae* 7448 and J protein extracts from OS and HS samples, along with the respective control samples, were evaluated and compared by SDS-PAGE. Similar electrophoretic profiles were observed for all OS, HS and control samples and in all replicates, with molecular masses of resolved proteins ranging from as low as 20 kDa to > 220 kDa (Figure 1).

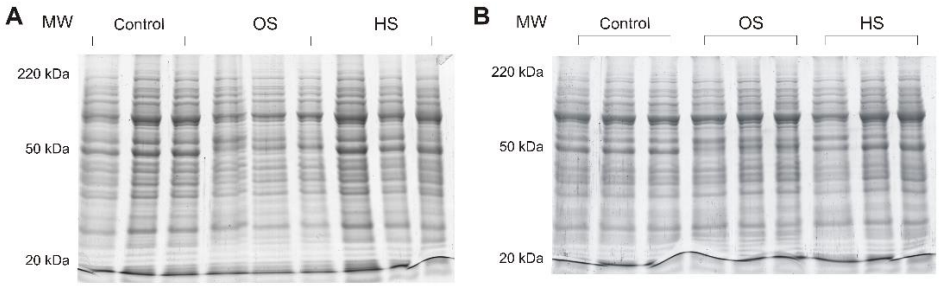

Fig. 1. SDS-PAGE protein profiles of **(A)** *M. hyopneumoniae* 7448 and **(B)** *M. hyopneumoniae* J under control (C) and stress (OS and HS) conditions. For all samples, the three used biological replicates are shown. Molecular weight markers (MW) are indicated (kDa).

3.2 MS-based protein identification in *M. hyopneumoniae* 7448 and J OS and HS protein extracts

LC-MS/MS analyses of proteins from *M. hyopneumoniae* 7448 and J strains identified overall totals of 267 and 278 protein species, respectively, considering proteins detected in all samples (OS, HS and controls). Detailed peptide and protein identification data are presented in Tables S1 and S2, respectively. Based on genome-wide analyses, the predicted proteome coverages obtained with these proteomic data was ~38%, for *M. hyopneumoniae* 7448, and ~41%, for *M. hyopneumoniae* J. The false discovery rates

(FDR) for the proteins and peptides of all samples were zero, validating all MS/MS results.

For the *M. hyopneumoniae* 7448 OS (Mh7448-OS), HS (Mh7448-HS) and control samples, 228, 261, and 208 proteins were identified respectively. Most (192) of the *M. hyopneumoniae* 7448 identified proteins were shared among these samples (Figure 2A). For the *M. hyopneumoniae* J OS (MhJ-OS), HS (MhJ-HS), and control, 262, 231, and 246 proteins were identified, respectively, with most of them (219) shared among these samples (Figure 2B).

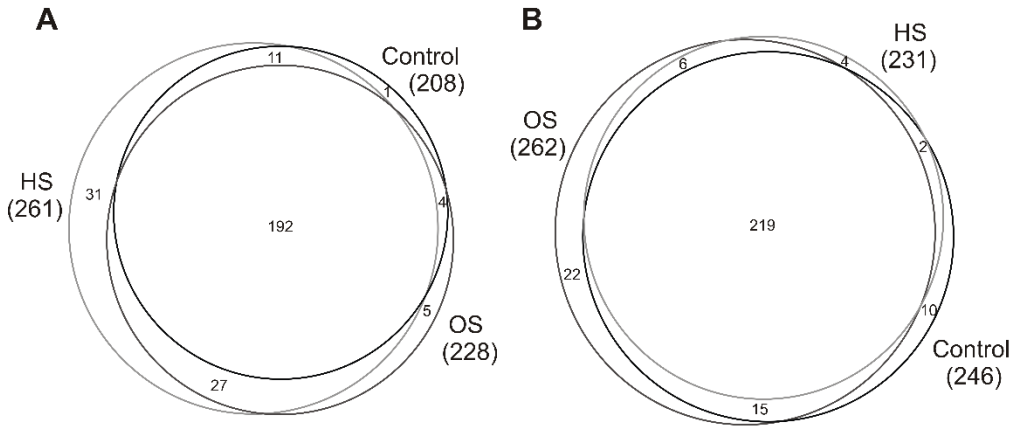

Fig. 2. Overall numbers of identified proteins in OS, HS and control samples from **(A)** *M. hyopneumoniae* 7448 and **(B)** *M. hyopneumoniae* J. The total of proteins identified in each are indicated outside the Venn diagram, between parentheses. The numbers of proteins exclusively detected in each sample or shared between them are indicated within the diagram.

3.3 LC-MS/MS protein profiling of *M. hyopneumoniae* 7448 and J strains under oxidative stress conditions

In order to understand the *M. hyopneumoniae* 7448 and *M. hyopneumoniae* J responses to oxidative stress, the protein repertoires of these strains under a hydrogen peroxide (H_2O_2) treatment were analyzed in comparison to the corresponding controls (untreated cultures) and to each other. For *M. hyopneumoniae* 7448, 40 out 228 (17.5%) proteins were differentially represented in Mh7448-OS samples in comparison to the control (Table 1). Among these proteins, 32 were exclusively detected, 7 were overrepresented (fold changes from 1.3 to 4.1), and only one was underrepresented (fold change of 0.68) in Mh7448-OS samples. Among the exclusively detected proteins in

Mh7448-OS, ribosomal proteins (3), membrane transporters (11), and uncharacterized proteins (7) were found. The set of overrepresented proteins included two ATP related proteins, one ribosomal protein, and two proteins involved with pyruvate metabolism, among others. Elongation factor EF-Tu was the only protein underrepresented in Mh7448-OS samples in comparison to the control. Interestingly, no proteins classically involved with oxidative stress, as OS-related enzymes or chaperones, were differentially represented in the OS condition in comparison to the control. However, functional domain analyses of uncharacterized proteins revealed that the MHP7448_0148 protein, which was only detected under stress conditions (OS and HS), bears an 'hsp33' functional domain.

GO analyses were performed to functionally categorize the 40 proteins differentially represented in Mh7448-OS samples. Twenty of them were categorized in 18 biological process (BP) subcategories; 24 were categorized in 5 cellular component (CC) subcategories; and 26 were categorized in 29 molecular function (MF) subcategories (Table S3A). No GOs could be assigned to 5 proteins. Functional subcategories related to DNA recombination, DNA repair, membrane transport, translation, and transcription were common for several of the Mh7448-OS differentially represented proteins.

In *M. hyopneumoniae* J protein sets, 45 out of 262 (17.1%) proteins were differentially represented in MhJ-OS samples in comparison to the control (Table 2). Among these proteins, 28 were exclusively detected, 5 were overrepresented (fold changes from 1.8 to 4.6), and 12 were underrepresented (fold changes from 0.25 to 0.66) in MhJ-OS samples. Among the exclusively detected proteins in MhJ-OS, ribosomal proteins (3), membrane transporters (7), uncharacterized proteins (6) and recombinant proteins (2) were found. The set of overrepresented proteins included GTP and ATP binding proteins (2), enzymes (2) and one uncharacterized protein. Ribosomal proteins (3), proteins involved with replication, transcription and translation were underrepresented in MhJ-OS sample in comparison to the control.

GO analyses were performed to functionally categorize the 45 proteins differentially represented in MhJ-OS samples. Eighteen of them were categorized in 20 BP subcategories; 16 were categorized in 4 CC subcategories; and 25 were categorized in 28 MF subcategories (Table S3b). No GOs could be assigned to only 4 proteins. As well as in *M. hyopneumoniae* 7448, functional subcategories related to DNA

recombination, DNA repair, membrane transport, translation, and transcription were common for several of the MhJ-OS differentially represented proteins.

Quantitative comparisons between ortholog proteins were performed based on emPAI values for the sets of proteins previously regarded as differentially represented in both *M. hyopneumoniae* 7448 and J OS-treated samples. Interestingly, among of the *M. hyopneumoniae* 7448 and J sets of 40 ad 45 differentially represented proteins, respectively, in comparison to the control, only 10 ortholog proteins were found (data not shown). Moreover, 3 of these proteins were differentially abundant between Mh7448-OS and MhJ-OS samples, namely a ribosomal protein (MHP7448_0122), a recombination protein (MHP7448_0258) and an ABC transporter (MHP7448_0315). These three proteins were overrepresented in Mh7448-OS samples.

3.4 LC-MS/MS protein profiling of *M. hyopneumoniae* 7448 and J strains under heat stress conditions

In order to investigate differences in heat shock responses of *M. hyopneumoniae* 7448 and *M. hyopneumoniae* J, the protein profiles of these strains were analyzed during a temperature shift (from 37°C to 42°C). Qualitative and quantitative differences were observed for both *M. hyopneumoniae* strains when compared to control (37ºC) and between each other.

LC-MS/MS analyses of *M. hyopneumoniae* 7448 revealed 62 out of 261 (23.7%) proteins differentially represented in Mh7448-HS samples in comparison to control (Table 3). Among these proteins, 57 were exclusively detected in HS, 4 were overrepresented (fold changes from 2.6 to 4.2), and only one was underrepresented (fold change of 0.5) in Mh7448-HS samples. Considering proteins exclusively detected in HS, several membrane transporters (11), ribosomal proteins (4) and uncharacterized ones (12) were found. Moreover, the adhesin P102-like (MHP7448_0271), the elongation factor EF-P (MHP7448_0427) and two uncharacterized proteins (MHP7448_0547 and MHP7448_0391) were found as overrepresented in HS samples. The 50S ribosomal L21 protein was the only protein underrepresented in Mh7448-HS samples in comparison to the control. Interestingly, no protein classically regarded as a heat shock response one, as DnaK or HSP70, was differentially represented in HS in comparison to control. Despite that, functional domain analyses of uncharacterized proteins revealed that the

MHP7448_0148 protein, which was exclusively detected in HS, bears an 'hsp33' functional domain.

GO analyses were performed to functionally categorize the proteins differentially represented in HS. Overall, 32 proteins were categorized in 36 biological process (BP) subcategories; 35 were categorized in 8 cellular component (CC) subcategories; and 43 were categorized in 47 molecular function (MF) subcategories (Table S3C). GO analyses demonstrated that functional subcategories related to membrane transport, translation and transcription were common for several proteins detected in HS.

In *M. hyopneumoniae* J protein sets, 15 out of 231 (6.4%) proteins were differentially represented in MhJ-HS samples in comparison to control (Table 4). Among these proteins, 10 were exclusively detected in HS, 2 were overrepresented (fold changes from 1.2 to 1.8), and three were underrepresented (fold changes from 0.5 to 0.6). Regarding proteins exclusively detected in MhJ- HS, there were 3 membrane transporters, one adhesin and 2 uncharacterized proteins, among others. Considering the proteins differentially abundant, four uncharacterized proteins were found: MHJ_0326 and MHJ_0662 were overrepresented, while MHJ_0445 and MHJ_0496 were underrepresented.

GO functional analyses of differentially represented proteins in HS revealed that 3 proteins were categorized in 5 BP subcategories; 9 were categorized in 5 CC subcategories; and 6 were categorized in 9 MF subcategories (Table S3D). Only MF subcategories of 'ATP binding' and 'ATPase activity' were found as common for some proteins found in HS samples. As well as in *M. hyopneumoniae* 7448, no protein classically regarded as a heat shock response was differentially represented in HS samples from *M. hyopneumoniae* J in comparison to control.

Quantitative comparisons of ortholog proteins of *M. hyopneumoniae* 7448 and J were performed based on emPAI values of proteins previously regarded as differentially represented in both *M. hyopneumoniae* 7448 and J HS samples. In this analysis, only five proteins were shared between *M. hyopneumoniae* 7448 and J, and just the putative ribose ABC transporter (MHP7448_0233) was found as differentially abundant, being 3.3 times more abundant in Mh7448-HS samples than in *M. hyopneumoniae* J. Furthermore, functional comparisons revealed that 73 GO subcategories (out of 91) were exclusively found for Mh7448-HS proteins, while only the MF 'serine-type endopeptidase activity' subcategory was exclusively detected in MhJ-HS proteins.

4. Discussion

Pathogenic bacteria face different stresses upon their contact with the host species. The host stress conditions triggers bacterial adaptive responses and directs the expression of several bacterial genes, many of them related to virulence [23]. Respiratory pathogens must deal with several host-derived antimicrobial mediators, including oxidative stress and temperature changes. H_2O_2 is a dangerous ROS involved in host-defense against infectious bacteria. For instance, macrophages produce H_2O_2 to damage and degrade bacterial cells. On the other hand, a variety of bacterial pathogens, including mycoplasmas, can produce H_2O_2 to damage host tissues [6, 24]. Moreover, pathogenic mycoplasmas, as other pathogens, also respond to heat stress [8, 25, 26], which is generated upon pyrogenic cytokine release by their hosts [4, 5].

In the case of *M. hyopneumoniae* infections, H_2O_2 production is induced in the swine respiratory tract, which results in host cell apoptosis [27]. Host immune cells attracted to the infection site produce ROS as a mechanism to eliminate *M. hyopneumoniae* [3]. *M. hyopneumoniae* response to survive the oxidative stress is still unknown, since its genome lacks genes coding for important antioxidant proteins, such as catalase, glutathione peroxidase and superoxide dismutase [28, 29]. Moreover, none of the ROS-related genes annotated in the *M. hyopneumoniae* genome were found as transcriptionally up-regulated during H_2O_2 exposure [7], suggesting that ROS-response may be more evident at the protein level, due to possible post-transcriptional regulation.

In the performed LC-MS/MS analyses, no abundance differences were found in the set of classical oxidative stress response proteins for both *M. hyopneumoniae* 7448 and J strains when comparing OS samples with non-exposed controls. However, for both *M. hyopneumoniae* strains, three proteins classically involved in oxidative stress response (thioredoxin, NADH oxidase and thiol peroxidase) are among the top 20 most abundant in both OS and control samples, suggesting high abundances of these proteins even in standard culture conditions. Moreover, a recent proteomics survey demonstrated that the enzyme thiol peroxidase was more abundant in the protein repertoire of *M. hyopneumoniae* 7448 than in the *M. hyopneumoniae* J one [30].

The oxidative stress response in mycoplasmas is still poorly understood, and other proteins that are not classically related to this process may be involved. In line with that, we found at least 40 proteins overrepresented in response to the H_2O_2 treatment that may participate in the *M. hyopneumoniae* mechanisms to cope with oxidative stress. For example, two lipoproteins detected exclusively in the Mh7448-OS sample, which suggest

that they can be regulated by oxidative stress and may be related to pathogenicity (as they were not detected at all in *M. hyopneumoniae* J). *Mycoplasma pneumoniae* lipoprotein genes were also identified as differentially expressed in response to exposure of several stress conditions, providing further evidence of lipoprotein regulation in response to changes in environmental conditions [31]. Moreover, among the proteins differentially represented between OS and control conditions for both *M. hyopneumoniae* strains there were many translation-related proteins. Mistranslation has been demonstrated as a bacterial strategy to overcome cell death caused by oxidative stress [32], and, since mistranslation is common in mycoplasmas [33], the overrepresentation of translation-related proteins upon exposure to H_2O_2 is suggestive of their involvement in mechanism that resist oxidative stress.

Mycoplasmas do not have orthologs for the typical sigma factors involved in heat stress responses found in other bacteria. However, transcriptional studies have demonstrated that mycoplasmas are able to regulate gene expression in response to environmental temperature changes. For *M. hyopneumoniae*, it was demonstrated that heat shock conditions affect the expression of several genes, including those coding for the heat shock proteins DnaK, DnaJ, Lon proteases, and ATP-dependent serine proteinase [8]. In the performed LC-MS/MS analyses, several proteins involved in HS responses were detected in both control and HS conditions, but quantitative analyses did not reveal significant differences in abundance of these proteins in comparison to control samples for both *M. hyopneumoniae* 7448 and J. A partial lack of correlation between transcriptomic and proteomic data is not unusual, due to post-transcriptional regulation and post-translational processing [34]. Interestingly, the DnaK chaperone was the most abundant protein in all tested conditions (HS, OS and controls) for both *M. hyopneumoniae* 7448 and J, which may indicate that the amounts of chaperones and other detected heat shock proteins are sufficient to deal with the temperature shift, without a requirement of upregulation in comparison to control conditions. Moreover, several proteins, non-classically related to heat stress protection, were found as differentially represented in HS conditions, suggesting that *M. hyopneumoniae* may present alternative mechanisms against heat stress.

The comparison between *M. hyopneumoniae* pathogenic and non-pathogenic strains under stress conditions (OS or HS) showed additional differences between these strains, some of them involving possible pathogenicity determinants. For instance, *M. hyopneumoniae* 7448 presented more known virulence factors as differentially

represented in response to stress including a P97-like protein and two lipoproteins, while *M. hyopneumoniae* J presented only the putative outer membrane protein (P95) as a differentially represented virulence factor. Moreover, under HS conditions, *M. hyopneumoniae* 7448 presented a larger set of differential proteins in comparison to the control than *M. hyopneumoniae* J. Among these *M. hyopneumoniae* 7448 proteins overrepresented under heat stress, there were potential virulence-related proteins including adhesins, membrane transporters and nucleases. These findings suggest that the expression of at least some *M. hyopneumoniae* 7448 virulence-related proteins is triggered by stress conditions, and that this does not occur in *M. hyopneumoniae* J.

Functional analyses of differentially represented proteins in OS and HS samples did not show extensive differences between these two stress conditions and between *M. hyopneumoniae* strains. Overall, functional subcategories involved in membrane transport, DNA repair, transcription, translation, ribosome biogenesis, and ATP binding and ATPase functions were common to both stress conditions and *M. hyopneumoniae* strains. Since all these functions are vital, the lack of overall differences is expected, even though some genes/proteins related to these functions were found as differentially regulated under oxidative and/or heat stress in the present study and/or in previous studies carried out with other mycoplasmas [7, 8, 35]. These findings confirm that OS and HS affect *M. hyopneumoniae* basic functions in order to maintain cell viability. Interestingly, our results provided the first evidence of regulation of DNA-repair functions in response to stress for a mycoplasma species. This suggests that *M. hyopneumoniae* has mechanisms to protect DNA against ROS or heat stress. This function may be post-transcriptionally regulated and executed by non-classic anti-stress proteins, such as recombination proteins.

Part of the stress response in *M. hyopneumoniae* may depend on roles played by currently unknown proteins, which still represent 37% of its genome coding capacity. Overall, around 10 unknown *M. hyopneumoniae* proteins with no function assigned (hypothetical proteins or conserved hypothetical proteins) were detected as differentially represented in response to OS or HS conditions for both strains. Five of the hypothetical proteins were differentially represented in both *M. hyopneumoniae* 7448 OS and HS samples, which suggests their involvement in general stress-response mechanisms. The search for domains among differentially abundant hypothetical proteins found an ‘hsp33’ functional domain in the *M. hyopneumoniae* 7448 hypothetical protein (AAZ53522.1 or MHP7448_0148) identified in both OS and HS samples. Hsp33 protein is a chaperone

whose activity is redox regulated [36], and interestingly, no ortholog for this protein was found as differentially represented in *M. hyopneumoniae* J. Large proportions of the genes coding for hypothetical proteins differentially expressed under OS and HS conditions have also been found for pathogenic *M. hyopneumoniae* 232 and *A. laidlawii* PG8 [7, 8, 35]. Therefore, the set of *M. hyopneumoniae* 7448 overrepresented or exclusive unknown proteins, including MHP7448_0148, may be part of a bacterial stress-response mechanism associated to pathogenicity.

5. Conclusion

Overall, our results provided a comprehensive profiling of the proteomes of two *M. hyopneumoniae* strains under oxidative and heat stress conditions. No differential abundance was found for most proteins classically related to stress responses, but, many of them were among the most abundant proteins in *M. hyopneumoniae*. This suggested that *in vivo*, *M. hyopneumoniae* may require constitutive expression of proteins involved with stress protection, due to the stresses of the host environment. Moreover, several proteins non-classically related to stress response were differentially represented under stress conditions, including proteins involved with protein expression and DNA repair. These findings indicated that the two *M. hyopneumoniae* strains may have alternative mechanisms to survival to stress conditions and differential stress-response capabilities. Pathogenic *M. hyopneumoniae* 7448 presented more virulence factors in the sets of differentially represented proteins during both stress conditions in comparison with non-pathogenic *M. hyopneumoniae* J, indicating that environmental stress may regulate pathogenicity determinants. Besides, as found in previous studies, a large set of proteins with unknown function was identified as differentially represented, and their roles in the bacterial stress response and pathogenicity remain to be elucidated.

Conflict of interest statement

The authors declare no conflict of interest.

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conclusions in this report are those of the authors and do not necessarily represent the views of CDC.

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Table 1. Proteins differentially represented in *M. hyopneumoniae* 7448 OS sample in comparison to control.

Identified Proteins	Accession Number[1]	T-Test ($p < 0.05$)[2]	Fold change[3]	NSAF Control[4]	NSAF H_2O_2[4]
Exclusive proteins[5]					
50S ribosomal protein L10	AAZ53982.1	-	-	-	0.0009
50S ribosomal protein L16	AAZ53561.1	-	-	-	0.0006
50S ribosomal protein L28	AAZ53497.1	-	-	-	0.0053
ABC transporter ATP-binding protein	AAZ53688.1	-	-	-	0.0012
ABC transporter ATP-binding protein	AAZ53398.1	-	-	-	0.0003
ABC transporter ATP-binding protein	AAZ53638.1	-	-	-	0.0003
ABC transporter permease protein	AAZ53820.2	-	-	-	0.0002
ABC transporter permease protein	AAZ53740.2	-	-	-	0.0001
conserved hypothetical protein	AAZ53849.2	-	-	-	0.0011
conserved hypothetical protein	AAZ53837.1	-	-	-	0.0003
conserved hypothetical protein	AAZ53846.2	-	-	-	0.0002
conserved hypothetical protein	AAZ53522.1	-	-	-	0.0005
DNA-directed RNA polymerase sigma factor	AAZ53437.2	-	-	-	0.0005
glucokinase	AAZ53883.2	-	-	-	0.0009
GTP-binding protein LepA	AAZ53450.1	-	-	-	0.0005
hydrolase of the HAD family	AAZ53582.1	-	-	-	0.0012
hypothetical protein MHP7448_0100	AAZ53477.2	-	-	-	0.0002
hypothetical protein MHP7448_0356	AAZ53727.1	-	-	-	0.0002
hypothetical protein MHP7448_0466	AAZ53833.2	-	-	-	0.0005
hypoxanthine phosphoribosyltransferase	AAZ53636.1	-	-	-	0.0010
inorganic pyrophosphatase	AAZ53966.1	-	-	-	0.0016
lipoprotein	AAZ53705.1	-	-	-	0.0005
lipoprotein	AAZ53871.1	-	-	-	0.0005
oligopeptide ABC transporter ATP-binding protein	AAZ53589.2	-	-	-	0.0006
oligopeptide ABC transporter system permease	AAZ53586.1	-	-	-	0.0005
P97-like protein	AAZ53646.2	-	-	-	0.0001
putative ribose ABC transporter	AAZ53607.2	-	-	-	0.0006
RecName: Full=Protein translocase subunit SecA	Q4A8S6.2	-	-	-	0.0006
recombination protein	AAZ53416.2	-	-	-	0.0005
transport protein sgaT	AAZ53747.1	-	-	-	0.0005
uridylate kinase	AAZ53901.1	-	-	-	0.0019
xylose ABC transporter ATP-binding protein	AAZ53880.2	-	-	-	0.0011
Overrepresented proteins					
recombination protein recR	AAZ53632.1	0.0085	4.1	0.0009	0.0036
pyruvate kinase	WP_020835572.1	0.013	2.1	0.0034	0.0073

ATP synthase beta chain	AAZ53430.1	0.021	2.1	0.0022	0.0047
periplasmic sugar-binding protein	AAZ53608.1	0.035	1.4	0.0033	0.0047
pyruvate dehydrogenase E1-alpha subunit	AAZ53490.1	0.037	1.3	0.0191	0.0252
ATP synthase alpha chain	AAZ53845.1	0.038	2.1	0.0037	0.0076
50S ribosomal protein L6	AAZ53553.1	0.04	1.3	0.0045	0.0061
Underrepresented proteins					
elongation factor EF-Tu	AAZ53889.1	0.01	0.68	0.0433	0.0297

[1] Accession numbers acquired from GenBank.
[2] T-test was performed in Scaffold software.
[3] Fold changes were calculated in Scaffold software, dividing NSAF values from OS-samples by NSAF values from control
[4] NSAF values correspond to the average of three biological replicates
[5] Dashes means that t-test and fold-changes were not calculated due to the corresponding proteins were not found in control samples

Table 2. Proteins differentially represented in *M. hyopneumoniae* J OS sample in comparison to control.

Identified Proteins	Accession Number[1]	T-Test ($p < 0.05$)[2]	Fold change[3]	NSAF Control[4]	NSAF H_2O_2[4]
Exclusive proteins[5]					
30S ribosomal protein S17	AAZ44272.2	-	-	-	0.0015
50S ribosomal protein L10	AAZ44704.1	-	-	-	0.0053
50S ribosomal protein L28	AAZ44210.1	-	-	-	0.0007
ABC transporter ATP-binding protein	WP_011284074.1	-	-	-	0.0184
ABC transporter ATP-binding protein	WP_011284207.1	-	-	-	0.0044
chromosomal replication initiator protein	AAZ44095.2	-	-	-	0.0002
conserved hypothetical protein	AAZ44515.2	-	-	-	0.0003
conserved hypothetical protein	AAZ44566.2	-	-	-	0.0011
conserved hypothetical protein	AAZ44717.2	-	-	-	0.0008
conserved hypothetical protein	AAZ44652.2	-	-	-	0.0003
conserved hypothetical protein	AAZ44350.1	-	-	-	0.0003
cytidylate kinase	AAZ44159.1	-	-	-	0.0006
glutamyl-tRNA amidotransferase subunit C	WP_011283844.1	-	-	-	0.0007
hexulose-6-phosphate isomerase	AAZ44521.2	-	-	-	0.0003
hypothetical protein MHJ_0291	AAZ44382.2	-	-	-	0.0004
hypoxanthine phosphoribosyltransferase	AAZ44345.1	-	-	-	0.0009
myo-inositol catabolism protein	AAZ44315.2	-	-	-	0.0008
NH(3)-dependent NAD+ synthetase	AAZ44556.1	-	-	-	0.0008
oligopeptide ABC transporter ATP-binding protein	AAZ44584.2	-	-	-	0.0008
PTS system, lichenan-specific IIA component	AAZ44130.2	-	-	-	0.0004
putative ABC transporter ATP-binding protein	AAZ44397.1	-	-	-	0.0001
putative ABC transporter ATP-binding protein	AAZ44347.1	-	-	-	0.0006
putative hexosephosphate transport protein	AAZ44223.2	-	-	-	0.0002
putative outer membrane protein - P95	AAZ44190.1	-	-	-	0.0001
putative PTS system galactitol-specific enzyme IIB component	AAZ44643.1	-	-	-	0.0020
putative sugar ABC transporter ATP-binding protein	AAZ44691.2	-	-	-	0.0004
recombination protein	AAZ44341.1	-	-	-	0.0025
recombination protein	AAZ44129.2	-	-	-	0.0005
Overrepresented proteins					
conserved hypothetical protein	AAZ44415.1	0.0073	1.8	0.0025	0.0044
uridylate kinase	AAZ44622.1	0.013	4.6	0.0003	0.0015
ATP-dependent protease binding protein	AAZ44192.2	0.015	3.8	0.0014	0.0053
phosphate acetyltransferase	AAZ44592.1	0.033	2.1	0.0086	0.0184
GTP-binding protein LepA	AAZ44163.1	0.043	2.5	0.0003	0.0007
Underrepresented proteins					

transketolase	AAZ44512.1	0.0027	0.70	0.0061	0.0043
50S ribosomal protein L19	AAZ44367.1	0.0042	0.86	0.0029	0.0025
hypothetical protein MHJ_0445	AAZ44531.2	0.0068	0.68	0.0016	0.0011
DNA-directed RNA polymerase subunit beta	AAZ44702.1	0.0075	0.66	0.0063	0.0042
conserved hypothetical protein	AAZ44183.2	0.013	0.35	0.0014	0.0005
30S ribosomal protein S9	AAZ44733.1	0.018	0.26	0.0026	0.0007
peptide chain release factor RF-1	AAZ44226.1	0.022	0.67	0.0028	0.0019
DNA topoisomerase I	AAZ44366.2	0.023	0.25	0.0012	0.0003
methionine aminopeptidase	AAZ44260.1	0.025	0.43	0.0023	0.0010
N-utilization substance protein A	AAZ44670.2	0.025	0.68	0.0044	0.0030
50S ribosomal protein L6	AAZ44266.1	0.031	0.71	0.0052	0.0037
glutamyl-tRNA amidotransferase subunit A	AAZ44118.2	0.046	0.56	0.0032	0.0018

[1] Accession numbers acquired from GenBank.

[2] T-test was performed in Scaffold software.

[3] Fold changes were calculated in Scaffold software, dividing NSAF values from OS-samples by NSAF values from control

[4] NSAF values correspond to the average of three biological replicates

[5] Dashes means that t-test and fold-changes were not calculated due to the corresponding proteins were not found in control samples

Table 3: Proteins differentially represented in *M. hyopneumoniae* 7448 HS samples in comparison to control.

Identified Proteins	Accession Number[1]	T-Test ($p < 0.05$)[2]	Fold change[3]	NSAF Control[4]	NSAF Heat shock[4]
Exclusive proteins[5]					
5"-3" exonuclease	AAZ53944.1	-	-	-	0.00066
50S ribosomal protein L10	AAZ53982.1	-	-	-	0.00131
50S ribosomal protein L16	AAZ53561.1	-	-	-	0.00112
50S ribosomal protein L28	AAZ53497.1	-	-	-	0.00381
50S ribosomal protein L9	AAZ54007.1	-	-	-	0.00078
ABC transporter ATP-binding protein	AAZ53398.1	-	-	-	0.00024
ABC transporter ATP-binding protein	AAZ53401.1	-	-	-	8.9E-05
ABC transporter ATP-binding protein	AAZ53679.1	-	-	-	9.1E-05
ABC transporter ATP-binding protein	AAZ53688.1	-	-	-	0.00094
ABC transporter permease protein	AAZ53820.2	-	-	-	0.00021
cell division protein ftsZ	AAZ53762.2	-	-	-	0.00039
conserved hypothetical protein	AAZ53522.1	-	-	-	0.00118
conserved hypothetical protein	AAZ53776.2	-	-	-	0.00032
conserved hypothetical protein	AAZ53846.2	-	-	-	0.0003
conserved hypothetical protein	AAZ53849.2	-	-	-	0.00057
conserved hypothetical protein	AAZ53991.1	-	-	-	0.00015
conserved hypothetical protein	AAZ54023.2	-	-	-	0.00034
conserved hypothetical protein	AAZ54038.1	-	-	-	0.00025
cytidylate kinase	AAZ53446.1	-	-	-	0.00041
DNA polymerase III alpha subunit	AAZ53945.2	-	-	-	0.00017
DNA-directed RNA polymerase sigma factor	AAZ53437.2	-	-	-	0.00024
glucokinase	AAZ53883.2	-	-	-	0.001
glucosamine-6-phosphate isomerase	AAZ53938.1	-	-	-	0.00027
glucose-6-phosphate isomerase	AAZ53897.1	-	-	-	0.00104
glycerol-3-phosphate dehydrogenase	AAZ53951.2	-	-	-	0.00036
glycine cleavage system H protein	AAZ53685.1	-	-	-	0.00103
glycyl-tRNA synthetase	AAZ53435.2	-	-	-	0.00018
GTP-binding protein LepA	AAZ53450.1	-	-	-	0.00041
GTP-binding protein YchF	AAZ53666.1	-	-	-	0.00062
hydrolase of the HAD family	AAZ53582.1	-	-	-	0.00127
hypothetical protein MHP7448_0100	AAZ53477.2	-	-	-	0.00021
hypothetical protein MHP7448_0356	AAZ53727.1	-	-	-	0.00016
hypothetical protein MHP7448_0499	AAZ53865.1	-	-	-	0.00015
hypoxanthine phosphoribosyltransferase	AAZ53636.1	-	-	-	0.00116
inorganic pyrophosphatase	AAZ53966.1	-	-	-	0.001
lipoate-protein ligase A	AAZ53628.2	-	-	-	0.00037
lipoprotein	AAZ53705.1	-	-	-	0.00056
lipoprotein	AAZ53871.1	-	-	-	0.00059

oligopeptide ABC transporter ATP-binding protein	AAZ53588.2	-	-	-	0.00029
oligopeptide ABC transporter ATP-binding protein	AAZ53589.2	-	-	-	0.00043
oligopeptide ABC transporter ATP-binding protein	AAZ53867.2	-	-	-	0.00029
P97-like protein	AAZ53646.2	-	-	-	0.00044
phenylalanyl-tRNA synthetase alpha chain	AAZ53647.2	-	-	-	0.00069
PTS system, lichenan-specific IIA component	AAZ53417.2	-	-	-	0.00058
putative ribose ABC transporter	AAZ53607.2	-	-	-	0.00075
RecName: Full=Protein translocase subunit SecA	Q4A8S6.2	-	-	-	0.00051
recombination protein	AAZ53416.2	-	-	-	0.0004
ribonuclease III	AAZ53767.1	-	-	-	0.00026
ribonucleoprotein NrdI	AAZ53596.1	-	-	-	0.00128
ribose-phosphate pyrophosphokinase	AAZ54016.2	-	-	-	0.00022
S-adenosylmethionine synthetase	AAZ53818.2	-	-	-	0.00043
site-specific DNA-methyltransferase	WP_051129931.1	-	-	-	0.00033
transport protein sgaT	AAZ53747.1	-	-	-	0.00055
tyrosyl tRNA synthetase	AAZ53440.1	-	-	-	0.00023
uridylate kinase	AAZ53901.1	-	-	-	0.00056
VACB-like ribonuclease II	AAZ53414.2	-	-	-	0.00038
xylose ABC transporter ATP-binding protein	AAZ53880.2	-	-	-	0.0012
Overrepresented proteins					
conserved hypothetical protein	AAZ53760.1	0.026	4.2	0.0005	0.00207
conserved hypothetical protein	AAZ53913.2	0.016	3.8	9E-05	0.00034
elongation factor EF-P	AAZ53795.1	0.048	2.6	0.00153	0.00405
P102-like protein	AAZ53645.2	0.02	3.1	0.00016	0.00048
Underrepresented proteins					
50S ribosomal protein L21	AAZ53506.1	0.036	0.5	0.00626	0.00327

[1] Accession numbers acquired from GenBank.
[2] T-test was performed in Scaffold software.
[3] Fold changes were calculated in Scaffold software, dividing NSAF values from HS-samples by NSAF values from control
[4] NSAF values correspond to the average of three biological replicates
[5] Dashes means that t-test and fold-changes were not calculated due to the corresponding proteins were not found in control samples.

Table 4. Proteins differentially represented in *M. hyopneumoniae* J HS sample in comparison to control.

Identified Proteins	Accession Number[1]	T-Test ($p < 0.05$)[2]	Fold change[3]	NSAF Control[4]	NSAF Heat shock[4]
Exclusive proteins[5]					
50S ribosomal protein L10	AAZ44704.1	-	-	-	0.00135
conserved hypothetical protein	AAZ44652.2	-	-	-	0.00081
hypothetical protein MHJ_0565	AAZ44650.2	-	-	-	0.00014
oligopeptide ABC transporter ATP-binding protein	AAZ44584.2	-	-	-	0.00017
putative hexosephosphate transport protein	AAZ44223.2	-	-	-	0.00037
putative outer membrane protein - P95	AAZ44190.1	-	-	-	0.00011
putative ribose ABC transporter	AAZ44317.2	-	-	-	0.00024
recombination protein	AAZ44129.2	-	-	-	0.00029
ribonucleoprotein NrdI	AAZ44307.1	-	-	-	0.00109
translation initiation factor IF-3	AAZ44213.2	-	-	-	0.0006
Overrepresented proteins					
conserved hypothetical protein	AAZ44415.1	0.0025	1.8	0.00248	0.00448
hypothetical protein MHJ_0662	AAZ44745.2	0.016	1.2	0.00538	0.00668
Underrepresented proteins					
hypothetical protein MHJ_0445	AAZ44531.2	0.023	0.6	0.0016	0.00108
hypothetical protein MHJ_0496	AAZ44582.1	0.023	0.5	0.00065	0.00036
sugar isomerase SgaE	AAZ44520.1	0.035	0.6	0.0025	0.00156

[1] Accession numbers acquired from GenBank.
[2] T-test was performed in Scaffold software.
[3] Fold changes were calculated in Scaffold software, dividing NSAF values from HS-samples by NSAF values from control
[4] NSAF values correspond to the average of three biological replicates
[5] Dashes means that t-test and fold-changes were not calculated due to the corresponding proteins were not found in control samples.

5. DISCUSSÃO GERAL

M. hyopneumoniae e *M. flocculare* colonizam o trato respiratório suíno, aderindo-se às células epiteliais ciliadas da traqueia, brônquios e bronquíolos. A interação *M. hyopneumoniae*-hospedeiro pode causar danos ao epitélio ciliado do trato respiratório suíno, que são característicos do quadro clínico da PES. Por outro lado, a interação *M. flocculare*-hospedeiro não produz danos, sendo virtualmente assintomática. Entretanto, *M. flocculare* possui ortólogos para a maioria dos genes codificadores de potenciais fatores de virulência presentes no genoma de *M. hyopneumoniae* (Siqueira *et al.*, 2013). Sabe-se que a patogênese bacteriana é um resultado dependente da interação entre a bactéria e seu hospedeiro, sendo que os danos ao hospedeiro são consequências de fatores microbianos, de fatores do próprio hospedeiro ou de ambos (Pirofski e Casadevall, 2018). Neste contexto, as naturezas patogênica de *M. hyopneumoniae* e comensal de *M. flocculare* podem ser resultantes da expressão diferencial de fatores microbianos e do hospedeiro induzida pela interação micoplasma-hospedeiro.

Os fatores microbianos relacionados ao dano são conhecidos como fatores de virulência ou determinantes de patogenicidade. Historicamente, a virulência tem sido estudada com foco nos efeitos induzidos no hospedeiro pela bactéria, sendo que fatores de virulência têm sido classicamente definidos como componentes do patógeno que impossibilitam o estabelecimento de uma doença quando deletados, mas não impossibilitam a viabilidade do patógeno (Casadevall e Pirofski, 1999; Casadevall e Pirofski, 2001). Entretanto, mesmo bactérias consideradas não-patogênicas podem ser capazes de causar doenças em indivíduos imunocomprometidos, o que reforça a importância dos fatores do hospedeiro para o estabelecimento da patogênese.

Como *M. hyopneumoniae* e *M. flocculare* compartilham o mesmo habitat e possivelmente o mesmo nicho, estudos comparativos entre estes dois micoplasmas foram relevantes para identificação de fatores microbianos diferenciais que possam estar relacionados com a determinação da patogenicidade de *M. hyopneumoniae*. Nestes estudos, foram identificadas algumas diferenças em nível genômico (Siqueira *et al.*, 2013), em nível gênico, isto é, entre ortólogos de *M. hyopneumoniae* e *M. flocculare* (Leal *et al.*, 2016) e em nível de expressão gênica transcricional e de RNAs não-codificantes (Siqueira *et al.*, 2014; Siqueira *et al.*, 2016). Apesar das diferenças encontradas, estes estudos também demonstraram o alto grau de similaridade entre *M. hyopneumoniae* e *M. flocculare*, não

sendo suficientes para explicar as naturezas diferenciais destes micoplasmas. Portanto, estudos comparativos entre *M. hyopneumoniae* e *M. flocculare* a nível pós-transcricional foram relevantes para identificação de fatores microbianos diferenciais que podem estar atuando como determinantes de patogenicidade. Na última década, diversos estudos proteômicos envolvendo o gênero *Mycoplasma* têm sido realizados para identificação de proteínas relacionadas a patogênese (Jores *et al.*, 2009; Catrein e Herrmann, 2011; Dietz *et al.*, 2016; Khan *et al.*, 2017). O estudo destas proteínas é relevante para compreensão da biologia destas bactérias na interação com o hospedeiro, sendo o produto final da expressão gênica e podendo atuar como efetoras da patogenicidade, e também para identificação de dezenas de alvos proteicos para o desenvolvimento de testes imunodiagnósticos e formulações vacinais.

A patogênese das infecções por micoplasmas envolve mecanismos complexos que englobam desde a adesão às células do tecido hospedeiro até a resposta imune desencadeada pelo hospedeiro infectado (He *et al.*, 2018). Estes mecanismos incluem a participação efetiva de diversas proteínas, que atuam como efetoras de processos metabólicos citoplasmáticos, ou como adesinas e antígenos de superfície e/ou como produtos de secreção. No contexto de *M. hyopneumoniae* e *M. flocculare*, o fracionamento celular combinado a análise proteômica permitiu a identificação de centenas de proteínas detectadas nos compartimentos citoplasmáticos, de superfície e/ou extracelulares destes micoplasmas, muitas das quais podem estar envolvidas no estabelecimento da patogênese de *M. hyopneumoniae*. Além disso, a análise comparativa destes repertórios proteicos de *M. hyopneumoniae* e *M. flocculare* possibilitou a detecção de diversas proteínas diferencialmente representadas entre estes micoplasmas, sendo que estas proteínas foram consideradas como potenciais determinantes de patogenicidade de *M. hyopneumoniae*.

Os processos biológicos potencialmente envolvidos na determinação da patogenicidade de *M. hyopneumoniae* incluem a adesão da bactéria às células ciliadas do epitélio respiratório do hospedeiro suíno, o processamento proteolítico de proteínas de superfície, o transporte de moléculas, a secreção de proteínas através da membrana plasmática, a proteção contra estresses e o metabolismo. Interessantemente, estes processos biológicos também podem ocorrer na interação entre *M. flocculare* e o hospedeiro suíno. Entretanto, as análises comparativas dos proteomas e secretomas de *M. hyopneumoniae* e *M. flocculare* evidenciaram diferenças qualitativas e quantitativas em diversas proteínas

envolvidas nestes processos celulares (seções 4.1, 4.2 e 4.3). Portanto, a abundância diferencial destas proteínas pode estar relacionada com a determinação da patogenicidade de *M. hyopneumoniae*, uma vez que muitas proteínas descritas como potenciais fatores de virulência (Ferreira e Castro, 2007; Machado *et al.*, 2009; Deutscher *et al.*, 2010; Bogema *et al.*, 2012; Deutscher *et al.*, 2012; Moitinho-Silva *et al.*, 2013; Jarocki *et al.*, 2015) foram detectadas exclusivamente ou em maior abundância no proteoma e/ou secretoma da linhagem patogênica *M. hyopneumoniae* 7448.

Considerando o processo de adesão micoplasmas-hospedeiro, a análise comparativa dos repertórios de adesinas nos proteomas de *M. hyopneumoniae* e *M. flocculare* demonstrou a abundância diferencial de algumas delas, que foram detectadas em maior quantidade em *M. hyopneumoniae* 7448 (Seção 4.1). Em contraponto, as adesinas P102-cópia 1 e P97-copy 2 foram mais abundantes em *M. hyopneumoniae* J e em *M. flocculare*, respectivamente, do que em *M. hyopneumoniae* 7448. A abundância diferencial das adesinas de linhagens de *M. hyopneumoniae* e de *M. flocculare* pode estar relacionada com as suas capacidades diferenciais de adesão. Sabe-se que mesmo linhagens não-patogênicas de *M. hyopneumoniae* e *M. flocculare* são capazes de se aderir ao epitélio ciliado do trato respiratório suíno, embora a capacidade de adesão sejam diferenciais quando comparadas com as de outras linhagens patogênicas de *M. hyopneumoniae* (Zielinski e Ross, 1993; Young *et al.*, 2000). Apesar das capacidades de adesão destes micoplasmas serem diferenciais, apenas a aderência ao epitélio respiratório suíno não é suficiente para explicar a patogênese da PES, pois mesmo *M. hyopneumoniae* J e *M. flocculare* são capazes de aderir ao epitélio respiratório suíno (Young *et al.*, 2000).

O sucesso da adaptação e da sobrevivência de micoplasmas nos seus hospedeiros está relacionado a variação antigênica de proteínas de superfície que resulta na evasão da resposta imune do hospedeiro (Citti *et al.*, 2010). O fenômeno da variação antigênica de micoplasmas tem sido associado a mecanismos genéticos dirigidos a genes codificadores de proteínas de superfície, que incluem mutações espontâneas, DNA *slippage* ou rearranjos de DNA envolvendo recombinação sítio-específica. Em *M. hyopneumoniae*, sequências de DNA codificadoras de proteínas de superfície contendo VNTRs foram identificadas, sugerindo que estas repetições podem estar relacionadas com mecanismos de variação antigênica nas proteínas correspondentes (De Castro *et al.*, 2006).

Além dos mecanismos genéticos de produção de variações antigênicas, mecanismos enzimáticos mediados por proteases também podem estar relacionados com a produção de diferentes antígenos e evasão/modulação da resposta imune do hospedeiro. A superfície celular de *M. hyopneumoniae* é extensivamente modificada por eventos de processamento proteolítico pós-traducionais que têm as adesinas como alvo principal (Tacchi *et al.*, 2016b). Evidências do processamento proteolítico de proteínas de superfície de *M. hyopneumoniae* e *M. flocculare* foram fornecidas pela análise comparativa de proteínas detectadas nos compartimentos citoplasmático e de superfície (Seção 4.1). Além disso, diferenças no processamento proteolítico de adesinas de *M. hyopneumoniae* e *M. flocculare* foram evidenciadas (Machado *et al.*, dados não publicados), indicando que apesar destes micoplasmas compartilharem mais de 90% dos genes codificadores proteínas de superfície, a composição peptídica da superfície destes micoplasmas é diferencial. Estes resultados podem estar relacionados com a patogenicidade de *M. hyopneumoniae*, uma vez que inferem na capacidade de adesão da bactéria às células do trato respiratório do hospedeiro e em mecanismos de evasão e/ou modulação da resposta imune.

O processamento proteolítico diferencial de proteínas de superfície de *M. hyopneumoniae* e *M. flocculare* também foi evidenciado na análise comparativa dos secretomas destes micoplasmas (Seção 4.2). A presença exclusiva de peptídeos correspondentes a oito adesinas (incluindo as P97-cópia 2 e P102-cópia 1) no compartimento extracelular de *M. hyopneumoniae* 7448 sugere que estes peptídeos podem ser produtos de clivagem executada por diferentes proteases que foram detectadas em maior abundância na superfície de *M. hyopneumoniae* 7448 (Seção 4.1). A identificação destes peptídeos de diferentes adesinas no repertório de proteínas secretadas por *M. hyopneumoniae* também é sugestiva de que estas proteínas podem exercer funções além da adesão, como a apresentação de diversos epítopos antigênicos ou ao hospedeiro e suas consequências para evasão/modulação da resposta imune.

Nas relações bactéria-hospedeiro, as proteínas secretadas representam a primeira linha de interação entre os organismos em interação. No caso de bactérias patogênicas, seus secretomas podem conter fatores de virulência ou determinantes de patogenicidade. A análise comparativa dos secretomas de *M. hyopneumoniae* 7448 e *M. flocculare* evidenciou que a composição proteica dos secretomas destas duas micoplasmas é distinta, uma vez que o repertório de proteínas secretadas por *M. hyopneumoniae* 7448 apresenta maior

complexidade e um conjunto maior de potenciais fatores de virulência, comparado ao de *M. flocculare* (seção 4.2). Entretanto, os mecanismos de secreção de proteínas por micoplasmas ainda são desconhecidos. A nível genômico, *M. hyopneumoniae* e *M. flocculare* apresentam seis de onze genes codificadores componentes da via de secreção Sec-dependente (Siqueira *et al.*, 2013). A nível proteômico, a expressão de cinco destes componentes foi validada experimentalmente, sendo que apenas a proteína SecA foi diferencialmente representada entre estes micoplasmas, sendo encontrada em maior abundância no repertório proteico de superfície de *M. hyopneumoniae* (Seção 4.2). A proteína SecA tem função de ATPase e é necessária para a translocação das proteínas secretadas através do canal de translocação Sec (Cranford-Smith e Huber, 2018). Assim, a maior abundância de SecA na superfície de *M. hyopneumoniae* pode estar relacionada com a maior complexidade (de espécies proteicas e abundância) do repertório de proteínas secretadas por este micoplasma. Além disso, os secretomas de *M. hyopneumoniae* e *M. flocculare* em interação com uma linhagem celular traqueal suína foram comparativamente analisados (Leal Zimmer *et al.*, 2018), e os resultados obtidos reforçam os dados discutidos na seção 4.2, uma vez que o repertório de proteínas secretadas por *M. hyopneumoniae* 7448 é rico em potenciais fatores de virulência. Interessantemente, em ambas as análises secretômicas, o repertório de proteínas secretadas por *M. hyopneumoniae* 7448 apresenta diversas proteínas envolvidas em processos celulares importantes, incluindo vias metabólicas relacionadas ao metabolismo energético, síntese de DNA e de proteínas.

Estudos metabolômicos demonstraram que as capacidades metabólicas de *M. hyopneumoniae* e *M. flocculare* são muito similares em termos de genoma (Ferrarini *et al.*, 2016). Neste contexto, diferenças metabólicas, que incluem taxas de reações diferenciais e produtos metabólicos distintos, podem ser resultantes da expressão diferencial de enzimas entre *M. hyopneumoniae* e *M. flocculare*. A expressão diferencial de enzimas relacionadas a diferentes vias metabólicas foi evidenciada nas análises proteômicas comparativas entre *M. hyopneumoniae* e *M. flocculare* (Seção 4.2), sugerindo que as capacidades metabólicas destes dois micoplasmas podem ser diferenciais em nível proteômico.

Apesar da similaridade metabólica de *M. hyopneumoniae* e *M. flocculare*, diferenças interessantes foram identificadas (Ferrarini *et al.*, 2016), podendo-se destacar o catabolismo do mio-inositol, cujos genes codificadores das enzimas envolvidas nesta via são exclusivamente encontrados no genoma de *M. hyopneumoniae*. O mio-inositol é encontrado

como um produto secundário na composição do surfactante pulmonar e pode ser usado como uma fonte de carbono e como uma via alternativa para produção de acetil-CoA. Desta forma, a exclusividade da via catabólica do mio-inositol em *M. hyopneumoniae* pode estar relacionada com a patogenicidade desta micoplasma. Entretanto, as enzimas relacionadas ao catabolismo do mio-inositol foram detectadas em quantidades equivalentes nos repertórios proteicos de *M. hyopneumoniae* 7448 e *M. hyopneumoniae* J (seção 4.2). A ausência da abundância diferencial entre as enzimas relacionadas ao catabolismo do mio-inositol em *M. hyopneumoniae* 7448 e J está de acordo com os resultados obtidos por Galvão-Ferrarini *et al.* (2018), uma vez que as duas linhagens são capazes de absorver mio-inositol *in vitro*, mas sem diferença significativa entre linhagens.

Além do catabolismo do mio-inositol, conversão de glicerol 3-fosfato em dihidroxiacetona-fosfato é um processo metabólico exclusivo de *M. hyopneumoniae*, uma vez que o gene codificador da enzima responsável por este processo, a glicerol 3-fosfato oxidase (GlpO), é exclusivamente encontrado no genoma desta micoplasma (Ferrarini *et al.*, 2016). O processo metabólico de conversão do glicerol 3-fosfato em dihidroxiacetona-fosfato produz peróxido de hidrogênio, que é altamente tóxico para a micoplasma e para o hospedeiro. Sabe-se que outros micoplasmas patogênicos, como *Mycoplasma pneumoniae* e *Mycoplasma mycoides*, utilizam a enzima GlpO para produção de peróxido de hidrogênio como mecanismo de patogenicidade (Vilei e Frey, 2001; Hames *et al.*, 2009). Análises comparativas entre linhagens patogênicas e não-patogênicas de *M. hyopneumoniae* também evidenciaram que apenas as linhagens patogênicas analisadas são capazes de produzir peróxido de hidrogênio *in vitro* (Galvao Ferrarini *et al.*, 2018). Além da produção endógena de ROS, a infecção por *M. hyopneumoniae* desencadeia uma resposta inflamatória no trato respiratório suíno que também induz a produção de ROS (Bai *et al.*, 2013; Bai *et al.*, 2015). Neste contexto, um dos potenciais pontos críticos para a determinação do quadro clínico da PES é a proteção contra o estresse oxidativo produzido pela interação micoplasmas-hospedeiro. As análises proteômicas comparativas entre *M. hyopneumoniae* e *M. flocculare* (seção 4.2) evidenciaram a expressão diferencial de enzimas relacionadas a proteção contra o estresse oxidativo, como a tiol peroxidase, a tiorredoxina e a tiorredoxina-redutase que foram mais representadas em *M. hyopneumoniae* 7448 do que em *M. flocculare*, sugerindo que estas enzimas podem exercer um papel importante na determinação da patogenicidade de *M. hyopneumoniae*.

As análises proteômicas comparativas evidenciaram a abundância e a localização subcelular diferenciais de diversas proteínas envolvidas em processos metabólicos nos repertórios proteicos de *M. hyopneumoniae* e *M. flocculare*. Dentre estas proteínas, enzimas da via glicolítica e fatores de tradução foram detectados em maior em maior abundância no repertório proteico de superfície *M. hyopneumoniae* 7448. Interessantemente, foi demonstrado que estas proteínas podem atuar alternativamente na adesão micoplasmas-hospedeiro (Tacchi *et al.*, 2016b; Widjaja *et al.*, 2017). Assim, é possível que estas proteínas atuem como potenciais determinantes de patogenicidade na superfície de *M. hyopneumoniae* através de funções *moonlighting* associadas à adesão (Henderson, 2014) ou a outros processos relevantes para patogenicidade, incluindo a atuação como antígenos de superfície e processos relacionados a modulação da resposta imune do hospedeiro.

No hospedeiro suíno, a infecção por *M. hyopneumoniae* é caracterizada histologicamente pela infiltração de neutrófilos e células mononucleares na lâmina própria do tecido colonizado e pela hiperplasia proeminente do tecido linfoide associado aos brônquios (BALT) (Rodríguez *et al.*, 2004). Análises imuno-histoquímicas de pulmões e do BALT de suínos naturalmente infectados por *M. hyopneumoniae* demonstraram um aumento da produção de citocinas pro-inflamatórias e imunorregulatórias, como interleucinas (IL)-2, IL-4 e o fator de necrose tumoral (TNF)-α. A secreção destas citocinas induz uma resposta imune inflamatória persistente que resulta nas lesões pulmonares características da PES.

Além da resposta imune inflamatória, proteínas de superfície e subprodutos do metabolismo de *M. hyopneumoniae* podem induzir morte celular em células suínas por apoptose (Bai *et al.*, 2013; Bai *et al.*, 2015; Paes *et al.*, 2017; Galvao Ferrarini *et al.*, 2018). Além da produção de citocinas pró-inflamatórias e da indução de apoptose em células suínas, outros fatores da interação *M. hyopneumoniae*-hospedeiro que são intrínsecos do hospedeiro têm sido recentemente apontados. Estes fatores incluem a secreção de proteínas suínas relacionadas a padrões moleculares associados ao dano (DAMPs) e alterações em vias relacionadas a homeostase de cálcio intracelular e de proteínas do retículo endoplasmático relacionadas a estresse durante ensaios de interação entre *M. hyopneumoniae* e células suínas em cultura (Leal Zimmer *et al.*, 2018; Leal Zimmer *et al.*, dados não publicados). Sabe-se que DAMPs atuam como "sinais de perigo" quando secretados, estimulando a resposta imune pró-inflamatória observada no quadro clínico da PES. Além disso, as alterações observadas nas vias relacionadas ao cálcio e ao retículo endoplasmático podem estar

relacionadas a mecanismos de morte celular por apoptose. Interessantemente, a secreção de proteínas relacionadas a DAMPs e as alterações das vias de relacionadas ao cálcio e ao retículo endoplasmático não foram observadas nas interações entre *M. hyopneumoniae* J ou *M. flocculare* e células suínas, indicando que estas respostas são fatores do hospedeiro específicos para a linhagem patogênica *M. hyopneumoniae* 7448 e estão relacionadas à determinação de patogenicidade desta bactéria.

Na tentativa de eliminar o patógeno, a resposta imune do hospedeiro impõe uma série de estresses à bactéria, que inclui o estresse oxidativo relacionado a produção de ROS pelos neutrófilos infiltrados no trato respiratório suíno infectado, e o estresse térmico relacionado a secreção de citocinas pirogênicas (Woolley *et al.*, 2013; Bai *et al.*, 2015). Considerando os mecanismos utilizados por *M. hyopneumoniae* para proteção contra estresse, análises proteômicas comparativas entre as linhagens *M. hyopneumoniae* 7448 e J demonstraram que não há diferença significativa na abundância de proteínas relacionadas a proteção contra ROS em condições de estresse oxidativo (Seção 4.3). De fato, foi observado que estas proteínas, como a tiol-peroxidase e a tiorredoxina, estão entre as mais abundantes, embora não haja abundância diferencial entre as linhagens. O mesmo resultado foi obtido em situações de estresse térmico, em que chaperonas foram detectadas entre as proteínas mais abundantes, mas sem apresentar abundância diferencial entre as linhagens de *M. hyopneumoniae*. Entretanto, os repertórios proteicos de *M. hyopneumoniae* 7448 e J identificados em condições de estresse oxidativo e estresse térmico foram diferenciais. Diversos potenciais determinantes de patogenicidade de *M. hyopneumoniae* foram detectados como exclusivos ou mais abundantes no repertório proteico da linhagem patogênica *M. hyopneumoniae* 7448 em comparação com a linhagem não-patogênica *M. hyopneumoniae* J. Estes resultados podem ser extrapolados para condições *in vivo* da relação micoplasmas-hospedeiro, uma vez que os estresses oxidativo e térmico são comumente ocasionados no estabelecimento do quadro clínico da PES (Deblanc *et al.*, 2013). Desta forma, as condições de estresse enfrentadas por estas micoplasmas no hospedeiro suíno podem atuar como potenciais gatilhos para expressão dos determinantes de patogenicidade.

As diferenças qualitativas e quantitativas encontradas nos repertórios proteicos de *M. hyopneumoniae* 7448, *M. hyopneumoniae* J, e *M. flocculare* sugerem que há diferenças na regulação da expressão gênica destes micoplasmas. A baixa correlação entre a abundância de transcritos (Madsen *et al.*, 2006; Schafer *et al.*, 2007; Siqueira *et al.*, 2013) e a abundância

de proteínas (seções 4.1, 4.2 e 4.3) de *M. hyopneumoniae* e *M. flocculare* indica a atuação de mecanismos pós-transcricionais e pós-traducionais de regulação da expressão gênica, incluindo a regulação da tradução destes transcritos e a degradação de proteínas. No contexto da regulação da tradução, proteínas ribossômicas, fatores de tradução e aminoacil-tRNA sintetases foram detectadas em maior abundância no repertório proteico de *M. hyopneumoniae* 7448 em comparação com *M. hyopneumoniae* J e *M. flocculare*, sugerindo uma maior eficiência de tradução na linhagem patogênica. Além disso, mecanismos alternativos de regulação pós-transcricionais baseados na interação sRNA-mRNA já foram evidenciados em *M. hyopneumoniae* (Siqueira *et al.*, 2016). Embora as diferenças qualitativas e quantitativas encontradas entre os repertórios proteicos de *M. hyopneumoniae* e *M. flocculare* possam estar relacionadas a determinação de patogenicidade de *M. hyopneumoniae*, novos estudos são necessários para compreender e elucidar os mecanismos moleculares de produção destas diferenças e as suas implicações para a patogenicidade.

6. CONCLUSÕES E PERSPECTIVAS

A análise dos proteomas intracelular e de superfície e dos secretomas de *M. hyopneumoniae* e *M. flocculare* evidenciou a expressão de mais de 50% dos genes preditos nos genomas destas espécies, incluindo a validação experimental e anotação funcional de dezenas de proteínas previamente consideradas como hipotéticas. Além disso, mais de uma centena de espécies proteicas foram detectadas como diferencialmente representadas entre *M. hyopneumoniae* e *M. flocculare*, incluindo potenciais determinantes de patogenicidade previamente descritos para *M. hyopneumoniae* e outros novos potenciais determinantes de patogenicidade a serem estudados. A identificação destes potenciais determinantes de patogenicidade também permitiu a identificação de diferentes processos biológicos de interação micoplasmas-hospedeiro potencialmente envolvidos com a determinação do quadro clínico da PES, que vão desde a adesão às células do trato respiratório suíno a vias metabólicas e de proteção contra estresses.

As análises proteômicas comparativas realizadas neste estudo permitirão a identificação de dezenas de potenciais determinantes de patogenicidade de *M. hyopneumoniae* cujos papéis como determinantes de patogenicidade efetivos podem ser confirmados experimentalmente. Para isso, ensaios de caracterização funcional, estrutural e/ou imunológica de versões recombinantes dos potenciais determinantes de patogenicidade podem ser inicialmente realizados, considerando principalmente proteínas previamente anotadas como hipotéticas. Atualmente, a versão recombinante da proteína MHP7448_0148, que contém o domínio funcional Hsp33 e foi detectada em maior abundância no proteoma de *M. hyopneumoniae* 7448, está sendo produzida para futuros ensaios de caracterização funcional. Além disso, podem ser realizados ensaios de infecção experimental de suínos com linhagens de *M. hyopneumoniae* mutantes para potenciais determinantes de patogenicidade, utilizando o método de mutagênese descrito por Maglennon *et al.* (2013).

Além da confirmação experimental dos potenciais determinantes de patogenicidade de *M. hyopneumoniae*, os estudos de caracterização funcional, estrutural e imunológica destas proteínas poderá contribuir para novas estratégias de controle, prevenção e tratamento da PES. Neste contexto, proteínas evidenciadas como antigênicas e/ou imunogênicas poderão ser aplicadas no desenvolvimento de testes imunodiagnósticos e/ou formulações vacinais recombinantes efetivos contra a PES. Além disso, análises estruturais destas

proteínas poderão fornecer dados importantes para o desenvolvimento ou reposicionamento de fármacos para o tratamento de suínos infectados por *M. hyopneumoniae*.

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ANEXO I

Curriculum vitae resumido

Dados pessoais

Nome	Jéssica Andrade Paes Vieira
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Formação acadêmica/Titulação

2013-2015 **Mestrado em Biologia Celular e Molecular (Conceito CAPES 7)**
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil
Orientador: Henrique Bunselmeyer Ferreira
Bolsista: CNPq

2009-2013 **Graduação em Ciências Biológicas**
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil
Título: Caracterização imunológica e funcional da sinal-peptidase I de *Mycoplasma hyopneumoniae*
Orientador: Henrique Bunselmeyer Ferreira
Bolsista: CNPq

Idiomas

Inglês Compreende bem, fala bem, lê bem, escreve bem
Espanhol Compreende razoavelmente, fala pouco, lê razoavelmente, escreve pouco

Estágios

Estágio voluntário 2009-2010	**Biologia molecular:** Caracterização imunológica e funcional da sinal-peptidase I de *Mycoplasma hyopneumoniae*. Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, UFRGS *Orientador:* Henrique Bunselmeyer Ferreira
Iniciação tecnológica PIBITI-CNPq 2010-2013	**Biologia molecular:** Caracterização imunológica e funcional da sinal-peptidase I de *Mycoplasma hyopneumoniae* & Antígenos recombinantes de *Mycoplasma hyopneumoniae* para formulação de vacinas contra a pneumonia enzoótica suína. Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, UFRGS *Orientador:* Henrique Bunselmeyer Ferreira

Prêmios e títulos

2011	**Talento Inovador**, XX Feira de Iniciação à Inovação e ao Desenvolvimento Tecnológico - UFRGS

Artigos completos publicados em periódicos

1. PAES, JÉSSICA A.; MACHADO, LAÍS, D.P.N.; LEAL, FERNANDA M. A.; DE MORAES, SOFIA N.; MOURA, HERCULES; BARR, JOHN R.; FERREIRA, HENRIQUE B. Comparative proteomics of two *Mycoplasma hyopneumoniae* strains and Mycoplasma flocculare identified potential porcine enzootic pneumonia determinants. Virulence, v. 9, p. 1230-1246, 2018

2. PAES, JÉSSICA A.; LORENZATTO, KARINA R.; DE MORAES, SOFIA N. ; MOURA, HERCULES ; BARR, JOHN R. ; FERREIRA, HENRIQUE B. . Secretomes of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* reveal differences associated to pathogenesis. Journal of Proteomics (Print), v. 154, p. 69-77, 2017.

3. PAES, JÉSSICA A.; VIRGINIO, VERIDIANA G. ; CANCELA, MARTIN ; LEAL, FERNANDA M.A. ; BORGES, THIAGO J. ; JAEGER, NATÁLIA ; BONORINO, CRISTINA ; SCHRANK, IRENE S. ; FERREIRA, HENRIQUE B. . Pro-apoptotic effect of a *Mycoplasma hyopneumoniae* putative type I signal peptidase on PK(15) swine cells. Veterinary Microbiology (Amsterdam. Print), v. 201, p. 170-176, 2017.

4. DOS ANJOS LEAL, FERNANDA MUNHOZ; VIRGINIO, VERIDIANA GOMES; MARTELLO, CAROLINA LUMERTZ; **PAES, JÉSSICA ANDRADE**; BORGES, THIAGO J.; JAEGER, NATÁLIA; BONORINO, CRISTINA; FERREIRA, HENRIQUE BUNSELMEYER. *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* differential

domains from orthologous surface proteins induce distinct cellular immune responses in mice. Veterinary Microbiology (Amsterdam. Print), v. 190, p. 50-57, 2016.

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6. COSTA, CAROLINE B. ; MONTEIRO, KARINA M. ; TEICHMANN, ALINE ; DA SILVA, EDILEUZA D.; LORENZATTO, KARINA R. ; CANCELA, MARTÍN ; **PAES, JÉSSICA A.**; BENITZ, ANDRÉ DE N. D.; CASTILLO, ESTELA ; MARGIS, ROGÉRIO ; ZAHA, ARNALDO ; FERREIRA, HENRIQUE B. . Expression of the histone chaperone SET/TAF-Iβ during the strobilation process of *Mesocestoides corti* (Platyhelminthes, Cestoda). Parasitology (London. Print), v. 142, p. 1-12, 2015.

7. VIRGINIO, VERIDIANA GOMES; GONCHOROSKI, TAYLOR ; **PAES, JÉSSICA ANDRADE**; SCHUCK, DESIRÉE CIGARAN ; ZAHA, ARNALDO ; FERREIRA, HENRIQUE BUNSELMEYER . Immune responses elicited by *Mycoplasma hyopneumoniae* recombinant antigens and DNA constructs with potential for use in vaccination against porcine enzootic pneumonia. Vaccine (Guildford), v. 32, p. 5832-5838, 2014.

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9. MOITINHO-SILVA, L.; HEINECK, B.; REOLON, L. A.; **PAES, J. A.;** KLEIN, C. S.; REBELATTO, R.; SCHRANK, IRENE SILVEIRA; ZAHA, A.; FERREIRA, HB. *Mycoplasma hyopneumoniae* type I signal peptidase: Expression and evaluation of its diagnostic potential. Veterinary Microbiology (Amsterdam. Print), v. 154, p. 282-291, 2012.

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2. MACHADO, LAÍS DPN; **PAES, J. A**. ; LEAL, F. M. A. ; MOURA, H. ; BARR, J. ; FERREIRA, H. B. . Comparative proteomics analyses of *M. hyopneumoniae* and *M. flocculare* surface proteins. In: 22nd Congress of the International Organization for

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3. LEAL, Fernanda M.A.; **PAES, Jéssica A.** ; LORENZATTO, K. R. ; de Moraes, S. N.; MOURA, Hercules ; BARR, J. ; FERREIRA, Henrique B. . Secretome characterization of *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare* and NPTr swine epithelial cells during interaction. In: ASM Microbe, 2017, New Orleans. ASM Microbe, 2017.

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