Veterinary Microbiology 154 (2012) 282–291

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Mycoplasma hyopneumoniae type I signal peptidase: Expression and evaluation of its diagnostic potential

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ARTICLE INFO

Article history:
Received 6 October 2010
Received in revised form 9 June 2011
Accepted 12 July 2011

Keywords:
Mycoplasma hyopneumoniae
Type I signal peptidase
Expression
Porcine enzootic pneumonia
Immunodiagnosis
ELISA

ABSTRACT

Type I signal peptidase (SPase I) is a membrane-anchored protease of the general secretory pathway, which is encoded by the *sipS* gene in *Mycoplasma hyopneumoniae*, the etiological agent of porcine enzootic pneumonia (PEP). In this study, the expression of the *M. hyopneumoniae* SPase I (MhSPase I) was analyzed in virulent and avirulent strains, and the recombinant protein (rMhSPase I), expressed in *Escherichia coli*, was evaluated regarding its potential as an immunodiagnostic antigen. It was demonstrated that the *sipS* coding DNA sequence (CDS) is most likely part of an operon, being co-transcribed along with four other CDSs. Quantitative reverse transcriptase PCR and immunoblot assays showed that MhSPase I is expressed by all three strains analyzed, with no transcriptional difference, but with evidence of a higher protein level in a pathogenic strain (7422), in comparison to another pathogenic (7448) and a non-pathogenic (J) strain. rMhSPase I was strongly immunogenic for mice, and the MhSPase I antigenicity was confirmed. Polyclonal serum anti-rMhSPase I presented no detectable cross-reaction with *Mycoplasma flocculare* and *Mycoplasma hyorhinis*. Moreover, phylogenetic analysis demonstrated a low conservation between MhSPase I and orthologous proteins from other porcine respiratory disease complex-related bacteria, Firmicutes and other *Mycoplasma* species. The potential of an rMhSPase I-based ELISA for PEP immunodiagnosis was demonstrated. Overall, we investigated the expression of *sipS* and the encoded MhSPase I in three *M. hyopneumoniae* strains and showed that this protein is a good antigen for use in PEP serodiagnosis and possibly vaccination, as well as a potential target for antibiotic development.

1. Introduction

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia (PEP) and one of the primary agents of the porcine respiratory disease complex (PRDC). In PRDC, *M. hyopneumoniae* infections are associated with colonization of other bacteria and viruses (Brockmeier et al., 2002). *M. hyopneumoniae* adheres to ciliated epithelium within the swine respiratory tract leading to a highly contagious chronic infection that causes major

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doi:10.1016/j.vetmic.2011.07.009

economic loss in the pig industry. ELISA serology is the most commonly used tool to assess the health status of herds regarding *M. hyopneumoniae* infection, even though ELISA-based tests have shown discrepancies of specificities and sensitivities in comparative studies (Sibila et al., 2009).

The genomes of four strains of *M. hyopneumoniae* (232, 7448, J and 168) have been sequenced so far (Minion et al., 2004; Vasconcelos et al., 2005; Liu et al., 2011). The *sipS* gene, coding for type I signal peptidase (SPase I) in all four strains, was identified as a possible virulence gene (Ferreira and Castro, 2007). SPase I is a membrane-bound endopeptidase that cleaves the signal peptide of proteins exported by the general secretory pathway. SPase I is believed to be essential for bacterial viability (van Roosmalen et al., 2004), being a target for vaccine and novel antibiotic development (Rafati et al., 2006; Bockstael et al., 2009).

In this work, we investigated the *sipS* gene expression in different *M. hyopneumoniae* strains and characterized the encoded SPase I (MhSPase I) as an antigen with the potential to be used for immunodiagnosis and vaccination. We show that *sipS* is most likely part of an operon, and that MhSPase I is differentially expressed among *M. hyopneumoniae* strains at the protein level. A recombinant version of MhSPase I (rMhSPase I) was strongly immunogenic to mice and an rMhSPase I-based ELISA efficiently discriminated between healthy and *M. hyopneumoniae* infected pigs in field conditions.

2. Materials and methods

2.1. Bacteria, culture conditions, protein and nucleic acid extractions

M. hyopneumoniae J (ATCC 25934), a non-pathogenic strain with reduced adhesion capacity to porcine cilia, and two pathogenic strains (7448 and 7422) were obtained as described elsewhere (Vasconcelos et al., 2005; Machado et al., 2009). *Mycoplasma flocculare* (ATCC 27716) and *Mycoplasma hyorhinis* (ATCC 17981) were acquired by Embrapa Suínos e Aves (Concórdia, Brazil) from the American Type Culture Collection. Mycoplasma cultures, protein extractions and sample preparation of biological replicates were performed according to Pinto et al. (2009). All protein quantifications were performed using a Quant-iT Protein Assay Kit and a Qubit Fluorometer (Invitrogen, USA).

Genomic DNA from *M. hyopneumoniae* was extracted from 5-mL cultures according to a standard protocol (Wilson, 2001). The removal of proteins and polysaccharides was performed using phenol:chloroform, and 3 μg of DNA was recovered. Total RNA was isolated from 25 mL of *M. hyopneumoniae* cultures using TRIzol reagent (Invitrogen, USA) according to the manufacturer's recommendations. Contamination with genomic DNA was avoided by treatment with RQ1 RNase-free DNase (Promega, USA). All nucleic acid quantifications were conducted by spectrophotometry.

2.2. Transcriptional analyses of sipS

For reverse transcriptase PCR (RT-PCR) analysis, primers (Table 1) were designed based on *sipS* co-oriented adjacent CDSs in the *M. hyopneumoniae* 7448 genome (Vasconcelos et al., 2005; see also Fig. 1). For cDNA synthesis, total RNA (200 ng) was reverse transcribed using M-MuLV reverse transcriptase (Fermentas, USA) and specific reverse primers (Fig. 1). The cDNAs were then amplified using Easy Taq DNA polymerase (LGCBIO, Brazil) and Platinum® *Taq* DNA polymerase high fidelity (Invitrogen, USA) for fragments longer than 1 kb. As a control for DNA contamination, total RNA was amplified by PCR without a previous reverse transcription step. Cycle conditions were optimized for each primer combination and all RT-PCR products were confirmed by automatic DNA sequencing in MegaBACE (Amersham Biosciences, Sweden).

Table 1
Oligonucleotide primers used for cloning and transcriptional analyses.

Name	Sequence (5′–3′)
	Cloning[a]
sipS-pGEX-5′	*tggttccgcgtggatcccc*gatgttaaaatctaaaaacttaagtaac
sipS-pGEX-3′	*gcgaggcagatcgtcagtcagtca*ttaggcccaaacacctttaatatc
	RT-PCR
A	cctcagcgaaatgatgtggtag
B	ttaggcccaaacacctttaatatc
C	gtggacaagcaaaaaatattaaaacttg
D	ggttttcactgctatgaatagaattg
O	caactgaaggtgtttctcatgcttc
P	gcacttccagatgaagatccgcc
Q	tagttggcaatccatcaattagtttg
R	atctagcttatctcggaactttacc
S	ggtcttgtaacaatttcaatcaaggg
T	gaagtagttagtgaagaatttgctcag
U	atggcattctgacttttaacaacctg
V	aagtgaagtttttgaagcctatttaac
	qRT-PCR
q-sipS-5′	tcctggtgataaattagaagttactg
q-sipS-3′	tccaaaagtacggctatcattac
q-MHP0333-5′	tgggcaatcaagaagcaac
q-MHP0333-3′	tgaaaacggaaaacaccttg

[a] Italic letters in the primers correspond to pGEX-4T-3 plasmid homologous sequences.

For *sipS* quantitative RT-PCR (qRT-PCR) analysis of *M. hyopneumoniae* 7448, 7422 and J strains, total RNA was reversed transcribed into cDNA with ImProm-II™ Reverse Transcription System (Promega, USA) according to the manufacturer's protocols using random hexamers. Specific qRT-PCR primers (Table 1) were designed to the target *sipS* gene and to the control MHP0333 CDS, which is an ortholog of *M. hyopneumoniae* 232 mhp345 CDS (Madsen et al., 2008). qPCR was performed on the StepOn Real-Time PCR System (Applied Biosystems, USA) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, USA). The relative gene expression was calculated using the comparative CT method (Livak and Schmittgen, 2001) and the value reported for each strain represents the average of three independent biological replicates. One-way ANOVA followed by Dunnett's multiple comparison test was used to determine if the expression values of *sipS* were significantly different. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, USA), with a 5% significance level.