Molecular Characterization of a Lipid-modified Virulence-associated Protein of *Rhodococcus equi* and its Potential in Protective Immunity

C. Tan, J.F. Prescott, M.C. Patterson and V.M. Nicholson

ABSTRACT

Virulent strains of *Rhodococcus equi* produce plasmid-mediated 15- and 17-kDa proteins, which are thermoregulated and apparently surface-expressed. We demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that *R. equi* produce three antigenically-related virulence-associated proteins, a diffuse 18-22-kDa, a 17.5-kDa and a 15-kDa protein. Phase partitioning of whole cells of *R. equi* strain 103 with Triton X-114 (TX-114) and labelling with [^{3}H]-labelled palmitic acid showed that the two higher molecular weight proteins are hydrophobic and lipid modified. The 15-kDa protein did not partition into TX-114 and was not lipid modified. Cloning and expression of a fragment of the *R. equi* virulence plasmid in *Escherichia coli* showed that the three proteins were expressed from a single gene. Sequence analysis of this gene (designated *vap*A) revealed a 570-bp open reading frame encoding a polypeptide of 189 amino acids with a calculated molecular mass of 19,175 Da. The mature, nonlipid modified protein had a calculated mass of 16,246 Da. The 17.5- and 18-22-kDa forms of the protein are therefore due to lipid modification. No significant sequence homology of the *vap*A gene with other reported nucleotide sequences were found. Opsonization of virulent *R. equi* with an IgG_1 mouse monoclonal antibody (MAb103) to the VapA protein significantly enhanced uptake in the murine macrophage cell line IC-21. Intraperitoneal injection of mice with Mab103 enhanced initial clearance from the liver of mice challenged intravenously with *R. equi*. Immunization of mice with the lipid-modified VapA purified by SDS-PAGE fractionation or with acetone precipitated VapA protein following TX-114 extraction resulted in significantly enhanced clearance from the liver and spleen following intravenous challenge. The VapA protein of *R. equi* appears therefore to be a protective immunogen.

RÉSUMÉ

Les sources virulentes de *Rhodococcus equi* expriment des protéines de 15 et 17 kDa qui sont codées par un plasmide. Ces protéines sont thermorégulées et apparemment exposées à la surface. À l'aide d'un gel de polyacrylamide en présence de dodécyl sulfate de sodium (SDS-PAGE), nous avons démontré que *R. equi* exprime trois protéines de 18-22, 17,5 et 15 kDa antigéniquement reliées et associées à la virulence. Une partition de phase d'un extrait cellulaire total de la souche 103 de *R. equi* à l'aide de Triton X-114 (TX-114) et marquage à l'aide de [^{3}H]-palmitate a démontré que les protéines de haut poids moléculaire sont de nature lipoprotéique et hydrophobe. Cependant, la protéine de 15 kDa n'était pas retrouvée dans le TX-114 et n'était pas une lipoprotéine. Le clonage et l'expression d'un fragment du plasmide de virulence de *R. equi* chez *Escherichia coli* a montré que ces trois protéines étaient codées par un gène unique. Le séquençage de ce gène (*vapA*) a révélé un cadre de lecture ouvert de 570 pb codant pour un polypeptide de 189 acides aminés avec un poids calculé de 19,175 Da. Le poids moléculaire calculé de la protéine sans lipide est de 16,246. Les protéines de 17,5 et 18-22 kDa représentent donc différentes formes de la protéine modifiées par la présence de lipides. Le gène *vapA* n'est homologue à aucune des séquences nucléotidiques connues. L'opsonisation d'une souche virulente de *R. equi* à l'aide d'un anticorps monoclonal de souris (AcM 103) dirigé contre la protéine VapA a augmenté de façon significative la phagocytose par la lignée IC-21 de macrophages murins. L'injection intra-péritonéale de souris avec AcM 103 a contribué à l'élimination de la bactérie au niveau du foie lorsque celle-ci était administrée par voie intraveineuse. La protéine VapA modifiée par la présence de lipides a été purifiée par SDS-PAGE et par précipitation à l'acétone à la suite d'une extraction avec TX-114. L'immunisation de souris avec cette protéine a augmenté le taux d'élimination du microorganisme au niveau du foie et de la rate à la suite d'une injection intraveineuse. Il appert que la protéine VapA est un immunogène protecteur. (*Traduit par Dr Daniel Dubreuil*)

INTRODUCTION

Rhodococcus equi is an important gram-positive bacterial cause of chronic suppurative bronchopneumonia in foals less than six months of age and is an emerging but sporadic cause of pneumonia in human

Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario N1G 2W1.

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immunodeficiency virus-infected patients with AIDS (1). Virulence is associated with 80–90 kb plasmids (2–5) which determine the expression of a thermoregulated, apparently surface-expressed, characteristically diffuse 17- and a 15-kDa protein (3,6). These proteins are prominent antigens since they are readily recognized in immunoblots using sera from foals with naturally-occurring *R. equi* pneumonia (6). Although nothing is known of the structure and function of these proteins, they are of interest as possible protective immunogens for foals, because of their apparent surface expression and high immunogenicity and the efficacy of immune plasma in protecting foals against *R. equi* pneumonia (7). While immunity to *R. equi* pneumonia in foals appears to depend on both the humoral and cellular components of the immune system (8), little is understood about the antigens involved. In the present paper, we describe the molecular characterization of these proteins and examine their possible role in protective immunity using experimentally infected mice and a mouse macrophage cell line.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

Rhodococcus equi strain 103, which possesses an 84 kb plasmid (pOTS), was used throughout this study. *Escherichia coli* strain XL1Blue was used for cloning and transformation. The plasmid cloning vector pBluescript II (SK+) (Stratagene, La Jolla, California) was used for cloning and transformation.

DNA METHODOLOGY

Restriction digestions, ligations, and gel electrophoresis were done essentially as described by Sambrook *et al* (9). In some cases, the boiling method of Holmes and Quigley (10) was used to prepare plasmid DNA for transformation screening and restriction enzyme analysis. Restriction enzymes and T_4 DNA ligase were obtained from Boehringer Mannheim (Laval, Quebec) or Bethesda Research Laboratories (Burlington, Ontario). Transformation of *E. coli* was done by electroporation (*E. coli* Pulser; Bio-Rad, Mississauga, Ontario). The virulence-associated plasmid pOTS was isolated from *R. equi* using the QIAGEN column (QIAGEN, Chatsworth, California), modified by treating cells with 5 mg/mL of lysozyme for 3 h at 37°C. A partial *Eco*RI library of the plasmid was constructed in pBluescript; *E. coli* XL1-Blue containing recombinant molecules were screened with polyclonal antibody prepared against the 17-kDa virulence-associated protein. A recombinant plasmid expressing immunoreactive proteins was subcloned using standard methods (9). Southern blotting by standard methods was done to confirm the plasmid origin of the cloned fragment using a digoxigenin-labelling system (Boehringer Mannheim) (9).

DNA SEQUENCING

A 1.6 kb *Bam*HI-*Pst*I fragment of pCT-C7 was cloned into the pBluescript sequencing vector. Unidirectional deletions used in sequencing were prepared using the Erase-a-Base system (Promega Corp., Madison, Wisconsin). The sequence of cloned *R. equi* DNA was determined from double-stranded plasmid templates by the dideoxy-chain termination method (11). Double stranded templates were denatured with alkali and the sequencing reactions carried out with the Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio). Synthetic primers designed from the DNA sequence were also used.

TRITON X-114 PHASE PARTITIONING

Rhodococcus equi was grown to stationary phase in nutrient broth (Difco, Detroit, Michigan) at 38°C for about 60 h with constant shaking at 150 rpm. Cells were harvested by centrifugation, washed with 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl (TBS buffer), recentrifuged and pellets stored in microcentrifuge tubes at −70°C. Extraction and phase separation with TX-114 was done essentially as described by Bordier *et al* (12). Briefly, 30–50 mg wet weight of cells were added per mL 2% TX-114 (Calbiochem, San Diego, California) in TBS with 1 mM phenylmethylsulfonylflouride (PMSF, Sigma Chemical Co., St Louis, Missouri) and shaken overnight at 4°C. Insoluble material was removed by centrifugation at 14,400 × *g* at 4°C for 15 min. The supernatant was recovered, and warmed to 37°C for 10 min before centrifugation at 25°C at 14,400 × *g* for 15 min. The upper aqueous layer was removed and reextracted with TX-114 to make a 2% solution. The lower TX-114 phase was washed three times with enough TBS to make a 2% TX-114 solution. The bacterial pellet was extracted with TX-114 a second time. The detergent phase was used in parallel with the aqueous phase for subsequent electrophoresis. Protein content was determined by the BCA Protein Assay (Pierce, Rockford, Illinois). TX-114 extraction of *E. coli* XL1Blue(pCT-C7) expressing the *vap*A gene products was done by an essentially similar process, except that the cells were sonicated before extraction. *Escherichia coli* XL1Blue(pBS) was used as a control. The triton extract was precipitated with 10 vol acetone overnight at −20°C, the pellet dissolved in Tris pH 7.4, boiled in sodium dodecyl sulfate (SDS) sample buffer for 10 min, and run on a 15% SDS-PAGE gel. The proteins were transferred to nitrocellulose and blotted with rabbit polyclonal antiserum or mouse monoclonal antibody (see below).

RADIOLABELLING OF LIPID-MODIFIED PROTEINS

Rhodococcus equi 103 proteins were labelled with [^{3}H]-palmitic acid according to the method of Nielsen and Lampen (13). *Rhodococcus equi* was grown to late log phase in 4 mL nutrient broth at 38°C for 35 h in a shaking waterbath. Twenty μCi [9,10(n)-[^{3}H]-palmitic acid (Amersham, Oakville, Ontario) was added and incubation continued for another 6 h. The cells were then harvested, washing once with phosphate buffered saline (PBS), pH 7.4, and twice with 100% methanol. The methanol was removed by drying the pellet in a vacuum oven. Sodium dodecyl sulfate reducing buffer was added to the dried pellet and the sample was run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below. The gel was fixed in isopropanol: water: acetic acid (25:65:10) for 30 min and then soaked in Amplify (Amersham) for 15 min.

The gel was then dried and exposed to Kodak XOMAT AR5 X-ray film at −70°C.

GEL ELECTROPHORESIS AND IMMUNOBLOTTING

For SDS-PAGE and immunoblotting, all samples were suspended in SDS reducing buffer and boiled for 10 min (14). Undissolved material was removed by centrifugation and samples separated in a mini-electrophoresis system (Bio-Rad) using 15% resolving and 5% stacking gels. Proteins were stained with Coomassie brilliant blue. For immunoblotting, gels were transferred to Biotrace NT (Gelman Sciences, Ann Arbor, Michigan) using a mini-blot electrophoretic transfer cell (Bio-Rad) (15). The membranes were blocked with 5% fish gelatin in TBS, incubated with an IgG_1 murine monoclonal antibody (Mab103) for 1 h or rabbit polyclonal antibody (1:12,000 dilution) prepared against the 17.5- and 18-22-kDa proteins, and then washed with PBS-0.05% Tween 20 (PBST). Membranes were incubated for 1 h with alkaline phosphatase-conjugated goat antimouse IgG F(ab)2 or antirabbit IgG F(ab)2 fragments (Bio/Can Scientific Inc., Mississauga, Ontario), as appropriate, then washed repeatedly with PBST. Naphthol ASBI phosphatase and Fast-Red (Sigma Chemical) in 0.01 M Tris-HCl pH 9.2 was used to visualize the proteins. Molecular weights of the *R. equi* virulence-associated proteins in *R. equi* or *E. coli* XL1Blue(pCT-C7) were determined using TX-114 extracts from these organisms run on 13% or 15% SDS-PAGE gels and transferred to nitrocellulose. Mouse polyclonal antiserum was used to identify the proteins of interest and the molecular weight calculated using linear regression by comparison of R_f values to low molecular weight marker standards (Bio-Rad).

MONOCLONAL OR POLYCLONAL ANTIBODY PRODUCTION

For monoclonal antibody production, whole cell proteins of *R. equi* strain 103 were separated by SDS-PAGE and electroblotted onto nitrocellulose. Horizontal strips containing the 15- and 17.5-22-kDa protein bands were excised. Two strips were ground in 1.5 mL PBS and homogenized with an equal volume of Freund's incomplete adjuvant. BALB/c mice were injected with 0.5 mL antigen intraperitoneally twice at ten day intervals and with 0.25 mL 21 days later. Serum was tested by immunoblot 14 days later for antibody production. A responder mouse was injected with 0.5 mL powdered nitrocellulose containing the proteins in PBS four days before sacrifice. Splenocytes were fused to NS-1 myeloma cells. Hybridoma supernates were tested by ELISA using sonicated whole *R. equi* as antigen and by immunoblot. An IgG_1-producing hybridoma (designated Mab103) was isotyped (Cedar Lane Laboratories, Hornby, Ontario) and cloned by limiting dilution. The cell culture supernatant was used in immunoblots undiluted or diluted by half with 5% gelatin in PBS. For opsonization studies and for passive immunization of mice, the IgG_1 was purified using membrane affinity chromatography-protein G capsule (Amicon, Oakville, Ontario) and quantified by protein assay (Bio-Rad). For polyclonal antibody production against the denatured 17.5-22-kDa virulence-associated proteins, the proteins were recovered from whole cell proteins from bacteria grown to stationary phase in brain heart infusion broth at 37°C for 72 h by solubilizing in SDS reducing buffer and separated by SDS-PAGE using continuous elution electrophoresis, as described below under mouse immunization. For polyclonal antibody production against the recombinant virulence-associated proteins expressed in *E. coli*, the proteins were extracted from *E. coli* XL1Blue-(pCT-C7) in TX-114 as described, precipitated with acetone at 4°C overnight, and suspended in 10 mM Tris, pH 7.0. For each of the proteins, the native *R. equi* and the recombinant

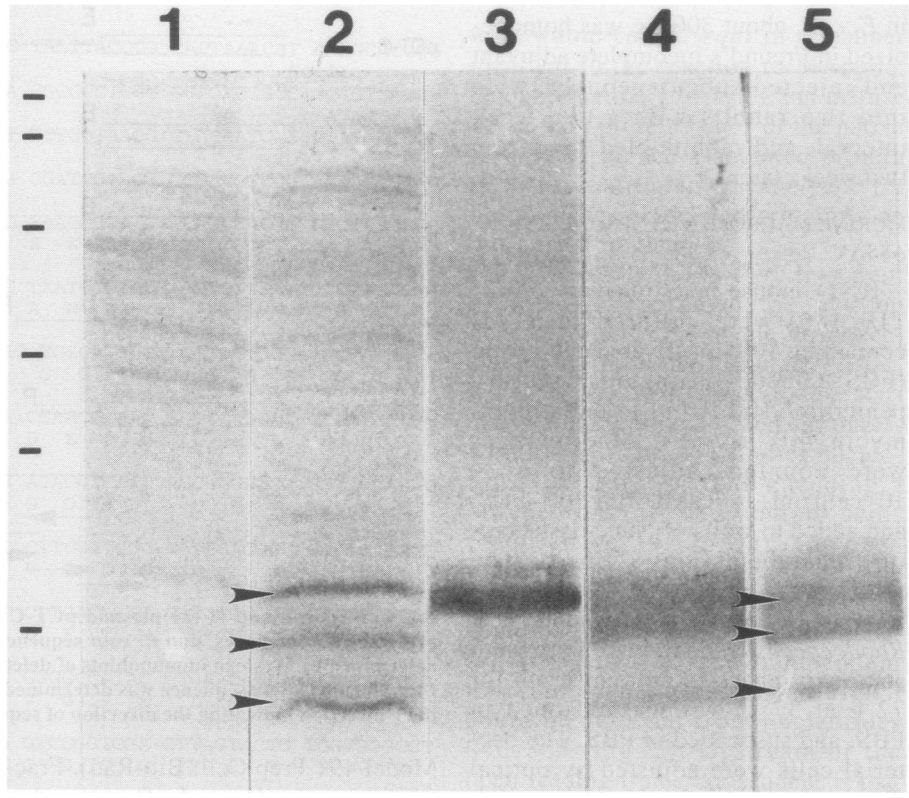

Fig. 1. Western immunoblots of recombinant and native virulence-associated protein of *R. equi*. Lane 1: *E. coli* XL1Blue (pBluescript) reacted with antiserum to virulence-associated protein of *R. equi*; Lane 2: *E. coli* XL1Blue(pCT-C7) reacted with the same antiserum showing three immunoreactive protein bands at 20-, 17.5-, and 15-kDa (arrows); Lane 3: TX-114 extracted, sonicated *E. coli* XL1Blue(pCT-C7) reacted with the same antiserum, showing the hydrophobicity of the heavier band; Lane 4: Whole cell preparation of *R. equi* reacted with antiserum against TX-114 extracted *E. coli* XL1Blue(pCT-C7) showing immunoreactivity with the three characteristic virulence-associated protein bands of *R. equi*; Lane 5: Whole cell preparation of *R. equi* reacted with MAb103, showing bands at 18-22-, 17.5, and 15-kDa (arrows). Molecular weight markers on left from top are: 106, 80, 49.5, 32.5, 27.5, 18.5 kDa.

in *E. coli*, about 300 μg was homogenized in Freund's incomplete adjuvant and injected subcutaneously twice into two rabbits with a two week interval, and rabbits bled for serum two weeks later.

MURINE MACROPHAGE OPSONIZATION ASSAY

IC-21 mouse macrophages (ATCC TIB 186) were cultured in RPMI containing 10% inactivated calf serum (FCS; Gibco, Burlington, Ontario), penicillin (100 IU/mL), and streptomycin (40 μg/mL). Macrophages were counted, adjusted to 1 × 10^6 cells/mL in RPMI with 10% FCS, and added to wells of Nunc tissue culture chamber slides (Gibco). The slides were incubated for 1.5 h at 37°C in 10% CO_2 to allow adherence. *Rhodococcus equi* strain 103 grown to stationary phase in nutrient broth for 72 h at 37°C was washed once in PBS, and suspended in PBS. The bacterial cells were adjusted by optical density to give a final concentration of 1 × 10^6/mL in hybridoma supernatant or appropriate dilution of purified Mab103. The antibody-bacterial suspensions were incubated for 15 min at 37°C before use to replace the media in the slide chambers. After incubation at 37°C in 10% CO_2 for 60 min, the slides were washed in three changes of PBS for 5 min each with stirring. Slides were stained with Wright's stain and the presence of bacteria in 300 macrophages (10 fields of 30 cells) determined.

MOUSE IMMUNIZATION AND CHALLENGE STUDIES

Female six to eight-week-old CD1 mice (Charles River, Montreal) were used. For passive immunization, mice were immunized intraperitoneally with 300 μg Mab103 one day before challenge. Immunized and unimmunized controls were injected intravenously with 5 × 10^5 *R. equi* strain 103. Five mice in each group were sacrificed on days 1, 4 and 7 postinfection and bacterial numbers in whole lung, liver and spleen enumerated. For active immunization, TX-114 extracted protein was precipitated with 10 vol of acetone overnight at −20°C and applied to a 15% PAGE resolving gel with a 4% stacking gel in SDS reducing buffer for continuous elution electrophoresis (Electrophor Model 491 Prep Cell; Bio-Rad). Fractions were collected after the dye front and concentrated (Centrifugal Ultrafree, 10,000 Da cutoff: Millipore Corp., Bedford, Massachusetts). These fractions were identified as the 17.5- and 18-22-kDa proteins by SDS-PAGE and immunoblotting with Mab103. Fractions which were identified as the 17.5-22-kDa proteins were combined, assayed for protein (BCA; Pierce) and adjusted to 400 μg/mL. The solution was mixed with an equal volume of Freund's incomplete adjuvant and 400 μL injected intraperitoneally into CD1 mice. The procedure was repeated 14 days later. Control mice received adjuvant mixed with an equal volume of Tris buffered saline, administered at the same schedule as vaccinates. All mice were challenged intravenously 17 days after the second immunization with *R. equi* strain 103 (10^5 organisms/mouse). Seven mice from each group were killed on days 2 and 3 after challenge and six mice on days 4 and 7, and bacteria enumerated in lung, liver and spleen. A second immunization study was done using the detergent phase, TX-114 extracted protein with overnight acetone precipitation as described. The protein concentration was adjusted to 0.5 mg/mL in 10 mM Tris (pH 7.0) and 100 μg mixed with an equal volume of Freund's incomplete adjuvant and injected intraperitoneally. A second immunization was given two weeks later. Control mice were immunized with Freund's incomplete adjuvant only. All mice were challenged intravenously 20 days later as described, euthanized four days after challenge, and bacteria in the liver and spleen enumerated.

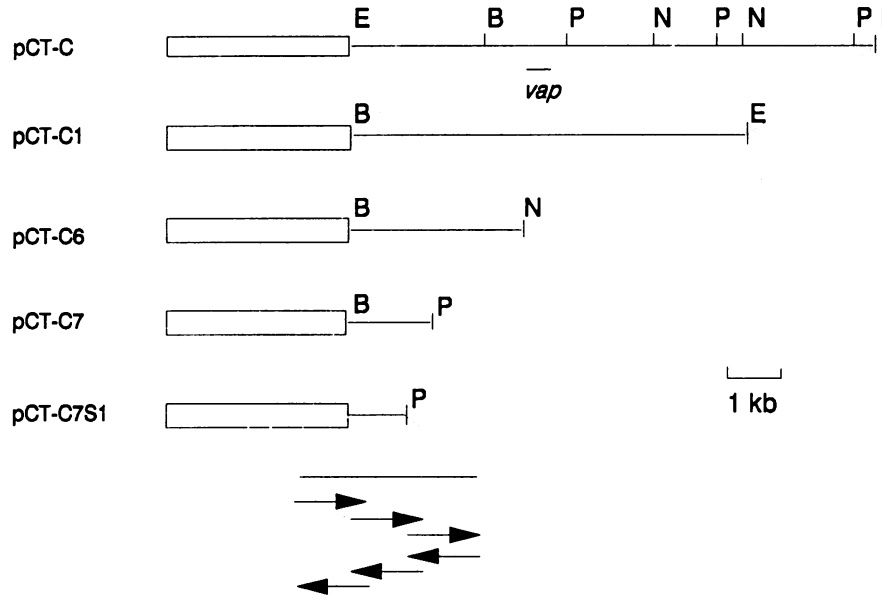

Fig. 2. Physical map of the plasmid pCT-C and deletion derivatives. Vector sequences are presented by the boxes, and *R. equi* sequences by the thin lines. The location of the ORF as determined by Western immunoblots of deletion derivatives and DNA sequencing is shown by *vap*. The nucleotide sequence was determined for the regions of the gene identified by arrows, their direction indicating the direction of sequencing. E, *Eco*RI; B, *Bgl*II; P, *Pst*I; N, *Not*I.

N-TERMINAL AMINO ACID SEQUENCING

To obtain N-terminal amino acid sequence from the native virulence–associated protein, the denatured protein was recovered by continuous elution electrophoresis as described above. The elution fraction containing the 17.5- and the 18-22-kDa proteins was separated on an SDS-PAGE gel, electroblotted to a polyvinylidene difluoride membrane (Bio-Rad) in 10 mM CAPS buffer and 10% methanol pH 11.0 at 14 V for 18 h, and excised for N-terminal amino acid sequencing using automated Edman degradation.

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

GenBank accession number is UO 5250.

PROTEIN SECONDARY STRUCTURE AND HYDROPHOBICITY ANALYSIS

Predicted protein secondary structure was determined using Protylze Predictor Version 3.0 software (16,17).

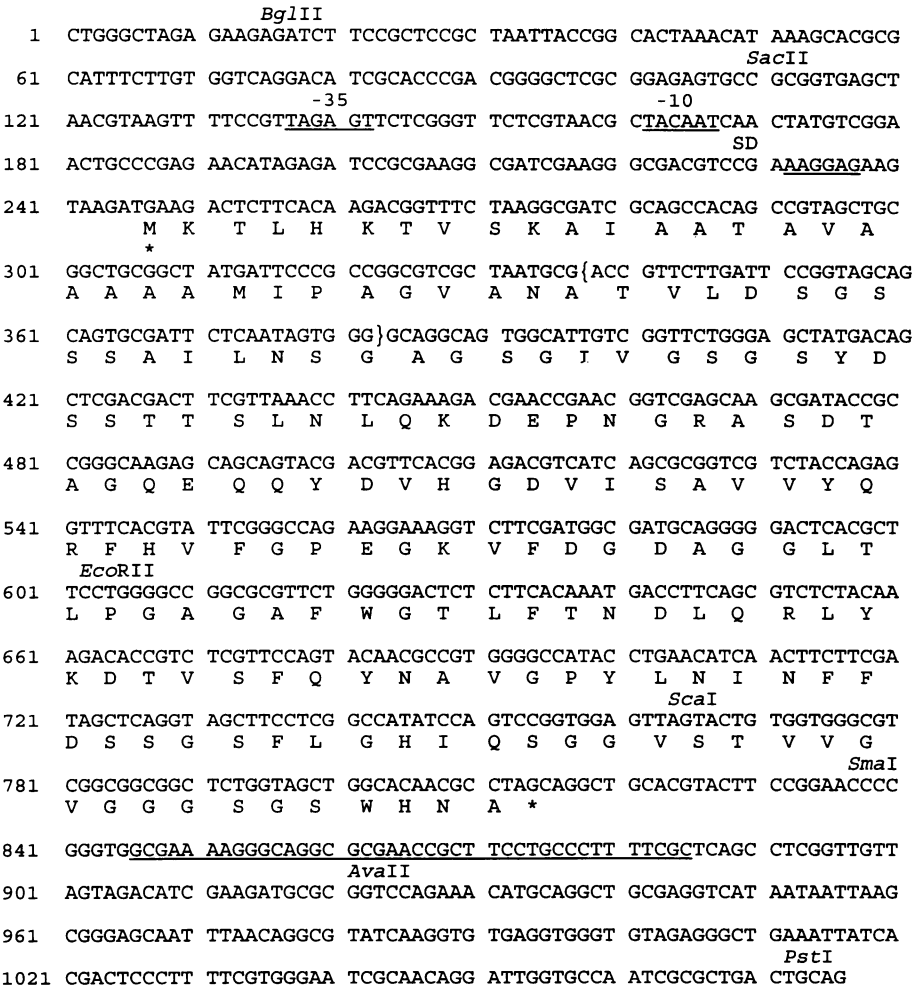

Fig. 3. Nucleotide sequence of the *R. equi* pOTS-derived DNA in pCT-C7S1 and the sequence of the *vap*A gene. The potential Shine-Dalgarno site of *vap*A is shown (SD), as is its start codon (ATG, *), its stop codon (TAG, *), and possible rho-independent termination termination signal (underlined). The N-terminal amino acid sequence of the mature protein determined by protein sequencing is also shown between the brackets. Positions of some of the restriction sites are indicated.

RESULTS

CLONING AND SEQUENCING OF THE *VAP*A GENE

A partial *Eco*RI library of pOTS, containing nine *Eco*RI fragments less than or equal to 10.5 kb, was constructed. Recombinants of each cloned fragment in *E. coli* were tested for production of virulence-associated proteins by Western immunoblotting using rabbit polyclonal antibody. A recombinant pCT-C, containing the 10.5 kb *Eco*RI fragment, was positive on Western immunoblot with and without IPTG induction. The monoclonal antibody Mab103 and foal convalescent sera failed to identify antigen in the recombinant. Three immunoreactive bands of approximately 15-, 17.5-, and 20-kDa were identified but the diffuse band characteristic of the *R. equi* 18-22-kDa virulence-associated protein was not present in the *E. coli* clone (Fig. 1). Southern blotting confirmed the plasmid origin of the fragment. Recombinant *E. coli* clones other than those carrying the 10.5 kb fragment were negative. The 10.5 kb fragment was mapped with restriction enzymes and subcloned in pBluescript. Several deletion derivatives were constructed and transformants containing the deletion derivative pCT-C1, pCT-C6, pCT-C7, and pCT-CS1 were found to produce the three immunoreactive proteins (Figs. 1 and 2). Antiserum prepared against TX-114 extracted *E. coli* XL1Blue(pCT-C7) reacted with whole cell *R. equi* in a manner identical to the reaction to MAb103, recognizing the 15-, 17.5- and diffuse 18-22-kDa protein bands of the native *R. equi* virulence-associated protein (Fig. 1), confirming that the gene for the virulence-associated protein is on this cloned fragment.

The 1.6 kb *Bam*H-*Pst*I fragment of pCT-C7 contained the 570 bp open reading frame of the virulence-associated protein, designated *vapA*, which begins with a methionine start codon at nucleotide 244 and terminates with a TAG stop codon after nucleotide 805 (Fig. 3), thus encoding a polypeptide of 189 amino acids (deduced Mr 19,175 Da). The open reading frame is preceded by the nucleotide sequences AAGGAG which could be transcribed into a ribosome-binding site. A rho-independent inverted repeat termination signal could be detected from nucleotides 846–885 (Fig. 3). Upstream of the open reading frame are sequences that resemble an *E. coli* and *Bacillus subtilis* promoter sequence (Fig. 3).

Protein folding prediction showed the first 31 amino acids to have an alpha-helical folding, corresponding to that expected of a leader peptide (16). Protein secondary structure analysis revealed the leader peptide to be the only significantly hydrophobic region (data not shown). N-terminal amino acid analysis of the combined 17.5- and 18-22-kDa proteins isolated from *R. equi* (TVLDSGSS?AILNSG) identified the start of the mature protein at nucleotide 337 (Fig. 3), 32 amino acids after the start codon and immediately following the predicted leader peptide (Fig. 3). N-terminal amino acid sequencing revealed also that the majority of the protein was blocked to the Edman reaction. The predicted mature polypeptide consists of 158 amino acids with a molecular mass of 16,246 Da. No significant nucleotide or amino acid sequence homologies were found between the *vapA* gene and other DNA and protein sequences contained in the GenBank, European Molecular Biology Laboratory (EMBL), National Biomedical Research Foundation-Protein Identification Resource (NBRF-PIR) and Swiss-Prot data bases. The overall G+C content of the *vapA* gene is 60%, with usage being 59.4%, 50%

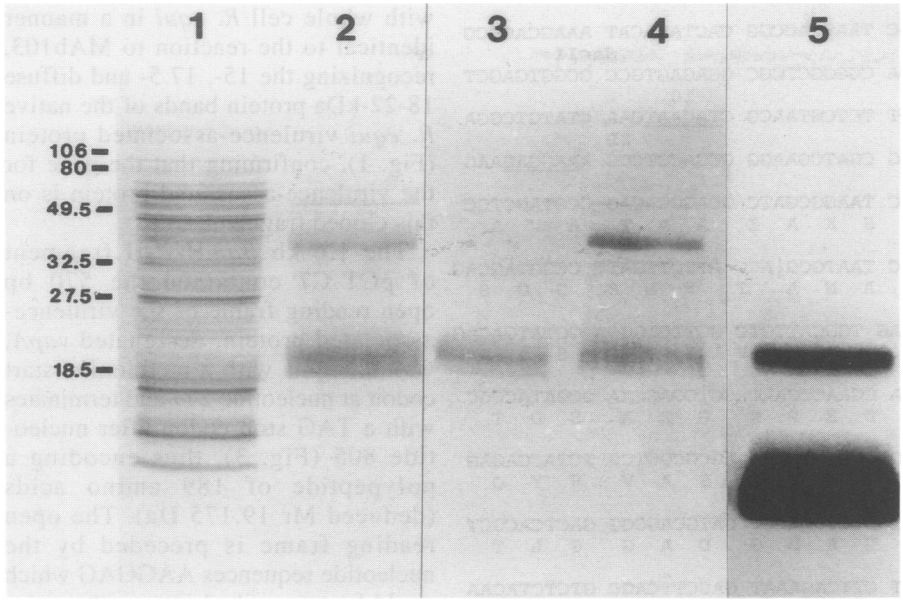

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins from *Rhodococcus equi* strain 103 (M30). Lane 1: Coomassie blue stained whole cell preparation showing weakly staining diffuse band at 17.5-22-kDa; Lane 2: Coomassie blue stained TX-114 detergent phase extract showing heavy protein bands at 17.5- and 18-22- (diffuse) and 44-kDa (diffuse); Lane 3: Immunoblot of whole cell preparation using murine monoclonal antibody identifying 17.5- and 18-22-kDa bands; Lane 4: Immunoblot of TX-114 detergent phase identifying relatedness of 17.5-, 18-22-, and 44-kDa bands; Lane 5: Autoradiograph of TX-114 detergent phase extract labelled with 20 μCi [^{3}H]-palmitic acid, showing fatty acid incorporation into the 17.5-22-kDa protein bands. Positions of prestained molecular weight markers are indicated on left.

and 64.2% at codon positions 1, 2 and 3, respectively.

TRITON X-114 EXTRACTION AND LIPID MODIFICATION OF VIRULENCE-ASSOCIATED PROTEINS

Whole cell preparations of *R. equi* separated on SDS-PAGE and stained with Coomassie blue show an often weakly staining diffuse protein band at approximately 18-22-kDa (Fig. 4), a narrow band which can sometimes be distinguished immediately below it at 17.5-kDa, and a band at about 15-kDa inconsistently. When whole cells of *R. equi* were extracted with TX-114, three major protein bands (44-, diffuse 18-22- and 17.5-kDa) were recovered in the detergent phase which reacted with the monoclonal antibody in Western immunoblot (Fig. 4). The 44-kDa band probably represents a TX-114 induced aggregation of the proteins or may be a protein-detergent micelle (18). No corresponding protein bands were observed in the aqueous phase (data not shown). The 15-kDa band was not observed in TX-114 preparations (Fig. 4) but was evident in some whole cell preparations (Fig. 1). In *E. coli* containing pCT-C7, but not in *E. coli* carrying pBluescript, protein bands of 20-kDa and sometimes of 17.6-kDa which reacted with rabbit polyclonal antiserum to the *R. equi* virulence-associated proteins were recovered in TX-114 (Fig. 1).

When the amount of [^{3}H]-palmitic acid was limited in *R. equi*, the 17.5- and 18-22-kDa proteins incorporated the radioactive fatty acid (Fig. 4). Incorporation into both the diffuse 18-22- and the 17.5-kDa protein bands could be distinguished on some gels (figure not shown).

MURINE MACROPHAGE OPSONIZATION ASSAY

Uptake of *R. equi* strain 103 by the mouse macrophage cell line was enhanced by opsonization with the monoclonal antibody (Table 1). The characteristic initial decline with subsequent rise compared to the controls observed was attributed to agglutination by the antibody.

MOUSE IMMUNIZATION AND CHALLENGE STUDIES

The monoclonal antibody administered intraperitoneally slightly enhanced liver clearance at one but not at four and seven days after intravenous challenge (immunized mice 3.69 ± 0.13 compared to 4.04 ± 0.13, five mice per group, $p < 0.003$, Duncan's multiple range test). There was no difference in splenic clearance. Clearance from liver, lung and spleen was enhanced in mice immunized with the TX-114 extracted 17.5- and 18-22-kDa proteins isolated by continuous elution electrophoresis (Table II). Antibody response in immunized mice determined by Western immunoblot against whole cell protein preparations was marked (titers >1:8000) and occurred predominantly to the virulence-associated proteins. An earlier study using a similar immunization protocol but with only 12 days from the second immunization to challenge showed statistically significantly enhanced clearance at day 3 but not at days 1, 5 and 7 following challenge. The second immunization study using TX-114 extracted, acetone precipitated protein and mice killed four days after intravenous challenge showed significant differences ($p < 0.0001$, Duncan's multiple range test) in numbers of $\log_{10}$ bacteria in the liver (5.17 ± 0.52, ten control mice; 3.54 ± 0.50, eight vaccinates) and spleen (5.07 ± 0.42, ten control mice; 3.71 ± 0.41, eight vaccinates) between vaccinated and control mice.

TABLE I. Uptake of *R. equi* by murine macrophage cell line (% cells) in presence of monoclonal antibody to *R. equi* VapA protein

Antibody dilution	% cells with *R. equi*		
	A[1]	B	C
No antibody	20[c]	15[ab]	25[c]
1:20	22[c]	9[c]	23[c]
1:40	—	11[bc]	24[c]
1:80	34[b]	17[a]	16[d]
1:320	54[a]	18[a]	36[b]
1:1280	34[b]	—	45[a]
1:10,240	—	—	30[bc]

[1]A, supernatant; B, C, two different studies with 500 μg/mL monoclonal antibody. Duncan's multiple range test; means with same superscript letter in same column no significant difference ($p > 0.05$)

DISCUSSION

The virulence-associated proteins of *R. equi* were first described by Takai and others, who also described

TABLE II. Bacterial clearance in CD1 mice immunized with SDS–PAGE eluted VapA protein and challenged intravenously with *R. equi*

Days post-infection	Liver		Spleen	
	Immunized	Control	Immunized	Control
2	3.21 ± 0.67[a]	4.17 ± 0.56	3.96 ± 0.31	4.25 ± 0.31
3	3.49 ± 0.68[a]	4.41 ± 0.30	4.01 ± 0.44[a]	4.95 ± 0.29
4	2.52 ± 1.36[a]	4.16 ± 0.19	3.38 ± 0.28[a]	4.44 ± 0.18
7	1.60 ± 1.33	2.39 ± 1.10	2.51 ± 0.35	3.03 ± 0.45

[a]$p < 0.05$, Duncan's multiple range test (5–7 mice per group)

their apparent surface localization and the intense humoral response in foals to these proteins (3,6). Our interest in them has focused on their nature and possible use as protective immunogens to prevent pneumonia in foals. The work described here establishes that these proteins are encoded by one plasmid-carried gene, which we call the *vap*A gene, and that lipid modification is responsible for the different forms observed. In addition, these proteins induce protective immunity in experimentally infected mice.

The 10.5 *Eco*RI fragment contained the gene for the plasmid-mediated virulence-associated protein (VapA protein), confirming the findings of others (19). Evidence that we cloned the gene for the *R. equi* virulence-associated proteins is: the presence of three protein bands in *E. coli* recombinants, similar in size to the *R. equi* virulence-associated proteins, which reacted with rabbit polyclonal antibodies prepared against the *R. equi* virulence-associated proteins (Fig. 1); the recognition by antiserum prepared against the recombinant protein of the virulence-associated protein complex in *R. equi* (Fig. 1); the identity of the N-terminal amino acid sequence of the *R. equi* protein with the sequence deduced from the cloned gene; the similar hydrophobic characterisitcs of proteins from *R. equi* or *E. coli* demonstrated by their partition into TX-114 (Figs. 1 and 4); the use of Southern blot to confirm the plasmid origin of the cloned gene.

The *Bam*HI-*Eco*RI and the *Not*I-*Eco*RI fragments cloned in opposite directions in pBluescript expressed the protein in the absence of IPTG induction. These findings are consistent with the notion that the promoter for *vapA* was contained in these fragments and that the promoter is functional in *E. coli*. Little is known about the genetics of rhodococci but this is an additional efficacy of a rhodococcal promoter in *E. coli* (20).

A DNA sequence analysis revealed an open reading frame corresponding to a polypeptide of molecular mass 19,175 Da. The first 31 amino acids had the characteristics of a signal sequence, starting with an N-terminal lysine followed by a alpha-helical hydrophobic region (21), and terminated in a possible alanine-X-alanine signal peptidase I cleavage site (21), which immediately preceded the N-terminal amino acid sequence of the mature protein identified by amino acid sequencing. The mass of the deduced mature protein of 16,246 Da corresponded closely to that determined by us from SDS-PAGE gels as 15-kDa, and is similar to that described by others (15-kDa) (6). Our finding that the *vap*A gene encodes a protein whose different sizes are due to lipid modifications explains why one monoclonal antibody recognizes all three protein bands on immunoblotting in *R. equi* (Figs. 1 and 4) and confirms the similar findings of others (22). Besides establishing the N-terminal sequence of the mature protein, N-terminal amino acid analysis of the combined 17.5- and 18-22-kDa proteins of *R. equi* also showed that the majority of the protein was blocked to the Edman degradation reaction, consistent with lipid modification. Failure of delipidation to remove radiolabel from the site of the VapA protein showed that lipid was covalently linked to the protein. The presence of three forms of the protein in both *R. equi* and the *E. coli* recombinant thus demonstrates that there are probably two lipid modifications of the VapA protein, and this was confirmed by incorporation of radiolabelled palmitic acid in both the 17.5- and 18-22-kDa proteins in *R. equi*. The heavier band and sometimes the 17.6-kDa band in the *E. coli* recombinant also extracted into TX-114, confirming their hydrophobic nature and suggesting their lipid modification also. Although the three immunoreactive proteins produced by recombinants in *E. coli* corresponded in size to the three forms of the protein in *R. equi*, the failure of the 18-22-kDa form to take its characteristically diffuse form in *E. coli* could be due to difference in lipid modification of the VapA protein between *E. coli* and *R. equi*. Antibody to the recombinant protein however recognized the diffuse band of *R. equi*. Failure of Mab103 to recognize the *E. coli* recombinant protein points to another difference in the protein. Diffuseness in SDS-PAGE gels due to lipid modifications of proteins have been described in *Mycobacterium tuberculosis* (23) and in *Treponema pallidum* (24). The heterogenous behavior of the diffuse 18-22-kDa protein on SDS-PAGE may result from either variability in lipid modifications with resulting differences in molecular mass or from the binding capacity of this lipid-modified form for SDS (23).

The lipid modifications determine the hydrophobic nature of the VapA protein since the unmodified mature 15-kDa protein is predominantly hydrophilic and does not extract into TX-114 in *R. equi*. The ease of TX-114 extraction of the 17.5- and 18-22-kDa proteins from whole bacteria supports the suggestion of Takai *et al* (3) that they are on the surface of the organism. The 15-kDa form of the protein may be largely intracellular. The site of lipid modifications is unclear but likely occurs at a site close to the N-terminal region of the mature protein. The protein lacks the consensus cleavage site of signal peptidase II and is not a typical lipoprotein (25). Besides lipoprotein modification, four other types of fatty acylation of proteins are recognized: palmitoylation, isoprenylation, myristoylation and glypiation (26). The first two involve cysteine residues, which are absent in the VapA protein. The absence of a glycine at the N-terminal end of the mature protein and the low molecular weight of myristic acid suggest that the VapA protein is not myristoylated. Glypiation, the attachment of a phos-

phatidylinositol-containing glycolipid, is a hydrophobic modification observed in eukaryotic proteins (26). The acylation of the VapA protein does not equate to any of these characterized examples.

The opsonization by *R. equi* with Mab103 in the mouse macrophage cell line was further evidence that the protein is expressed on the surface of the organism (3). Opsonization in the macrophage cell line justified the use of mice to further examine the immunogenic potential of the protein in mice. The temporarily and slightly enhanced clearance following passive immunization suggested that antibody plays a role in protecting immunocompetent mice from experimental infection but that this role is limited. Active immunization with denatured protein recovered by continuous elution from SDS-PAGE resulted in marked antibody response and in significant enhancement of tissue clearance. The increased effect of active immunization with time, the relatively poor clearance shown by Mab103, and the immunogenic effect of what is likely SDS-denatured antigen suggests that cell-mediated immune mechanisms produced by the VapA protein may be largely responsible for enhanced clearance from mice. TX-114, acetone precipitated VapA was also shown to enhance clearance of intravenously injected bacteria but whether this effect was the result of antibody or cellular immune mechanisms, or both, was not determined. Earlier studies of killing of *R. equi* by equine macrophages suggested that immunity was the result of both antibody and cellular immune mechanisms (8). More recently, pulmonary clearance studies in $CD4^+$ T-lymphocyte-deficient transgenic mice have established the importance of $CD4^+$ rather than $CD8^+$ lymphocyte in immunity to *R. equi* infection in mice (27). It will be important to determine the basis of immunity to the *R. equi* VapA proteins in both mice and foals.

Lipid modifications may be important in attaching the protein to the surface of *R. equi* and in ensuring survival within foal macrophages by resisting intracellular killing or in another manner. Lipid modification might be one explanation for the apparently intense immunogenicity of the virulence-associated proteins (6), since high immunogenicity often appears to be a feature of lipoproteins (23,24). There is a rapidly increasing list of lipid-modified proteins in prokaryotic organisms (24,24,28), including intracellular pathogens (29). The role of the lipid modified VapA protein in immunomodulation by *R. equi* will also be of interest since, for example, synthetic lipopeptides can prime cytotoxic T-lymphocytes *in vivo* (30) and lipid modified proteins have B-cell mitogenic and cytokine-stimulatory properties (28,31,32). Potential B-cell and T-cell epitopes can be determined from the amino acid composition of proteins but such analyses may not be useful in lipid modified proteins.

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