Virulence Plasmid of *Rhodococcus equi* Contains Inducible Gene Family Encoding Secreted Proteins

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*Rhodococcus equi* causes severe pyogranulomatous pneumonia in foals. This facultative intracellular pathogen produces similar lesions in immunocompromised humans, particularly in AIDS patients. Virulent strains of *R. equi* bear a large plasmid that is required for intracellular survival within macrophages and for virulence in foals and mice. Only two plasmid-encoded proteins have been described previously; a 15- to 17-kDa surface protein designated virulence-associated protein A (VapA) and an antigenically related 20-kDa protein (herein designated VapB). These two proteins are not expressed by the same *R. equi* isolate. We describe here the substantial similarity between VapA and VapB. Moreover, we identify three additional genes carried on the virulence plasmid, vapC, -D, and -E, that are tandemly arranged downstream of vapA. These new genes are members of a gene family and encode proteins that are approximately 50% homologous to VapA, VapB, and each other. vapC, -D, and -E are found only in *R. equi* strains that express VapA and are highly conserved in VapA-positive isolates from both horses and humans. VapC, -D, and -E are secreted proteins coordinately regulated by temperature with VapA; the proteins are expressed when *R. equi* is cultured at 37°C but not at 30°C, a finding that is compatible with a role in virulence. As secreted proteins, VapC, -D, and -E may represent targets for the prevention of rhodococcal pneumonia. An immunologic study using VapA-specific antibodies and recombinant Vap proteins revealed no evidence of cross-reactivity despite extensive sequence similarity over the carboxy terminus of all four proteins.

The nocardioform actinomycete, *Rhodococcus equi*, is an important pulmonary pathogen in foals and in human patients with AIDS. This gram-positive bacterium is a facultative intracellular pathogen that persists and multiplies within macrophages. Intracellular survival is considered to be necessary for the development of disease, which is characterized by severe and sometimes fatal pneumonia in both humans and foals (5, 17).

Clinical isolates of *R. equi* contain a large plasmid ranging in size from 80 to 90 kb in equine or 30 to 100 kb in human AIDS isolates (25, 30). The large plasmid is essential for virulence in mice and foals and for intracellular survival in murine and equine macrophages (9). Plasmid curing by repeated passage during in vitro culture at 37°C eliminates the virulent phenotype (9, 27). These findings indicate that the plasmid encodes proteins that are necessary for virulence.

Only two proteins encoded by *R. equi* virulence plasmids have been described to date. Equine isolates express a 15- to 17-kDa protein (VapA) that appears as a characteristic broad band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (20, 26, 29). Human *R. equi* isolates from AIDS patients generally express either VapA or an antigenically related 20-kDa protein (25). The 20-kDa protein is expressed by isolates from pigs as well as humans. No *R. equi* isolate has been shown to express both VapA and the 20-kDa protein. However, antigenic cross-reactivity between VapA and the 20-kDa protein has been demonstrated using immunoblots and serum from infected foals (25). The similarity of VapA and the 20-kDa plasmid-encoded proteins raises the possibility that the two proteins have analogous functions in different strains of *R. equi*.

VapA and the 20-kDa protein are located on the bacterial surface, and expression is reported to be thermally and pH regulated (22, 24). Specifically, VapA and the 20-kDa protein can be detected when *R. equi* is cultured at 38°C but not when cultured at 30°C, and expression at 38°C is observed only if the pH of the medium is decreased below 8 (22). These characteristics suggest that expression is upregulated in the mammalian host and intracellularly where VapA or the 20-kDa protein would play a role in the pathogenesis of rhodococcal pneumonia. In support of this premise, expression of VapA can be detected within macrophages in the pulmonary lesions of affected foals (13).

Although VapA expression correlates with increased virulence in mice and appears to be required for pathogenicity in horses, the expression of VapA alone is not sufficient for virulence. Transfer of *vapA* to an avirulent, plasmid-negative strain of *R. equi* using a shuttle vector resulted in VapA expression but did not confer virulence. This replacement strain did not cause pneumonia in foals and was unable to survive within macrophages (7). These results strongly suggest that the
virulence plasmid encodes additional genes required for pathogenicity.

The work described in this report identifies a multigene family encoded by the R. equi virulence plasmid. The gene family includes vapA, the gene encoding the 20-kDa protein (herein designated vapB), and three newly identified vap genes. The new genes encode proteins that have a high degree of similarity to VapA and VapB. Further characterization demonstrates that these molecules, VapC, -D, and -E, are secreted proteins and coordinately regulated with VapA.

MATERIALS AND METHODS

Bacteria. All R. equi strains were stored at −80°C in glycerol. Prior to use, bacteria were grown for 48 h in brain heart infusion broth (BHIB). BHIB with 0.1% yeast extract or, for the temperature regulation studies, tryptic soy broth with 0.1% yeast extract.

Sequencing and analysis. Virulence plasmids from R. equi strains 33701 and 103 (both fowl isolates) were isolated as previously described and digested with EcoRI (29). The 10.1-kb and adjacent 4.9-kb EcoRI fragments were each cloned into the Escherichia coli vector pBluescript (Stratagene, La Jolla, Calif.). Plasmid was isolated from E. coli clones using a Qiagen Plasmid Maxi Kit (Valencia, Calif.) according to manufacturer’s instructions. Double-stranded sequencing was carried out using sequentially derived primers with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, Calif.) and read with an ABI Prism 373 Genetic Analyzer. Identification of homologous sequences was performed using computation by the National Center for Biotechnology Information on the BLAST network service. Direct sequence comparisons utilized the University of Wisconsin Genetics Computer Group (GCG) software (Bestfit, Gap, Pileup, Findpatterns, and Pedi- tisstructure). Additional computer analyses were carried out using Motifs (from Proust-protein motif prediction), PSORT, and SOUSI (prediction of a soluble secreted protein) (8; K. Nakai and M. Kanehisa, PSORT [http://psort.nibb.ac.jp/index.html]).

RT-PCR. Total RNA was isolated from R. equi strain 33701 grown for 48 h in BHIB at 37°C at pH 7 using the Qiagen RNeasy Mini Kit. Modifications of the manufacturer’s instructions were necessary to obtain adequate amounts of RNA. Bacteria from 5 ml of culture broth were collected by centrifugation and the pellet was resuspended in 20 ml of lysosome (Sigma, St. Louis, Mo.) per ml of 10 mM Tris–1 mM EDTA and incubated for 3 h at room temperature. Then, 600 μl of RLT solution was added, and the lysate was centrifuged through a QiaShredder (Qiagen) to aid cell lysis. Samples were applied to the RNeasy column according to the manufacturer’s directions. RNA was reverse-transcribed to DNA using downstream priming. Oligonucleotide primer pairs used for the PCR were designed to amplify a gene fragment that spanned unique and conserved regions for each open reading frame (ORF). The sequences for the primers were as follows: vapA, forward (5′-TCCACCGGGCGTCAGAAGCGAATCGGAGC) and reverse (5′-ATGACTGGATCCACGCGGTAGGATGTG GCC) (the PCR fragment starts at nucleotide 12 of the coding sequence and ends at nucleotide 529); vapC, forward (5′-CCGTGTGCGGCAAGCTTCTA) and reverse (5′-AAGAATGGGACGTACTATG) (nucleotides 36 to 450); vapD, forward (5′-GGCGTTTATTCACTTTCTTG) and reverse (5′-AGTCGT TGCCCTTCTTGGTC) (nucleotides 23 to 425); and vapE, forward (5′-TGGG TTCTAATCGACCCGGTAC) and reverse (5′-TGCCACCAACTAGTATA) (nucleotides 84 to 591). The reverse transcription-PCR (RT-PCR) reactions were carried out on a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. The sequences of the RT-PCR products were sequenced to confirm the identity.

PCR to characterize conservation of vap genes. Virulence plasmids from R. equi isolates were isolated as previously described (29). Oligonucleotide primer pairs were those used for the RT-PCR. One-half microgram of plasmid DNA was used in each reaction.

Cloning of ORFs. Primer pairs were used to amplify each ORF, including the start and stop codons from R. equi 33701. Each PCR product was ligated into pBluescript (Stratagene), and recombinant plasmids were transformed into E. coli XL1-Blue (Stratagene). Clones were sequenced to confirm identity. Expression of recombinant VapC (rVapC), rVapD, and rVapE was demonstrated by immunoblotting (see below).

Generation of protein-specific antiserum. Unique regions of the predicted amino acid sequences of vapC, -D, or -E were identified, and corresponding peptides were synthesized. The peptide sequences were VapC (AWGGAQSA ADKEEGEGVTG; amino acids 36 to 54 of the predicted sequence), VapD (DAALLSGKAAIPEDK; amino acids 40 to 55), and VapE (RMAVHDDST HTREFKEDDS; amino acids 76 to 94). The peptides were coupled to keyhole limpet hemocyanin according to the manufacturer’s instructions (Pierce, Rockford, Ill.). Rabbits were immunized with 100 μg of coupled protein using complete Freund’s adjutant (first immunization) and boosted with coupled peptide and incomplete Freund’s adjutant (Sigma, St. Louis, Mo.). Immunoblotting of the recombinant bacterial antigen and plasmid-cured R. equi strains was used to determine if the antiserum was specific. Rabbit antiserum was used at a 1:250 dilution.

Antigen preparation. E. coli clones grown in Luria broth containing 50 μg of ampicillin per ml overnight, and IPTG (isopropyl-β-D-thiogalactopyranoside) at 1 mM was added during the last 2 h of incubation. Bacteria were pelleted, washed twice in cold phosphate-buffered saline (PBS), and frozen overnight at −20°C. Pellets were thawed and 50 mM Tris, 5 mM EDTA, and 1% NP-40 was added to an optical density of 600 nm of 1.7 to 2.0. Cell lysates were sonicated three times for 10 s with 30-s intervals between sonications. Lysates were centrifuged at 20,000 × g for 30 min, and the supernatant was collected for cellular antigen. R. equi cellular antigen was prepared as for E. coli except that R. equi was grown for 48 h (stationary phase) in BHIB-0.1% yeast or TSB-0.1% yeast, was not treated with IPTG, and was frozen overnight at −20°C prior to centrifugation and collection of cellular antigen. To collect secreted antigen, R. equi was grown in a culture volume of 300 ml for 48 h and then pelleted by centrifugation, and the culture supernatant was collected. The culture supernatants were concentrated by dialysis with sucrose using Spectra/Por 12,000 to 14,000 molecular weight cutoff (MWCO) dialysis tubing (Spectrum, Houston, Tex.). Subsequently, the concentrated supernatants were dialyzed against PBS using a Slide-A-Lyzer (10,000 MWCO; Pierce) and then concentrated using a 10,000 MWCO column (Centriconp 10; Millipore, Bedford, Mass.) according to the manufacturer’s instructions. Protein concentrations of all antigen preparations were quantitated using the micro-BCA Protein assay with an albumin standard according to the manufacturer’s instructions (Pierce).

Antibodies and immune serum. To produce polyclonal anti-VapA serum, mice were immunized with gel-purified VapA using complete Freund’s adjutant (initial immunization) and incomplete Freund’s adjutant (booster immunizations). Briefly, VapA was isolated from other R. equi proteins using electrophoresis in a 4 to 20% Tris-glycine polyacrylamide gel. Proteins were stained with Coomassie blue, and the characteristic band corresponding to VapA was cut from the gel. Protein in the excised gel fragment was electroeluted and dialyzed against PBS. The postimmunization serum was characterized by immunoblot where it recognized a 15- to 17-kDa band in R. equi 33701 and E. coli expressing recombinant VapA. This serum did not detect any protein in immunoblots of plasmid-cured R. equi 33701. Monoclonal antibodies to VapA were Mab103 and 10G5 (23, 29).

Infected cell lysate was collected from four 1.5- to 4-month-old foals naturally infected with VapA-positive R. equi strains. Infected mouse serum was generated by infecting mice with 2 × 107 R. equi strain 33701 intraperitoneally. Serum was collected 3 to 6 weeks postinfection and pooled.

Immunoblotting. Whole bacterial antigen or concentrated culture supernatants were boiled with 50 mM Tris-Cl, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol; separated by SDS-PAGE using a 4 to 20% Tris-glycine gel; and transferred electrophoretically to a nitrocellulose membrane. Nitrocellulose was incubated with rabbit serum, infected foal serum, polyclonal mouse serum, or anti-VapA monoclonal antibodies (23, 29). Horsedrash peroxidase-conjugated goat anti-horse or goat anti-rabbit immunoglobulin G (IgG; Kirkegaard & Perry, Gaithersburg, Md.) were used as secondary antibodies at a 1:5,000 dilution. Horsedrash peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry) was used at 1:7,500 dilution. Bound primary and secondary antibodies were detected using an enhanced chemiluminescent substrate (Amersham Life Sciences, Arlington Heights, Ill.).

RESULTS

Homology of VapA and the 20-kDa protein (VapB). An examination of the entire nucleic acid sequences of vapA and the gene encoding the 20-kDa protein expressed by human and porcine isolates of R. equi shows 83.6% identity. Using the University of Wisconsin GCG program Bestfit, the amino acid sequence of these proteins was found to be 79% similar with the greatest similarity, 94%, in their carboxy termini (Table 1).
The 20-kDa protein has been designated as VapB based on these findings.

Identification of additional ORFs encoding vap genes. vapA is located on a 10.1-kb EcoRI fragment of the virulence plasmid of R. equi strains 33701 and 103 (foal isolates). The 10.1-kb and adjacent 4.9-kb EcoRI fragments of the virulence plasmid from R. equi strains 33701 and 103 were cloned and sequenced. Five regions with similarity to vapA were identified by amino acid sequence analysis using BLAST (Fig. 1) (1). Four of these regions represent ORFs based on the presence of predicted ribosome-binding sites (RBS), promoters, start, and stop codons. These ORFs have been designated vapC, vapD, vapE, and vapF. We focus here on vapC, -D, and -E. The GenBank accession numbers for vapC, vapD, and vapE are AF118813, AF118814, and AF118815, respectively. Using the sequence for previously identified genes in R. equi (AAGGAG for VapA, AGGAGG for a rifampin resistance protein) (3, 29), an RBS was found for each ORF that was either identical or had up to two mismatches with the search sequences. The predicted RBS are as follows: vapC, CTAGGA; vapD, GAAGAG; and vapE, AAGGAG. The expression of vapC, -D, and -E was demonstrated initially by using RT-PCR from bacteria cultured at 37°C in BHIB (Fig. 2).

Sequence analysis. The predicted sizes of the ORF gene products are 19.1 (VapC), 18.0 (VapD), and 22.7 (VapE) kDa, which are similar to the sizes of VapA and -B. All three ORFs are located downstream of vapA and are transcribed in the same direction as vapA (Fig. 1). The overall amino acid similarity is approximately 50%, but the similarity to VapA and B in the carboxy-terminal regions of all ORFs approaches 70 to 80% (Table 1, Fig. 3). The identity of the ORF nucleic acid sequences to vapA is approximately 50%. The region with similarity to vapA downstream of vapE, i.e., vapF, is also an ORF; however, the area of homology is located in the central region of the ORF rather than the carboxy terminus. The fifth region, located just downstream of vapA, appears to be a pseudogene based on the lack of an ATG start codon. However, it is possible that an alternative start codon could be used, in which case the region would constitute an ORF.

Each VapC, -D, and -E amino acid sequence includes a probable signal sequence. Although the amino termini of the predicted proteins share little similarity, they all have the characteristics of a signal sequence: positively charged amino acid(s) followed by a hydrophobic region and several potential cleavage sites for signal peptidase I (19). Furthermore, computer analysis using PSORT supports the identification of a signal sequence that is cleaved (Nakai and Kanehisa, PSORT). These findings suggest that, like VapA and -B, VapC, -D, and -E are transported to the bacterial surface. In addition, SOSUI software, used to predict secondary structures of membrane proteins, identified all five Vap proteins as soluble and not as integral membrane proteins (8). No significant homology of any of the Vap proteins with any other bacterial proteins was detected using BLAST. Using a Swiss Pro analysis of protein motifs, an ATP-GTP binding site motif A (P-loop) was detected in VapD and VapE. The consensus sequence for this binding site is: (A, G) × 4 GK (S, T). The sequence in VapD runs from amino acid 84 to 91 and is AEDKKGKT; for VapE it is GRGGPGKT (amino acids 126 to 133).

Gene family conservation. The presence of the gene family in R. equi isolates was examined in two ways: (i) detection of vapC, -D, and -E in multiple R. equi isolates by PCR and (ii) sequencing the region of the virulence plasmid containing the vap genes from two VapA-positive equine isolates (strains 33701 and 103). PCR was performed on the virulence plasmids purified from 11 equine R. equi isolates, 19 human R. equi isolates (11 isolates from AIDS patients and 8 isolates from non-AIDS patients), and one porcine strain.

All but one of the equine isolates contain vapA and all vapA-positive isolates contained vapC, -D, and -E. Five human R. equi isolates were vapA positive: two from AIDS patients and three from non-AIDS patients. As with the horse isolates, human isolates that carried vapA were also positive for vapC, -D, and -E. One human isolate (isolate 201) was positive for vapF only. Sequencing of the vapC PCR product from this isolate showed that it is 86% identical on a nucleotide basis to the vapC sequence described in horse isolates. Most differ-

<table>
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<tr>
<th>Vap protein</th>
<th>% Identity</th>
<th>% Similarity</th>
<th>% Similarity, Carboxy terminus* (amino acid positions)</th>
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<tr>
<td>VapB</td>
<td>76</td>
<td>79</td>
<td>94 (82–187)</td>
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<tr>
<td>VapC</td>
<td>39</td>
<td>46</td>
<td>97 (85–196)</td>
</tr>
<tr>
<td>VapD</td>
<td>43</td>
<td>50</td>
<td>76 (53–164)</td>
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<tr>
<td>VapE</td>
<td>41</td>
<td>51</td>
<td>79 (95–206)</td>
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*Similarity determined by the GCG program GAP using default settings.

FIG. 1. Diagram showing regions of similarity with vapA on the R. equi virulence plasmid. Open boxes are ORFs with homology to the carboxy terminus of VapA. The stippled box indicates ORF with homology to the central region of VapA. The cross-hatched box designates a region of similarity that is a pseudogene. Arrows indicate the direction of transcription. The length of each ORF is given in base pairs under each box. The numbers above the boxes are the lengths of intergenic regions between ORFs. The predicted sizes of the ORF encoded proteins are given in kilodaltons. EcoRI indicates restriction sites on the virulence plasmid. The region containing vapA, -C, -D, -E, and -F is approximately 7.8 kb.

TABLE 1. Predicted amino acid similaritya of VapB, -C, -D, and -E with VapAa

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<tr>
<th>Vap protein</th>
<th>% Identity</th>
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<tr>
<td>VapB</td>
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<td>VapE</td>
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a Similarity determined by the GCG program GAP using default settings.
ences were single base substitutions scattered throughout the sequence, although they were more frequent near the 5' end.

Comparison of the nucleic acid sequences of the three ORFs in R. equi strains 33701 and 103 showed only one base difference. It was in the wobble position for amino acid 47 in vapC of strain 103 and did not result in an amino acid change. The pseudogene and vapF were found in both strains; the nucleic acid and the predicted amino acid sequences were identical.

Identification of VapC, -D, and -E as secreted proteins. Protein-specific antiserum was developed in rabbits to demonstrate the expression of VapC, -D, and -E. Each serum recognized only the appropriate recombinant protein that matched the predicted molecular weight of VapC, -D, or -E. In addition, rabbit serum did not recognize any proteins of the proper size from plasmid-cured R. equi 33701 in the cellular lysate or concentrated supernatant, thus confirming that they identify proteins unique to plasmid-positive strains. Attempts to detect VapC, -D, or -E in R. equi cellular antigen preparations from culture in BHIB plus 0.1% yeast by immunoblot analysis were unsuccessful. Since the predicted amino acid sequences of

FIG. 2. RT-PCR demonstrating transcription of vap genes with homology to VapA. RNA from R. equi 33701 incubated at 37°C at pH 7 in BHIB was amplified by RT-PCR using ORF-specific primers for vapC, vapD, and vapE. +RT, reverse transcriptase present in the RT-PCR reaction; -RT, no reverse transcriptase added; PC, RT-PCR reaction carried out on RNA collected from plasmid-cured R. equi 33701 incubated under the same conditions; -RNA, no RNA added to the RT-PCR reaction. The expected sizes of each RT-PCR product were as follows: vapA, 517 bp; vapC, 414 bp; vapD, 402 bp; vapE, 517 bp (100-bp ladder).

FIG. 3. Comparison of the predicted amino acid sequences of VapA, VapB, VapC, VapD, and VapE using Pileup and Prettybox from the GCG sequence analysis package (default parameters). Black regions indicate identical amino acids, gray regions represent similar amino acids, and white boxes indicate nonconserved residues.
these genes indicated a signal sequence, secretion of the proteins was a possible explanation. Therefore, culture supernatants were examined for protein expression. Supernatants from *R. equi* cultures grown for 48 h at 37°C in BHI supplemented with 0.1% yeast extract were collected and concentrated. Immunoblot analysis using protein-specific rabbit antisera demonstrated that VapC, -D, and -E can be found in culture supernatants, indicating that all three proteins are secreted into the culture media (Fig. 4). A wide band that extends to the edge of the immunoblot can be seen in the anti-VapC lane (lane 2). This band is of lower molecular weight than VapC, is seen only in culture supernatant from the plasmid-cured *R. equi* strain, and most likely represents a nonspecific background. The sizes calculated based on migration in SDS-PAGE are 15.5 kDa for VapC and 15.9 kDa for VapD. VapE has a characteristic appearance consisting of two to three bands of between 14.8 and 18.8 kDa. These sizes are smaller than those predicted from the gene sequence (VapC, 19.1 kDa; VapD, 18.0 kDa; and VapE, 22.3 kDa), possibly due to cleavage of the signal peptide to form the mature secreted protein. VapA was not detected in the culture supernatants but was identified in cellular antigen preparations by immunoblot, indicating that it was expressed but not secreted (data not shown).

**Temperature regulation of VapA, -C, -D, and -E expression.** To determine if VapC, -D, and -E are coordinately regulated with VapA, the expression of these secreted proteins in culture supernatants (VapC, -D, and -E) or cell lysates (VapA) from *R. equi* cultured at 30 and 37°C were evaluated using protein-specific rabbit sera or anti-VapA monoclonal antibody. The effect of temperature on expression of VapC, -D, and -E paralleled the expression of VapA. All Vap proteins were detected at 37°C but minimally or not at all at 30°C (Fig. 5).

**Antigenic cross-reactivity of Vap proteins.** The characteristic appearance of VapA as a broad band at between 15 and 17 kDa in both immunoblots and protein stains is postulated to be a result of lipid modification and has been used as an indirect marker for strains that carry the virulence plasmid. However, VapC, -D, and -E are of similar predicted sizes and migrate to a similar location as VapA in SDS-PAGE gels (20, 29). It is possible that the 15- to 17-kDa VapA band seen in immunoblots is actually composed of multiple Vap proteins and that these proteins are recognized through shared B-cell epitopes in their highly conserved carboxy termini.

In order to examine for the cross-reaction of antibodies with proteins encoded by the gene family, we used recombinant protein. The expression of each recombinant Vap protein in *E. coli* was confirmed by immunoblotting using the protein-specific rabbit antiserum described previously. These clones were used in immunoblots reacted with serum from horses or mice infected with *R. equi*, two anti-VapA monoclonal antibodies, or polyclonal anti-VapA serum. Serum from two infected foals recognized rVapA and VapD (Fig. 6A), whereas serum from another infected foal recognized native VapA and rVapE (Fig. 6B). This observation is consistent with the in vivo expression and immune recognition of at least two of the new Vap proteins. The anti-VapA monoclonal antibodies recognized only rVapA and no other recombinant Vap proteins (Fig. 6C). Likewise, polyclonal murine anti-VapA serum failed to react with any recombinant protein except VapA, as did sera from mice infected with *R. equi* by intraperitoneal injection (data not shown).

**DISCUSSION**

The large plasmid found in virulent strains of *R. equi* is required for intracellular survival and persistent infection in foals and mice. Only two plasmid-encoded proteins, VapA and VapB, have been identified previously. Clinical isolates express one or the other but not both proteins and presumably carry the gene for only one protein (25). VapA and VapB have been considered to be structurally related based on their antigenic cross-reactivity using infected foal serum (25). Each gene has been sequenced; however, direct comparison of the two genes and encoded proteins has not been reported (20, 25, 29). In this study, we demonstrate that VapA and VapB are encoded by closely related genes and share significant amino acid similarity. We also identify three additional plasmid-encoded genes that are members of the *vapA* gene family. These new genes, designated *vapC*, *vapD*, and *vapE*, are broadly conserved in all isolates that express VapA.
The distribution and conservation of the *vapA* gene family was determined from PCR analysis of multiple *R. equi* strains and direct sequence comparison of two virulent strains. The gene family is found in all *VapA*-positive *R. equi* strains examined. The gene family is also conserved across *R. equi* serotypes; isolates 33701 and 103 belong to two different serotypes, serotypes 1 and 6, respectively, yet both contain the *vap* genes without amino acid changes. These data are compatible with previous restriction enzyme analysis and cross-hybridization experiments showing that plasmids from *VapA*-positive strains, regardless of source, are related (15, 25, 28). Moreover, these data support the contention that the virulence plasmids of *R. equi* encode multiple proteins, as well as *VapA*, that may play a role in virulence (7).

In addition to their conservation, the organization of the *vapA*, -C, -D, and -E genes is noteworthy. These genes are restricted to a small region of the virulence plasmid, tandemly arranged, and all transcribed in the same direction. This is an arrangement consistent with a regulon or operon. Teleologically, the configuration would be advantageous for the control of multiple genes that are coordinately regulated and has been described in other pathogens such as *Salmonella* spp. and *Listeria* spp. (14, 21, 64). Also, the arrangement of the *Vap* genes may reflect the generation of closely related genes via gene duplication (6).

Since *vapC*, -D, and -E are closely related to *vapA* by homology and location, we investigated whether they were regulated like *vapA*. Protein expression of *VapC*, -D, and -E parallels that of *VapA*. All are expressed at 37°C but not at 30°C, a result indicative of coordinate regulation. Since 37°C is similar to temperatures encountered in its mammalian host, expression would occur as the bacterium enters the lung, consistent with a role for *VapC*, -D, and -E in virulence. Frequently, more than one virulence protein is required for a pathogenic event, such as invasion or alteration of the phagolysosome, and multiple proteins must be activated simultaneously. Coordinate regulation of virulence proteins has been observed in a number of pathogenic bacteria; this appears to be true for the *vap* gene family (21).

VapC, -D, and -E are each approximately 50% similar to *VapA* and VapB. Consequently, VapC, -D, and -E are likely to have divergent functions with respect to *VapA*. This proposition is supported by the observation that these proteins are secreted whereas *VapA* is not. VapC, -D, and -E may have an essential role in virulence since these genes are strictly conserved in all isolates of *R. equi* that express *VapA*. However, conservation alone does not prove that these *vap* genes are required for rhodococcal virulence.

We concluded that the presence of VapC, -D, and -E in the culture supernatant was due to secretion rather than to the breakdown of bacteria because they were not detected in cellular lysates and *VapA*, a nonsecreted protein produced in abundant amounts, was not detected in culture supernatants. Secreted proteins have been reported to have an important role in the pathogenesis of several intracellular pathogens such as *Yersinia* spp. and *Mycobacterium tuberculosis* (4, 10). Moreover, soluble proteins can escape the phagosome to enter the major histocompatibility complex class I pathway, where they can be presented to cytotoxic CD8+ T lymphocytes (31). Perhaps as a result, secreted antigens of intracellular bacteria appear to be primary targets of protective T-cell responses. Likewise, studies performed in a variety of animal models indicate that proteins secreted by *M. tuberculosis* may represent major components of an improved tuberculosis vaccine (12).

Vaccination against *M. tuberculosis* using secreted proteins can induce cytotoxic CD8+ lymphocyte immunity and T-helper type 1 immunity and can induce protective memory responses (2, 12). Since T-helper type 1 immunity has been demonstrated to be sufficient for the protection from *R. equi* challenge in mice (11), these studies in *R. equi* and closely related actinomycete pathogens suggest that *VapC*, -D, and -E could be useful in immunization strategies to prevent rhodococcal pneumonia.

Recombinant proteins and defined antisera were used to determine whether the characteristic *VapA* band seen in SDS-PAGE gels actually represents multiple *Vap* proteins. This issue is relevant because serologic reactivity with *VapA* has been utilized to study the epidemiology and development of *R. equi* infection in foals (18). Likewise, immunization with purified and semipurified *VapA* has been proposed as a strategy for preventing rhodococcal pneumonia (16, 18). Therefore, we evaluated several sera and anti-*VapA* monoclonal antibodies for cross-reaction using recombinant *Vap* proteins. The broad band seen on immunoblots seems to consist only of *VapA*. There was no detectable cross-reaction between r*VapA* and r*VapC*, -D, or -E by anti-*VapA* specific antisera or monoclonal

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**FIG. 6.** Recognition of recombinant Vap proteins by serum from infected foals or a monoclonal antibody. Immunoblots of cellular antigen from *E. coli* expressing recombinant *VapA* (r*VapA*), r*VapC*, r*VapD*, or r*VapE*. PBs, lysates from *E. coli* containing vector alone. All cultures were induced with IPTG, and 5 μg of protein was used in all lanes. The expression of recombinant *Vap* proteins was previously confirmed using monospecific antipeptide rabbit sera. Immunoblots were probed with serum from a foal naturally infected with *R. equi* that recognizes r*VapE* (B), or monoclonal antibody (10G5) to VapA (C). Similar results were demonstrated with monoclonal antibody Mab103.
antibodies. Therefore, immunodominant B-cell epitopes appear to be localized to unique regions encoded by the 5' end of the genes. In contrast, serum from three of four foals infected with R. equi detected either rVapD or rVapE in addition to VapA. This finding is consistent with VapD and VapE expression in vivo and supports the contention that these putative virulence proteins are also targets of the humoral immune response in horses. It is possible that equine immune serum recognized shared, linear epitopes in the carboxy terminus of the proteins; however, if this were the case, the serum should have cross-reacted with all Vap proteins.

In summary, we have identified, cloned, and expressed proteins encoded by a virulence plasmid gene family in R. equi. The genes in this family have a high degree of similarity to each other, especially in the carboxy termini. Two previously identified members, VapA and VapB, have been characterized as putative virulence proteins in horses and humans based on their regulation, surface location, and close association with virulent R. equi strains (24-26). The similarities and regulation of VapC, -D, and -E to VapA and VapB are also consistent with a role in virulence. Studies to examine the function and molecular regulation of the gene family are in progress.

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