Proteomic analysis, immunogenicity of secreted proteins from *Rhodococcus equi* ATCC 33701

Corinne Barbey, Aurélie Budin-Verneuil, Séverine Cauchard, Axel Hartke,
Claire Laugier, Vianney Pichereau, Sandrine Petry

► To cite this version:

Corinne Barbey, Aurélie Budin-Verneuil, Séverine Cauchard, Axel Hartke, Claire Laugier, et al.. Proteomic analysis, immunogenicity of secreted proteins from *Rhodococcus equi* ATCC 33701. *Veterinary Microbiology*, 2009, 135 (3-4), pp.334. 10.1016/j.vetmic.2008.09.086 . hal-00532518

HAL Id: hal-00532518

<https://hal.science/hal-00532518>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Proteomic analysis, immunogenicity of secreted proteins from *Rhodococcus equi* ATCC 33701

Authors: Corinne Barbey, Aurélie Budin-Verneuil, Séverine Cauchard, Axel Hartke, Claire Laugier, Vianney Pichereau, Sandrine Petry

PII: S0378-1135(08)00466-5
DOI: doi:10.1016/j.vetmic.2008.09.086
Reference: VETMIC 4219

To appear in: *VETMIC*

Received date: 7-8-2008
Revised date: 19-9-2008
Accepted date: 24-9-2008

Please cite this article as: Barbey, C., Budin-Verneuil, A., Cauchard, S., Hartke, A., Laugier, C., Pichereau, V., Petry, S., Proteomic analysis, immunogenicity of secreted proteins from *Rhodococcus equi* ATCC 33701, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.09.086

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Proteomic analysis and immunogenicity of secreted proteins from *Rhodococcus equi*
ATCC 33701

Corinne Barbey¹, Aurélie Budin-Verneuil², Séverine Cauchard¹, Axel Hartke², Claire
Laugier¹, Vianney Pichereau², and Sandrine Petry^{1*}

AFSSA, Laboratoire d'Etudes et de Recherches en Pathologie Equine, IPC, Goustranville,
14430 Dozulé, France¹ and Laboratoire de Microbiologie de l'Environnement, USC INRA
2017, EA956, IFR ICORE 146, Université de Caen, 14032 Caen Cedex, France²

*Corresponding author. Mailing address: AFSSA, Laboratoire d'Etudes et de Recherches en
Pathologie Equine, IPC, Goustranville, 14430 Dozulé, France. Phone: 33 (0)2 31 79 22 76.
Fax: 33 (0)2 31 39 21 37. E-mail: s.petry@dozule.afssa.fr.

19 Abstract

20 *Rhodococcus equi* is one of the most important causes of mortality in foals between 1 to 6
 21 months of age. Although rare, infection also occurs in a variety of other mammals including
 22 humans, often following immunosuppression of various causes. Secreted proteins are known
 23 to mediate important pathogen-host interactions and consequently are favored candidates for
 24 vaccine development as they are the most easily accessible microbial antigens to the immune
 25 system. Here, we describe the results of a proteomic analysis based on SDS PAGE,
 26 immunoblot and mass spectrometry, which was carried out aiming the identification of
 27 secreted proteins that are differently expressed at 30°C versus 37°C and at mid-exponential
 28 versus early-stationary growth phase and antigenic proteins from *R. equi* ATCC 33701. A
 29 total of 48 proteins was identified regardless of growth conditions. The cholesterol oxidase
 30 ChoE appears to be the major secretory protein. Moreover, four proteins revealed high
 31 homologies with the mycolyl transferases of the Ag85 complex from *Mycobacterium*
 32 *tuberculosis*. The sequence analysis predicted that 24 proteins are transported by a signal
 33 peptide-dependent pathway. Moreover, five antigenic proteins of *R. equi* were identified by
 34 immunoblot, including a novel strongly immunoreactive protein of unknown function. In
 35 conclusion, the elucidation of the secretome of *R. equi* identified several proteins with
 36 different biological functions and a new candidate for developing vaccines against *R. equi*
 37 infection in horse.

38
 39 Keywords: *Rhodococcus equi*; Secretome; Mass spectrometry; Signal peptide; Antigenic
 40 proteins

1. Introduction

Rhodococcus equi is the common cause of pyogranulomatous pneumonia with a high mortality rate in foals up to 6 months of age (Muscatello et al., 2007). Even if the pathogenic mechanisms of *R. equi* remain largely unknown, there is evidence that virulence is dependent on a large plasmid with a pathogenicity island (Takai et al., 2000; Takai et al., 1991b), containing nine virulence-associated protein (*vap*) genes (Muscatello et al., 2007). Among them, *vapA* encodes a surface-expressed protein (Takai et al., 1991a; Tan et al., 1995), which plays a crucial role in intracellular growth in macrophages and for disease development (Giguère et al., 1999; Jain et al., 2003).

Secreted proteins are of high interest for functional investigations of bacterial pathogens because they come into direct contact with host tissues. Particularly, secreted proteins of intracellular pathogens such as *R. equi* represent the primary antigen targets of host immune response (Kaufmann and Hess, 1999). Thus, they may be key molecules to induce immune protection and, consequently, key candidates for vaccine development. Few data are available about *R. equi* and only five secreted proteins were described such as the cholesterol oxidase ChoE and the phospholipase C, two membrane-damaging exoenzymes, originally called equi factors (Machang'u and Prescott, 1991a), and three virulence-associated proteins VapC, VapD and VapE (Byrne et al., 2001) of still unknown function.

The goal of the present study was to identify proteins secreted by *R. equi* ATCC 33701 at different temperatures (30°C, the optimal growth temperature, versus 37°C, the host temperature) and at different growth phases (mid-exponential versus early-stationary phase of growth). Bioinformatic study was carried out to predict the functions and to confirm the extracellular localization of the identified proteins. Furthermore, immunogenic secreted proteins expressed during equine rhodococcosis were characterized by using Western blot probed with pooled sera from nine foals which died from *R. equi* infection.

66

67 **2. Materials and methods**68 *2. 1. Bacteria and culture conditions*

69 The bacterial strain used in experiments was *R. equi* ATCC 33701, a virulence plasmid-
 70 bearing strain. A single colony was selected and grown in 10 ml of Luria Bertani (LB, Difco,
 71 Le Pont de Claix, France) medium for 16 h at 30°C or 37°C, at pH 7.0 with shaking at 200
 72 rpm. Cultures were inoculated at a 1:100 dilution with the preculture. Samples (500 ml) were
 73 taken in mid-exponential (OD₆₀₀ of 0.4 to 0.5) and early-stationary (OD₆₀₀ of 0.7 to 0.8)
 74 growth phases, respectively after 15 and 22 h of incubation with shaking at 200 rpm.

75 *E. coli* BL21(DE3) (Novagen, Merck, Darmstadt, Germany) and *E. coli* M15 (Qiagen,
 76 Courtaboeuf, France) were used for protein expression.

77

78 *2. 2. Extracellular sample preparation for the proteome analysis*

79 Cultures were centrifuged at 20,000 g for 30 min at 4°C and the supernatants were sterile
 80 filtered by using a 0.2-µm nitrocellulose filter (Millipore, Molsheim, France). Then a final
 81 concentration of 10% (w/v) trichloroacetic acid (TCA, Sigma, St Quentin Fallavier, France)
 82 was added to the filtrate supernatant, mixed well and placed on ice at 4°C for 16 h. The
 83 precipitated proteins were harvested by centrifugation (20,000 g for 30 min at 4°C), washed
 84 four times in cold methanol (Fluka, quality upgraded, St Quentin Fallavier, France) dried with
 85 speed-vac and dissolved in sample solution consisting of 0.5 M Tris base pH 8.0 (Sigma).
 86 Protein concentration was determined according to the Lowry method (Lowry et al., 1951),
 87 using bovine serum albumine (BSA, Sigma) as a standard.

88

89 *2. 3. SDS PAGE*

Proteins samples were denatured in Laemmli Buffer 6% (v/v) SDS (Eurobio, Les Ulis, France), 16% (v/v) glycerol (Eurobio), 0.02% (v/v) β -mercaptoethanol (Sigma), and 0.08% (w/v) bromophenol blue (Electran, BDH Laboratory Supplies, Poole, UK) in 100 mM Tris-HCl Buffer pH6.8 by heating to 100°C for 5 min. Then samples (150 μ g in each well) were separated by one-dimensional denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 7% acrylamide (29:1 acrylamide/bisacrylamide, Eurobio) stacking gel and a 12% acrylamide resolving gel. The Bio-Rad prestained broad standard marker was used as a protein molecular weight marker. After electrophoresis using the PROTEAN[®] II xi Cell (Bio-Rad Laboratories, Marnes-la-Coquette, France) at 150 V for 15 h, the gel was stained with Coomassie Brilliant Blue R250 (Reactifs Ral, Martillac, France) to visualize the proteins.

2. 4. *In gel-trypsin digestion*

Protein spots to be identified were manually excised from Coomassie stained gels and excised gel fractions were washed in ultra-pure water. These fractions were then dehydrated by several incubations in 50 mM ammonium bicarbonate (BICAM, Fluka) and acetonitrile (CAN, Merck, Fontenay-sous-Bois, France), followed by rehydration in 50 mM BICAM. 0.5 μ g modified porcine trypsin (sequencing grade modified trypsin, Promega Corporation, Charbonnières, France) was added to each sample, which was then incubated during 16 h at 37°C. The supernatant was recovered by centrifugation (5,000 g for 5 min). A second extraction from these gel pieces was carried out by incubation in 50 mM BICAM/ACN (1/1 : v/v) and then by incubation in 5% acetic acid (Carlo Erba, Val de Reuil, France). Peptide-containing liquid fractions were pooled after each incubation and vacuum dried. Peptides were resuspended in 20 μ l 0.1% acetic acid prior to storage at -70°C until mass spectrometry (MS) analysis.

115

116 *2. 5. Trypsin digestion of crude extracts of secreted proteins*

117 Crude extracts of secreted proteins were obtained as described above from culture
 118 supernatants at the early-stationary growth phase (OD₆₀₀ of 0.7 to 0.8) and 30°C or 37°C.
 119 Proteins were resuspended in 50 mM BICAM pH 8.0 containing 0.5 µg trypsin, incubated at
 120 37°C during 16 h, before being vacuum dried and resuspended in 0.1% acetic acid. Peptides
 121 were stored at –70°C until MS analysis.

122

123 *2.6. MS/MS analysis*

124 An electrospray ion trap mass spectrometer (LCQ DECA XP, Thermo Finnigan) coupled
 125 on line with an HPLC (Surveyor LC, Thermo Finnigan) was used. Peptides were separated
 126 and analysed mainly as previously described (Sauvageot et al., 2002). Recorded MS/MS
 127 spectra were compared to theoretical fragmentations of a trypsinolysed database deduced
 128 from the *R. equi* 103 DNA genomic sequence, using the Sequest software (version 2, Thermo
 129 Finnigan).

130

131 *2. 7. Bioinformatic tools to analyze amino acid sequences*

132 Protein sequences were obtained thanks to the non-annotated genome sequence of *R. equi*
 133 strain 103 produced by the *Rhodococcus equi* Sequencing Group at the Sanger Institute
 134 (http://www.sanger.ac.uk/Projects/R_equi/). For unknown proteins, homologous proteins
 135 were identified using the BLASTP program available at the website
 136 <http://www.ncbi.nlm.nih.gov/BLAST/sequence>. Conserved protein domains were identified
 137 by using CDD software based on NCBI
 138 (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>).

The secretory (Sec-type) signal peptides were determined with the SignalP 3.0 algorithm (<http://www.cbs.dtu.dk/services/SignalP/>) while the Twin-arginine (Tat-type) and the lipoprotein signal peptides were determined with the TatP 1.0 (<http://www.cbs.dtu.dk/services/TatP/>) and the LipoP 1.0 (<http://www.cbs.dtu.dk/services/LipoP/>) algorithms respectively. TMHMM 2.0 algorithm was used to predict transmembrane domains (<http://www.cbs.dtu.dk/services/TMHMM/>). The different softwares cited below are all available from www.expasy.org.

2. 8. Western blot analysis

Two SDS PAGE gels were run in parallel. One was used for identification and the other for immunoblot. 150 µg of proteins were loaded in each well to compare each culture condition. Proteins resolved by SDS PAGE were electroblotted onto nitrocellulose membranes (Amersham Biosciences, Orsay, France) with Towbin transfer buffer using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad Laboratories) at 15 V for 40 min. Blotted membranes were blocked with 5% (w/v) skimmed milk powder in TBS buffer (10 mM Tris, 0.2 M NaCl, 0.05% (v/v) Tween 20) overnight at 4°C and incubated with pooled sera from nine foals that had died of rhodococcosis at 1:200 dilution in blocking solution for 1 h at 37°C. Membranes were then washed three times for 15 min each with TBS buffer. They were incubated with rabbit anti-horse IgG, conjugated to alkaline phosphatase, at 1:5000 dilution (Bethyl Laboratories, Montgomery, TX, USA) for 1 h at 37°C and washed as described above. Antigen-antibody reactions were developed with AP Conjugate Substrate kit (Bio-Rad Laboratories) according to the manufacturer.

2. 9. Cloning and expression of twelve genes

The primers and vectors used for PCR amplification and cloning are listed (Table 1). The primers were designed on the available *R. equi* genome sequence. PCR products obtained with *R. equi* ATCC 33701 DNA were cloned into pQE30 (Qiagen) or pET-30 Ek/LIC vector (Novagen) to obtain recombinant plasmids following the recommendations of the supplier (Table 1). Recombinant pQE30 vectors were transformed into *E. coli* M15 while recombinant pET-30 Ek/LIC vectors were transformed into *E. coli* BL21(DE3). The presence of appropriate insert was confirmed by sequencing.

To assess expression of the cloned genes, recombinant *E. coli* M15 or *E. coli* BL21(DE3) were grown overnight at 37°C in 10 ml of LB medium supplemented with 30 µg/ml of kanamycin (Sigma) for the two *E. coli* strains and 100 µg/ml of ampicillin (Sigma) for *E. coli* M15 only. One ml of the bacterial suspension was then subinoculated into 100 ml of fresh LB medium and the bacteria were allowed to grow at 37°C until the optical density of the medium at 600 nm reached 0.6. Thereafter, the expression of the cloned genes was induced by addition of IPTG (ABgene, Courtaboeuf, France) to a final concentration of 1 mM. After a further 3 h incubation at 37°C, the bacterial cells were collected by centrifugation for 10 min at 5,000 g. The cell lysis was performed by incubation for 1 h with a lysis buffer containing 100 mM NaH₂PO₄ (Merck), 10 mM Tris base and 8 M urea (Eurobio). The cell debris were removed by centrifugation at 10,000 g for 10 min. Supernatants were used for Western blot analysis.

3. Results

3. 1. Analysis of secreted proteins by SDS PAGE

Secreted proteins were extracted from the supernatant of cultures of *R. equi* grown in the poor protein containing LB medium in order to limit contamination by medium derived polypeptides. Secreted proteins obtained at 30°C or 37°C and at mid-exponential or early-

stationary phase were analyzed by SDS PAGE (Fig. 1). According to standard marker proteins, secreted polypeptides had molecular weight ranging from 7 to 100 kDa. At 30°C, the migration patterns of secreted proteins did not appear to have significant differences depending on the growth phase and at least 21 distinct bands were detected. At 37°C, three additional bands around 29 kDa (bands No. 17 to 19) were present in supernatants harvested in early-stationary phase compared to mid-exponential phase. Furthermore, regardless of the growth phase, six additional bands between 7 and 20 kDa (bands No. 23 to 27 and 30) were detected in supernatants from cultures grown at 37°C compared to 30°C. Finally, several intensively Coomassie-stained bands were observed: four bands (No. 1, 3, 6 and 15) in all tested supernatants and three additional bands (No. 25 to 27) expressed in supernatants harvested at 37°C.

3. 2. Proteins identification

30 bands were cut out of gels (bands No. 1 to 30, Fig. 1), subjected to in-gel trypsin digestion and analyzed by LC-ESI tandem mass spectrometry. In parallel, the secretome of *R. equi* was also analyzed without previous electrophoretic separation by injecting a trypsinolysed crude preparation of secreted proteins into LC-ESI tandem mass spectrometer (see Materials and Methods for details). These two approaches led to the identification of 44 and 16 proteins, respectively (Table 2). The method without previous electrophoretic separation allowed us to identify fewer secreted proteins than the other method but led to the rapid identification of dominant proteins present in the secretome. Since we found twelve identical proteins in both methods, a total of 48 proteins was finally identified. All identified proteins were encoded by the chromosome except for the Vap proteins. Because of the non-annotated genome sequence of *R. equi*, homologous proteins were identified using BLASTP program and the homology with the first hit was indicated for each protein (Table 2). Six

proteins arbitrarily named proteins X1 to X6 did not share any similarity with proteins from other organisms. For the majority of the proteins (73%), a potential function could be assigned due to the presence of conserved domains detected by NCBI CDD algorithm. All proteins identified in the culture filtrate could be classified into several major functional categories: pathogenicity (ChoE, VapA), stress adaptation (alkyl hydroperoxide reductase, catalase, Cpn10, GroEL, CspA, probable Stage II sporulation protein), export (MspA), cell wall biosynthesis (four probable mycolyl transferases arbitrarily named 1 to 4, N-acetyl muramoyl-L-alanine amidase), reactivation of bacteria in stationary phase (probable resuscitation promoting factor), energy metabolism (dihydrolipoyl dehydrogenase, phosphomannomutase), fatty acid metabolism (lipid-transfer protein), general cell processes and hypothetical proteins without known function.

The molecular weights of most proteins detected in SDS PAGE were consistent with the theoretical values (Table 2). However, several proteins displayed a significantly higher mass than expected. Six proteins were represented by several isoforms of different molecular weights. The cholesterol oxidase ChoE was presented in six, VapD in four, VapA and mycolyl transferase 3 (MT3) in three and elongation factor Tu and protein X4 in two different fractions. By taking into account the growth conditions, the three additional bands around 29 kDa (No. 17 to 19, Fig. 1) in supernatants harvested in early-stationary phase compared to mid-exponential phase at 37°C were composed of one isoform of mycolyl transferase 3, four hypothetical proteins and a probable protease from *Rhodococcus* sp. strain RHA1. Moreover, the six additional bands between 7 and 20 kDa (No. 23 to 27 and 30, Fig. 1) in supernatants from cultures grown at 37°C compared to 30°C contained three isoforms of VapA, two isoforms of VapD, protein X1 and probable mycolyl transferase 3 (no result was obtained for the band No. 26). The unfractionated protein extract analysis revealed that VapA and VapD were detected only at 37°C.

Concerning the most intensively Coomassie-stained bands, cholesterol oxidase and probable mycolyl transferase 1 (No. 1, 3, 6 and 15, Fig. 1) were present in all tested supernatants whereas protein X1, VapA and VapD (No. 25 to 27, Fig. 1) were only expressed in supernatants harvested at 37°C. Cholesterol oxidase, VapA and VapD were also identified among the unfractionated protein extract directly analyzed by mass spectrometry.

3.3. Protein structure and localization

SignalP 3.0 algorithm revealed the presence of cleavage site for signal peptidases (SPases) for 24 out of the 48 identified proteins (Table 3), suggesting that these are secreted proteins. Three proteins were recognized by the TatP 1.0 algorithm as harboring a twin-arginine (Tat) signal peptide. The consensus sequence recognized by this algorithm is RRX[FGAVML][LITMVF]. It contains the two invariant arginine residues and variable amino-acids indicated between parentheses or by the X letter. Nine additional proteins contained a complete or incomplete Tat motif with at least the two arginines in their signal peptide but they were not recognized by the TatP 1.0 algorithm. The other 12 proteins contained a signal peptide specific for the general protein secretion (Sec) pathway (Table 3). In our study, the Sec and Tat signal peptides had a length varying from 26 to 63 amino acids. Both signal peptides were composed of three domains, a positively charged N-terminal domain (n-region), a hydrophobic domain (h-region) and a hydrophilic C-terminal domain (c-region) usually carrying the consensus AXA at position -1 to -3 relative to the cleavage site for SPases I (Tjalsma et al., 2000). This consensus was observed for twenty out of the 24 signal peptides (Table 3). If the 24 signal peptides were taken into consideration, the general consensus sequence at position -3 to -1 relative to the SPases I cleavage site was (A/V)-X-(A/K/T). The consensus sequence L-(A/S)-(A/G)-C at position -3 to +1 relative to the SPases

II cleavage site had never been found, suggesting the absence of lipoprotein signal peptides (Table 3). At last, 24 proteins contained no obvious signal sequence.

According to the TMHMM 2.0 algorithm, we could predict a putative membrane localization for two proteins. The polypeptide homologous to the hypothetical protein RHA1_ro05477 contained, in addition to a signal peptide, two transmembrane domains at central position. The polypeptide homologous to the hypothetical protein RHA1_ro03156, which did not possess a signal peptide, contained a single transmembrane domain localized at the N-terminus (Table 3). These two proteins may correspond to a membrane protein and a surface protein, respectively. Furthermore, according to the NCBI CDD algorithm, the probable mycolyl transferase 1 contained at its C-terminus three LGFP tandem repeats, which may be involved in anchoring the protein to the cell wall.

3. 4. Antigenic profiles of *R. equi* secretome

Antigenic profiles were explored by immunoblot performed with pooled sera from nine *R. equi* infected foals (Fig. 2). No band was detected in the control medium. Moreover, Western blot control experiments using antisera from healthy foals did not reveal any immunogenic protein (data not shown). Independent of growth conditions, immunogenic proteins were present in four regions with molecular weights about 99, 54, 29 and 20 kDa, respectively. A protein band of a monodimensional gel may harbor more than one polypeptide and probably not all of them would correspond to immunoreactive proteins. Analysis of the four bands by mass spectrometry revealed the presence of the following proteins: cholesterol oxidase, catalase, hypothetical proteins RHA1_ro06188, RHA1_ro03156, RHA1_ro05216, RHA1_ro05669, probable protease, probable secreted protein named PSP, VapA, VapD, probable mycolyl transferases 3 and 1. In order to test which of them reacts actually with the serum, the twelve proteins were first overexpressed in *E. coli* (Fig. 3A) and then used for a

Western blot analysis using the same pooled horse sera as in Fig. 2 (Fig. 3B). No *E. coli* immunoreactive proteins were observed in the negative controls with empty pQE30 and pET-30 vectors. In contrast, the serum reacted strongly with the PSP and the cholesterol oxidase. The proteins VapA, VapD and the probable mycolyl transferase 1 were less immunoreactive and the serum reacted only weakly with the proteins homologous to the hypothetical proteins RHA1_ro06188 and RHA1_ro03156. The other recombinant proteins did not react with the serum.

4. Discussion

In order to characterize new *R. equi* candidate antigens associated with rhodococcosis among secreted proteins, we used a proteomic approach combined with serum profiling by using sera from foals that had died of rhodococcosis. This approach enabled identification of 48 proteins released by the *R. equi* ATCC 33701 strain during growth. As expected, 24 polypeptides contained a signal peptide sequence required for their translocation across the cytoplasmic membrane *via* the Sec-pathway (Keyzer et al., 2003) or the Tat-pathway (Lee et al., 2006), the two most important secretion systems distributed in all bacterial genera. Among them, half the proteins were predicted to possess a potential Tat signal peptide, suggesting that the Tat pathway could be as significant as the Sec pathway in *R. equi*. In contrast to the latter, the former pathway is involved in the secretion of exclusively folded proteins and protein translocation has no requirement for ATP as an energy source but relies solely on the proton motive force. It is interesting to note that all proteins identified in the most intensively Coomassie-stained bands contained a Sec signal peptide (VapA and VapD) or a Tat signal peptide (cholesterol oxidase, protein X1, mycolyl transferases 1 and 3).

The majority of the 24 remaining proteins without identifiable signal peptides contained proteins usually considered as strictly cytoplasmic and their presence in the secretome

requires further investigation. Abundant cytoplasmic proteins are often found in the secretome of other pathogens such as *M. tuberculosis* (Malen et al., 2007), *Listeria monocytogenes* (Trost et al., 2005) or *Staphylococcus aureus* (Sibbald et al., 2006) and extracellular roles have been suggested. For example, the cytoplasmic elongation factor Tu (EF-Tu) is described as a surface protein, which may exhibit an unexpected and novel function, the adhesion of bacteria to host tissue (Dallo et al., 2002). A similar activity has been reported for GroEL in several pathogens (Bergonzelli et al., 2006). Two *R. equi* *groEL2*- based DNA vaccines were used in immunization of mice and were found to elicit a predominantly Th1 biased immune response (Phumoonna et al., 2008; Vanniasinkam et al., 2004). But their protective efficacy against *R. equi* infection was not demonstrated. The mode of secretion for these proteins is unknown but they can be released by cell lysis or by hitherto unknown non-classical secretion pathways or signal peptide-independent pathways (Bendtsen et al., 2005). Such non-classical systems have been reported in *M. tuberculosis* and *L. monocytogenes* for the export of virulence factors without signal peptide (Bendtsen et al., 2005). For many years, it has been known that *M. tuberculosis* secretes antigenic proteins without apparent signal peptide such as ESAT-6 (early secretory antigenic target) (Bendtsen et al., 2005).

The cholesterol oxidase was, under all conditions tested, the major secreted protein of *R. equi* ATCC 33701. Despite its membranolytic activity, this enzyme was demonstrated not to be an important virulence factor in the natural host (Pei et al., 2006). It may be important in providing carbon sources for *R. equi* inside macrophages, as they are for other intracellular bacteria such as *Mycobacterium avium* and *M. tuberculosis*. The variants or isoforms of cholesterol oxidase may be attributed to biologically important post-translational modifications, ranging from chemical modifications, such as glycosylations for isoforms of higher molecular weight than the calculated one, to proteolytic cleavage for polypeptides with molecular weight significantly lesser than the expected one. VapA, an atypical lipoprotein, is

another virulence factor involved in the intracellular growth in macrophages and identified among secreted proteins in our experimental conditions, contrary to previous studies which detected VapA in the cell pellet only (Byrne et al., 2001; Kohler et al., 2003). Its presence in the supernatant can be explained by the fact that lipoproteins are vulnerable to proteolytic cleavage relatively close to the N-terminal cysteine and are consequently released as soluble protein in the culture filtrate (Malen et al., 2007). Moreover, it has been suggested that some lipoproteins may be alternatively processed by signal peptidase I or II, and this mechanism may explain their dual localization in the extracellular environment or as lipoproteins in the cell wall (Malen et al., 2007). In our work, VapA was observed in three isoforms, in accordance with the study of Tan et al. (1995). Indeed, in that work, the polypeptides isolated from the 18-22 kDa and 17.5 kDa fractions were hydrophobic and lipid modified whereas the 15-kDa protein did not show this modification. VapD was detected in four isoforms which correspond most probably to degradation products. VapA and VapD were observed only at 37°C in our study which is in accordance with previous findings (Byrne et al., 2001).

Some proteins identified in the present study were predicted to be involved in the cell wall biosynthesis. Indeed, four out of at least 13 *R. equi* probable mycolyl transferases predicted by genome analysis (Sydor et al., 2007) were detected in our study. These four enzymes were homologous to the mycobacterial antigen 85 complex (Ag85 complex), major secretion products of *M. tuberculosis* (Content et al., 1991; Wiker and Harboe, 1992). This Ag85 complex is made of three mycolyl transferases (FbpA, FbpB and FbpC), pivotal for the synthesis of the mycolic acid layer in the cell wall (Belisle et al., 1997). The probable mycolyl transferases 1 and 2 are encoded by the gene *fbpA* (Sydor et al., 2007) and *fbpB* (Pei et al., 2007) of *R. equi*, respectively. In *R. equi*, FbpA could be involved in capsule anchorage since a *fbpA* mutant is characterized by a non-mucoid phenotype (Sydor et al., 2007). Since synthesis of the capsule is constitutive in *R. equi*, it is not surprising that FbpA was one of the

most abundant proteins of the secretome regardless of growth conditions (Band No 3, Fig 1).

It should be noted that recent virulence studies in a murine model showed that both FbpA and FbpB might not be involved in *R. equi* pathogenesis (Pei et al., 2007; Sydor et al., 2007).

Immunological characterization of *R. equi* secreted proteins by sera from foals that had died of rhodococcosis revealed antigenic reactivity with four 99, 54, 29 and 20 kDa immunogenic regions, similar to the immunoblots obtained by Kohler et al. (2003). By using recombinant proteins, we demonstrated that five proteins (VapA, VapD, cholesterol oxidase, probable mycolyl transferase 1 and the PSP) were indeed involved in the immunogenicity of these four regions. Contrary to previous studies which identified Vap proteins as strongly immunoreactive proteins (Byrne et al., 2001; Kohler et al., 2003), the two most strongly immunoreactive proteins detected in the present study were the cholesterol oxidase and the PSP. Machang'u and Prescott (1991b) showed that antibodies against the cholesterol oxidase associated to the phospholipase C of *R. equi* did not prevent foals from developing lung abscesses following experimental infection. However, this result has to be examined by taking into account the infective dose, the dose and the nature of antigens which correspond to a combination of two proteins, the immunization and infection routes and the type of adjuvant. Indeed, recent studies demonstrated that these various parameters may influence the polarization of immune response which must be a cellular type response to confer protection against *R. equi* infection (Lunn and Townsend, 2000). The strong immune response to the PSP suggests that this protein was readily accessible to the immune system *in vivo* and consequently supports the fact that it was actively secreted despite the lack of a signal peptide. This protein needs to be further characterized. Finally, the latter protein, cholesterol oxidase and VapA that has been shown highly immunogenic (Kohler et al., 2003) in foals and protective in a murine model (Haghighi and Prescott, 2005) may represent promising antigen candidates for the vaccine development against equine rhodococcosis.

387

388 **5. Conclusion**

389 To our knowledge, this is the first report of a comprehensive proteomic analysis of
390 secreted proteins of *R. equi*. The associated immunological characterization allowed us to
391 identify several proteins recognized by the host immune system, including a novel strongly
392 immunoreactive protein without known function. Studies to examine their immunoprotective
393 potential against *R. equi* infections are in progress.

394

395 **Acknowledgements**

396 This research was supported by grants from the Région Basse-Normandie, Haras
397 Nationaux and ADREF (Association pour le Développement de la Recherche Equine en
398 France).

399 We thank Doctor J. Cauchard (AFSSA Dozulé, France) for providing sera from nine foals
400 that had died of rhodococcosis.

References

- Belisle, J.T., Vissa, V.D., Sievert, T., Takayama, K., Brennan, P.J., Besra, G.S., 1997. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* 276, 1420-1422.
- Bendtsen, J.D., Kiemer, L., Fausboll, A., Brunak, S., 2005. Non-classical protein secretion in bacteria. *BMC Microbiol.* 58, 1-13.
- Bergonzelli, G.E., Granato, D., Pridmore, R.D., Marvin-Guy, L.F., Donnicola, D., Cortesy-Theulaz, I.E., 2006. GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. *Infect. Immun.* 74, 425-434.
- Byrne, B.A., Prescott, J.F., Palmer, G.H., Takai, S., Nicholson, V.M., Alperin, D.C., Hines, S.A., 2001. Virulence plasmid of *Rhodococcus equi* contains inducible gene family encoding secreted proteins. *Infect. Immun.* 69, 650-656.
- Content, J., de la Cuvelier, A., De Wit, L., Vincent-Levy-Frebault, V., Ooms, J., De Bruyn, J., 1991. The genes coding for the antigen 85 complexes of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG are members of a gene family: cloning, sequence determination, and genomic organization of the gene coding for antigen 85-C of *M. tuberculosis*. *Infect. Immun.* 59, 3205-3212.
- Dallo, S.F., Kannan, T.R., Blaylock, M.W., Baseman, J.B., 2002. Elongation factor Tu and E1 beta subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in *Mycoplasma pneumoniae*. *Mol. Microbiol.* 46, 1041-1051.
- Giguère, S., Hondalus, M.K., Yager, J.A., Darrah, P., Mosser, D.M., Prescott, J.F., 1999. Role of the 85-kilobase plasmid and plasmid-encoded virulence-associated protein A in intracellular survival and virulence of *Rhodococcus equi*. *Infect. Immun.* 67, 3548-3557.
- Haghighi, H.R., Prescott, J.F., 2005. Assessment in mice of vapA-DNA vaccination against *Rhodococcus equi* infection. *Vet. Immunol. Immunopathol.* 104, 215-225.
- Jain, S., Bloom, B.R., Hondalus, M.K., 2003. Deletion of vapA encoding Virulence Associated Protein A attenuates the intracellular actinomycete *Rhodococcus equi*. *Mol. Microbiol.* 50, 115-128.
- Kaufmann, S.H., Hess, J., 1999. Impact of intracellular location of and antigen display by intracellular bacteria: implications for vaccine development. *Immunol. Lett.* 65, 81-84.
- de Keyzer, J., van der Does, C., Driessen, A.J., 2003. The bacterial translocase: a dynamic protein channel complex. *Cell. Mol. Life Sci.* 60, 2034-2052.
- Kohler, A.K., Stone, D.M., Hines, M.T., Byrne, B.A., Alperin, D.C., Norton, L.K., Hines, S.A., 2003. *Rhodococcus equi* secreted antigens are immunogenic and stimulate a type 1 recall response in the lungs of horses immune to *R. equi* infection. *Infect. Immun.* 71, 6329-6337.
- Lee, P.A., Tullman-Ercek, D., Georgiou, G., 2006. The bacterial twin-arginine translocation pathway. *Annu. Rev. Microbiol.* 60, 373-395.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
- Lunn, D.P., Townsend, H.G., 2000. Equine vaccination. *Vet. Clin. North Am. Equine Pract.* 16, 199-226.
- Machang'u, R.S., Prescott, J.F., 1991a. Purification and properties of cholesterol oxidase and choline phosphohydrolase from *Rhodococcus equi*. *Can. J. Vet. Res.* 55, 332-340.

- Machang'u, R.S., Prescott, J.F., 1991b. Role of antibody to extracellular proteins of *Rhodococcus equi* in protection against *R. equi* pneumonia in foals. *Vet. Microbiol.* 26, 323-333.
- Malen, H., Berven, F.S., Fladmark, K.E., Wiker, H.G., 2007. Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. *Proteomics* 7, 1702-1718.
- Muscatello, G., Leadon, D.P., Klayt, M., Ocampo-Sosa, A., Lewis, D.A., Fogarty, U., Buckley, T., Gilkerson, J.R., Meijer, W.G., Vazquez-Boland, J.A., 2007. *Rhodococcus equi* infection in foals: the science of 'rattles'. *Equine Vet. J.* 39, 470-478.
- Pei, Y., Dupont, C., Sydor, T., Haas, A., Prescott, J.F., 2006. Cholesterol oxidase (ChoE) is not important in the virulence of *Rhodococcus equi*. *Vet. Microbiol.* 118, 240-246.
- Pei, Y., Parreira, V., Nicholson, V.M., Prescott, J.F., 2007. Mutation and virulence assessment of chromosomal genes of *Rhodococcus equi* 103. *Can. J. Vet. Res.* 71, 1-7.
- Phumoonna, T., Barton, M.D., Vanniasinkam, T., Heuzenroeder, M.W., 2008. Chimeric vapA/groEL2 DNA vaccines enhance clearance of *Rhodococcus equi* in aerosol challenged C3H/He mice. *Vaccine* 26, 2457-2465.
- Sauvageot, N., Pichereau, V., Louarme, L., Hartke, A., Auffray, Y., Laplace, J.M., 2002. Purification, characterization and subunits identification of the diol dehydratase of *Lactobacillus collinoides*. *Eur. J. Biochem.* 269, 5731-5737.
- Sibbald, M.J., Ziebandt, A.K., Engelmann, S., Hecker, M., de Jong, A., Harmsen, H.J., Raangs, G.C., Stokroos, I., Arends, J.P., Dubois, J.Y., van Dijl, J.M., 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. *Microbiol. Mol. Biol. Rev.* 70, 755-788.
- Sydor, T., von Bagen, K., Becken, U., Spuerck, S., Nicholson, V.M., Prescott, J.F., Haas, A., 2007. A mycolyl transferase mutant of *Rhodococcus equi* lacking capsule integrity is fully virulent. *Vet. Microbiol.* 128, 327-341.
- Takai, S., Hines, S.A., Sekizaki, T., Nicholson, V.M., Alperin, D.A., Osaki, M., Takamatsu, D., Nakamura, M., Suzuki, K., Ogino, N., Kakuda, T., Dan, H., Prescott, J.F., 2000. DNA sequence and comparison of virulence plasmids from *Rhodococcus equi* ATCC 33701 and 103. *Infect. Immun.* 68, 6840-6847.
- Takai, S., Koike, K., Ohbushi, S., Izumi, C., Tsubaki, S., 1991a. Identification of 15- to 17-kilodalton antigens associated with virulent *Rhodococcus equi*. *J. Clin. Microbiol.* 29, 439-443.
- Takai, S., Sekizaki, T., Ozawa, T., Sugawara, T., Watanabe, Y., Tsubaki, S., 1991b. Association between a large plasmid and 15- to 17-kilodalton antigens in virulent *Rhodococcus equi*. *Infect. Immun.* 59, 4056-4060.
- Tan, C., Prescott, J.F., Patterson, M.C., Nicholson, V.M., 1995. Molecular characterization of a lipid-modified virulence-associated protein of *Rhodococcus equi* and its potential in protective immunity. *Can. J. Vet. Res.* 59, 51-59.
- Tjalsma, H., Bolhuis, A., Jongbloed, J.D., Bron, S., van Dijl, J.M., 2000. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiol. Mol. Biol. Rev.* 64, 515-547.
- Trost, M., Wehmhoner, D., Karst, U., Dieterich, G., Wehland, J., Jansch, L., 2005. Comparative proteome analysis of secretory proteins from pathogenic and nonpathogenic *Listeria* species. *Proteomics* 5, 1544-1557.
- Vanniasinkam, T., Barton, M.D., Heuzenroeder, M.W., 2004. The immunogenicity of *Rhodococcus equi* GroEL2-based vaccines in a murine model. *Vet. Immunol. Immunopathol.* 98, 91-100.
- Wiker, H.G., Harboe, M., 1992. The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. *Microbiol. Rev.* 56, 648-661.

Figure legends

Fig. 1. Separation of secreted proteins from *R. equi* by SDS-PAGE. Proteins extracted from *R. equi* ATCC 33701 grown in LB medium at 30°C (lanes 2, 3) or 37°C (lanes 4, 5) and at the mid-exponential (lanes 2, 4) or the early-stationary (lanes 3, 5) growth phase. Lane 1 contains proteins extracted from non inoculated LB medium (negative control). All lanes are loaded with 150 µg of protein. The spots 1 to 30 indicate bands analyzed by LC ESI MS/MS. The numbers on the left indicate molecular masses (in kilodaltons).

Fig. 2. Detection of immunogenic proteins in the culture supernatant of *R. equi*. Immunoblot of secreted proteins from *R. equi* ATCC 33701 was probed with pooled sera from nine foals that had died of rhodococcosis. Lanes were loaded with 150 µg of secreted proteins *R. equi* ATCC 33701 as follows: lane 1, non inoculated LB medium (negative control medium); lane 2, 30°C mid-exponential phase; lane 3, 30°C early-stationary phase; lane 4, 37°C mid-exponential phase; lane 5, 37°C stationary phase. The numbers on the left indicate molecular masses (in kilodaltons).

Fig. 3. Overexpression and Western blot analysis of recombinant *R. equi* proteins. A) SDS-PAGE of lysates of *E. coli* M15 or *E. coli* BL21(DE3) expressing the recombinant proteins. Lane 1, molecular mass marker; lane 2, empty pQE30 vector; lane 3, *E. coli* lysate containing recombinant cholesterol oxidase; lane 4, *E. coli* lysate containing recombinant catalase; Lane 5, empty pET-30 vector; Lanes 6 to 15, the following recombinant proteins: hypothetical proteins RHA1_ro06188, RHA1_ro03156, RHA1_ro05216, RHA1_ro05669, probable protease, PSP (probable secreted protein), VapA, VapD, probable mycolyl transferase 3, probable mycolyl transferase 1. B) Western blot showing reactivity of sera with the twelve recombinant proteins. Sera were obtained from foals that had died of rhodococcosis.

Fig. 1

Fig. 2

Fig. 3**A)** **B)**

Table 1. Primers and vectors used in gene cloning

Gene of	Primers ^a	Vector
catalase	5' ATGACGTCCATTTCAGCCACGT 3' 5' GTTCTGCCGACCGAACTACC 3'	pQE30
ChoE	5' TCCGCCTCCGAGCTCCCAGCC 3' 5' TCTCATAAGCTTCGACGCGT 3'	pQE30
hypothetical protein RHA1_ro03156	5' <u>GACGACGACAAGATCCAGCAGGAGAACACCTC</u> 3' 5' <u>GAGGAGAAGCCCGGTCTAGCCGTA</u> ACTGACGACGAC 3'	pET-30 Ek/LIC
hypothetical protein RHA1_ro05216	5' <u>GACGACGACAAGATCGTGGCTGCAATGACCTCC</u> 3' 5' <u>GAGGAGAAGCCCGGTCTACTCGCAGGCGATCCCCGTC</u> 3'	pET-30 Ek/LIC
hypothetical protein RHA1_ro05669	5' <u>GACGACGACAAGATCCAGCACAAGACGATCACACTC</u> 3' 5' <u>GAGGAGAAGCCCGGTTTACCGGAGACCCA</u> ACTTGCT 3'	pET-30 Ek/LIC
hypothetical protein RHA1_ro06188	5' <u>GACGACGACAAGATACCGCTGCCGGCGTCGAT</u> 3' 5' <u>GAGGAGAAGCCCGGTCTAGCGCAGTGTGGCGAG</u> 3'	pET-30 Ek/LIC
probable antigen 85 protein precursor (mycolyl transferase 3)	5' <u>GACGACGACAAGATCTCGACGCCCCGGCGCGACCGAC</u> 3' 5' <u>GAGGAGAAGCCCGGTCTACCGGGGAGTGCCCATG</u> 3'	pET-30 Ek/LIC
probable antigen 85 complex protein (mycolyl transferase 1)	5' <u>GACGACGACAAGATGGCACAGTCCCTCGGCACCCCC</u> 3' 5' <u>GAGGAGAAGCCCGGTTACGGCGATGGTCGCCTTTCC</u> 3'	pET-30 Ek/LIC
probable protease	5' <u>GACGACGACAAGATCGAACCCGCCGCGTCGGGT</u> 3' 5' <u>GAGGAGAAGCCCGGTATTACCGATGTCGGTCAGTG</u> 3'	pET-30 Ek/LIC
probable secreted protein (PSP)	5' <u>GACGACGACAAGATTGGTGTGGCGGACGCGG</u> 3' 5' <u>GAGGAGAAGCCCGGTCTACGACAGCTCCCATCC</u> 3'	pET-30 Ek/LIC
VapA	5' <u>GACGACGACAAGATGACCGTTCTTGATTCCGGTAGC</u> 3' 5' <u>GAGGAGAAGCCCGGTCTAGGCGTTGTGCCAGCTACC</u> 3'	pET-30 Ek/LIC
VapD	5' <u>GACGACGACAAGATGCAGGAGCTAGCTGGCACCAAG</u> 3' 5' <u>GAGGAGAAGCCCGGTCTACTCCCACCCGCCAGTGCC</u> 3'	pET-30 Ek/LIC

^a The underlined letters in primers are complementary extensions compatible to sequences in the pET-30 Ek/LIC cloning vector.

Table 2. Identification of secreted proteins by mass spectrometry on SDS PAGE (bands noted by a number) or in unfractionated protein extract

Band No. ^a	Sp score ^b	Identified protein ^c				Observed MW (kDa) ^f	Theoretical MW (kDa) ^g
		Description	Bacteria ^d (% Identity)	NCBI Accession No.	Protein domain ^e		
1	1442	Cholesterol oxidase ChoE	<i>R. equi</i> 103	(100) CAC44897	choline dehydrogenase / related flavoproteins	100	54.5
2	1627	Cholesterol oxidase ChoE	<i>R. equi</i> 103	(100) CAC44897	choline dehydrogenase / related flavoproteins	70	54.5
	2009	30S ribosomal protein	<i>R. sp</i> RHA1	(95) YP_118107	ribosomal protein S1 like RNA binding domain	70	54.0
3	798	Probable antigen 85 complex protein (mycolyl transferase 1)	<i>R. sp</i> RHA1	(71) YP_704140	putative esterase	65	62.8
4		No result			3 LGFP repeat motifs	60	
5	1388	Catalase	<i>R. equi</i> 103	(100) AY779519.1	catalase	55	55.0
	1058	Cholesterol oxidase ChoE	<i>R. equi</i> 103	(100) CAC44897	choline dehydrogenase / related flavoproteins	55	54.5
	2104	Hypothetical protein RHA1_ro06188	<i>R. sp</i> RHA1	(75) YP_706123		55	58.5
6	1440	Cholesterol oxidase ChoE	<i>R. equi</i> 103	(100) CAC44897	choline dehydrogenase / related flavoproteins	54	54.5
7	676	Elongation factor Tu	<i>N. farcinica</i>	(95) YP_121291	EF Tu family (II and III domains)	50	43.5
8	1007	Elongation factor Tu	<i>N. farcinica</i>	(95) YP_121291	EF Tu family (II and III domains)	48	43.5
9	922	DNA-directed RNA polymerase α -subunit	<i>R. sp</i> RHA1	(98) YP_706097	RNA polymerase Rbp3 / Rpb11 dimerisation domain / RpoA insert domain / α -chain C terminal domain	46	38.1
	647	Hypothetical protein RHA1_ro05154	<i>R. sp</i> RHA1	(77) YP_705093		46	21.0
	1035	Probable stage II sporulation protein	<i>R. sp</i> RHA1	(51) ABG95848	stage II sporulation protein	46	54.8
	448	Short-chain dehydrogenase/reductase	<i>S. tropica</i>	(67) YP_001159019	short chain dehydrogenase / 3 ketoacyl (acyl carrier protein) reductase	46	33.0
10	1004	Cholesterol oxidase ChoE	<i>R. equi</i> 103	(100) CAC44897	choline dehydrogenase / related flavoproteins	45	54.5
	1056	Hypothetical protein RHA1_ro01045	<i>R. sp</i> RHA1	(54) YP_701030	secretory lipase	45	36.5
	1795	Hypothetical protein RHA1_ro04854	<i>R. sp</i> RHA1	(60) YP_704797	cutinase	45	39.0
11	715	Alkyl hydroperoxide reductase	<i>R. sp</i> RHA1	(87) YP_703236	peroxiredoxin family	42	21.0
	703	Cell division initiation protein	<i>R. sp</i> RHA1	(78) YP_701065	DivIVA protein (cell division initiation protein)	42	31.0
	714	Probable thioesterase	<i>R. sp</i> RHA1	(66) YP_707177	thioesterase protein	42	17.0
	957	Not matched (protein X3)				42	24.0
	645	Short-chain dehydrogenase/reductase	<i>S. tropica</i>	(67) YP_001159019	short chain dehydrogenase / 3 ketoacyl (acyl carrier protein) reductase	42	33.0
12	1895	Not matched (protein X4)				40	37.0
	1589	Hypothetical protein RHA1_ro05704	<i>R. sp</i> RHA1	(64) YP_705641	glucose dehydrogenase	40	37.2
13	1435	Probable antigen 85-A precursor (mycolyl transferase 2)	<i>R. sp</i> RHA1	(43) YP_704946	putative esterase	38	36.8
	1906	Not matched (protein X4)				38	37.0
	739	Not matched (protein X5)				38	37.0
14	701	Probable antigen 85 protein precursor (mycolyl transferase 3)	<i>R. sp</i> RHA1	(57) YP_702108	putative esterase	37	34.5
	816	Hypothetical protein RHA1_ro04050	<i>R. sp</i> RHA1	(56) YP_704005	N-acetylmuramoyl-L-alanine amidase domain	37	68.0
15	1678	Cholesterol oxidase ChoE	<i>R. equi</i> 103	(100) CAC44897	choline dehydrogenase / related flavoproteins	36	54.5
16	450	Amidase AmiC_1	<i>M. ulcerans</i>	(49) YP_906566	amidase	34	49.2
17	701	Probable antigen 85 protein precursor (mycolyl transferase 3)	<i>R. sp</i> RHA1	(57) YP_702108	putative esterase	32	34.5
18	1672	Hypothetical protein RHA1_ro03156	<i>R. sp</i> RHA1	(35) YP_703117	MspA (porin)	29	30.2
	1379	Hypothetical protein RHA1_ro05216	<i>R. sp</i> RHA1	(66) YP_705155		29	30.2
	1151	Hypothetical protein RHA1_ro05669	<i>R. sp</i> RHA1	(67) YP_705606	transglycosylase-like domain	29	36.0
19	1803	Probable protease	<i>R. sp</i> RHA1	(63) YP_705835		28	42.8
	1124	Probable secreted protein (PSP)	<i>R. erythropolis</i>	(46) CAC37046		28	29.8
20	817	Probable mycolyl transferase (mycolyl transferase 4)	<i>R. sp</i> RHA1	(65) YP_704013	putative esterase	25	33.0
	1625	Hypothetical protein nfa45220	<i>N. farcinica</i>	(73) YP_120737	transglycosylase like domain	25	8.0
	2135	Hypothetical protein RHA1_ro06358	<i>R. sp</i> RHA1	(69) YP_706293		25	8.0
	840	Cold shock protein A	<i>R. sp</i> RHA1	(98) YP_704299	cold shock protein	25	7.0
	713	Not matched (protein X6)				25	53.3
21	2579	Not matched (protein X2)				22	18.5

	1033	Probable lipoprotein	<i>R. sp</i> RHA1	(60)	YP_704138	peptidase M23	22	18.0
	881	Probable resuscitation-promoting factor	<i>R. sp</i> RHA1	(67)	YP_704901	transglycosylase like domain	22	17.5
22	1543	Peptidyl-prolyl cis-trans isomerase	<i>R. sp</i> RHA1	(88)	YP_704938	cyclophilin	20	22.0
23	678	VapA (intracellular multiplication)	<i>R. equi</i> 103	(100)	BAB16621	<i>R. equi</i> virulence-associated protein	19	16.2
24	762	VapA (intracellular multiplication)	<i>R. equi</i> 103	(100)	BAB16621	<i>R. equi</i> virulence-associated protein	18	16.2
25	793	VapA (intracellular multiplication)	<i>R. equi</i> 103	(100)	BAB16621	<i>R. equi</i> virulence-associated protein	17	16.2
	1604	Probable antigen 85 protein precursor (mycolyl transferase 3)	<i>R. sp</i> RHA1	(57)	YP_702108	putative esterase	17	34.5
	666	Not matched (protein X1)					17	11.0
26		No result					16	
27	1045	VapD	<i>R. equi</i> 103	(100)	BAB16624	<i>R. equi</i> virulence-associated protein	14	14.0
28	1705	VapD	<i>R. equi</i> 103	(100)	BAB16624	<i>R. equi</i> virulence-associated protein	12	14.0
	1109	Hypothetical protein RHA1_ro05477	<i>R. sp</i> RHA1	(37)	YP_705415		12	18.5
	1055	Chaperonin Cpn10	<i>M. sp</i> JLS	(94)	ZP_01280279	chaperonin Cpn10	12	11.0
29	2117	VapD	<i>R. equi</i> 103	(100)	BAB16624	<i>R. equi</i> virulence-associated protein	10	14.0
	1607	Chaperonin Cpn10	<i>M. sp</i> JLS	(94)	ZP_01280279	chaperonin Cpn10	10	11.0
	581	Phosphomannomutase	<i>R. sp</i> RHA1	(82)	YP_706251	Phosphoglucomutase / phosphomannomutase	10	48.6
	930	Probable extracellular protein	<i>R. sp</i> RHA1	(37)	YP_700670		10	14.7
30	1475	VapD	<i>R. equi</i> 103	(100)	BAB16624	<i>R. equi</i> virulence-associated protein	9	14.0
unfractionated protein extract	450	Catalase	<i>R. equi</i> 103	(100)	AY779519.1	catalase		55.0
	669	Chaperonin Cpn10	<i>M. sp</i> JLS	(94)	ZP_01280279	chaperonin Cpn10		11.0
	1898	Cholesterol oxidase ChoE	<i>R. equi</i> 103	(100)	CAC44897	choline dehydrogenase / related flavoproteins		54.5
	793	Cold shock protein A	<i>R. sp</i> RHA1	(98)	YP_704299	cold shock protein		7.2
	895	Dihydrolipoyl dehydrogenase	<i>R. sp</i> RHA1	(89)	YP_706197	pyridine nucleotide-disulphide oxidoreductase		21.4
	545	Elongation factor G	<i>R. sp</i> RHA1	(95)	YP_701891	EF G subfamily (II and IV domains)		76.9
	630	Elongation factor Tu	<i>N. farcinica</i>	(94)	YP_121291	EF Tu family (II and III domains)		43.5
	1205	Chaperonin GroEL2	<i>R. equi</i> 103	(100)	AAK95493	chaperonin GroEL		56.5
	687	Hypothetical protein RHA1_ro04050	<i>R. sp</i> RHA1	(56)	YP_704005	N-acetylmuramoyl-L-alanine amidase domain		68.0
	1268	Hypothetical protein RHA1_ro05477	<i>R. sp</i> RHA1	(37)	YP_705415			21.1
	1843	Hypothetical protein RHA1_ro06188	<i>R. sp</i> RHA1	(75)	YP_706123			58.5
	625	Probable nonspecific lipid-transfer protein	<i>R. sp</i> RHA1	(89)	YP_704432	thiolase domain		40.1
	2337	Probable antigen 85 protein precursor (mycolyl transferase 3)	<i>R. sp</i> RHA1	(57)	YP_702108	putative esterase		34.5
	1339	Not matched (protein X2)						18.4
	976	VapA	<i>R. equi</i> 103	(100)	BAB16621	<i>R. equi</i> virulence-associated protein		16.2
	549	VapD	<i>R. equi</i> 103	(100)	BAB16624	<i>R. equi</i> virulence-associated protein		14.0

^a The band numbers were shown in Fig. 1.

^b Sp score given by Sequest for the most significant peptide (Sp ≥ 450) found using a DNA database prepared from the non annotated genome sequence of *R. equi* 103.

^c Best matching proteins with identities ≥ 35% were taken into consideration.

^d *M. sp* JLS, *Mycobacterium sp* JLS; *M. ulcerans*, *Mycobacterium ulcerans*; *N. farcinica*, *Nocardia farcinica*; *R. sp* RHA1, *Rhodococcus sp* RHA1; *R. erythropolis*, *Rhodococcus erythropolis*; *S. tropica*, *Salinispora tropica*.

^e Protein domain as deduced from sequence analysis with the NCBI conserved domain algorithm.

^f Deduced from the gels.

^g The molecular weight of proteins without signal peptide.

Table 3. Subcellular localization of proteins based on sequence analysis

Identified proteins	Export pathway ^a	Signal Peptide sequence (SP) ^b	TMH ^c (except for SP)
Probable extracellular protein	Sec	MRKVTLAAGLVAAAGLVVPATA ANA	
Protein X2	Sec	MQKLRTIVVAAVAALPVVAAP ANA	
Hypothetical protein RHA1_ro05477	Sec	MKLRFKAATSALVIAAMGVGAGT AYA	140-162 169-191
VapD	Sec	MVRARAFGRFLTFLAVAVIATVSMGG ANA	
VapA	Sec	MKTLHKTVSKAIAATAVAAAAAMIPAGV ANA	
Hypothetical protein RHA1_ro04854	Sec	MGRRVERMVGTAALGGLVAAATVFASGGV AAA	
Hypothetical protein nfa45220	Sec	MAKFDIKRTIGTVAATAALVAAPFALSTGT ANA	
Protein X4	Sec	VLKTTPLRLWLLVAVLSVVTMASCAGLAPQ AQA	
Hypothetical protein RHA1_ro05669	Sec	VSPFARINTARSPLLYVVVAALFATLLVGGGL VVT	
Probable resuscitation-promoting factor	Sec	MSGRHRKPSTTTGRTVAKVAVTGAIMGVGGVAFAGT ANA	
Probable mycolyl transferase 4	Sec	MRTHRPGSTHRLKKRLIGLVAAALLPALTAVTGTTT ANA	
Hypothetical protein RHA1_ro05216	Sec	VPAPLHLKSCRPLSFHRVRSTLLGAAVAATVAASLAACGSIDSAGNTLTVPSPSSSTTT PAK	
Probable mycolyl transferase 2	Tat	MWGS DPRRAARRRLRGVVGASLAALVALPLAGE AAA	
Cholesterol oxidase ChoE	Tat	MTDSRANRADATRGVASVSRRRFLAGAGLTAGAIALSSMSTS ASA	
Probable mycolyl transferase 1	Tat	MRSGLRNSYRAGTEGRWRRRRLSVAAAALVMPAAGITSVT AGA	
Hypothetical protein RHA1_ro04050	Tat	LPHRRPKNSIVLAAVA AVAVATPFV VQA	d
Hypothetical protein RHA1_ro01045	Tat	MRRSIGESLRRATAVGAALFALTALVPAV ASA	
Probable mycolyl transferase 3	Tat	MRVTQKSRRRLKALIGATTAAVAALPVFVGAGV ASA	
Hypothetical protein RHA1_ro06188	Tat	MNRRTFGLGAAGVGALGISALPLVGNRSWNPLAAQ ARA	
Probable protease	Tat	MRIPLAPRAAVHSFARRVAVVGSSALLVLVPLSAS AQA	
Hypothetical protein RHA1_ro05704	Tat	VSSSTPADREGSFVRRSTLGAGA AVAAAIIGIGVAPT TACA	
Probable lipoprotein	Tat	MKGC MAGSKWRLGVRRMVAGVVAAGLTAGVAVMSAPA ASA	
Protein X1	Tat	MIRRWRCSSKSSMMMPRRKNGPIRKQAGSFANAFCRFI IATA	
Probable stage II sporulation protein	Tat	MRSEPMATPRLRISADRVLRRRRPGRRPSRSVGRALALGLVPALAV VGA	
Hypothetical protein RHA1_ro03156	None		61-83 (N-terminal)
Alkyl hydroperoxide reductase	None		
Amidase AmiC_1	None		
Catalase	None		
Cell division initiation protein	None		
Chaperonin Cpn10	None		
Cold shock protein A	None		
Dihydrolipoyl dehydrogenase	None		
DNA-directed RNA polymerase α -subunit	None		
Elongation factor G	None		
Elongation factor TU	None		
Chaperonin GroEL2	None		
Hypothetical protein RHA1_ro05154	None		
Hypothetical protein RHA1_ro06358	None		
Peptidyl-prolyl cis-trans isomerase	None		
Phosphomannomutase	None		
Probable nonspecific lipid-transfer protein	None		
Probable thioesterase	None		
Protein X3	None		
Protein X5	None		
Protein X6	None		
Probable secreted protein (PSP)	None		
30S ribosomal protein S1	None		
Short-chain dehydrogenase/reductase	None		

^a Sec, protein secretion system; Tat, twin-arginine translocation system.

^b Signal peptide as deduced from sequence analysis with the SignalP 3.0 and TatP 1.0 algorithms (www.expasy.org). Only results with score ≥ 0.8 were taken into consideration. The consensus motif recognized by TatP1.0 algorithm RRX[FGAVML][LITMVF] is underlined. The two invariant arginines are enlarged. The recognition sequence of the SPases 1 at position -1 to -3 relative to the cleavage site is indicated in bold.

^c TMH, transmembrane helix as predicted from sequence analysis with the TMHMM 2.0 algorithms (www.expasy.org). Their position in the sequence is indicated.

^d These signal peptides contain a complete or incomplete Tat motif, not recognized by the TatP1.0 algorithm.