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

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1 **Proteomic analysis and immunogenicity of secreted proteins from *Rhodococcus equi***

2 **ATCC 33701**

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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

## 41 1. Introduction

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

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

59 The goal of the present study was to identify proteins secreted by *R. equi* ATCC 33701 at  
60 different temperatures (30°C, the optimal growth temperature, versus 37°C, the host  
61 temperature) and at different growth phases (mid-exponential versus early-stationary phase of  
62 growth). Bioinformatic study was carried out to predict the functions and to confirm the  
63 extracellular localization of the identified proteins. Furthermore, immunogenic secreted  
64 proteins expressed during equine rhodococcosis were characterized by using Western blot  
65 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 (OD<sub>600</sub> of 0.4 to 0.5) and early-stationary (OD<sub>600</sub> 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*

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

101

#### 102 2. 4. *In gel-trypsin digestion*

103 Protein spots to be identified were manually excised from Coomassie stained gels and  
104 excised gel fractions were washed in ultra-pure water. These fractions were then dehydrated  
105 by several incubations in 50 mM ammonium bicarbonate (BICAM, Fluka) and acetonitrile  
106 (CAN, Merck, Fontenay-sous-Bois, France), followed by rehydration in 50 mM BICAM. 0.5  
107  $\mu$ g modified porcine trypsin (sequencing grade modified trypsin, Promega Corporation,  
108 Charbonnières, France) was added to each sample, which was then incubated during 16 h at  
109 37°C. The supernatant was recovered by centrifugation (5,000 g for 5 min). A second  
110 extraction from these gel pieces was carried out by incubation in 50 mM BICAM/ACN (1/1 :  
111 v/v) and then by incubation in 5% acetic acid (Carlo Erba, Val de Reuil, France). Peptide-  
112 containing liquid fractions were pooled after each incubation and vacuum dried. Peptides  
113 were resuspended in 20  $\mu$ l 0.1% acetic acid prior to storage at -70°C until mass spectrometry  
114 (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 ( $OD_{600}$  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/](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>).

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

146

#### 147 2. 8. *Western blot analysis*

148 Two SDS PAGE gels were run in parallel. One was used for identification and the other  
149 for immunoblot. 150 µg of proteins were loaded in each well to compare each culture  
150 condition. Proteins resolved by SDS PAGE were electroblotted onto nitrocellulose  
151 membranes (Amersham Biosciences, Orsay, France) with Towbin transfer buffer using a  
152 Trans-Blot<sup>®</sup> SD semi-dry transfer cell (Bio-Rad Laboratories) at 15 V for 40 min. Blotted  
153 membranes were blocked with 5% (w/v) skimmed milk powder in TBS buffer (10 mM Tris,  
154 0.2 M NaCl, 0.05% (v/v) Tween 20) overnight at 4°C and incubated with pooled sera from  
155 nine foals that had died of rhodococcosis at 1:200 dilution in blocking solution for 1 h at  
156 37°C. Membranes were then washed three times for 15 min each with TBS buffer. They were  
157 incubated with rabbit anti-horse IgG, conjugated to alkaline phosphatase, at 1:5000 dilution  
158 (Bethyl Laboratories, Montgomery, TX, USA) for 1 h at 37°C and washed as described  
159 above. Antigen-antibody reactions were developed with AP Conjugate Substrate kit (Bio-Rad  
160 Laboratories) according to the manufacturer.

161

#### 162 2. 9. *Cloning and expression of twelve genes*

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

170 To assess expression of the cloned genes, recombinant *E. coli* M15 or *E. coli* BL21(DE3)  
171 were grown overnight at 37°C in 10 ml of LB medium supplemented with 30 µg/ml of  
172 kanamycin (Sigma) for the two *E. coli* strains and 100 µg/ml of ampicillin (Sigma) for *E. coli*  
173 M15 only. One ml of the bacterial suspension was then subinoculated into 100 ml of fresh LB  
174 medium and the bacteria were allowed to grow at 37°C until the optical density of the  
175 medium at 600 nm reached 0.6. Thereafter, the expression of the cloned genes was induced by  
176 addition of IPTG (ABgene, Courtaboeuf, France) to a final concentration of 1 mM. After a  
177 further 3 h incubation at 37°C, the bacterial cells were collected by centrifugation for 10 min  
178 at 5,000 g. The cell lysis was performed by incubation for 1 h with a lysis buffer containing  
179 100 mM NaH<sub>2</sub>PO<sub>4</sub> (Merck), 10 mM Tris base and 8 M urea (Eurobio). The cell debris were  
180 removed by centrifugation at 10,000 g for 10 min. Supernatants were used for Western blot  
181 analysis.

182

### 183 **3. Results**

#### 184 *3. 1. Analysis of secreted proteins by SDS PAGE*

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

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

199

### 200 3. 2. *Proteins identification*

201 30 bands were cut out of gels (bands No. 1 to 30, Fig. 1), subjected to in-gel trypsin  
202 digestion and analyzed by LC-ESI tandem mass spectrometry. In parallel, the secretome of *R.*  
203 *equi* was also analyzed without previous electrophoretic separation by injecting a  
204 trypsinolysed crude preparation of secreted proteins into LC-ESI tandem mass spectrometer  
205 (see Materials and Methods for details). These two approaches led to the identification of 44  
206 and 16 proteins, respectively (Table 2). The method without previous electrophoretic  
207 separation allowed us to identify fewer secreted proteins than the other method but led to the  
208 rapid identification of dominant proteins present in the secretome. Since we found twelve  
209 identical proteins in both methods, a total of 48 proteins was finally identified. All identified  
210 proteins were encoded by the chromosome except for the Vap proteins. Because of the non-  
211 annotated genome sequence of *R. equi*, homologous proteins were identified using BLASTP  
212 program and the homology with the first hit was indicated for each protein (Table 2). Six

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

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

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

243

### 244 3.3. Protein structure and localization

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

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

264 According to the TMHMM 2.0 algorithm, we could predict a putative membrane  
265 localization for two proteins. The polypeptide homologous to the hypothetical protein  
266 RHA1\_ro05477 contained, in addition to a signal peptide, two transmembrane domains at  
267 central position. The polypeptide homologous to the hypothetical protein RHA1\_ro03156,  
268 which did not possess a signal peptide, contained a single transmembrane domain localized at  
269 the N-terminus (Table 3). These two proteins may correspond to a membrane protein and a  
270 surface protein, respectively. Furthermore, according to the NCBI CDD algorithm, the  
271 probable mycolyl transferase 1 contained at its C-terminus three LGFP tandem repeats, which  
272 may be involved in anchoring the protein to the cell wall.

273

#### 274 3. 4. Antigenic profiles of *R. equi* secretome

275 Antigenic profiles were explored by immunoblot performed with pooled sera from nine *R.*  
276 *equi* infected foals (Fig. 2). No band was detected in the control medium. Moreover, Western  
277 blot control experiments using antisera from healthy foals did not reveal any immunogenic  
278 protein (data not shown). Independent of growth conditions, immunogenic proteins were  
279 present in four regions with molecular weights about 99, 54, 29 and 20 kDa, respectively. A  
280 protein band of a monodimensional gel may harbor more than one polypeptide and probably  
281 not all of them would correspond to immunoreactive proteins. Analysis of the four bands by  
282 mass spectrometry revealed the presence of the following proteins: cholesterol oxidase,  
283 catalase, hypothetical proteins RHA1\_ro06188, RHA1\_ro03156, RHA1\_ro05216,  
284 RHA1\_ro05669, probable protease, probable secreted protein named PSP, VapA, VapD,  
285 probable mycolyl transferases 3 and 1. In order to test which of them reacts actually with the  
286 serum, the twelve proteins were first overexpressed in *E. coli* (Fig. 3A) and then used for a

287 Western blot analysis using the same pooled horse sera as in Fig. 2 (Fig. 3B). No *E. coli*  
288 immunoreactive proteins were observed in the negative controls with empty pQE30 and pET-  
289 30 vectors. In contrast, the serum reacted strongly with the PSP and the cholesterol oxidase.  
290 The proteins VapA, VapD and the probable mycolyl transferase 1 were less immunoreactive  
291 and the serum reacted only weakly with the proteins homologous to the hypothetical proteins  
292 RHA1\_ro06188 and RHA1\_ro03156. The other recombinant proteins did not react with the  
293 serum.

294

#### 295 **4. Discussion**

296 In order to characterize new *R. equi* candidate antigens associated with rhodococcosis  
297 among secreted proteins, we used a proteomic approach combined with serum profiling by  
298 using sera from foals that had died of rhodococcosis. This approach enabled identification of  
299 48 proteins released by the *R. equi* ATCC 33701 strain during growth. As expected, 24  
300 polypeptides contained a signal peptide sequence required for their translocation across the  
301 cytoplasmic membrane *via* the Sec-pathway (Keyzer et al., 2003) or the Tat-pathway (Lee et  
302 al., 2006), the two most important secretion systems distributed in all bacterial genera.  
303 Among them, half the proteins were predicted to possess a potential Tat signal peptide,  
304 suggesting that the Tat pathway could be as significant as the Sec pathway in *R. equi*. In  
305 contrast to the latter, the former pathway is involved in the secretion of exclusively folded  
306 proteins and protein translocation has no requirement for ATP as an energy source but relies  
307 solely on the proton motive force. It is interesting to note that all proteins identified in the  
308 most intensively Coomassie-stained bands contained a Sec signal peptide (VapA and VapD)  
309 or a Tat signal peptide (cholesterol oxidase, protein X1, mycolyl transferases 1 and 3).

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

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

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

337 another virulence factor involved in the intracellular growth in macrophages and identified  
338 among secreted proteins in our experimental conditions, contrary to previous studies which  
339 detected VapA in the cell pellet only (Byrne et al., 2001; Kohler et al., 2003). Its presence in  
340 the supernatant can be explained by the fact that lipoproteins are vulnerable to proteolytic  
341 cleavage relatively close to the N-terminal cysteine and are consequently released as soluble  
342 protein in the culture filtrate (Malen et al., 2007). Moreover, it has been suggested that some  
343 lipoproteins may be alternatively processed by signal peptidase I or II, and this mechanism  
344 may explain their dual localization in the extracellular environment or as lipoproteins in the  
345 cell wall (Malen et al., 2007). In our work, VapA was observed in three isoforms, in  
346 accordance with the study of Tan et al. (1995). Indeed, in that work, the polypeptides isolated  
347 from the 18-22 kDa and 17.5 kDa fractions were hydrophobic and lipid modified whereas the  
348 15-kDa protein did not show this modification. VapD was detected in four isoforms which  
349 correspond most probably to degradation products. VapA and VapD were observed only at  
350 37°C in our study which is in accordance with previous findings (Byrne et al., 2001).

351 Some proteins identified in the present study were predicted to be involved in the cell wall  
352 biosynthesis. Indeed, four out of at least 13 *R. equi* probable mycolyl transferases predicted by  
353 genome analysis (Sydor et al., 2007) were detected in our study. These four enzymes were  
354 homologous to the mycobacterial antigen 85 complex (Ag85 complex), major secretion  
355 products of *M. tuberculosis* (Content et al., 1991; Wiker and Harboe, 1992). This Ag85  
356 complex is made of three mycolyl transferases (FbpA, FbpB and FbpC), pivotal for the  
357 synthesis of the mycolic acid layer in the cell wall (Belisle et al., 1997). The probable mycolyl  
358 transferases 1 and 2 are encoded by the gene *fbpA* (Sydor et al., 2007) and *fbpB* (Pei et al.,  
359 2007) of *R. equi*, respectively. In *R. equi*, FbpA could be involved in capsule anchorage since  
360 a *fbpA* mutant is characterized by a non-mucoid phenotype (Sydor et al., 2007). Since  
361 synthesis of the capsule is constitutive in *R. equi*, it is not surprising that FbpA was one of the

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

363 It should be noted that recent virulence studies in a murine model showed that both FbpA and

364 FbpB might not be involved in *R. equi* pathogenesis (Pei et al., 2007; Sydor et al., 2007).

365 Immunological characterization of *R. equi* secreted proteins by sera from foals that had

366 died of rhodococcosis revealed antigenic reactivity with four 99, 54, 29 and 20 kDa

367 immunogenic regions, similar to the immunoblots obtained by Kohler et al. (2003). By using

368 recombinant proteins, we demonstrated that five proteins (VapA, VapD, cholesterol oxidase,

369 probable mycolyl transferase 1 and the PSP) were indeed involved in the immunogenicity of

370 these four regions. Contrary to previous studies which identified Vap proteins as strongly

371 immunoreactive proteins (Byrne et al., 2001; Kohler et al., 2003), the two most strongly

372 immunoreactive proteins detected in the present study were the cholesterol oxidase and the

373 PSP. Machang'u and Prescott (1991b) showed that antibodies against the cholesterol oxidase

374 associated to the phospholipase C of *R. equi* did not prevent foals from developing lung

375 abscesses following experimental infection. However, this result has to be examined by taking

376 into account the infective dose, the dose and the nature of antigens which correspond to a

377 combination of two proteins, the immunization and infection routes and the type of adjuvant.

378 Indeed, recent studies demonstrated that these various parameters may influence the

379 polarization of immune response which must be a cellular type response to confer protection

380 against *R. equi* infection (Lunn and Townsend, 2000). The strong immune response to the

381 PSP suggests that this protein was readily accessible to the immune system *in vivo* and

382 consequently supports the fact that it was actively secreted despite the lack of a signal peptide.

383 This protein needs to be further characterized. Finally, the latter protein, cholesterol oxidase

384 and VapA that has been shown highly immunogenic (Kohler et al., 2003) in foals and

385 protective in a murine model (Haghighi and Prescott, 2005) may represent promising antigen

386 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

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400 that had died of rhodococcosis.

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498 **Figure legends**

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

505

506 **Fig. 2.** Detection of immunogenic proteins in the culture supernatant of *R. equi*. Immunoblot  
507 of secreted proteins from *R. equi* ATCC 33701 was probed with pooled sera from nine foals  
508 that had died of rhodococcosis. Lanes were loaded with 150 µg of secreted proteins *R. equi*  
509 ATCC 33701 as follows: lane 1, non inoculated LB medium (negative control medium); lane  
510 2, 30°C mid-exponential phase; lane 3, 30°C early-stationary phase; lane 4, 37°C mid-  
511 exponential phase; lane 5, 37°C stationary phase. The numbers on the left indicate molecular  
512 masses (in kilodaltons).

513

514 **Fig. 3.** Overexpression and Western blot analysis of recombinant *R. equi* proteins. A) SDS-  
515 PAGE of lysates of *E. coli* M15 or *E. coli* BL21(DE3) expressing the recombinant proteins.  
516 Lane 1, molecular mass marker; lane 2, empty pQE30 vector; lane 3, *E. coli* lysate containing  
517 recombinant cholesterol oxidase; lane 4, *E. coli* lysate containing recombinant catalase; Lane  
518 5, empty pET-30 vector; Lanes 6 to 15, the following recombinant proteins: hypothetical  
519 proteins RHA1\_ro06188, RHA1\_ro03156, RHA1\_ro05216, RHA1\_ro05669, probable  
520 protease, PSP (probable secreted protein), VapA, VapD, probable mycolyl transferase 3,  
521 probable mycolyl transferase 1. B) Western blot showing reactivity of sera with the twelve  
522 recombinant proteins. Sera were obtained from foals that had died of rhodococcosis.

Fig. 1

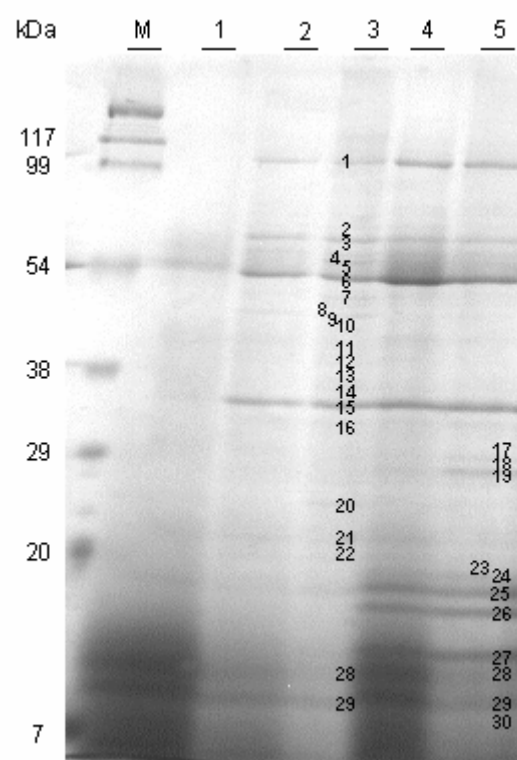
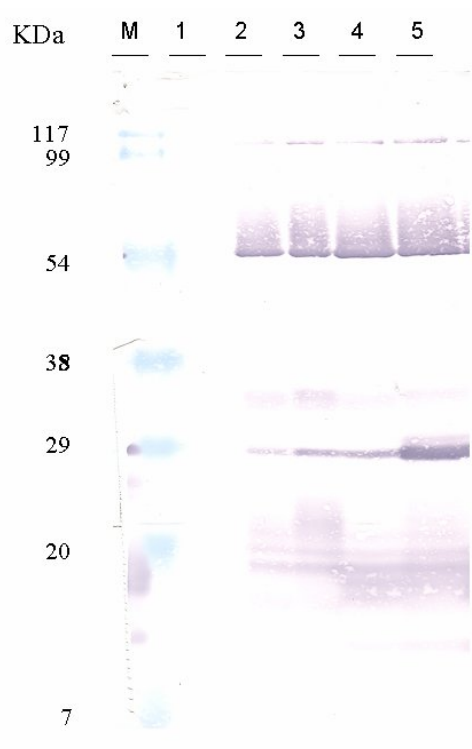


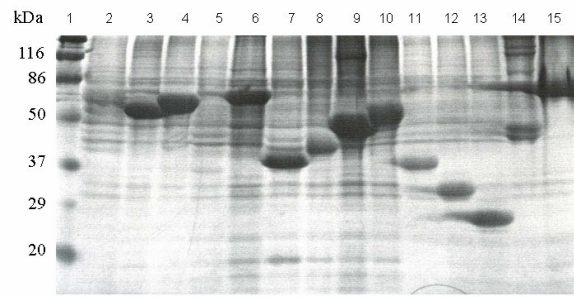
Fig. 2



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Fig. 3

A)



B)

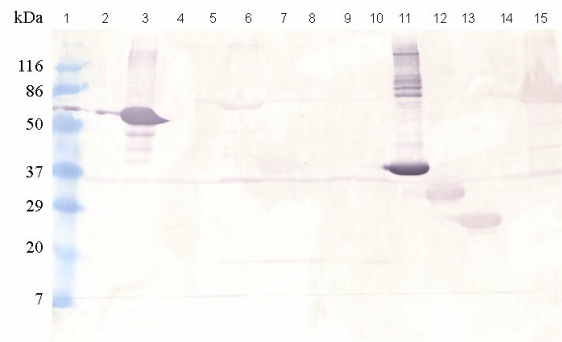


Table 1. Primers and vectors used in gene cloning

Gene of	Primers <sup>a</sup>	Vector
catalase	5' ATGACGTCCATTCAGCCACGT 3' 5' GTTCTGCCGACCGAACTACC 3'	pQE30
ChoE	5' TCCGCCTCCGGAGCTCCAGCC 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>GAGGAGAAGCCCGGTCTACTCGCAGGCGATCCCGTC</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>GAGGAGAAGCCCGGTCTAGCCGAGTGTGGCGAG</u> 3'	pET-30 Ek/LIC
probable antigen 85 protein precursor (mycolyl transferase 3)	5' <u>GACGACGACAAGATCTCGACGCCCGGCGCGACCGAC</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>GAGGAGAAGCCCGGTT</u> CAGGCGATGGTCGCCTTTCC 3'	pET-30 Ek/LIC
probable protease	5' <u>GACGACGACAAGATCGAACCCGCCGCTCGGGT</u> 3' 5' <u>GAGGAGAAGCCCGGTATT</u> CACCGATGTCGGTCAGTG 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

<sup>a</sup> 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. <sup>a</sup>	Sp score <sup>b</sup>	Description	Identified protein <sup>c</sup>			Protein domain <sup>c</sup>	Observed MW (kDa) <sup>f</sup>	Theoretical MW (kDa) <sup>g</sup>
			Bacteria <sup>d</sup> (% Identity)		NCBI Accession No.			
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					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 $\alpha$ -subunit	<i>R. sp</i> RHA1	(98)	YP_706097	RNA polymerase Rbp3 / Rpb11 dimerisation domain / RpoA insert domain / $\alpha$ -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

<sup>a</sup> The band numbers were shown in Fig. 1.

<sup>b</sup> Sp score given by Sequest for the most significant peptide (Sp  $\geq$  450) found using a DNA database prepared from the non annotated genome sequence of *R. equi* 103.

<sup>c</sup> Best matching proteins with identities  $\geq$  35% were taken into consideration.

<sup>d</sup> *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*.

<sup>e</sup> Protein domain as deduced from sequence analysis with the NCBI conserved domain algorithm.

<sup>f</sup> Deduced from the gels.

<sup>g</sup> The molecular weight of proteins without signal peptide.

Table 3. Subcellular localization of proteins based on sequence analysis

Identified proteins	Export pathway <sup>a</sup>	Signal Peptide sequence (SP) <sup>b</sup>	TMH <sup>c</sup> (except for SP)
Probable extracellular protein	Sec	MRKVTLAAGLVAAAGLVVPATA <b>ANA</b>	
Protein X2	Sec	MQKLRTIVVAAAVALPVVAAP <b>ANA</b>	
Hypothetical protein RHA1_ro05477	Sec	MKLRKFAATSALVIAAMGVGAGT <b>AYA</b>	140-162 169-191
VapD	Sec	MVRARAFGRFLTFFLLAVAVIATVSMGG <b>ANA</b>	
VapA	Sec	MKTLHKTVSKAIAATAVAAAAAMI <b>PAGVANA</b>	
Hypothetical protein RHA1_ro04854	Sec	MGRRVERMVGTAALGGLVAAATVFASGGV <b>AAA</b>	
Hypothetical protein nfa45220	Sec	MAKFDIKRTIGTVAATAALVAAPFALSTGT <b>TANA</b>	
Protein X4	Sec	VLKTTPLRLWLLVAVLSVVTMASCAGLAP <b>QAQA</b>	
Hypothetical protein RHA1_ro05669	Sec	VSPFARINTARSPLLYVVVAALFATLLVGGGL <b>VVT</b>	
Probable resuscitation-promoting factor	Sec	MSGRHRKPTTTGRVAVAVTGAIMGVGGVAFAGT <b>TANA</b>	
Probable mycolyl transferase 4	Sec	MRTHRPGSTHRLKRLIGLVAAALLPALTAVTGTT <b>TANA</b>	
Hypothetical protein RHA1_ro05216	Sec	VPAPLHLKSCRPLSFHRVRSSTLLGAAVAATVAASLAACGSDSAGNTLTVSPSSSTTT <b>PAAK</b>	
Probable mycolyl transferase 2	Tat	MWGS DPRRAARRRL <b>RRGVV</b> GASLAALVALPLAGE <b>AAA</b>	
Cholesterol oxidase ChoE	Tat	MTDSRANRADATRGVASVSR <b>RREFLAGAGL</b> TAGAIALSSMSTS <b>ASA</b>	
Probable mycolyl transferase 1	Tat	MRSGLERSYRAGTEGRW <b>RRRALS</b> VAAALVMP <b>IAAGITSVT</b> AGA	
Hypothetical protein RHA1_ro04050	Tat	LPH <b>RR</b> PKNSIVLAAVAAVAVATPF <b>AVQA</b>	} <sup>d</sup>
Hypothetical protein RHA1_ro01045	Tat	MRRSIGESL <b>RR</b> ATAVGAALFALTALVPAV <b>ASA</b>	
Probable mycolyl transferase 3	Tat	MRVTQKSR <b>RLKAL</b> IGATTAAVAALPVFVGAGV <b>ASA</b>	
Hypothetical protein RHA1_ro06188	Tat	MNRR <b>TFLGA</b> AGVGALGISALPLVGNRSWNPLAAQ <b>ARA</b>	
Probable protease	Tat	MRIPLAPRAAVHSF <b>ARRVAV</b> VGSSALLVLPVLS <b>ASAQA</b>	
Hypothetical protein RHA1_ro05704	Tat	VSSSTPADREGSFV <b>RRSTL</b> GAGAAVAAAIIGIGVAPT <b>TACA</b>	
Probable lipoprotein	Tat	MKGCGMAGSKWRLGV <b>RRMVAG</b> VVAAAGLTAGVAVMSAPA <b>ASA</b>	
Protein X1	Tat	MIRRWRCSSKSMMP <b>RRKNGP</b> IRK <b>PQAGS</b> FANAFCRFI <b>IATA</b>	
Probable stage II sporulation protein	Tat	MRSEPMATPRLRISADRVLR <b>RRRPGRR</b> PSRSVGR <b>LALGLVPALAVGA</b>	
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 $\alpha$ -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		

<sup>a</sup> Sec, protein secretion system; Tat, twin-arginine translocation system.

<sup>b</sup> Signal peptide as deduced from sequence analysis with the SignalP 3.0 and TatP 1.0 algorithms ([www.expasy.org](http://www.expasy.org)). Only results with score  $\geq 0,8$  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 I at position -1 to -3 relative to the cleavage site is indicated in bold.

<sup>c</sup> TMH, transmembrane helix as predicted from sequence analysis with the TMHMM 2.0 algorithms ([www.expasy.org](http://www.expasy.org)). Their position in the sequence is indicated.

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