THE MAKING OF A PATHOGEN: CONVERSION OF A SOIL SAPROPHYTE TO A MACROPHAGE PARASITE THROUGH FOREIGN DNA ACQUISITION AND ALTERATION OF CHROMOSOMAL GENE EXPRESSION IN *RHODOCOCCUS EQUI*

by

GARRY BRIAN COULSON

(Under the Direction of Mary Hondalus)

ABSTRACT

Rhodococcus equi is an opportunistic facultative intracellular pathogen of macrophages that causes severe pyogranulomatous pneumonia in young foals and immunocompromised people. Virulence of this organism is dependent on a plasmid-encoded pathogenicity island (PAI) that houses a unique family of highly related proteins of unknown function termed the virulence-associated proteins (VapACDEFGHIX). VapA, a surface-localized lipoprotein, is essential but not sufficient for *R. equi* replication in macrophages and establishment of a chronic infection in the Severe Combined Immunodeficient (SCID) mouse infection model system. To determine the identity of other plasmid-derived genes necessary for intramacrophage replication, we constructed and analyzed a series of deletion mutant strains. The first assessed was a PAI deletion mutant which was used a molecular tool for narrowing the search for additional plasmid-derived virulence factors aside from *vapA*. Expression of *vapA* in the attenuated PAI mutant from a constitutive promoter, thus eliminating the requirement of the PAI-encoded *vapA* regulators, did not restore the capacity of the PAI strain to replicate intracellularly, indicating

that additional virulence genes resided within the deleted PAI region. Subsequently, several more deletion strains were constructed and analyzed to establish the requirement of other PAI genes for intramacrophage growth, most notably, a multiple-deletion mutant (MDM) in which 14 out of 26 PAI-encoded genes were removed including every full length *vap* gene apart from *vapA.* Experimental assessment of the MDM mutant demonstrated that the collective deletion of these genes did not impair the intracellular growth capacity of *R. equi.* This finding indicated that VapA was the only Vap family member essential for intramacrophage growth, and that most of the PAI region was dispensable for the same. We subsequently hypothesized that the minimum virulence plasmid-encoded genes necessary for *R. equi* replication in macrophages was *vapA* and its regulators, *virR* and *orf8*. Expression of these three genes from their native promoters in an avirulent virulence plasmid-cured strain of *R. equi* (strain 103-) promoted intracellular replication in macrophages. This finding implied that the combined effect of the presence of VapA and regulator-induced alteration of chromosomal gene expression allows *R. equi* adaption to life within the restrictive macrophage environment.

INDEX WORDS: *Rhodococcus equi*, Vap, pathogen, pathogenesis, virulence, pathogenicity island (PAI), horizontal gene transfer (HGT), cooption, genetic crosstalk

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DEDICATION

This dissertation is dedicated to my father and mother, Brian and Marilyn Coulson, who have supported and encouraged me every single step of the way from my very first year in college to the end of my PhD degree some fifteen years later. This degree is as much mine as it is yours. I hope I made you proud.

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CHAPTER 1

INTRODUCTION

Rhodococcus equi is a Gram-positive bacterium capable of causing a life-threatening necrotizing bronchopneumonia in foals that are less than six months of age. It is also recognized as an opportunistic pathogen of humans, predominantly in high-risk populations with some form of immunosuppressive or immunocompromised state. In humans the disease presents as a tuberculosis-like pyogranulomatous lesion in the lungs that can be fatal in up to 55% of AIDS patients. In both foals and humans, significant morbidity and mortality occur despite aggressive antibiotic therapy. *R. equi* is a facultative intracellular pathogen that survives and replicates within host macrophages, the end result of which is necrotic death of the host cell and dissemination of the bacterium. The ability of virulent *R. equi* to survive within the inhospitable environment of the professional phagocyte is dependent on the possession of an 80 kb extra-chromosomal virulence plasmid. Located within this virulence plasmid is a 21 kb pathogenicity island (PAI) whose genes mediate the intracellular replication capability. A unique family of genes of unknown function, referred to as the virulence-associated protein (*vap*) genes (*vapACDEFGHIX*), encoded by the PAI has been implicated for a role in virulence of *R. equi*, although only *vapA* has been experimentally shown to be essential for replication in macrophages and in mice. The contribution of the other *vap* genes and the non-*vap* PAI-encoded genes towards *R. equi* survival and growth in host macrophages was unknown at the outset of this research effort and is the focus of the work presented in this dissertation.

CHAPTER 2

LITERATURE REVIEW

BACKGROUND

History and Taxonomy

Rhodococcus equi, a causative agent of pyogranulomatous pneumonia in foals and humans, is a facultative intracellular pathogen belonging to the order Actinomycetales, suborder Corynebacterinae (Hondalus & Mosser, 1994). The organism was first described in 1923 when it was isolated from pulmonary lesions of a foal with purulent bronchopneumonia (Magnusson, 1923). Originally classified in the genus *Corynebacterium* and named *C. equi*, the organism has subsequently been reclassified and renamed *Rhodococcus equi* and now belongs in the family Nocardiacaea (Goodfellow & Alderson, 1977). Within this family it is a member of a distinct phyletic taxon of mycolic-acid producing bacteria known as the mycolata which encompasses the genera *Mycobacterium, Nocardia, Tsukamurella, Streptomyces, Gordonia* and *Corynebacteria* with whom *R. equi* shares certain intrinsic biological and biochemical features (Mosser & Hondalus, 1996; Muscatello *et al.*, 2007).

Microbiological Characteristics

The bacterium is a weakly acid-fast, non-motile, non-sporulating Gram-positive coccobacillus with distinct pleomorphic characteristics depending on growth conditions and phase of the growth cycle (Weinstock & Brown, 2002). It forms colonies that are round, smooth and mucoid on solid agar and exhibit a characteristic salmon-pink color, from which the name

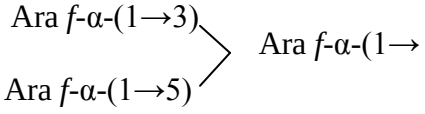
"*Rhodococcus*" was originally coined (Goodfellow *et al.*, 1982; Goodfellow, 1987). *R. equi* is furthermore biochemically characterized by the presence of catalase, urease, lipase and phosphatase, and the absence of oxidase, elastase and protease (Weinstock & Brown, 2002).

Capsule

R. equi is encapsulated with a polysaccharide layer, with at least 27 polysaccharide capsule serotypes identified to date (Prescott, 1991). Of these, the structures of *R. equi* capsular polysaccharides 1, 2, 4 and 7 have been well described (Leitch & Richards, 1990; Masoud & Richards, 1994; Severn & Richards, 1990; Severn & Richards, 1999). The capsule of *R. equi* has been proposed to play a role in diminishing phagocytosis of the bacterium (Prescott, 1991), as has been reported for other encapsulated organisms (e.g. *E. coli*, *S. pneumonia* and *H. influenzae*) (Curtis *et al*., 2005). Capsules also impart resistance to complement-mediated and oxidative killing mechanisms and to desiccation (Sydor *et al*., 2008). Capsule-deficient mutants of *R. equi*, however, are fully resistant to complement-mediated lysis (Hondalus *et al*., 1993) and can replicate normally in macrophages and maintain full virulence in mice, suggesting that the capsule is not an important virulence factor for this bacterium, but may only be necessary for survival during its saprophytic lifecycle (Sydor *et al*., 2008). In addition to the capsule, *R. equi* also possesses a rather complex cell envelope consisting of peptidoglycan (PG), arabinogalactan (AG), lipoarabinomannan (LAM) and mycolic acids, as will be described in more detail in the following section.

Cell Envelope

Gram-positive bacteria typically possess a peptidoglycan cell wall composed of repeating NAM (N-acetyl muramic acid) and NAG subunits (N-acetyl glutamic acid) joined via a β1-4 linkage and cross-linked by an adjoining penta-peptide moiety (Navarre & Schneewind, 1999). However, the peptidoglycan of *Rhodococcus*, like that of other members of the mycolata (e.g. Mycobacteria), differs from the prototypic peptidoglycan structure in that it contains an N-glycolylmuramic acid rather than the acetylated N-acetyl muramic acid derivative (Minnikin & O'Donnell, 1984; Sutcliffe, 1997). Covalently attached to this peptidoglycan cell wall is a layer of arabinogalactan (AG) polysaccharide that accounts for ~35% of the cell wall mass in *Rhodococcus*, *Mycobacteria* and *Nocardia* (Daffe *et al*., 1993). The AG layer is attached to the peptidoglycan layer via a phosphodiester link involving a unique linker disaccharide, →4-Rha *p*-(1→3)-GlcNAc, as is present in the mycobacteria (Daffe *et al*., 1993). Whilst the gross structure of the AG layer in *R. equi* resembles that of closely-related genera members, it has distinct differences, mainly with regard to the glycosyl linkages. In *R. equi*, the arabinogalactan is a homogalactan core polymer containing a mixture of (1→3), (1→5), and (1→6) Gal-*f* (galactofuranose) linkages with the following motif ("motif G"): [→5)-Gal *f*-β-(1→5)-Gal *f*-β-(1→3)-Gal *f*-(1→]$_q$ (Daffe *et al*., 1993). Branching off from this homogalactan polymer are either unbranched linear Ara-(1→5) termini or terminal arabinan side chains with 3→5 Ara-*f* linkages with the following trisaccharide motif ("motif A"):



Both linear and branched arabinans are capped with mannose residues (Daffe *et al*., 1993). The predominance of the trisaccharide motif suggests that each of the termini can carry two mycolic

acids, in contrast to the pentarabinosyl motifs found in mycobacteria, which can carry four mycolic acids each (Besra *et al*., 1995; Sutcliffe, 1998).

Anchored perpendicularly to the arabinogalactan polysaccharide through ester-linkages is a layer of bound mycolic acids of variable length (30-60 carbons in length, compared to 60-90 carbons in mycobacteria) (Prescott, 1991) which account for 30-40% of the cell wall in the closely related *R. erythropolis* and *R. opacus* (Dufrene *et al*., 1997). These mycolic acids are composed of two chains: the longer meromycolate main chain and a shorter alkyl branch side chain (Sutcliffe, 1998). While the majority of the mycolic acids are covalently bound directly to the arabinogalactan layer, there are also "free lipids" that are packed within the spaces found between the covalently bound mycolic acids. These free mycolic acid-containing lipids are typically shorter carbon chain extractable lipids, of which two types are present in *R. equi*, glucose monomycolate and the more abundant trehalose dimycolate ("cord factor") (Gotoh *et al*., 1991; Sutcliffe, 1997). Together, the bound and free mycolic acids form an asymmetric outer lipid permeability layer. In order to gain access to hydrophilic solutes on the distal side of the thick lipid layer, *R. equi* possesses at least two channel-forming proteins (porins), a cation-selective channel and a complementary anion-selective channel, which would allow for the passage of different hydrophilic compounds into the cell (Riess *et al*., 2003).

Another important component of the *R. equi* cell envelope is the lipoarabinomannan (LAM) layer, a layer of lipoglycans that is widely distributed throughout the mycolata. These complex macromolecules consist of polysaccharides covalently attached to a terminal phosphatidylinositol mannosyl (PIM) lipid moiety that anchors the structure to the cell membrane (Sutcliffe, 1998). The structure of *R. equi* LAMs is a linear α-1,6 mannan backbone with side chains containing one 2-linked α-D-Man*p* residue (Garton *et al*., 2002), with Man*p*

accounting for 86% of the LAM. In contrast to mycobacterial LAM, there is no extensive arabinan domain, but rather a single terminal α-D-Ara*f* residue capping the 2-linked α-D-Man*p*.

Genome

The chromosome of *R. equi* strain 103S, a clinical isolate from a foal with pneumonia, was recently sequenced by the Wellcome Trust Sanger Institute (*http://www.sanger.ac.uk/Projects/R_equi/*). The circular genome is 5,043,170 bp in length and has a G+C content of approximately 68%. Analysis of the annotated genome has revealed that the genome encodes ~4,525 predicted genes with an overall coding percentage of 90% (Letek *et al*., 2010; Takai *et al*., 1986a). A remarkable aspect of the *R. equi* chromosome composition is that approximately one-third of the genes are involved in transport, secretion or regulation, which is consistent with the fact that *R. equi* must adapt to existence within quite diverse environmental niches, including both saprophytic and intracellular stages (Letek *et al*., 2010). Another one-third of the genome encodes proteins of unknown function, and the remainder of the genome is involved in central and intermediate metabolism.

Interestingly, *R. equi* does not possess any genes involved in carbohydrate transport and thus cannot utilize sugars as a primary carbon source. Rather, it appears as though the organism is dependent on organic acids (e.g. succinate, malate and fumarate) and fatty acids for carbon assimilation. With over 36 lipases and an extensive array of enzymes involved in lipid metabolism, it appears that lipids are the predominant carbon substrate for the organism. *R. equi* also possesses the required pathways for the *de novo* synthesis of all amino acids, allowing the organism to grow in amino acid-starved environments such as the macrophage vacuole and thus

is not dependent on the host for amino acids. In contrast, *R. equi* is thiamine auxotrophic and completely dependent on host-derived thiamine for growth during infection.

In addition to the circular chromosome, virulent *R. equi* also possess a single 80kb circular plasmid. Recent computational analysis and reannotation of the *R. equi* virulence plasmid by Letek *et al* (2008) suggests that the virulence plasmid contains 73 coding sequences (CDS), including 8 pseudogenes (Letek *et al*., 2008; Takai *et al*., 2000b). The function of only 39% of the proteins encoded by the plasmid can be predicted by bioinformatic analysis. The plasmid consists of four large, fairly distinct genetic regions: specifically a conjugation region and a plasmid replication/partitioning region which together account for ~75% of the total plasmid size, a region of unknown function, and a pathogenicity island (Letek *et al*., 2008; Takai *et al*., 2000b). The pathogenicity island (PAI) region of the *R. equi* virulence plasmids from clinical isolates obtained from foals is a 21.3kb region (spanning *orf1* through *orf21*) likely acquired through horizontal gene transfer from a source of unknown origin. The evidence in support for this region being a pathogenicity island is that i) it is present in all pathogenic strains (associated with the virulence plasmid) and absent in avirulent (plasmid-cured) strains, ii) the GC content of the genes in this region is lower than the rest of the plasmid (60.8% vs 66.6%), iii) it is bound by insertion elements (e.g. transposon resolvases), and iv) it contains a tRNA gene (*orf1*: *lsr2*: lysyl-tRNA synthetase gene), all of which are hallmarks of PAIs (Hacker *et al*., 1997). Computational analysis of the PAI has revealed 26 putative CDSs, 7 of which are believed to be pseudogenes. Of particular interest on this PAI is the presence of a novel family of genes that are unique to *R. equi* termed the virulence-associated protein (*vap*) genes. There are six full-length functional *vap* genes (*vapA*, *-C*, *-D*, *-E*, *-G* and *–H*) which all share extensive homology at their C-termini (Takai *et al*., 2000b), and three *vap* pseudogenes (*vapI*, *-F* and *–X*) that are believed to

encode non-functional protein as they either exhibit substantial truncations and/or frameshift mutations in their coding sequences (Letek *et al.*, 2008; Polidori & Haas, 2006; Takai *et al.*, 2000b). The relevance of this unique family of proteins will be discussed in detail later.

One particularly interesting observation of the *R. equi* genome is the finding that the chromosome and virulence plasmid do not operate in transcriptional isolation, but rather under certain environmental conditions engage in regulatory cross-talk (Letek *et al*., 2010). Using a microarray approach, Letek and colleagues showed that under non-inducing conditions (i.e. 30°C pH 8.0) there was no significant difference in gene expression of chromosomal genes in virulence plasmid-containing and plasmid-cured strains. However, when the strains were cultured in inducing conditions (i.e. 37°C, pH 6.5), a significant change in gene expression was observed for a large number of chromosomal genes (n = 88) in plasmid-containing strains, but not plasmid-cured strains. This suggests that plasmid-encoded genes are capable of directly or indirectly influencing transcription of chromosomal genes, a finding which has important implications for virulence.

EPIDEMIOLOGY AND CLINICAL PERSPECTIVES

Introduction

Like many of the bacteria to which it is related, *R. equi* is a saprophytic bacterium found ubiquitously in the soil and in fecal matter of grazing animals worldwide (Barton & Hughes, 1984). It is capable of surviving in these ecological niches provided that certain environmental conditions are met (i.e. neutral to moderately alkaline pH, temperatures around 30°C, and appropriate nutrient availability in the form of volatile fatty acids such as those present in fecal material) (Hughes & Sulaiman, 1987; McNeil & Brown, 1994; Prescott, 1991). Through

inhalation or ingestion of contaminated soil or feces susceptible individuals (e.g. foals, pigs and humans) can acquire *R. equi* organisms (Johnson *et al.*, 1983a; Johnson *et al.*, 1983b). Direct host-to-host transmission via inhalation of respiratory droplet aerosols generated by individuals with *R. equi* pneumonia has recently been proposed as an alternate means of infection in foals (Muscatello *et al*., 2009). Host susceptibility is a prerequisite for disease development, an occurrence which is believed to depend on immunological naiveté or immaturity of neonatal foals, or is a consequence of a pre-existing immunocompromised state in humans (Prescott, 1991; Yager, 1987). Thus, *R. equi* is an opportunistic pathogen.

Epidemiology of *R. equi* Disease in Horses

R. equi is recognized as a potential pathogen of a relatively wide range of herbivorous animal hosts (e.g. cattle, goats, sheep) (Prescott, 1991), but the greatest burden of *R. equi* disease is in young foals between the ages of 1 and 6 months (Yager, 1987). The most common clinical presentation of *R. equi* disease in foals is chronic bronchopneumonia (Yager, 1987). Mortality rates can vary from 1-30% depending on the study (Cohen *et al*., 2005; Giguere *et al*., 2004; Muscatello *et al*., 2007; Zink *et al*., 1986). The reason for the preponderance of disease in young foals will be discussed later in this review.

Although *R. equi* can be isolated from the soil on most horse farms worldwide, the prevalence of *R. equi* infection in foals on farms can vary markedly, with morbidity rates fluctuating from 5% to over 50% depending on the farm (Chaffin *et al.*, 2003b). *R. equi* infection can occur endemically on some horse farms, sporadically on others, and is absent on most (Prescott, 1991). Even on farms with endemic disease, there is marked year-to-year variability in the number of foals that present with pneumonia due to *R. equi* (Chaffin *et al*.,

2003c). The reason for this great variation in prevalence is still unclear, although several risk factors have been identified. These include poor grass cover and low soil moisture, both of which contribute to formation of dust and aerosolization of the bacterium. Additionally, increase in stocking densities on farms may contribute to increased incidence of disease. This can occur either directly as a result of bringing large numbers of foals into close contact with one another, thus potentially facilitating foal-to-foal transmission (Muscatello *et al*., 2009), or indirectly through the buildup of large quantities of manure, in which *R. equi* can thrive (Barton & Hughes, 1984). It has been reported that foals in general can shed 1000-10,000 *R. equi*/g of feces, whereas foals with *R. equi* pneumonia are capable of shedding up to 10^8 *R. equi*/g feces (Takai *et al*., 1986a; Takai *et al*., 1986b). Nonetheless, soil concentrations of *R. equi* are not significantly associated with the prevalence of pneumonia on farms with endemic *R. equi* (Cohen *et al*., 2008; Muscatello *et al*., 2006b). Molecular epidemiologic data from farms with *R. equi* have shown that various genotypes of *R. equi* disease can be isolated from affected foals and their environment, suggesting that no single genotype is responsible for the recurrence of disease on a particular farm (Cohen *et al*., 2003; Morton *et al*., 2001). Thus, the incidence of *R. equi* disease appears to be multi-factorial, dependent on the virulence of the organism, the susceptibility of the host and the environmental conditions.

Epidemiology of *R. equi* Disease in Humans

In addition to being an important cause of life-threatening disease in foals, *R. equi* has also emerged as an opportunistic pathogen of immunocompromised or immunosuppressed people (Emmons *et al*., 1991; Puthucheary *et al*., 2006). The first reported case of *R. equi* infection in humans was in 1967 in a patient on immunosuppressive therapy for chronic hepatitis (Golub *et*

al., 1967). Since then, there has been a significant increase in the number of case reports of human *R. equi* infection, predominantly in immunocompromised individuals (Weinstock & Brown, 2002). Conditions such as AIDS, organ transplantation, chemotherapy, steroid therapy, diabetes mellitus and alcoholism are seen as host risk factors for disease (Arlotti *et al*., 1996; Borghi *et al*., 2008; Lasky *et al*., 1991; Munoz *et al*., 1998; Speck *et al*., 2008), with HIV-infected patients accounting for approximately two-thirds of the cases of *R. equi* infection in humans (Weinstock & Brown, 2002). Although human infection is predominantly associated with an immunocompromised status, rare cases of *R. equi* infection in immunocompetent patients have also been described (Gabriels *et al*., 2006; Kedlaya *et al*., 2001). Acquisition of the organism in people may occur via exposure to animals carrying the organism (Prescott, 1991; Verville *et al*., 1994), exposure to contaminated soil or feces (Takai *et al*., 1991b) or consumption of contaminated foods (Kedlaya *et al*., 2001), although many patients have no history of any such exposure. Possible modes of infection include inhalation, ingestion or wound inoculation. Person-to-person transmission has also been proposed, but has not yet been confirmed. The mortality rate associated with *R. equi* infection is 50-55% in HIV-infected individuals and 20-25% in patients with other immunocompromised conditions (Cornish & Washington, 1999; Harvey & Sunstrum, 1991). In contrast, the mortality rate is about 11% in immunocompetent patients (Kedlaya & Ing, 2008).

Clinical Disease

Foals infected with *R. equi* may show very little in the way of clinical signs of disease until the latter stages of infection, making early diagnosis and treatment difficult. In foals that do develop respiratory disease, the most common clinical manifestation of *R. equi* infection is a suppurative

bronchopneumonia with associated abscessation of the lungs and regional lymph nodes (Prescott, 1991; Yager, 1987; Zink *et al*., 1986). As an intracellular pathogen, *R. equi* persists in host alveolar macrophages (Hondalus & Mosser, 1994), which are ultimately destroyed if conditions are favorable for bacterial replication. Early lung lesions are characterized by massive influx of phagocytic cells into the alveolar spaces. These cells are predominantly large macrophages often in the form of multinucleated giant cells. *R. equi* is found in large numbers within the macrophages and giant cells, but rarely elsewhere. The eventual degeneration of the macrophages coincides with the development of lytic lesions in the lungs and caseous necrosis (Prescott, 1991). Although predominantly presenting as a chronic disease in foals, a lesser seen acute form of *R. equi* pneumonia can occur with foals dying within a few hours or days of showing signs of respiratory distress (Martens *et al*., 1982; Muscatello *et al*., 2007).

In addition to the inhalation route of infection, foals may become infected through ingestion of the organism. The resultant manifestation of infection may include intestinal involvement in the form of enterocolitis and typhlitis with gross inflammation of the mesenteric and/or colonic lymph nodes (Zink *et al*., 1986). Approximately half of all foals with clinical *R. equi* pneumonia also develop ulcerative colitis (as Peyer's patches become ulcerated and destroyed) and/or lymphadenitis (Zink *et al*., 1986). In up to half of infected foals, bacteremic spread of the organism to other sites in the body may occur, resulting in polysynovitis, osteomyelitis, nephritis and uveitis (Giguere & Lavoie, 1994; Prescott, 1991; Sweeney *et al.*, 1987; Zink *et al.*, 1986). In adult horses, disease due to *R. equi* is rare, but in reported cases the illness appears similar to that observed in foals (Giguere & Prescott, 1997). Immunodeficiency in adult horses appears to be a risk factor for disease (Freestone *et al*., 1987).

In humans, the most common clinical manifestation of *R. equi* infection is cavitary pneumonia, occurring in ~80% of immunocompromised patients and ~40% of immunocompetent hosts with *R. equi* disease (Harvey & Sunstrum, 1991; Kedlaya *et al.*, 2001; Weinstock & Brown, 2002). Clinical pneumonia is often associated with fever, chest pain, dyspnea and cough (Kedlaya *et al*., 2001). In addition to necrotizing pneumonia, *R. equi* infection may also present as a lymphadenitis, septic arthritis, pericarditis or meningitis, often as a result of direct dissemination from the lung (Corne *et al.*, 2002; Kedlaya & Ing, 2008). In most cases, extrapulmonary disease is a late manifestation seen in immunocompromised patients (Corne *et al*., 2002; Prescott, 1991).

Diagnosis

As mentioned previously, many foals infected with *R. equi* have subclinical signs of the disease, making early diagnosis and initiation of treatment a challenge. However, certain clinical signs including fever, increased respiratory rate, decreased appetite and weight loss, lethargy, tachypnea, nostril flaring and wheezing are consistent with *R. equi* disease in foals (Giguere & Prescott, 1997). In cases where *R. equi* infection is suspected in foals, a variety of diagnostic tests can be performed to positively identify *R. equi*. Such tests may include thoracic radiography or ultrasound to detect nodular lung abscesses and lymphadenopathies (Falcon *et al*., 1985), serologic tests such as agar gel immunodiffusion (AGID) to detect *R.equi*-specific membranolytic exoenzymes (Prescott *et al*., 1982), or enzyme-linked immunosorbent assays (ELISA) to detect antibodies to *R. equi* (Takai *et al*., 1985). Additionally, polymerase chain reaction (PCR) assays based on the detection of *vapA* (virulence associated protein A), *aceA* (isocitrate lyase), *IdeR* (iron-dependent regulator gene) and *choE* (cholesterol oxidase gene) have also been developed to identify *R. equi* in specimens (Rodriguez-Lazaro *et al*., 2006; Sellon *et*

al., 2001; Venner *et al*., 2007; Vivrette *et al*., 2000). In most clinical settings, diagnosis of *R. equi* disease is based on a combination of both clinical signs and laboratory tests. A positive PCR for *vapA* in foal tracheal aspirates coupled with either signs of respiratory disease or evidence of nodular lesions by X-ray or ultrasound is commonly used to diagnose *R. equi* disease

In humans with pneumonic *R. equi* infection, isolation and culture of the organism from bronchial brushing or percutaneous thoracic aspiration is the preferred method of diagnosing infection (Prescott, 1991). Blood culture can also be performed; however, great variation in the sensitivity of this assay has been reported (Donisi *et al.*, 1996; Harvey & Sunstrum, 1991). Radiologic examination of the lungs of patients with pulmonary infection showing features characteristic of nodules, cavitations or abscesses may also be used as a preliminary step in the diagnostic process, however additional diagnostic tests are required to exclude other possible causative agents of nodular pulmonary involvement (e.g. *M. tuberculosis, P. jiroveci*, actinomycosis, pulmonary cancer) (Speck *et al.*, 2008; Weinstock & Brown, 2002). On the basis of its variable acid-fast staining and morphological characteristics, the organism can also easily be mistaken for *Mycobacterium* spp, or diptheroid contaminants (Golub *et al*., 1967; Prescott, 1991).

Prevention and Treatment

Since no vaccine for *R. equi* disease in foals exists to date, prevention of *R. equi* infection in foals relies heavily on sound farm management practices to limit the exposure of foals to the bacterium. Such practices may include maintaining good pasture cover in paddocks and yards to limit dust and aerosolization of the organism, reducing the time foals spend in stables, thorough and regular cleansing of stables, and limiting the amount of time foals spend crowded together to

prevent direct foal-to-foal aerosol transmission (Chaffin *et al.*, 2003a; Chaffin *et al.*, 2003c; Cohen *et al.*, 2003; Muscatello *et al.*, 2007). When management practices fail and foals acquire *R. equi* disease, treatment of infected foals is typically done via oral administration of a combination of drugs, often erythromycin and rifampin (Prescott, 1991). These drugs penetrate well into macrophages and have been shown to have synergistic activity *in vivo* and *in vitro* (Prescott & Nicholson, 1984). Use in combination also reduces the likelihood of resistance to either drug (Nordmann & Ronco, 1992). Alternatives to erythromycin have been proposed, including azithromycin and clarithromycin, which are more stable and have a greater bioavailability (Jacks *et al.*, 2003). Successful treatment of *R. equi* pneumonia in foals requires prolonged treatment (4 to 9 weeks), typically until the animals appear clear of the infection by radiological and cytological evidence (Giguere & Prescott, 1997). During treatment, foals are monitored for signs of drug intolerance and toxicity, which may manifest as depression, diarrhea and dehydration and may necessitate intensive fluid replacement therapy (Giguere & Prescott, 1997; Stratton-Phelps *et al.*, 2000).

Combination antibiotic therapy is also the mainstay approach to treating *R. equi* infections in people (Weinstock & Brown, 2002). Depending on the clinical manifestation of the disease, drug tolerance issues in the patient and other complicating factors in specific cases, the type of drugs administered and duration of treatment varies considerably in people with *R. equi* disease. Consequently, there is no standardized protocol currently established for the treatment of *R. equi* infections in humans. Similar to treatment of foals, a combination of potent bactericidal drugs (e.g. vancomycin, imipenem, amikacin) and drugs with intracellular penetration (e.g. rifampin, erythromycin, doxycycline) are used in conjunction to target both extracellular and intracellular organisms (Gabriels *et al.*, 2006; Rouquet *et al.*, 1991).

Vancomycin, imipenem and rifampin are the most effective single antimicrobial agents, with rifampin-erythromycin, rifampin-minocycline, erythromycin-minocycline and imipenem-amikacin proving to be effective drug combinations (Nordmann *et al.*, 1992a; Nordmann & Ronco, 1992). The duration of therapy can range from a few weeks to 1 year depending on the site and severity of infection, as well as the underlying immunocompetence and clinical response of the patient to therapy (McNeil & Brown, 1994; Weinstock & Brown, 2002). Generally, *R. equi* isolates are resistant to β-lactams (e.g. penicillin, ampicillin, carbenicillin) and thus the use of drugs in this class for treatment is not recommended (Barton, 1986; McNeil & Brown, 1992; Verville *et al.*, 1994).

In addition to antibiotic therapies, surgical intervention (drainage of suppurative lesions or surgical resection of granulomatous tissue) may be performed to reduce the microbial burden in patients who do not respond to antimicrobial therapy, although this is controversial and not common practice.

HOST-PATHOGEN INTERACTION

Virulence

Although *R. equi* is ubiquitous in the environment on a geographically widespread scale, the prevalence of disease in foals varies greatly from farm-to-farm, region-to-region and year-to-year (Muscatello *et al*., 2006a). The reasons for this variation are poorly understood, as has been discussed previously, but one of the possible contributing factors may be related to the virulence *R. equi* isolates at each location. Virulence factors of *R. equi* include both chromosomally encoded gene products and plasmid-encoded factors (Hondalus, 1997; Prescott, 1991), and a combination of both is required for disease development.

Isocitrate Lyase

Many bacteria (e.g. *M. tuberculosis, E. coli*) make use of the glyoxylate cycle as an efficient strategy for converting C2 compounds (e.g. acetyl-CoA, acetate) into a form that bacteria can use as an energy source, such as C4 dicarboxylic acids (e.g. succinate, malate) (Mdluli & Spigelman, 2006). One of the key enzymes that catalyzes an initial step in this process is isocitrate lyase which facilitates the cleavage of isocitrate to yield succinate and glyoxylate (Ensign, 2006). Additionally, fatty acids can also be converted to acetyl-CoA via β-oxidation, which then enters the normal glyoxylate pathway, thus linking the glyoxylate pathway to fatty acid metabolism. Using such a pathway, it has been hypothesized that intracellular bacteria may be able to make use of lipid-derived fatty acids from host membranes as a potential source for carbon when the environment within the macrophage becomes more hostile and nutrient deprivation occurs (Honer zu Bentrup & Russell, 2001). In *M. tuberculosis*, an isocitrate lyase mutant was attenuated for growth both in macrophages and in mice (Honer Zu Bentrup *et al*., 1999; McKinney *et al*., 2000), suggesting that lipids are actually a major source of carbon for intracellular *M. tuberculosis*. Similar findings have also been described in *Rhodococcus fascians* and *Candida albicans* (Lorenz & Fink, 2001; Vereecke *et al*., 2002). To assess whether isocitrate lyase was required for virulence in *R. equi*, Wall and colleagues constructed an isocitrate lyase (*aceA* in *R. equi*) deletion mutant (Wall *et al*., 2005). The mutant was severely attenuated for replication in macrophages and for *in vivo* growth in CD1 mice. The virulence of the mutant was also assessed in 3-week old foals. Foals challenged via the intrabronchial route with the *aceA* mutant showed no change in heart rate and respiration rate post infection, and displayed no presence of lesions in their lungs upon post-mortem examination. In contrast, foals similarly infected with wildtype *R. equi* developed elevated heart and respiratory rates after 9

days post-challenge, and severe pneumonic lesions were found at necropsy (Wall *et al*., 2005). These data demonstrate the importance of isocitrate lyase in establishment of *R. equi* disease, and suggest that host membrane lipids are an important source of carbon for intracellular *R. equi*.

Mycolic Acids

Mycolic acids, which have been well studied in the closely related organism *M. tuberculosis*, are β-hydroxy fatty acids with long hydrocarbon α-side chains that contribute significantly to the structural integrity of the cell wall (Takayama *et al*., 2005). Mycolic acids create a thick, hydrophobic layer of lipid on the outer surface of the cell which can provide protection from drugs and hostile host immune responses (e.g. oxidative stress) (Takayama *et al*., 2005) and can modulate immune responses (Ryll *et al*., 2001). The importance of these mycolic acids in virulence was shown in *M. tuberculosis*, where disruption of the mycolates led to attenuation in growth *in vitro* in macrophages (Yuan *et al*., 1998), and also in mice (Dubnau *et al*., 1997; Glickman *et al*., 2000). *R. equi* possesses a cell wall with characteristics similar to the mycobacteria in that mycolic acid-containing glycolipids are a major component of the cell wall (Garton *et al*., 2002; Sutcliffe, 1997). In a mouse model system, *R. equi* strains with longer carbon chain mycolic acids were shown to induce a greater number of granulomas and be more lethal to the mice than those strains with shorter carbon chains, demonstrating the importance of mycolic acids in the virulence of *R. equi* (Gotoh *et al*., 1991).

Polysaccharide Capsule

Polysaccharide capsule is believed to contribute to virulence by diminishing phagocytosis of the bacterium (Prescott, 1991), as has been reported for other encapsulated organisms (e.g. *E. coli*, *S.*

pneumonia and *H. influenzae*) (Curtis *et al*., 2005). In addition, capsules enable bacteria to resist desiccation and the potential killing effects of complement and oxidative radicals (Sydor *et al*., 2008). At least 27 polysaccharide capsule serotypes have been identified in *R. equi*, yet there is no evidence to suggest a direct link between serotype and virulence (Prescott, 1991). In a recent study, capsule production in a virulent *R. equi* strain was disrupted by transposon inactivation of a mycolyl transferase gene (*fbpA*) (Sydor *et al*., 2008). The *fbpA* mutant multiplied normally in macrophages cultured *in vitro* and retained virulence in the mouse model of infection, suggesting that the capsule may not be an important virulence factor of *R. equi*. Furthermore, the mutation had no significant effect on the production of mycolic acids, another putative virulence factor of *R. equi*, nor was the pattern of surface glycolipids altered, presumably because *R. equi* possesses 13 putative mycolyl transferases (Sydor *et al*., 2008).

Cholesterol Oxidase

Cholesterol oxidase (CO), an *R. equi* exoenzyme (formerly known as "*equi* factor"), has been proposed to be a putative virulence factor. It is speculated that cholesterol oxidase may oxidize host cholesterol, thereby leading to subsequent disruption of host membranes and ultimately to macrophage death (Navas *et al*., 2001). Alternatively, it may play some role in the protective response of *R. equi* to reactive oxygen species, yet this assertion still remains to be confirmed (Fuhrmann *et al*., 2002). The role of CO as a virulence factor is controversial, with conflicting reports in the literature. In one report, cholesterol oxidase (CO) was found to be required for intracellular survival of the bacterium in macrophages, since an *R. equi* strain (strain 4219) lacking cholesterol oxidase was cleared from host macrophages (Linder & Bernheimer, 1997). In contrast, Prescott *et al* (1982) reported that both virulent and avirulent strains of *R. equi* can

express CO (Prescott *et al*., 1982). Furthermore, a cholesterol oxidase (*choE*) mutant is neither attenuated in the ability to replicate in macrophages, nor in the ability to survive and grow in mouse livers *in vivo* (Pei *et al*., 2006). While these data suggest that CO may not be important for the virulence of *R. equi*, it is possible that deletion of *choE* could be compensated for by the action of other chromosomal cholesterol oxidases, such as *choD*. To truly determine the role of CO in virulence, the entire CO genetic operon would need to be deleted to avoid the confusing effects of potential redundancy.

Nitrate Reductase

Nitrate reductase, encoded by *narG*, is a metabolic enzyme that plays an important role in respiration in the absence of oxygen. The enzyme catalyzes the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), which is further reduced to ammonia. This process involves both oxidative and reductive reactions with ammonia utilized as electron donor and nitrite as electron acceptor. This pathway is also important for detoxification and is an important step in the addition of nitrogen into biomolecules (Gonzalez *et al*., 2006). Deletion of *narG* in *R. equi* results in complete attenuation of growth in the livers of infected CD1 mice, indicating the importance of this gene in virulence *in vivo* (Pei *et al*., 2007), and also suggests that *R. equi* encounters an anaerobic environment at some stage during infection, presumably within granulomas. The importance of nitrate reductase in virulence for other bacterial pathogens (e.g. *M. bovis* and *P. aeruginosa*) has been well described (Fritz *et al*., 2002; Van Alst *et al*., 2007; Weber *et al*., 2000).

High Temperature Requirement A Protein

High temperature requirement A (*htrA*) proteins are stress-induced serine proteases that scavenge misfolded or damaged proteins before they reach toxic levels that interfere with normal biological functions in the cell (Clausen *et al*., 2011). Typical stresses that may induce expression of HtrA include heat shock (*E. coli*) (Lipinska *et al*., 1990) and oxidative stress (*S. typhimurium*) (Johnson *et al*., 1991). HtrA proteins have been found to be essential for virulence in both these pathogens and others (e.g. *B. melitensis, K. pneumonia* and *H. pylori*) (Cortes *et al*., 2002; Hoy *et al*., 2010; Phillips *et al*., 1995). Similar to these aforementioned pathogens, deletion of *R. equi htrA* results in complete attenuation of growth in the livers of infected mice, indicating the essentiality of this gene *in vivo* (Pei *et al*., 2007).

Chorismate Mutase and Anthranilate Synthase

Both chorismate mutase (AroQ*) and anthranilate synthase (TrpEG-like) are key metabolic enzymes that participate in the shikimate pathway responsible for the biosynthesis of aromatic amino acids. Chorismate mutase converts chorismate into prephenate, which is subsequently converted into tyrosine and phenylalanine. In contrast, anthranilate synthase converts anthranilate to tryptophan (Letek *et al*., 2010). Deletion of either of these metabolic genes in *R. equi* results in complete attenuation of intracellular bacterial growth in macrophages, demonstrating the necessity of *de novo* synthesis of aromatic amino acids, which may be in limited supply in host *R. equi*-containing vacuoles (Letek *et al*., 2010). The importance of the shikimate pathway for survival and intracellular replication has been reported in other model intracellular pathogens (e.g. *M. tuberculosis* and *F. tularensis*) (Asare & Abu Kwaik, 2010; Asare *et al.*, 2010; Kraemer *et al.*, 2009; Parish & Stoker, 2002).

Sensor Kinase MprB

One of the methods by which bacteria respond to changes in the environment is by altering gene expression. Two-component regulatory systems play an important role in sensing these changes through sensor kinase proteins which, upon activation, phosphorylate and activate their cognate response regulators which then bind to DNA and alter the transcriptional activity of target genes (Galperin, 2010). The *R. equi* genome encodes 24 sensor kinases and 26 response regulators, 23 of which are encoded in pairs (Letek *et al*., 2010). Mutation of one sensor kinase, MprB, abolished the ability of *R. equi* to replicate in macrophages, suggesting that MprB is important in sensing the macrophage environment and initiating downstream events required for intramacrophage survival (Macarthur *et al*., 2011).

Virulence Plasmid

All clinical isolates of *R. equi* derived from foals or swine with *R. equi* disease possess a large circular plasmid approximately of 80-90 kb, or 79-100 kb in size respectively (Takai *et al.*, 1991c; Takai *et al.*, 2000a). These plasmids are absolutely necessary for virulence, since strains cured of the plasmid are completely unable to replicate in macrophages or cause disease in mice and foals (Giguere *et al.*, 1999; Hondalus & Mosser, 1994). Plasmids from equine and porcine isolates possess a pathogenicity island (PAI), although the PAI in equine isolates is significantly larger at 21 kb versus the 15 kb PAI of porcine isolates (Letek *et al*., 2008). Of particular interest on these PAIs is the presence of a novel family of genes that are unique to *R. equi* termed the virulence-associated protein (*vap*) genes. In plasmids from equine isolates, there are six full-length functional *vap* genes (*vapA, -C, -D, -E, -G* and *–H*) and three *vap* pseudogenes (*vapI, -F* and *–X*) (Letek *et al.*, 2008; Polidori & Haas, 2006; Takai *et al.*, 2000b). Worthy of note is that

virulence plasmids of foal and pigs isolates differ significantly in the composition of their respective pathogenicity island regions. The main difference is that pig isolates possess a gene, *vapB*, in place of *vapA* (sharing ~84% identity), and also encode four distinct Vap proteins (VapJ, VapL, VapM and VapK, of which there are two copies). It has been postulated that the particular assemblage of Vap proteins contributes to the species-specific niche of *R. equi* isolates (Letek *et al*., 2008). For the sake of this review, the focus will be on VapA-encoding plasmids present in equine clinical isolates.

VapA is a surface-localized lipoprotein with a molecular weight of 17-22kDa depending on the degree of covalent lipid-modification of the protein (Byrne *et al*., 2001; Sekizaki *et al*., 1995; Takai *et al*., 1991c; Tan *et al*., 1995). Deletion of *vapA* results in significant attenuation of the organism; *vapA* mutants are unable to replicate within *in vitro* grown macrophages and are also cleared rapidly *in vivo* in the SCID mouse infection model (Jain *et al*., 2003). In both *in vitro* and *in vivo* models, complementation of the *vapA* mutant with an exogenous plasmid expressing VapA at wildtype levels from its native promoter restored full virulence to the mutant (Jain *et al*., 2003). This study provided the first definitive evidence for the essential role of *vapA* in virulence. To date, however, the precise function of *vap* remains unknown. While there is some evidence perhaps consistent with a role for VapA in preventing acidification of *R. equi* containing vacuoles and fusion with lysosomal compartments, this remains controversial and requires further investigation (von Bargen *et al*., 2009). *VapA* is necessary for replication in macrophages, but it is not sufficient. Expression of wildtype levels of VapA in a plasmid-cured strain of *R. equi* does not restore virulence to the attenuated strain (Giguere *et al*., 1999). These data strongly suggest that there are additional virulence determinants on the *R. equi* virulence

plasmid, aside from *vapA*, that are required for virulence. At the outset of this dissertation, the identity and location of these factors was unknown.

The *vapA* gene, and its homologs *vapI*, *vapC* and *vapD* are clustered together in an operon that is expressed as a 2.3 kb polycistronic transcript (Byrne *et al*., 2008). This transcript is processed, leading to the formation of a 700 bp *vapA* transcript (Russell *et al*., 2004), which is more stable (~4-5fold greater half-life) than the *vapICD* transcripts, allowing for differential gene expression of the genes in this operon (Byrne *et al*., 2008). The *vapE* gene *vapF* pseudogene are found together downstream of the *vapAICD* operon, whereas *vapG* is independently located ~10.5 kb upstream of *vapA*. The remaining *vap* family member, *vapH*, is also found upstream of *vapA* and is part of a five-gene operon (*virR*, *orf5*, *vapH*, *orf7* and *orf8*). Similar to *vapA*, expression of *vapC*, *vapD* and *vapE* is induced by temperature (>37°C) and pH (< pH6.5) (Byrne *et al*., 2001), environmental conditions similar to those found *in vivo* and consistent with the possibility that these genes play a role in pathogenesis. However, Jain *et al* (2003) reported that a *vap*-locus mutant (deletion spanning *vapA*-*vapF*), which was strongly attenuated for growth in macrophages and in mice since it lacked *vapA*, could be restored to full virulence if transformed with a *vapA*-expressing shuttle plasmid (Jain *et al*., 2003). Transformation of the locus mutant with *vapC*, *vapD*, *vapE* or *vapF* did not complement the growth defect of the *vap*-locus mutant. These data suggest that while *vapA* is crucial for virulence, the remaining *vap* genes in the locus (*vapC*, *vapD*, *vapE*, *vapF* and *vapI*) are not.

As mentioned previously, *vapA* expression is induced in response to various environmental cues (e.g. increased temperature, low pH, low magnesium and low iron concentrations), conditions which may reflect those encountered by *R. equi* when it enters a host. In response to these signals, *vapA* expression is regulated through the concerted action of at least

two transcriptional regulators, *virR* (*orf4*) and *orf8*, which are the first and last genes respectively of a five-gene operon that includes another *vap* gene, *vapH* (*orf6*). The *virR* gene encodes for a LysR-type transcriptional regulator, while *orf8* is an orphan response regulator partner of a two-component type regulatory system. The *vapA* gene is transcribed from a promoter 226 bp upstream of the gene, with transcription dependent on the binding of VirR to the *vapA* promoter (Russell *et al*., 2004). When a plasmid-cured strain of *R. equi* was transformed with an expression plasmid encoding *vapA* and its native promoter alone, no VapA protein could be detected. However, when the plasmid-cured strain was transformed with a plasmid encoding both *vapA* and *virR*, *vapA* expression could be detected, although at levels less than that seen in the wildtype strain. When the entire *virR* operon (*orf4-8*) was used to drive *vapA* expression, levels of *vapA* message were restored to that of wildtype, suggesting that a gene/s aside from *virR* in the *virR* operon was necessary to allow maximal expression of *vapA*. The finding that the deletion of either *virR* or *orf8* profoundly decreases the expression of *vapA* under inducing conditions is consistent with the requirement for both *virR* and *orf8* for optimal *vapA* expression (Ren & Prescott, 2004).

Transcriptional regulation of the genes in the *virR* operon is complex and involves the combined participation of two distinct promoters, P_{virR} and P_{orf5} (Byrne *et al*., 2007). The P_{virR} promoter is located 53 bp upstream of the *virR* translation initiation codon, whereas the P_{orf5} promoter is located internally within the *virR* coding sequence 585 bp downstream of the *virR* translation initiation codon. Under non-inducing conditions, the *virR* operon is transcribed at low constitutive levels from the P_{virR} promoter, which is likely recognized by the principle sigma factor of *R. equi*, presumably σ^{70}. As reported for other LysR-type transcriptional regulators (Schell, 1993), VirR binds to its own promoter and represses transcription, thus acting as a

negative autoregulator of the *virR* operon, explaining its low transcription levels. However, under inducing condition (high temperature, low pH), P_{orf5} becomes activated and is recognized by an alternative sigma factor. This activation of P_{orf5} results in a significant increase (67-fold) in the transcription of downstream genes in the operon (*orf*5, *vapH*, *orf7* and *orf8*) (Byrne *et al.*, 2007; Ren & Prescott, 2003).

Pathogenesis

When a susceptible foal or person is exposed to a sufficient aerosolized *R. equi* challenge, a chronic bronchopneumonia associated with significant morbidity and mortality is the likely outcome. As a facultative intracellular bacterium, establishment of disease in foals and humans is dependent on the ability of *R. equi* to successfully invade host alveolar macrophages and multiply therein. Optimal adherence to and uptake by host macrophages requires *R. equi* to fix host complement by activating the alternative complement pathway, and subsequently binding to cells expressing complement receptor type 3 (CR3 or Mac-1) (Hondalus *et al*., 1993). The presence of this receptor on only a small subset of cells (e.g. monocytes and macrophages) may partially explain the limited tropism of this bacterium for certain host tissues (Mosser & Hondalus, 1996). *R. equi* may also enter macrophages through Fc-receptors if the organism is coated with specific antibody. However, the intracellular fate of the bacteria differs markedly upon entry into macrophages through this route, as it is associated with significantly higher killing linked to enhanced phagosome-lysosome fusion (Hietala & Ardans, 1987; Zink *et al.*, 1987). Thus, the ability of *R. equi* to survive within host macrophages is influenced profoundly by the route of entry into host macrophages. Alternative receptors such as scavenger receptors, mannose receptors and surfactant protein (SP-A and SP-B) receptors that have all been

implicated in binding and phagocytosis of the closely-related organism, *M. tuberculosis*, may also presumably play a role in *R. equi* infection, yet their contribution remains unstudied to date (Ernst, 1998; Schafer *et al*., 2009).

The capacity of virulent *R. equi* to grow within host macrophages is dependent on the organism's resistance to or disruption of innate macrophage microbiocidal responses. Under normal circumstances, once bacteria are internalized the phagosome typically undergoes a sequential maturation process, ultimately culminating in the fusion with lysosomal vesicles resulting and destruction of bacteria by acid-resistant hydrolases (Haas, 1998; Meijer & Prescott, 2004). However, several intracellular bacteria (e.g. *M. tuberculosis*, *F. tularensis*, *L. pneumophila*) actively block this process from occurring, ensuring their survival in macrophages (Isberg *et al*., 2009; Philips, 2008; Rohde *et al*., 2007). Similarly, several reports have suggested that virulent *R. equi* may also inhibit phagolysosomal fusion (Hietala & Ardans, 1987; Zink *et al.*, 1987). After phagocytosis, *R. equi*-containing vacuoles (RCVs) mature from early endosomal vacuoles to late endosomal vacuoles, demonstrated by the loss of early endosomal markers PIP3 (phosphatidylinositol-3-phosphate), EEA1 (early endosomal antigen-1) and Rab5, and the acquisition of late endosomal markers LAMP-1 (lysosome-associated membrane protein 1), LBPA (lysobisphosphatidic acid) and Rab7 (Fernandez-Mora *et al*., 2005). However, RCVs do not fuse with pre-labeled lysosomes, nor do they acquire hallmark lysosomal markers such as cathepsin D, vATPase, β-glucoronidase activity. Thus, these data support previous reports that *R. equi* blocks phagolysosomal fusion. Whilst, no significant difference in phagolysosomal blockage was evident between virulent plasmid-containing strains and avirulent plasmid-cured strains, virulent strains maintain non-acidified compartments whereas vacuoles containing avirulent plasmid-cured strains acidify progressively. Thus, prevention of acidification of RCVs

and maintaining a vacuolar pH ~6.5 through the exclusion of vATPase, may be a strategy employed by virulent *R. equi* to avoid killing (Fernandez-Mora *et al.*, 2005; Meijer & Prescott, 2004). Maintenance of a neutral vacuolar pH is essential, since *R. equi* can only withstand acid environments milder than pH 4.0, below which the pH is bactericidal to *R. equi* (Benoit *et al*., 2000).

Another advantage *R. equi* possesses is its resistance to reactive oxygen intermediates (ROIs), which are a long appreciated component of the innate immune response of the macrophage. Toxic ROIs (e.g. superoxide [0_2^-], hydrogen peroxide [H_20_2], hydroxyl radical [OH^-]) are produced through the combined action of NADPH oxidase which converts molecular oxygen into superoxide, and superoxide dismutase which converts superoxide to hydrogen peroxide (Benoit *et al*., 2002). *R. equi* is resistant to killing by superoxide and hydrogen peroxide presumably through the action of bacterial superoxide dismutases and catalases. Likewise, *R. equi* is resistant to the direct bactericidal effects of nitric oxide (NO) (Darrah *et al*., 2000) produced by macrophages through the catalytic oxidation of L-arginine by inducible nitric oxide synthase (iNOS) (Xie & Nathan, 1994).

Provided the macrophage remains inactivated, *R. equi* can circumvent acidification of the RCV and subsequent fusion with lysosomes, allowing intracellular proliferation that ultimately leads to death of the macrophage. By a variety of molecular methods (i.e. transmission electron microscopy, intracellular staining for annexin V and 7AAD, and western blot analysis of PARP cleavage), it has been shown that *R. equi*-mediated cytotoxicity is necrotic in nature, rather than apoptotic (Luhrmann *et al*., 2004). Infected macrophages lack classical morphological hallmarks of apoptosis, such as densely-packed chromatin, membrane blebbing and nuclear fragmentation (Konkel & Mixter, 2000). This induction of host cell necrosis may be an important contributing

factor to the damage observed in host lung tissues, characterized by necrotic granulomas and cavitation (Meijer & Prescott, 2004). Virulent plasmid-containing strains of *R. equi* showed 20-70% greater cytotoxic effects than plasmid-cured strains, again demonstrating the importance of the virulence plasmid in pathogenesis (Luhrmann *et al*., 2004).

Protective Host Response

As mentioned earlier in this report, *R. equi* is an important pathogen of significant morbidity and mortality in foals less than 6 months of age. Almost all foals are exposed to *R. equi* early in life, yet not all foals develop disease. The reasons for this are still unclear, but are believed to be multi-factorial in nature relating to the infective dose, the virulence of the infecting strain, and the development of an appropriate immune response.

Antibody-mediated Immunity

While antibody may play a role in opsonizing *R. equi*, thus promoting bacterial phagocytosis and subsequent clearance by macrophages and neutrophils (Cauchard *et al.*, 2004; Hietala & Ardans, 1987; Martens *et al.*, 1988; Martens *et al.*, 2005), the extent of the role of humoral immunity in protection against *R. equi* infection is unclear. In foals, a declining maternal antibody coincides with susceptibility to *R. equi* disease, suggesting the importance of antibody in protection. This is supported by the finding that passive transfer of hyperimmune sera to naïve foals prior to a subsequent challenge can reduce or prevent the development of pneumonia in infected foals (Madigan *et al*., 1991; Martens *et al*., 1989). However, the use of hyperimmune serum to protect foals is controversial; many subsequent reports have shown no beneficial effects on foal protection following hyperimmune plasma treatment (Giguere *et al.*, 2002; Hurley & Begg,

1995). Passive transfer of *R. equi*-specific antibody also does not protect mice, as it does in foals (Nordmann *et al*., 1992b). Thus, the importance of an antibody-mediated immune response is unclear.

Cell-mediated Immunity

Once *R. equi* has bound and entered macrophages, the bacterium is no longer accessible to antibody, thus primary control of the infection is likely dependent on a robust cell-mediated host immune response (Hines *et al*., 1997). The susceptibility of immunodeficient mice and AIDS patients (with low CD4+ T cell counts) to *R. equi*-induced pneumonia emphasizes the importance of the cellular immune response in clearance. Both severe combined immunodeficient (SCID) mice and T cell-deficient athymic nude mice (*nu/nu*) are highly susceptible to *R. equi* disease and are unable to clear infection (Bowles *et al*., 1987; Yager *et al*., 1991), in contrast to immunocompetent mice which clear *R. equi* readily after infection (Yager *et al*., 1991). The importance of CD4+ and CD8+ T cell subsets for clearance of *R. equi* infection have been shown unequivocally by either antibody-mediated depletion of CD4+ and CD8+ lymphocytes *in vivo* or through the use of the respective T lymphocyte-deficient mice (Kanaly *et al*., 1993; Nordmann *et al*., 1992b). The absence, or depletion, of either CD4+ or CD8+ T cell subsets renders normally resistant mice highly susceptible to *R. equi* disease. Furthermore, the importance of both CD4+ and CD8+ T lymphocytes can be demonstrated by experiments in which splenocytes from previously-infected, immunocompetent BALB/c mice can be transferred to SCID/beige mice, significantly protecting the mice from subsequent intranasal challenge (Ross *et al*., 1996). Thus, it is clear that functional T cell responses are required for clearance of *R. equi*.

In mice and horses, CD4+ T lymphocytes can be subdivided into Th1 and Th2 subsets based on distinct patterns of cytokine secretion (Hines *et al*., 1997). Differentiation of CD4+ T cells into either Th1 or Th2 lymphocytes is driven by IL-12 and IL-18, or IL-4, respectively (Rao & Avni, 2000). Th1 lymphocytes are characterized by the expression of IL-2, IFN-γ, TNF-α and lymphotoxin (LT), whereas Th2 lymphocytes characteristically express IL-4, IL-5 and IL-10 (Mosmann & Coffman, 1989). Differentiation of CD4+ T cells into either Th1 or Th2 populations can profoundly determine the outcome of an infection. Through MAb-depletion experiments it was determined that IFN-γ and TNF-α are essential for clearance of *R. equi*, suggesting that a Th1 response is required for protection (Kanaly *et al*., 1995; Nordmann *et al*., 1993). These results were supported by subsequent studies that demonstrated that adoptive transfer of Th1 lymphocytes into nude mice could induce expression of IFN-γ mRNA locally and enhance clearance of *R. equi* from the lungs, whereas transfer of Th2 cells was detrimental to the mice, which could not clear infection and developed large granulomas in the lung (Kanaly *et al*., 1996). Thus, Th1 lymphocytes are necessary for resistance to and clearance of *R. equi*, a finding that has been well characterized in other intracellular bacteria (e.g. *Mycobacteria* SPP, *Salmonella* SPP) (Harris *et al*., 2008; Jouanguy *et al*., 1999).

The mechanism by which Th1 lymphocytes mediate their protective effects is primarily through IFN-γ secretion which activates host macrophages, the main effector cells of the immune response to intracellular bacteria. A direct consequence of macrophage activation is a significant increase in the bactericidal activity of the macrophages and their efficiency in clearing infections (Zhang & Mosser, 2008). This increased bactericidal activity is believed to be related to upregulation of ROI and RNI production, as well as enhanced phagolysosomal fusion (Pieters, 2008). Macrophage activation can also lead to the induction of autophagy which may promote

fusion with lysosomes (Gutierrez *et al*., 2004). While wildtype *R. equi* can readily survive and replicate within non-activated macrophages, IFN-γ induced activation of infected macrophages has been shown to restrict the intracellular growth of *R. equi*, possibly due to the production of bactericidal peroxynitrite ($ONOO^-$) formed via the combination of O_2^- and NO (Darrah *et al*., 2000). Peroxynitrite is capable of killing *R. equi* rapidly *in vitro*, and deficiencies of the NADPH oxidase and or iNOS enzyme render normally resistant C57BL/6 mice hypersusceptible to infection by *R. equi*, indicating that components of both pathways are essential for protection against *R. equi* infection (Darrah *et al*., 2000). If mice are treated with monoclonal anti-IFN-γ antibody, they are no longer able to clear infection with *R. equi* and develop significant granulomas in the lung (Kanaly *et al*., 1995; Kasuga-Aoki *et al*., 1999). In addition, *R. equi* infection of IFN-γ knockout mice is lethal (Darrah *et al*., 2000).

While CD4+ T lymphocytes promote clearance of *R. equi* via IFN-γ secretion and subsequent macrophage activation, CD8+ T lymphocytes promote clearance both through cytokine secretion and also direct cytotoxic activity. *R. equi*-infected adult horses develop strong *R. equi*-specific CD8+ CTL responses in the blood which can recognize and kill infected targets (Patton *et al*., 2004). When lysis of targets infected with virulent or avirulent *R. equi* was compared, no significant difference was observed, suggesting that virulent *R. equi* do not interfere with antigen presentation to CTLs. Interestingly, the authors also showed that recognition of *R. equi* infected macrophages occurred in a MHC-Class I unrestricted fashion suggesting a non-classical method of antigen processing and presentation (Patton *et al*., 2004). Comparison of CTL responses in the lungs of foals compared with adult horses demonstrated that CTL activity was deficient in foals at 3 weeks of age, and significantly lower than that of adults in foals between 3-6 weeks of age (Patton *et al*., 2005). These data suggest that

deficiencies in the development of *R. equi*-specific CTL in the lungs at the time foals are presumably first exposed to the organism may contribute to the susceptibility of foals to development of *R. equi* disease.

CONCLUSION

Rhodococcus equi is the most important bacterial pathogen of foals and is also an opportunistic infectious agent of immunocompromised people. In both populations of susceptible individuals, infection results in bronchopneumonia, which can be fatal if not diagnosed early or treated appropriately. Even with antibiotic therapy, significant mortality is observed in infected individuals emphasizing the need for an efficacious vaccine. Unfortunately, no commercial vaccine exists to date. Much remains to be understood regarding the pathogenesis of this organism and the genetic components essential for disease development. The goal of this dissertation has been to identify the specific virulence plasmid-encoded genes required for replication in macrophages and the establishment of chronic disease in the host.

CHAPTER 3

CHARACTERIZATION OF THE ROLE OF THE PATHOGENICITY ISLAND AND *vapG* IN VIRULENCE OF THE INTRACELLULAR ACTINOMYCETE PATHOGEN, *RHODOCOCCUS EQUI*[1]

[1] Coulson G.B., S. Agarwal and M.K. Hondalus. 2010. *Infect. Immun.* 78(8):3323-3334
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ABSTRACT

Rhodococcus equi, a facultative intracellular pathogen of macrophages, causes severe, life-threatening pneumonia in young foals and people with underlying immune deficiencies. *R. equi* virulence is dependent on the presence of a large virulence plasmid that houses a pathogenicity island (PAI) encoding a novel family of surface-localized and secreted proteins of largely unknown function termed the virulence associated proteins (VapACDEFGHI). To date, *vapA* and its positive regulators *virR* and *orf8* are the only experimentally established virulence genes residing on the virulence plasmid. In this study, a PAI-deletion mutant was constructed, and as anticipated, was attenuated for growth both in macrophages and in mice due to the absence of *vapA* expression. Expression of *vapA* in the PAI mutant from a constitutive promoter, thereby eliminating the requirement of the PAI-encoded *vapA* regulators, resulted in delayed bacterial clearance *in vivo,* yet full virulence was not restored, indicating that additional virulence genes are indeed located within the deleted pathogenicity island region. Based on previous reports demonstrating that the PAI-encoded gene, *vapG*, is highly upregulated in macrophages and in the lungs of *R. equi* infected foals, we hypothesized that *vapG* could be an important virulence factor. However, analysis of a marked deletion mutant of *vapG* determined the gene to be dispensable for growth in macrophages and *in vivo* in mice.

INTRODUCTION

Rhodococcus equi is a soil-borne, facultative intracellular Gram-positive pathogen belonging to the order *Actinomycetales*. While *R. equi* has been reported to infect a varying number of different animal hosts (e.g. pigs, sheep and cattle) (Prescott, 1991), the greatest burden of *R. equi* disease is in its primary host, young foals between the ages of 1 and 6 months, in whom mortality rates if untreated can approach 80% (Cohen *et al*., 2005; Elissalde *et al*., 1980; Muscatello *et al*., 2007; Zink *et al*., 1986). Even despite treatment, ~30% fatality rates have been reported in foals infected with this organism (Ainsworth *et al*., 1998; Giguere *et al*., 2004). Currently, there are no commercially-available vaccines licensed to prevent disease caused by *R. equi*. In addition to being an important cause of serious disease in foals, this bacterium has also emerged as an opportunistic pathogen of immunocompromised humans with conditions such as HIV infection, organ transplantation, chemotherapy, steroid therapy, diabetes mellitus and alcoholism seen as risk factors for disease (Arlotti *et al*., 1996; Borghi *et al*., 2008; Lasky *et al*., 1991; Munoz *et al*., 1998; Speck *et al*., 2008). In immunocompromised patients, the mortality rate is 50-55% among HIV-infected individuals, compared to 11% in immunocompetent patients (Cornish & Washington, 1999; Harvey & Sunstrum, 1991; Kedlaya & Ing, 2008). In both susceptible foals and humans, *R. equi* disease commonly presents as a pyogranulomatous pneumonia (Hondalus & Mosser, 1994), although numerous other clinical manifestations may occur concomitantly. Extrapulmonary disorders (EPDs) in foals infected with *R. equi* such as abdominal lymphadenitis, ulcerative enterotyphlocolitis and pyogranulomatous hepatitis are associated with a significant reduction in survival rates (Reuss *et al*., 2009).

The ability of *R. equi* to resist macrophage killing and replicate intracellularly is linked to possession of large plasmid which, in all equine isolates and 30% of human isolates, is

approximately 80 kb in size. *R. equi* strains cured of the plasmid are avirulent in foals and murine models of infection and are unable to replicate in macrophages cultured *in vitro* (Hondalus & Mosser, 1994; Takai *et al.*, 1991c). Sequencing and annotation of the virulence plasmids from two distinct foal isolates, *R. equi* strains 103+ and 33701, has revealed that the plasmid has 73 coding sequences (CDS), including 8 pseudogenes (Letek *et al*., 2008; Takai *et al*., 2000b). Based on homology searches, the virulence plasmid can be divided into 4 regions, specifically the areas of replication/partitioning, conjugation, unknown function, and a ~21 kb region with features typical of a pathogenicity island (PAI) (Letek *et al*., 2008; Takai *et al*., 2000b). The PAI is believed to have been acquired through horizontal gene transfer from a source of unknown origin. Computational analysis of the PAI has revealed that it contains 26 putative CDSs, including the unique and *R. equi*-specific family of proteins, the Vap family (virulence associated protein) (Letek *et al*., 2008). There are six full length *vap* genes, (*vapA*, *-C, -D, -E, -G, -H*) and three *vap* pseudogenes (*vapF*, *-I* and *-X*) (Letek *et al.*, 2008; Polidori & Haas, 2006; Takai *et al.*, 2000b). These pseudogenes are thought not to encode functional protein as they either exhibit substantial truncations and/or frameshift mutations in their coding sequences.

All strains of *R. equi* isolated from infected foals are positive for VapA, an immunodominant lipoprotein located on the bacterial surface (Takai *et al*., 1991a; Takai *et al*., 1991c). VapA is required for intracellular growth in macrophages (Jain *et al*., 2003), wherein it may aid in preventing fusion of *R. equi*-containing vacuoles with lysosomes (Fernandez-Mora *et al*., 2005; Toyooka *et al*., 2005; von Bargen *et al*., 2009). An *R. equi vapA*-deletion mutant is unable to establish a persistent infection and is rapidly cleared by Severe Combined Immunodeficient (SCID) mice (Jain *et al*., 2003). Two genes, *virR* and *orf8*, also encoded in the pathogenicity island region are needed for optimal *vapA* transcription. (Ren & Prescott, 2003;

Russell *et al.*, 2004). VirR is a LysR-type transcriptional regulator which binds to the *vapA* promoter (Russell *et al*., 2004), whilst homology analyses suggest that Orf8 is an orphan two component response type regulator (Takai *et al*., 2000b). Loss of either regulator results in attenuation of the organism (Ren & Prescott, 2003).

Although *vapA* is necessary, it is not sufficient for virulence, since expression of wildtype levels of VapA in a plasmid-cured derivative of a virulent strain does not restore virulence in mice or foals (Giguere *et al*., 1999), indicating that virulence determinants in addition to *vapA*, reside on the virulence plasmid. To date, with the exception of the key regulators of *vapA* expression, *virR* and *orf8*, (Ren & Prescott, 2004; Russell *et al.*, 2004), the location and identity of these other virulence determinants remain unknown and determining such has been the focus of this work. Herein we describe the construction of a PAI deletion mutant which was used as a molecular tool to determine the location of plasmid-encoded virulence factors, aside from *vapA*. Our data show that these determinants do indeed reside within the PAI region. Based on sequence and expression data, we and others have hypothesized that *vapG* could be an important virulence determinant of *R. equi* (Benoit *et al.*, 2001; Benoit *et al.*, 2002; Jacks *et al.*, 2007; Ren & Prescott, 2003). To assess this hypothesis experimentally, a marked deletion mutant of *vapG* was constructed using a two-step allelic exchange strategy and the effect of the deletion on the ability of the mutant strain to survive and replicate in macrophages and *in vivo* in mice was evaluated.

MATERIALS AND METHODS

Bacterial strains. Strains used are listed in **Table 1**. Virulent *R. equi* strain 103+ was originally isolated from a foal with *R. equi* pneumonia and was kindly provided by J. Prescott

(Guelph, Ontario, Canada). Avirulent *R. equi* strain 103- was derived by serial subculture of strain 103+ at 37°C to facilitate loss of the virulence plasmid (Jain *et al*., 2003). The *vapA* deletion mutant on the 103+ strain background was derived as described earlier (Jain *et al*., 2003). The standard culture medium used was brain heart infusion (BHI; Difco Laboratories, Detroit, MI) broth or agar, and unless otherwise noted, cultures were incubated at 30°C to help maintain the presence of the *R. equi* virulence plasmid. Antibiotics, when necessary, were used at the following concentrations: apramycin, 80 μg/ml; hygromycin, 180 μg/ml**.**

Antisera. Rabbit polyclonal serum to *R. equi* was raised as previously described (Hondalus & Mosser, 1994). Rabbit polyclonal serum to VapA was generated as reported earlier (Jain *et al*., 2003).

Plasmid construction. Plasmids used are listed in **Table 1**. The first cloning step to construct the suicide plasmid used in making the pathogenicity island mutant was restriction digestion of pBlueScript (Stratagene; La Jolla, CA) with *Ssp*I and *Dra*III to remove all restriction sites between them. The remaining vector fragment was re-circularized to get pSJ42. Next, a 700bp DNA fragment located immediately downstream of *orf22* was amplified with primers ORF22(f) and ORF22(r) **(Table 2)**, and cloned into the *Eco*RV site of pSJ42 to yield pSJ47. A 583bp amplicon covering the region upstream of *orf62* was amplified with primers ORF62u(f) and ORF62u(r) **(Table 2)** and cloned into pSJ47 digested with *Xba*I to give pSJ48. Subsequently, a 1.1 kb DNA fragment encoding the apramycin resistance gene, *aacC4*, was obtained by digesting pSC146 (Ashour & Hondalus, 2003) with *Pst*I and *Pml*I, and was end-repaired with T4 DNA polymerase. It was then cloned into pSJ48 which had been digested with *Age*I and *Sgr*AI. This resulted in pSJ49 which carried *aac4* flanked by DNA upstream of *orf62* on its 5' end and downstream of *orf22* on its 3' end. Finally, a 3.5 kb DNA fragment containing the *E. coli lacZ*

gene was obtained by digesting pMV261-lacZ2 (Steyn *et al*., 2003) with *Pst*I and *Dra*I followed by end repair. It was cloned in the *Sca*I site of pSJ49 after filling its sticky ends with Klenow fragment to get pSJ53.

The *vapA*-expression plasmid, pMV261-*vapA* (Hong & Hondalus, 2008), was constructed by in-frame cloning of a *vapA*-containing fragment immediately downstream of the constitutive mycobacterial *hsp60* promoter in the vector pMV261 (Stover *et al*., 1991) to allow for *vapA*-expression independently of its two regulators, *virR* and *orf8*, which would be absent in the PAI-deletion mutant strain.

To generate the suicide plasmid used to construct the *vapG* mutant, an 858 bp region downstream of *vapG* was amplified by PCR using primer pairs *vapG*-up-F and *vapG*-up-R **(Table 2)**. The PCR product was digested with restriction enzymes *EagI* and *BamHI* and cloned into pSelAct (van der Geize *et al*., 2008) (a gift generously provided by R. van der Geize) which had been digested by the same enzymes, giving rise to pJM10. A 669 bp region of DNA upstream of *vapG* was amplified using the primers *vapG*-down-F and *vapG*-down-R **(Table 2)** and digested with *BamHI* and *EcoRI*. This fragment was then ligated to the downstream fragment by digesting pJM10 with *BamHI* and *EcoRI*, giving rise to the plasmid pJM11. The mycobacterial shuttle plasmid pMV261.hyg (Giguere *et al*., 1999) was digested with *DraI* and *HpaI* to yield a blunt-ended 1.4kb fragment containing a hygromycin resistance cassette (*hyg*). The hygromycin-containing fragment was then cloned in between the two *vapG* flanks in pJM11 by digesting pJM11 with *BamHI* and blunt-ending the overhangs by Klenow treatment, giving rise to pJM11.hyg. The plasmid pJM11.hyg was then digested with *PvuII* to yield a blunt-ended 3.3 kb fragment containing both *vapG* flanks ligated to the hygromycin resistance cassette. This fragment was then cloned into pMV261.lacZ2 which had been previously digested with *SgrAI*

and *KpnI* and Klenow treated to create compatible blunt ends. Digestion with *SgrAI* and *KpnI* removed the *oriM* from pMV261.lacZ2, thus allowing the resulting plasmid, pGBC14.1lacZ to be used as a suicide plasmid to generate the *vapG* mutant. To confirm the deletion of *vapG*, we performed PCR using several primer pairs including vapG-up-F:*vapG*-down-R; *vapG*-F:*vapG*/*podG*-R and *vapG*-int-F:*vapG*-int-R. Further confirmation of the deletion of *vapG* was achieved by reverse-transcriptase PCR (RT-PCR) and DNA sequencing.

To create the complementing plasmid for the *vapG* mutation, a 36 bp fragment from pETDuet-1 (Novagen; Madison, WI) containing the multiple-cloning site (MCS-2) was amplified using primers MCS-F and MCS-R. The PCR product was digested with *BamHI* and *DraI* and cloned into pSET152 (Hong & Hondalus, 2008), which had been previously digested with *BamHI* and *EcoRV*, giving rise to pSET152-AM. The plasmid pSET152-AM was subsequently digested with *BamHI* and *NdeI* to allow for the insertion of a constitutive *hsp60* promoter as a 442 bp fragment amplified from pMV261 using primers F_HSP60 and HSP60_R, giving rise to the integrating plasmid, pSET152-AMH. Next, a 553bp *vapG*-containing fragment was amplified by PCR using the primers *vapG*-comp-*NdeI* and *vapG*-comp-*EcoRV*. The resulting PCR fragment was digested with *NdeI* and *EcoRV* and cloned in-frame immediately downstream of the *hsp60* promoter in the integrating plasmid, pSET152-AMH. PCR and RT-PCR were used to confirm the complementation of the *vapG* mutant with a wildtype copy of *vapG*.

Construction of *R. equi* mutants. The suicide vector pSJ53, containing flanking regions of the PAI ligated to either side of the gene encoding apramycin resistance and the *lacZ* gene encoding β-galactosidase on its backbone, was used to derive the pathogenicity island mutant of *R. equi* 103+ in which nearly 25.6 kb spanning ORF62 through ORF22 (coordinates: 77178-

23501) was deleted, using the method described in Jain *et al*., 2003 (Jain *et al*., 2003). In brief, pSJ53 was electroporated into *R. equi* and transformants were selected on BHI plates supplemented with apramycin (80µg/ml) and X-gal (40µg/ml), the chromogenic substrate of β-galactosidase. Blue transformants were screened by PCR (data not shown) to confirm them as single crossover intermediates in which the entire pSJ53 plasmid had integrated into the virulence plasmid at either of the flanks of the PAI. Two blue colonies that were PCR positive for the single crossover were serially subcultured in BHI containing apramycin at 30°C to facilitate a second recombination event between the wildtype PAI and its mutated version resulting in a looping out of the intervening vector sequence, including the *lacZ* gene. Following plating on BHI agar supplemented with apramycin and X-gal, the resulting white colonies were analyzed by PCR to confirm the loss of the PAI region and retention of the virulence plasmid backbone (Jain *et al*., 2003). A similar two-step allelic exchange procedure was performed to construct the *vapG* deletion mutant using the suicide vector pGBC14.1lacZ except that hygromycin at 180µg/ml was used for antibiotic selection.

Electroporation of *R. equi*. *R. equi* strains were grown in 200ml BHI broth to an optical density at 600nm of ~0.8. Bacteria were pelleted and washed twice with an equal volume of cold distilled water, before finally being resuspended in 10ml of cold 10% glycerol made in dH_20. Aliquots (400µl) of the cells were made and stored at -80°C. For electroporation, an aliquot of cells were mixed with 0.5µg of plasmid DNA and placed in a prechilled 0.2cm electroporation cuvette. Electroporation was performed using a Gene Pulser (Bio-Rad) set at 2.5kV, 25µF, 1000Ω and single pulse. Immediately after electroporation, 1ml of BHI broth supplemented with 0.5M sucrose was added to the cuvette. Bacteria were then incubated for 1hr at 30°C and subsequently plated on BHI agar supplemented with the appropriate antibiotics.

RNA isolation. 5ml bacterial cultures were grown to mid-logarithmic phase (OD_{600nm} of 0.8), after which 1ml of culture (~1 x 10^8 bacteria) was removed and incubated with 2 volumes RNAprotect® Bacteria Reagent (Qiagen; Valencia, CA) and bacteria subsequently harvested by centrifugation at 4000 X g for 10 min. The cells were resuspended in 700µl RLT buffer (RNeasy® mini kit; Qiagen) and added to 0.1mm diameter acid-washed zirconia/silica beads (BioSpec; Bartlesville, OK). The bacterial samples were lysed for 5min at maximum speed with a Mini BeadBeater-1 (BioSpec) cell disruptor. Total RNA was subsequently isolated using the RNeasy® Mini Kit (Qiagen) according to the manufacturers' instruction.

Reverse-Transcriptase (RT) PCR. Total RNA in each sample was quantified by UV spectrophotometry and cDNA was synthesized using the ThermoScript™ RT-PCR system (Invitrogen; Carlsbad, CA) from equal amounts (1µg) of RNA template using 50ng of the supplied random hexamer primers and 15 U of ThermoScript™ RT enzyme according to manufacturers' instructions. One-tenth of the cDNA reaction mixture (2µl) was then used as template for standard PCR amplification with *Pfu* DNA polymerase (Stratagene; La Jolla, CA) using primer pairs specific for *vapG* (vapG-int-F and vapG-int-R) or the internal housekeeping gene control, *gyrB* (gyrB-qPCR-F and gyrB-qPCR-R) as listed in **Table 2**.

Western blot analysis. Bacterial strains were grown overnight at 37°C in 5ml BHI and optical density at 600nm adjusted to 1.0. A 1ml aliquot from each suspension was pelleted in a microcentrifuge set at 13,000rpm for 5min. The supernatant was discarded and the bacterial pellet resuspended in 100µl SDS gel-loading buffer (100mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200mM DTT) and boiled for 10min at 100°C. After allowing the sample to cool, 10µl of each sample was loaded onto a precast 8-16% Tris-Glycine 1.0mm PAGE Gel (Invitrogen; Carlsbad, CA), and run at 80V for 30min, followed by 1hr at 120V.

Following electrophoresis, proteins were transferred to a 0.45 micron nitrocellulose membrane using the Bio-Rad Transblot apparatus set at 150mA for 3h. Once transfer was complete, the membrane was immersed in Blocking Buffer (Pierce Scientific; Rockford, IL) overnight at 4°C. Labeling of VapA protein was done with 1:1000 dilution of rabbit-anti-VapA antisera for 60min at room temperature, followed by repeated washing with Tris Wash Buffer (Pierce Scientific) to remove unbound antibody. The membrane was then incubated in 1:20,000 dilution of HRP-conjugated goat anti-rabbit antibody (Pierce Scientific) for 60min at room temperature. Following repeated washes, detection of protein was performed using Pierce SuperSignal Pico ECL kit (Pierce Scientific) as per manufacturer's instructions.

Flow cytometry. After overnight culture at 37°C in BHI, bacteria were washed twice and resuspended in cation-free phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). Approximately 10^8 CFU of bacteria were incubated at room temperature with a 1:500 dilution of rabbit immune serum containing α-VapA polyclonal antisera. After being washed three times with PBS containing 1% BSA, bacteria were stained by incubation at room temperature with a 1:500 dilution of $Alexa^{488}$-conjugated goat anti-rabbit immunoglobulin G (IgG; Invitrogen) for 45min. Bacteria were then washed three times with PBS supplemented with 1% BSA, fixed in 2% paraformaldehyde, and analyzed on a Becton Dickenson LSR II Flow Cytometer (BD; Franklin Lakes, NJ). Negative controls included unstained bacteria, the addition of secondary antibody without prior incubation in primary antibody and the use of preimmune serum as IgG control.

Macrophages and macrophage cell lines. Murine RAW264.7 macrophages were maintained in DMEM supplemented with 10% FCS and 2mM glutamine, and incubated at 37°C in 5% CO_2. For intracellular growth assays, macrophages were seeded in 24-well tissue culture

plates at $1x10^5$ cells per well. Equine alveolar macrophages were obtained by bronchoalveolar lavage (BAL) of healthy adult horses. Horses were sedated intravenously with xylazine (0.4 mg/kg; Butler Co., Dublin, OH) and butorphanol tartrate (Torbugesic, 0.02 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). A sterile 1.8 m bronchoalveolar lavage (BAL) catheter (Jorvet, Loveland, CO) was passed via nasal approach until wedged into a bronchus. The lavage solution consisted of 4 aliquots of 60 mL physiologic saline (0.9% NaCl) solution infused and aspirated immediately. The resulting BAL exudate cells were washed twice in PBS supplemented with 9mM EDTA, penicillin G and streptomycin (100 U/ml and 100µg/ml, respectively). Cells ($5 x 10^5$) were incubated for 4 h at 37°C and 6% CO_2 in Minimal Essential Media Alpha (αMEM, Hyclone, Waltham, MA) supplemented with 12% DHS (donor horse serum, Hyclone) 2mM glutamine and penicillin G/streptomycin in DHS-coated 24-well plates. Following incubation, adherent macrophages were washed twice with warm αMEM to remove non-adherent cells and incubated for a further 3h in antibiotic-free media. Macrophages were then washed again prior to infection with *R. equi*.

Intracellular growth of *R. equi* in macrophages. Overnight broth cultures of bacteria at an optical density at 600nm of 1.0 (~$2x10^8$ CFU/ml) were pelleted, washed once with PBS and resuspended in PBS. Murine macrophage monolayers were washed once with warm DMEM, and the medium was replaced with fresh DMEM supplemented with 10% FCS and 2mM glutamine. Bacteria were added at a multiplicity of infection (MOI) of ~10 bacteria per macrophage. Bacterial incubation with macrophages proceeded for 45 min at 37°C, and then the monolayers were washed repeatedly with pre-warmed DMEM to remove unbound bacteria. After an additional 15 min incubation period to allow bound bacteria to be internalized, the monolayers were washed again, and the medium was replaced with complete DMEM supplemented with

20µg/ml of amikacin sulfate. At various times postinfection, macrophage monolayers were washed repeatedly, and 500µl sterile water was then added to lyse the macrophages upon further incubation at 37°C for 20min. Bacterial growth was determined by dilution plating of lysates onto BHI agar plates. CFU were enumerated after a 48 h incubation at 37°C.

Bacterial survival in equine macrophages was determined by immunofluorescent staining, as previously described (Jain *et al*., 2003). Basically, *R. equi* strains were grown in BHI broth overnight at 37°C. The bacteria were pelleted and diluted in αMEM supplemented with non-heat inactivated DHS to a concentration of 1×10^7 cfu/ml. Equine alveolar macrophage monolayers were cultured in 24-well plates (~1×10^5/well) and infected at an MOI of 1:1 as described above. At 1, 12 and 24 h after infection, monolayers were washed once with PBS and fixed with 100% methanol for 45 min at 4°C, washed twice and stored in 1× PBS with 5% FCS. Bacteria associated with macrophages were stained by incubating monolayers with rabbit anti-*R. equi* antibodies for 1 h at room temperature (RT). After washing with 1× PBS-5% FCS three times, monolayers were incubated with Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) for 1 h in the dark at RT. Finally, the macrophage monolayers were washed four times as before and examined under a Zeiss Axiovert 200M fluorescence microscope. Two hundred macrophages per well were counted, and the number of bacteria associated with these cells was determined for each strain at each time point. Any cell containing 10 or more bacteria was simply scored as having 10 bacteria due to the difficulty in reliably quantifying large bacterial numbers within an individual macrophage.

Infection of mice. Female Severe Combined Immunodeficient (SCID) mice were obtained from either Charles River (Wilmington, MA) or Jackson Laboratories (Bar Harbor, MN). Mice were received at 6 weeks of age and were used when they were between the ages of

8 and 10 weeks. For the infection of mice, frozen aliquots of the bacterial strains were thawed and grown for 1h at 37°C in BHI broth. Bacteria were pelleted and resuspended in PBS. Depending on the experiment, groups of mice were infected intravenously through the tail vein with approximately 5 x 10^5 or 5x10^6 bacteria. The total number of bacteria injected was confirmed retrospectively by dilution plating of the injection stock. At various times postinfection, four mice from each group were euthanized, and their spleens, livers and lungs were removed. Each organ was placed in sterile PBS and homogenized with a tissue homogenizer (Seward, Bohemia, NY). Serial 10-fold dilutions of the homogenate were plated onto BHI agar, and CFU counts were determined after 48h of incubation at 37°C.

Statistical analysis. Statistical analyses were performed using the SPSS Inc. statistical package (SPSS version 17.0.1, Chicago, IL). Normal distribution and equal variance of the data were assessed using the Shapiro-Wilk and Levene test, respectively. Comparison of the means in fold replication of intracellular bacterial numbers between bacterial strains was assessed using a one-way Analysis of Variance (ANOVA). When appropriate, multiple pairwise comparisons were done using Tukey's HSD test. Significance was set at a *P* value of <0.05.

RESULTS

Creation of a pathogenicity island deletion mutant of *R. equi*. With the exception of the virulence factor, *vapA*, and its two regulators *virR* and *orf8*, no other virulence determinants on the virulence plasmid have been conclusively identified to date. It is known that *vapA* is not sufficient for virulence since expression of *vapA* alone in a plasmid-less strain does not restore the virulence phenotype. We sought to narrow the search for other plasmid-encoded virulence factors by addressing whether additional virulence determinants reside within the pathogenicity

island (PAI) or are, alternatively, positioned outside of this area within the plasmid backbone. Therefore, an *R. equi* strain in which we deleted a ~26kb stretch of genetic sequence that encompassed the entire pathogenicity island extending from *orf62* to *orf22*, was constructed. A two-step knockout strategy employing a suicide vector with a single crossover intermediate step was used as previously described (Jain *et al*., 2003). To confirm the absence of PAI-encoded genes (i.e. *vapA, vapC, vapD, vapF, vapG* and *vapH*) in the PAI-mutant, PCR analysis using primer pairs (**Fig. 1A**) that would specifically and individually amplify these genes, respectively, was performed. As shown in **Fig 1B**, no amplicons were obtained for any of these primer pairs in the PAI mutant, just as seen with the plasmid-cured strain 103- used as a negative control, thus confirming their absence in the ΔPAI strain. In contrast, all primer pairs yielded an amplicon of the expected size when wildtype 103+ DNA was used as template. To ensure that no gross alterations had taken place in the virulence plasmid backbone during the recombination events leading to the recovery of the PAI mutant, we performed PCR analysis using four primer pair sets annealing to different regions outside of the PAI. As shown in **Fig. 1C**, both wildtype 103+ and the PAI mutant gave PCR products of the correct length for each of the primer pairs used, whereas no specific products were obtained with 103- DNA as template. Having deleted the entire pathogenicity island, the PAI-deletion mutant was then used as a vehicle for constitutive expression of *vapA* to assist in pinpointing the likely location of plasmid regions required for virulence. To do so, the PAI mutant was transformed with an episomal recombinant plasmid, pMV261-*vapA,* in which the virulence determinant *vapA* was expressed from the mycobacterial heat shock promoter *hsp60,* thus allowing for constitutive expression of *vapA* independently of its two regulators, *virR* and *orf8.* The resulting strain, ΔPAI/*vapA,* was analyzed by Western blotting using specific polyclonal antiserum to VapA to confirm the presence of VapA protein.

As shown in **Fig. 2A**, the PAI mutant (lane 6) lacks VapA, whereas the PAI mutant transformed with pMV261-*vapA* (lane 7) produces VapA protein at levels comparable to *R. equi* wildtype strain 103+ exposed to inducing conditions (lane 1). To ensure that the VapA protein produced in the PAI mutant was located on the bacterial cell surface, and was not inappropriately confined to the cytosol due to possible disruption of normal protein targeting in the mutant, surface-localized VapA was quantified by flow cytometry. Using rabbit polyclonal antiserum as primary antibody and fluorescently-conjugated goat anti-rabbit IgG as secondary antibody, the ΔPAI/*vapA* strain was confirmed to have wildtype amounts of VapA on its surface (**Fig. 2B**).

VapA does not restore the virulence phenotype to the PAI mutant. Given that the PAI mutant does not possess the crucial virulence determinant *vapA*, the expression of which is essential for replication of *R. equi* in *in vitro* cultured macrophages and *in vivo* in mice and foals (Jain *et al*., 2003), it was anticipated that the strain would be compromised in both settings. As expected, the PAI mutant was fully attenuated, displaying intramacrophage growth kinetics that mirrored those of the avirulent plasmid-cured strain 103- (**Fig. 3A**), both of which showed ~10-fold or greater reduction in CFUs at 48h post-infection (**Fig. 3B**). The latter is in contrast to the growth of wildtype bacteria wherein a ~10-fold increase in macrophage associated CFU was apparent at 48h. We next assessed whether or not the intracellular replication potential of the PAI mutant could be restored by expression of *vapA* in the mutant (**Fig. 2A and 2B**). It was reasoned that if production of wildtype levels of VapA protein in the PAI mutant did not restore intracellular growth, it would indicate that additional virulence determinants reside within the pathogenicity island of *R. equi*. The results show that the production of VapA protein allowed the PAI mutant to persist in macrophages, with the *vapA*-expressing strain showing a significant positive difference in fold growth compared to the PAI mutant (3-fold increase vs 10-fold

decrease, respectively; $p < 0.001$) at 48h post-infection. Although expression of *vapA* in the PAI mutant allowed the strain to survive better in macrophages than the PAI mutant without *vapA*, the strain was still not able to efficiently replicate, with a significant difference ($p = 0.001$) in CFUs at 48hpi compared to wildtype 103+ **(Fig. 3A and 3B)**. Similarly, production of VapA protein by the PAI mutant also allowed it to survive slightly longer *in vivo* in SCID mice which are incapable of clearing an infection of wildtype *R. equi* **(Fig. 4)**. While the PAI mutant showed rapid clearance at a rate almost identical to the plasmid-cured strain 103-, the *vapA*-expressing PAI mutant displayed delayed clearance in the spleens and livers **(Fig. 4A and 4C)** relative to the PAI mutant. At 14 days post-infection, the *vapA*-expressing PAI mutant had a 158-fold higher CFU count in the liver (**Fig. 4C)** and a ~20-fold higher CFU in the spleen relative to the PAI mutant. Nonetheless, production of VapA was not enough; bacterial numbers of the *vapA*-expressing PAI mutant still showed a ~3-log decline in both the spleen (**Fig. 4A**) and liver (**Fig. 4C**), and complete clearance in the lung (**Fig. 4B**) by 7 days post-infection. In contrast, wildtype bacteria had increased 454-fold in the spleen (**Fig. 4A**); 30-fold in the liver (**Fig. 4C**) and held steady in the lung (**Fig. 4B**) during this same time frame. The inability of *vapA* expression to restore the virulence phenotype to the PAI mutant either in macrophages or *in vivo* in mice was not due to some defect in the pMV261-*vapA* expression plasmid, as complete restoration of bacterial growth in macrophages (data not shown) and replication and persistence in mice **(Fig. 4)** was observed in a *vapA* deletion mutant transformed with this complementing plasmid.

Creation of a *vapG* mutant and *vapG* complementing strain of *R. equi*. The apparent failure of *vapA* expression to restore wildtype virulence to the PAI mutant suggested that *vapA* is not the only virulence determinant within the pathogenicity island region, and that additional virulence determinants reside in the island. Because it possessed characteristics typical of

virulence factors, it was hypothesized that another *vap* gene, *vapG*, was a logical candidate for an *R. equi* virulence determinate. To test this hypothesis, an *R. equi* strain with the *vapG* gene deleted by means of a two-step knockout strategy similar to that as described earlier for the PAI mutant, was constructed. PCR analysis with specific diagnostic primer pair sets confirmed that the gene for *vapG* had been replaced by the 1.4kb DNA fragment encoding for hygromycin resistance (**Fig. 5A**). For example, PCR analysis using primer pair vapG-up-F and vapG-down-R yielded a 2.1kb amplicon in wildtype 103+ and the anticipated 2.9kb product in the mutant (**Fig 5A**). Likewise, primer pair *vapG*-F:*vapG*/*pod*G-R gave ~700bp and ~1.6kb amplicons in the wildtype and the Δ*vapG* mutant, as expected. Finally, using primer pairs *vapG*-int-F and *vapG*-int-R which anneal at sites internal to *vapG*, the expected ~250bp amplicon was observed using wildtype DNA as template while no product was obtained with Δ*vapG* mutant DNA. To further confirm deletion of the *vapG* gene, we performed RT-PCR on total RNA extracts using an internal *vapG* primer pair (*vapG*-int-F and -R) as a probe for the presence or absence *vapG* transcripts. The results of the RT-PCR (**Fig. 5B**) confirmed the absence of *vapG* transcripts in the *vapG* mutant. Furthermore, it was verified that transformation of the *vapG* mutant with a wildtype copy of *vapG* resulted in restoration of *vapG* gene expression (**Fig. 5B**).

***vapG* is dispensable for growth in macrophages.** To evaluate whether deletion of *vapG* affected the ability of *R. equi* to replicate intracellularly in macrophages, murine RAW264.7 macrophages were infected with the *vapG* mutant and its intracellular growth capacity relative to that of wildtype *R. equi* strain 103+ and the virulence plasmid-cured strain, 103-, was assessed (**Fig. 6A and 6B**). As expected, the plasmid-cured strain failed to replicate in the macrophages (**Fig. 6A**), whereas the virulent strain 103+ multiplied ~15-fold over a 48h period. The growth of the *vapG* mutant closely mimicked that of wildtype, as did the *vapG* complemented strain

whose numbers increased ~13-fold and 10-fold, respectively, over the same time period (**Fig. 6B**). No significant difference was observed between 103+ and the *vapG* mutant at any time point post-infection, demonstrating that the deletion of *vapG* does not adversely affect the growth of *R. equi* in murine macrophages. Identical results were obtained using murine primary bone marrow-derived macrophages (data not shown). To exclude the possibility that VapG is required solely for growth in macrophages of its native host, we assessed the growth of the *vapG* mutant in equine alveolar macrophages. Similar to findings in the murine macrophages, the *vapG* mutant grew as well as wildtype bacteria in equine macrophages. In these cells, the two bacterial strains exhibited indistinguishable growth curves reflected by identical increases in the total number of bacteria associated with the macrophage monolayer ($p > 0.05$; **Fig. 7A**) and in the number of macrophages with >10 bacteria per macrophage over time ($p > 0.05$; **Fig. 7B**) when quantified by immunofluorescent counting as previously described (Hondalus & Mosser, 1994). In contrast, the plasmid-cured strain failed to replicate in the macrophages (**Fig. 7A, 7B)**. Representative photomicrographs of equine macrophage monolayers infected with wildtype 103+, the *vapG* mutant, the complemented *vapG* mutant strain or the plasmid-cured strain are provided in **Fig. 7C**

***vapG* is dispensable for survival and growth *in vivo*.** Although loss of *vapG* did not affect the macrophage growth capabilities of the bacteria, we reasoned that *vapG* might be necessary specifically for survival *in vivo,* as has been observed to be the case with some virulence proteins of *Mycobacterium tuberculosis* (Rengarajan *et al*., 2005). To determine such, SCID mice were challenged with the *vapG* mutant and the clearance of the mutant relative to the wildtype and plasmid-cured controls was monitored. Analysis of bacterial organ burden showed the *vapG* mutant replicated as efficiently as its wildtype parent, displaying a ~40-fold increase in

bacterial number in the spleen, (**Fig 8A**) and a 9-fold increase in numbers in the liver (**Fig. 8C**) at 48 hpi, a level of burden which persisted for the duration of the infection. Furthermore, the *vapG* mutant was able to persist in the lungs (**Fig. 8B**) of infected mice to the same degree as wildtype *R. equi*. Clearance of the *vapG* mutant complemented with a wildtype copy of *vapG* integrated on the chromosome was indistinguishable from the *vapG* mutant and wildtype *R. equi* 103+ at all timepoints post-infection, whereas the plasmid-cured strain was rapidly cleared. The data demonstrate that *vapG* is dispensable for bacterial survival and replication *in vivo* in mice.

DISCUSSION

Although necessary, VapA is not sufficient for *R. equi* virulence. Microarray analysis by Ren and Prescott (2003) demonstrated that only genes within the pathogenicity island were differentially regulated during intramacrophage growth, as compared to virulence plasmid genes outside of this region which showed no significant changes in transcription (Ren & Prescott, 2003). This led to the hypothesis that the additional plasmid-derived virulence factors were most likely to reside within the pathogenicity island, rather than the plasmid backbone. To address the hypothesis, a pathogenicity island deletion strain of *R. equi*, removing ~26kb of plasmid sequence encompassing *orf62* to *orf22*, was constructed. The mutant strain was then used as the background for constitutive expression of *vapA*, reasoning that the phenotype of the VapA producing PAI strain would yield insight into the location of these additional virulence factors. Attenuated growth of the pathogenicity island mutant in macrophages and accelerated clearance of the mutant *in vivo* in SCID mice was observed, as expected, since the strain lacked *vapA*. While constitutive expression of *vapA* by the PAI-deletion mutant did help to delay its clearance in both macrophages and mice, the virulence phenotype was not restored. The findings therefore,

were supportive of the hypothesis that still further virulence determinants were to be found within the deleted PAI region.

We had earlier shown that a mutant strain deleted for the pathogenicity island genes *vapA, -I, -C, -D, -E, -F* and 5 genes of unknown function (*orfs 16-18*, *0680* and *0740*), is complemented by *in trans* expression of *vapA* alone, suggesting that the latter 10 genes are not essential for virulence (Jain *et al*., 2003). Likewise, *orf10*, another gene of unknown function, is similarly dispensable, since its deletion had no effect on *R. equi* clearance in mice (Ren & Prescott, 2003; Ren & Prescott, 2004). Of the remaining unanalyzed PAI genes, it was noted that *vapG* possessed a number of characteristics similar to that of established virulence determinants. Specifically it is an extracellular protein sharing 55% similarity with VapA. It possesses a clear signal sequence indicating it is secreted, as are many proteins involved in host-pathogen interactions, although it remains to be established whether VapG is surface-localized. Expression of *vapG* is induced by many environmental signals, including oxidative stress (Benoit *et al*., 2002), increased temperature (Ren & Prescott, 2003), low iron and magnesium concentrations, and reduced pH (Ren & Prescott, 2003), conditions found within the local environment of host phagosomes. Induction of *vapG* in response to any of these signals could perhaps indicate a role for this protein in the bacterial response to such stresses within the macrophage. Further support for this idea is provided by the findings that *vapG* expression is highly upregulated in foal macrophages (Ren & Prescott, 2003), and is the highest expressed *vap* gene (~5-fold higher than *vapA*) in the lungs of foals infected with *R. equi* (Jacks *et al*., 2007). Therefore, *vapG* was considered by us, and others, to be a highly probable virulence determinant candidate. To test this, a *vapG* deletion stain was constructed using an allelic exchange methodology (16) established by this laboratory. Analysis of the *vapG* mutant in both murine and equine

macrophages showed that deletion of the gene had no significant effect on the ability of the strain to replicate intracellularly, a somewhat surprising finding given that expression of the gene is significantly increased in that environment (Ren & Prescott, 2003). When assessed in the SCID mouse infection model, it was observed that the *vapG* mutant displayed *in vivo* survival and replication kinetics indistinguishable from wildtype *R. equi*. Thus, *vapG* is dispensable for growth in *in vitro* cultured macrophages and *in vivo* in mice, data which upon first pass suggests it plays little if any role in virulence of the organism.

Certain caveats exist which preclude the complete exclusion of a role for VapG in disease pathogenesis. It is possible that VapG is important during the earliest phase of infection, for example during initial interactions with the host respiratory tract (e.g. epithelial cells), perhaps facilitating initial attachment and adhesion events. Alternatively, VapG may function in aiding dissemination from the lung following respiratory infection. Either of these two possibilities cannot be assessed by direct infection of *in vitro* grown macrophages or intravenously challenged animals. Furthermore, while VapG appears to be dispensable in the murine infection model, it may play a role in survival and propagation of bacteria in the lungs of infected foals, the natural hosts of *R. equi*. The significantly high level of *vapG* expression in the lungs of infected foals is supportive of the hypothesis that VapG is needed during infection of susceptible horses. An *in vivo* equine infection model could definitely evaluate the requirement for this protein in *R. equi* disease, however such analysis is beyond the scope of this work.

The data suggest that virulence determinants, aside from *vapA* and its two regulators (*virR* and *orf8*), reside within the pathogenicity island. While it is formally a possibility that VirR and Orf8 influence genes on the plasmid backbone, the probability is viewed to be unlikely given that expression of these backbone genes remains unchanged during intracellular growth,

whilst the expression of the regulators is increased (Ren & Prescott, 2003). Full-length PAI genes that to the authors' knowledge have yet to be assessed for a role in virulence include *orf1* (putative Lsr2 protein), *orf3* (putative SAM dependent methyltransferase) (Letek *et al*., 2008), *orf5* (putative major facilitator superfamily transporter) (Letek *et al*., 2008), *orf6* (*vapH*; unknown function*), orf7* (unknown function), *orf9* (hypothetical protein), *orf11* (hypothetical protein), and *orf21* (chorismate mutase, *scm2*) (Letek *et al*., 2008). Work is currently underway to establish which of those genes are needed for virulence.

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Table 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Source or reference
Escherichia coli		
DH5 α	F-φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK- mK+) phoA supE44 thi-1 gyrA96 relA1, λ-	Zymo Research
Rhodococcus equi		
103+	Wildtype strain with 81kb virulence plasmid	Giguere *et al* 1999
103-	Plasmid-cured variant of strain 103+	Jain *et al* 2003
103-/*vapA*	Strain 103- transformed with pMV261-*vapA*	This study
Δ*vapA*	Strain 103+ with deletion of the *vapA* gene	Jain *et al* 2003
Δ*vapA*/*vapA*	*VapA* deletion mutant transformed with pMV261-*vapA*	This study
ΔPAI	Strain 103+ with deletion of pathogenicity island	This study
ΔPAI/*vapA*	PAI-mutant transformed with pMV261-*vapA*	This study
Δ*vapG*	Strain 103+ with deletion of the *vapG* gene	This study
Δ*vapG/vapG*	*VapG* deletion mutant transformed with pSET152.vapG	This study
Plasmids		
pBluescriptII	Amp^R, αlacZ'	Stratagene
pSJ42	pBluescriptII digested with *SspI* and *DraIII*	This study
pSJ47	pSJ42 with fragment downstream of ORF22 (co-ordinates: 22802-23501)	This study
pSJ48	pSJ47 with fragment upstream of ORF62 (co-ordinates: 77178-77742)	This study
pSJ49	pSJ48 with 1.1kb Apr^R-containing fragment from pSC146	This study
pSJ53	pSJ49 with 3.5kb *lacZ*-containing fragment from pMV261-lacZ2	This study
pSC146	*Mycobacterium-E. coli* shuttle plasmid, *ts oriM*, Apr^R, *Himar*1	Ashour *et al* 2003
pMV261	*Mycobacterium-E. coli* shuttle plasmid with Hyg^R, *oriM*, *Hsp60*	Stover *et al* 1991
pMV261-lacZ2	pMV261 with *lacZ*	Steyn *et al* 2003
pMV261-*vapA*	pMV261 with *vapA* cloned in-frame downstream of *Hsp60* promoter	Hong *et al* 2008
pSelAct	Apr^R, *lacZ*, *codA*:*upp*	vd Geize *et al* 2008
pJM10	pSelAct with fragment downstream of *vapG* (co-ordinates: 693-1545)	This study
pJM11.1	pJM10 with fragment upstream of *vapG* (co-ordinates: 2042-2711)	This study
pJM11.1hyg	pJM11.1 with 1.4kb Hyg^R-containing fragment from pMV261	This study
pMV261-lacZ2Δ*oriM*	pMV261-lacZ2 lacking *oriM*	This study
pGBC14.1lacZ	pMV261-lacZ2Δ*oriM* with flanks of *vapG*, Hyg^R	This study
pSET152	phiC31 integrase, *att*P, Apr^R	Hong *et al* 2008
pETDuet-1	Amp^R, lacI, MCS1, MCS2	Novagen
pSET152-AMH	pSET152 with *Hsp60* from pMV261 and MCS2 from pETDuet-1 MCS2	This study
pSET152.vapG	pSET152-AMH with *vapG*-containing fragment (co-ordinates: 1516-2061)	This study

Table 2

Primers used in this study

Primer name[a]	Sequence[b]	Reference
VapA-hsp60-F[I]	5'- CGC *TGG CCA* CTC TTC ACA AGA CG-3'	This study
VapA-hsp60-R[II]	5'- CTA *TGG CCA* CTA GGC GTT GTG CCA-3'	This study
VapC1[III]	5'- TAT CAA *TCT AGA* TTG ATG ACG ACG GTC GAG-3'	Jain *et al* 2003
VapC1c[IV]	5'- ATA TAT *GTT AAC* TCA CAC CAA ATG CCA TCG-3'	Jain *et al* 2003
VapD1[V]	5'- ATA TAT *TCT AGA* ATG GGT CGG AAG GTA AAC-3'	Jain *et al* 2003
VapD1c[VI]	5'- ATA TAT *GTT AAC* ACT TGT TCC TCA CGC AGC-3'	Jain *et al* 2003
VapE1	5'- ATA TAT *TCT AGA* GTC GCG CTT GAA GTG CGG-3'	Jain *et al* 2003
VapE1c	5'- ATA TAT *GTT AAC* CAG CTA TCG CCA GGC G-3'	Jain *et al* 2003
VapF1[VII]	5'- ATA TAT *TCT AGA* CTG ACG ATA GCT GGG CCT-3'	Jain *et al* 2003
VapF1c[VIII]	5'- ATA TAT *GTT AAC* CAA TCA TTG CGC TAA CAC-3'	Jain *et al* 2003
REVP2	5'- GAC CTG TTC ATA GCC GAG-3'	Jain *et al* 2003
REVP2c	5'- TCG TCC TCG ATC CGC TGC-3'	Jain *et al* 2003
REVP4	5'- ATC GTC GCG ATC TGC TGA-3'	Jain *et al* 2003
REVP4c	5'- TCA TTC TCG CGG TTG TGC-3'	Jain *et al* 2003
REVP5	5'- CTT TGC AGT CGG CCT GAG-3'	Jain *et al* 2003
REVP5c	5'- GAA GAT GAG TAG CAC TGT C-3'	Jain *et al* 2003
REVP6	5'- GAG AGT TCA GTT TCG CGG-3'	Jain *et al* 2003
REVP6c	5'- CCT TTC CAT TGG TGT CTT C-3'	Jain *et al* 2003
REtrbA1	5'- GCG TCA GTG CGA CAG TGA TG-3'	Jain *et al* 2003
REtrbA1c	5'- TCG GAG TCA GGT CGG AGG-3'	Jain *et al* 2003
ORF61(f)	5' - TGC GTC CTC CCG CCC GTC -3'	This study
ORF61(r)	5' - ATG CGC CCA TCC TAT TGC -3'	This study
ORF62-1(f)	5' - GTT CGA TCT TCC GTT CTG -3'	This study
ORF22-1(r)	5' - CAT CGC TAC GGC ACA ACC -3'	This study
ORF22(f)	5'- AGT GCA GAG AAG CAG AGC-3'	This study
ORF22(r)	5'-ACC CAC ATC AGC CAA CT-3'	This study
ORF62u(f)	5'- ACG CGC TCT ACC TGC TCG-3'	This study
ORF62u(r)	5'- CTT TTG CCC TTC TGG CAT GG-3'	This study
vapG-up-F	5'- AAT T*CG GCC G*TC TCT GGC TTC ATA ACA C-3'	This study
vapG-up-R	5'- AAT A*GG ATC C*AA TAG GAC GGC GCA CTC-3'	This study
vapG-down-F	5'- AAT A*GG ATC C*AA GGG TCC GAA CAC TCA C -3'	This study
vapG-down-R	5'- AAG A*GA ATT C*AG TGC CGC TCC TAC GTT G -3'	This study
vapG-comp-NdeI	5'- GCG C*CA TAT G*TC GTG AGT GTT CGG ACC C-3'	This study
vapG-comp-EcoRV	5'- ATA A*GA TAT C*AC GCG CCG ACT GTG AG-3'	This study
vapG-F	5' - AAG A*AC ATG T*GC GCC GTC CTA TTG -3'	This study
vapG-int-F[IX]	5' - CCG CCA GAA TCA CCA GTA AAC -3'	This study
vapG-int-R[X]	5' - GCG AAC GCG GAA ACT TCA ATG -3'	This study
vapG/podG-R	5' - AAT A*GG TAC C*CG TTC GGA GGT ATT CG -3'	This study

Table 2 (cont.)

Primer name[a]	Sequence[b]	Source
HSP60_R	5'- CC*C ATA TG*C CAT TGC GAA GTC -3'	This study
F_HSP60	5'- AT*G GAT CC*C TCC GTT GTA GTG CTT GTG -3'	This study
gyrB-qPCR-F	5' - GTC GAG CAG GGT CAC GTG TA -3'	This study
gyrB-qPCR-R	5'- AGC TCC TTT GCG TTC ATC T -3'	This study
MCS-F	5'- CG*G GAT CC*G GGA TAT ACA TAT GG -3'	This study
MCS-R	5'- CGG CCG *TTT AAA* TTA ACT CGA GGG TAC CG -3'	This study
ORF6F-RTPCR[XI]	5'- AGC TGT GCC TGC AAC ATT CG-3'	This study
ORF6R-RTPCR[XII]	5'- CTA CGC TAC ATC GCC TAT CC-3'	This study

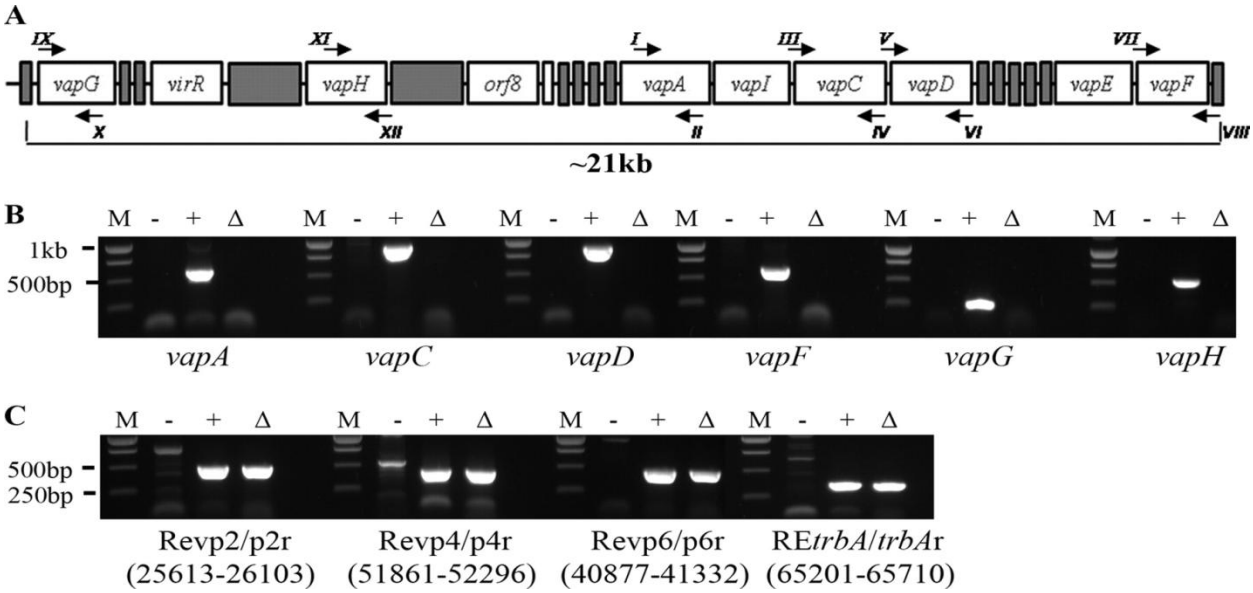

Figure 1. Confirmation of the PAI deletion. (A) Schematic of the pathogenicity island and locations of the PCR primers (arrows) used in analysis of the PAI mutant. (B) Results of PCR analysis of the PAI mutant using primers annealing within the deleted region. Primers specific for either *vapA*, *-C*, *-D*, *-F*, *-G*, or *-H* were used to confirm the deletion in the mutant. PCR amplicons for these primer pairs are shown. Total DNA from *R. equi* 103– (lanes –), *R. equi* 103+ (lane +), and the PAI mutant (lanes Δ) was used as the template in PCRs. DNA standards (lanes M) are shown to the left of each group of lanes. (C) Results of PCR analysis of the virulence plasmid backbone in the PAI mutant. Primer pairs annealing to different regions spanning the virulence plasmid outside the deleted pathogenicity island region were used in PCRs using the same template DNAs as for panel A. Annealing site coordinates are shown in parentheses under the respective primer pair.

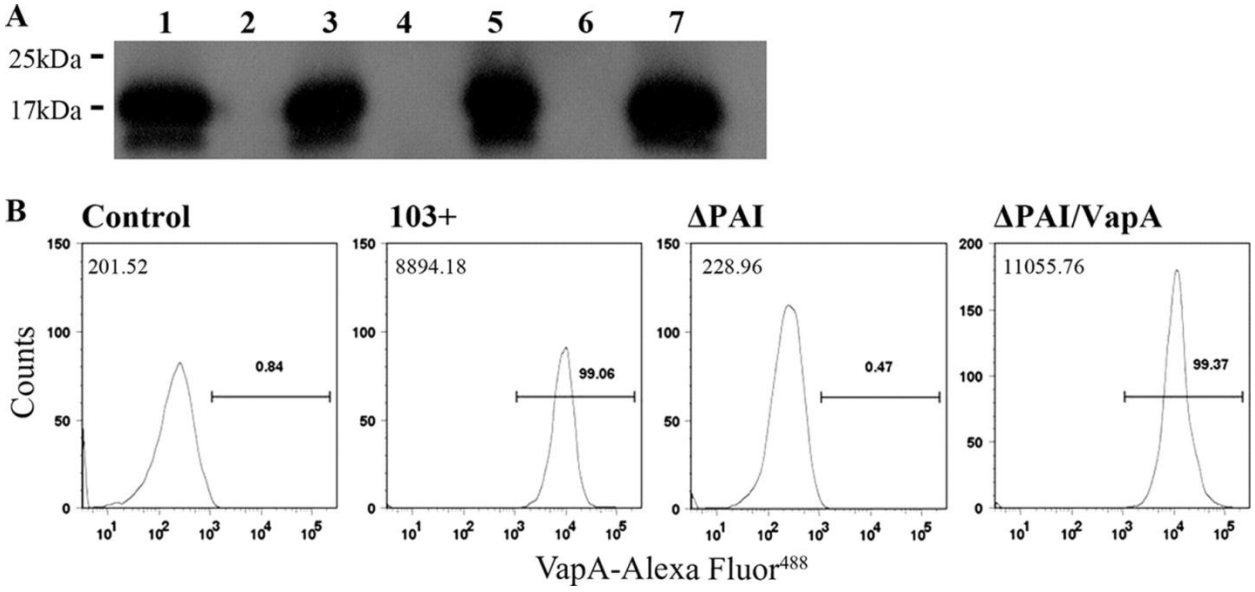

Figure 2. Expression of VapA in the PAI mutant. (A) Western blot analysis. Whole-cell extracts were prepared from *R. equi* 103+ (lane 1), virulence plasmid-cured 103– (lane 2), 103–/*vapA* (lane 3), 103+ Δ*vapA* (lane 4), 103+ Δ*vapA/vapA* (lane 5), ΔPAI (lane 6), and ΔPAI/*vapA* (lane 7), all cultured at 37°C as described in Materials and Methods. The presence of VapA protein was detected with rabbit polyclonal antiserum to VapA. Sizes of protein molecular mass standards are indicated on the left. (B) Flow cytometry profile showing the expression of VapA on the surface of *R. equi*. Bacteria were grown in liquid broth at 37°C, washed, and stained with a polyclonal antiserum to VapA. The relative median fluorescence values of wild-type isolate 103+, the PAI mutant strain (ΔPAI), and the recombinant pMV261-*vapA*-transformed PAI mutant strain (ΔPAI/*vapA*) were compared. Analyses included a comparison to bacteria stained with an irrelevant antibody (control; preimmune serum). The number in the upper left corner of each histogram represents the median fluorescence intensity for each strain. The values above the gate reflect the percentage of VapA-positive cells within the population analyzed.

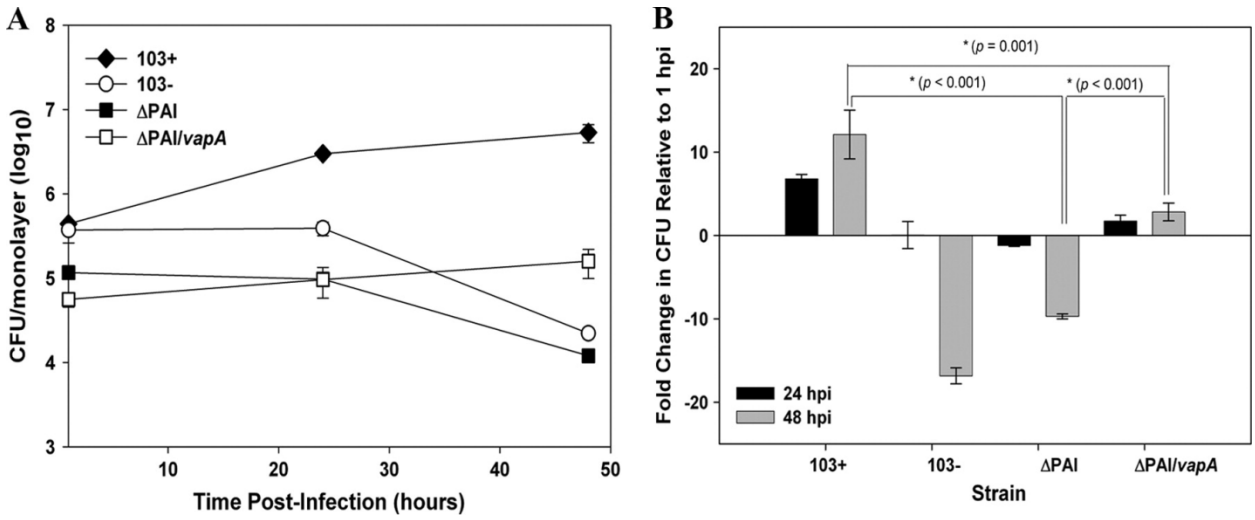

Figure. 3. Expression of *vapA* fails to restore the intracellular growth defect of the PAI mutant. (A) RAW264.7 macrophage monolayers were infected with *R. equi* strains 103+, 103–, ΔPAI, and ΔPAI/*vapA* at an MOI of 10:1. Following uptake and repeated washing to remove unbound bacteria and the addition of antibiotic to kill any remaining extracellular bacteria, triplicate macrophage monolayers were lysed at 1 h, 24 h, and 48 h postinfection. Lysates were plated onto BHI agar plates to determine the associated CFU. The data are expressed as means ± standard deviations. The graphs shown here are representative of three independent experiments. (B) Fold change in the CFU of intracellular bacteria at 24 h and 48 h postinfection relative to 1 h postinfection (hpi). A positive ratio reflects an increase in bacterial CFU over time resulting from bacterial replication in macrophages, whereas a negative ratio reflects a decrease in bacterial number over time. Values shown are the means ± standard deviations for triplicate monolayers from an individual experiment. The data shown are representative of three independent experiments.

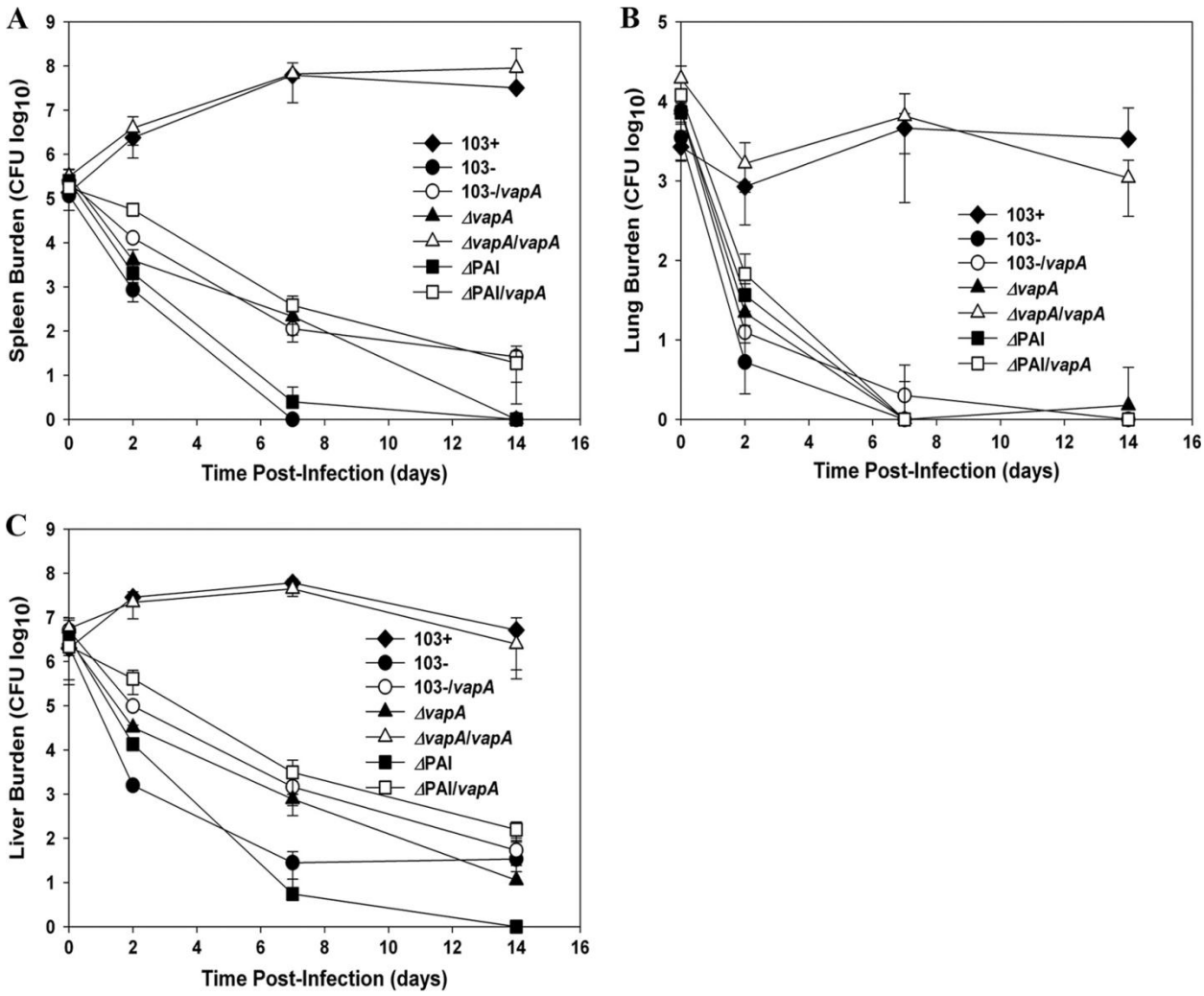

Figure 4. Expression of *vapA* fails to rescue the PAI mutant of *R. equi* from clearance in SCID mice. SCID mice were injected intravenously with 5 x 10^6 CFU of *R. equi* 103+, 103–, 103–/*vapA*, 103+ Δ*vapA*, 103+ Δ*vapA/vapA*, ΔPAI, and ΔPAI/*vapA*. At 0, 2, 7, and 14 days postinfection, mice were humanely sacrificed and organs removed aseptically. The total numbers of bacteria in the spleens (A), lungs (B), and livers (C) were determined by dilution plating of organ homogenates. Each point on the graph represents the mean ± standard deviation of bacterial counts for four mice.

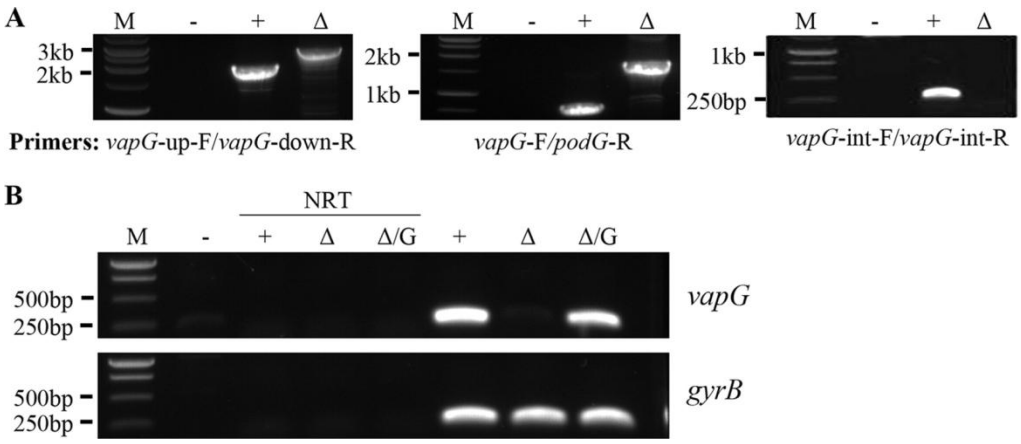

Figure 5. Confirmation of deletion of *vapG*. (A) PCR analysis of the *vapG* mutant. Primer pairs that specifically amplify regions across the deletion site were used in PCRs in which total DNA from *R. equi* 103+ (+) and the *vapG* mutant (Δ) served as the template. Standard DNA markers are indicated on the left (M). A no-template control (–) is also shown. (B) RT-PCR analysis of *vapG* expression. Total RNA was extracted from *R. equi* strains 103+ (+), the *vapG* mutant (Δ), and the complemented *vapG* mutant (Δ/G). cDNA was synthesized using equivalent concentrations of total RNA as the template. The presence of *vapG* and *gyrB* (internal control) was assessed by PCR using primer pairs specific for internal regions of each of the genes. Standard DNA markers are indicated on the left (M). No-template controls (–) and no-RT controls (NRT) are shown.

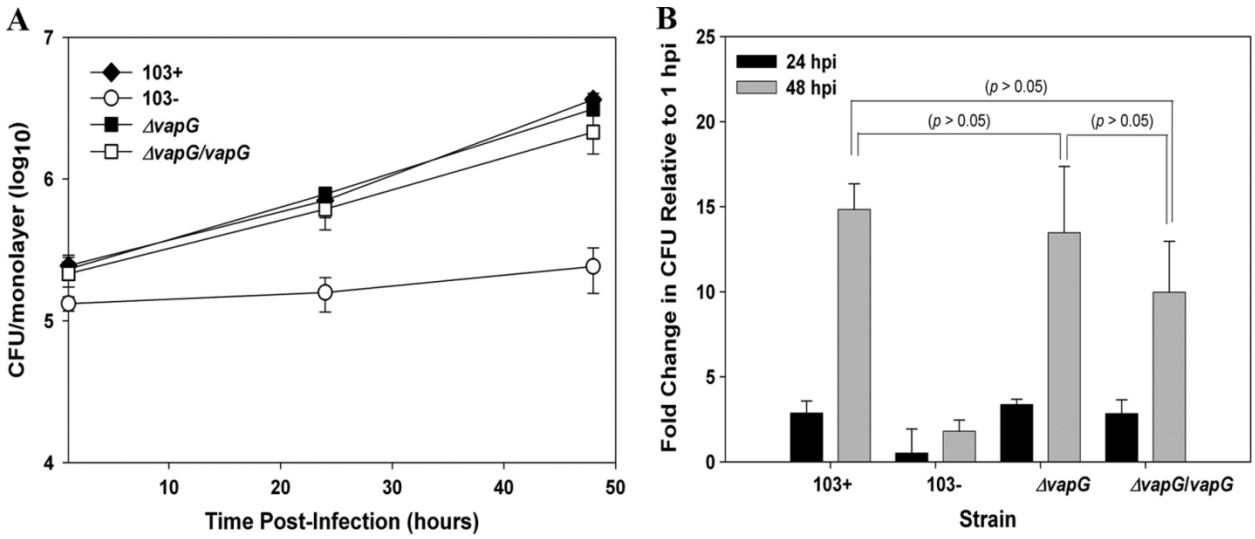

Figure 6. *vapG* is dispensable for growth in murine macrophages. (A) Intracellular replication of *R. equi* strains in RAW264.7 macrophages. Macrophage monolayers were infected with *R. equi* 103+, 103–, 103+ Δ*vapG*, and 103+ Δ*vapG/vapG*, and bacterial intracellular replication was followed as described in the legend to Fig. 3. (B) Fold change in intracellular bacterial CFU among the various strains at 24 h and 48 h postinfection (hpi). Values shown are the means ± standard deviations for monolayers assessed in triplicate. The data shown here are representative of three independent experiments.

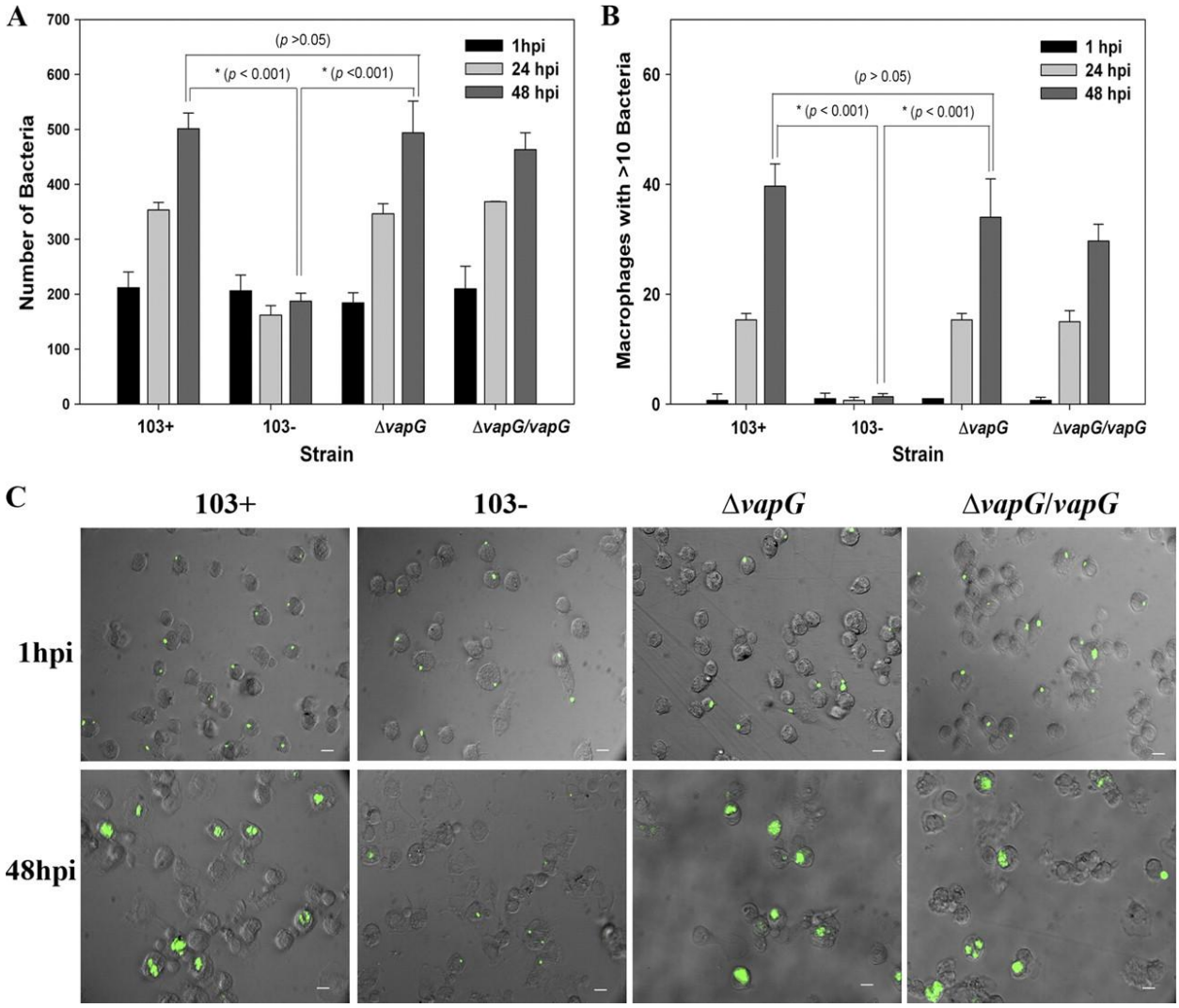

Figure 7. *vapG* is dispensable for growth in equine alveolar macrophages. Intracellular replication of *R. equi* strains in equine alveolar macrophages is shown. (A) The number of bacteria associated with 200 macrophages was determined at 1 h, 24 h, and 48 h postinfection by fluorescence microscopy. (B) Number of macrophages with 10 or more intracellular bacteria at the indicated time points postinfection. Values shown are the means ± standard deviation for monolayers assessed in triplicate. The data shown here are representative of three independent experiments. (C) Representative epifluorescence microscopy images of equine macrophages infected with *R. equi* strains 103+, 103–, 103+ Δ*vapG*, and 103+ Δ*vapG/vapG* taken at 1 h, 24 h, and 48 h postinfection. Scale bar, 10 µm; magnification, x400.

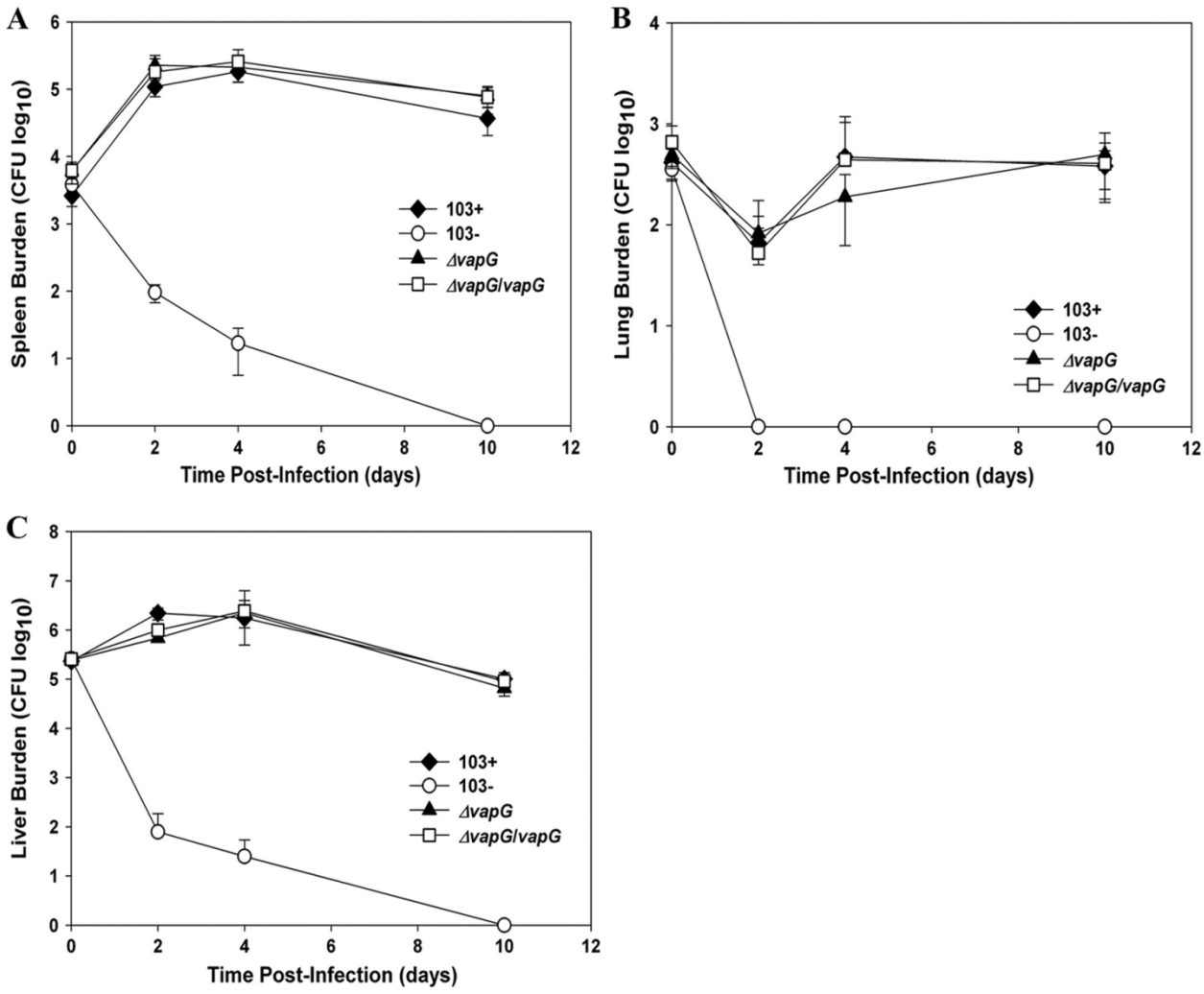

Figure 8. *vapG* is dispensable for survival *in vivo*. SCID mice were infected intravenously with 5 x 10^5 CFU of *R. equi* 103+, 103–, 103+ Δ*vapG*, and 103+ Δ*vapG/vapG*. At 0, 2, 7, and 10 days postinfection, mice were humanely sacrificed and organs removed and homogenized. The total numbers of bacteria in the spleens (A), lungs (B), and livers (C) were determined by dilution plating of the organ homogenates. Each point on the graph represents the mean ± standard deviation of bacterial CFU of four mice.

CHAPTER 4

ACQUISITION OF THE FOREIGN GENES *vapA, virR* AND *orf8* IS ALL THAT IS REQUIRED FOR CONVERSION OF THE ENVIRONMENTAL SAPROPHYTE *RHODOCOCCUS EQUI* TO AN INTRACELLULAR PATHOGEN[1]

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[1] **Coulson G.B.**, R. Miranda-CasoLuengo, A. Gnojska, X. Wang, J. Adair, J. Willingham, W. Meijer and M.K. Hondalus. Manuscript in preparation.

ABSTRACT

Rhodococcus equi is an opportunistic facultative intracellular pathogen of macrophages that causes severe pyogranulomatous pneumonia in young foals and immunocompromised people. Virulence of this organism is dependent on a plasmid-encoded pathogenicity island (PAI) that houses the unique Vap family of highly related proteins of unknown function termed the virulence-associated proteins (VapACDEFGHIX). *VapA* has been shown to be essential but not sufficient for *R. equi* replication in macrophages and establishment of a chronic infection in the Severe Combined Immunodeficient (SCID) mouse model system. In contrast, the highly similar *vapG* gene is dispensable for *R. equi* growth in macrophages and in mice. To determine whether any of the other full-length *vap* genes are necessary for intramacrophage replication, we constructed and analyzed a series of in-frame deletion mutant strains including a multiple-deletion mutant (MDM) in which every *vap* gene aside from *vapA* was removed. Experimental assessment of the MDM mutant demonstrated that the collective deletion of all *vap* genes aside from *vapA* did not impair the intracellular growth capacity of *R. equi*. These data indicate that VapA is the only Vap family member essential for intramacrophage growth. We subsequently hypothesized that the minimal subset of virulence plasmid-encoded genes necessary to allow *R. equi* replication in macrophages was *vapA* and its regulators, *virR* and *orf8*. Expression of these three genes from their native promoters in an avirulent virulence plasmid-cured strain of *R. equi* (strain 103-) promoted intracellular replication in macrophages, suggesting that the combined effects of the presence of VapA, and VirR- or Orf8-induced alteration of chromosomal gene expression allows bacterial adaption to life within the restrictive macrophage environment.

INTRODUCTION

Rhodococcus equi is an important Gram-positive pathogen of foals less than six months of age. Since this saprophytic organism is endemic in the soil of most farms worldwide, (Johnson *et al.*, 1983a; Johnson *et al.*, 1983b) newborn foals are exposed to the bacterium almost immediately following birth (Horowitz *et al*., 2001; Muscatello *et al*., 2007). Infection is initiated primarily through inhalation of contaminated soil or feces, and depending on a number of environmental, host, and organism factors, the infected foal may progressively develop chronic respiratory disease that commonly manifests as a suppurative bronchopneumonia with associated pulmonary abscessation (Prescott, 1991; Yager, 1987; Zink *et al*., 1986). Despite aggressive antibiotic treatment, ~30% case fatality rates have been reported in foals with *R. equi* pneumonia (Ainsworth *et al*., 1998; Giguere *et al*., 2004). In addition to being an important veterinary pathogen, *R. equi* is recognized as an infectious agent of immunocompromised people, in whom mortality rates can approach 50%, as has been reported in the case of AIDS patients.

Virulence of *R. equi* is associated with the possession of a ~81 kb virulence plasmid (Giguere *et al*., 1999; Takai *et al*., 2000b). Within this virulence plasmid is a ~21kb pathogenicity island (PAI) that encodes 26 open reading frames (ORFs) including the novel virulence-associated protein (Vap) gene family. There are six full-length *vap* genes (*vapA, -C, -D, -E, -G* and *–H*) and three additional *vap* pseudogenes (vap*I, -F* and *–X*), whose expression is induced *in vitro* by an increase in temperature (from 30°C to 37°C), a decrease in pH (from pH 8.0 to pH 6.5) or upon uptake by macrophages (Letek *et al.*, 2008; Polidori & Haas, 2006; Ren & Prescott, 2003; Takai *et al.*, 2000b). Furthermore, several *vap* genes (*vapA, -D* and *–G*) are also significantly upregulated *in vivo* in the lungs of *R. equi* infected foals (Jacks *et al*., 2007).

While the increased expression of the *vap* genes under specific *in vitro* conditions mimicking the intracellular environment of the host has implicated a role for these genes in disease pathogenesis, to date only *vapA* has definitively been shown to be required for intramacrophage replication (Jain *et al*., 2003). VapA is a lipoprotein expressed on surface of the bacterium (Takai *et al*., 1991a; Takai *et al*., 1991c), and it may function to perturb phagosome development (von Bargen *et al*., 2009). VapA expression is controlled by two PAI-encoded regulators, VirR and Orf8, both shown to be a requisite for *R. equi* virulence (Ren & Prescott, 2004; Russell *et al.*, 2004). Deletion of *vapA* leads to attenuation of the organism, observed experimentally by the loss of ability of the *vapA* mutant to replicate in macrophages or establish a chronic infection *in vivo* in the Severe Combined Immunodeficient (SCID) mouse model (Jain *et al*., 2003). Although *vapA* is absolutely required for *R. equi* virulence, it is not sufficient. Expression of wildtype levels of VapA protein in a virulence plasmid-cured strain of *R. equi* (103-) does not restore the capacity of that avirulent strain to replicate in macrophages, mice or foals (Giguere *et al*., 1999), suggesting that one or more virulence determinants, in addition to *vapA,* reside on the virulence plasmid. The location of those additional plasmid-encoded determinants was later narrowed to the PAI region (Coulson *et al*., 2010), although their specific identities remained unknown. Earlier work has likely excluded a role for the PAI-encoded gene, *orf10*, a gene of unknown function highly induced in macrophages but shown to be unnecessary for *in vivo* growth in mice (Ren & Prescott, 2003; Ren & Prescott, 2004). Likewise, deletion of a closely-related homolog of *vapA*, *vapG*, produces no phenotype in macrophages or mice suggesting *vapG* is dispensable for *R. equi* virulence (Coulson *et al*., 2010).

To determine whether the remaining *vap* genes (vapC, -D, -E, -F, -H and –I) are needed for *R. equi* survival in the macrophage environment, we constructed a series of deletion mutants

that lacked various combinations of the *vap* family members and assessed the mutants for intracellular growth potential. Herein we show that simultaneous deletion of all the *vap* genes, excluding *vapA*, did not impair the capacity of virulent *R. equi* to grow in macrophages, suggesting that VapA is the only Vap protein essential for intramacrophage replication. Significantly, a complementation analysis was done in which plasmid-cured *R. equi* (strain 103-) was engineered to express *vapA* and its regulators, *virR* and *orf8*, revealing that these three genes are the minimal subset of plasmid-encoded components required for growth in macrophages.

MATERIALS AND METHODS

Bacterial strains. Strains used are listed in **Table 1**. Virulent *R. equi* strain 103+ was originally isolated from a foal with *R. equi* pneumonia and was kindly provided by J. Prescott (Guelph, Ontario, Canada). Avirulent *R. equi* strain 103- was derived by serial subculture of strain 103+ at 37°C to facilitate loss of the virulence plasmid (Jain *et al*., 2003). The *vapA* and *vapG* deletion mutants on the 103+ strain background were derived as described earlier (Coulson *et al*., 2010; Jain *et al*., 2003). The standard culture medium used was brain heart infusion (BHI; Difco Laboratories, Detroit, MI) broth or agar, and unless otherwise noted, cultures were incubated at 30°C to help maintain the presence of the *R. equi* virulence plasmid. Antibiotics, when necessary, were used at the following concentrations: apramycin, 80 μg/ml; hygromycin, 180 μg/ml and vancomycin, 5μg/ml.

Plasmid construction. Plasmids used are listed in **Table 1**. To generate the suicide plasmid to construct the in-frame Δ*orf5-7* mutant, a 734 bp region upstream of *orf5* was amplified by PCR using primer pairs *orf5*-up-F and *orf5*-up-R **(Table 2)**. The PCR product was digested with restriction enzymes *EcoRI* and *BamHI* and cloned into pSelAct (van der Geize *et*

al., 2008) (a gift generously provided by R. van der Geize) which had been digested by the same enzymes, giving rise to the plasmid pGBC10. A 726 bp region of DNA downstream of *orf7* was amplified using the primers *orf7*-down-F and *orf7*-down-R **(Table 2)** and digested with *BamHI* and *EagI*. This fragment was then ligated to the upstream fragment by digesting pGBC10 with *BamHI* and *EagI*, giving rise to the plasmid pGBC11, which was subsequently used as the suicide plasmid to generate the Δ*orf5-7* mutant.

To construct the individual unmarked deletion mutants of *orf5*, *orf6* and *orf7*, a similar approach as outlined above was employed. A ~1.3 kb DNA fragment upstream of the gene of interest was amplified using primer pair orf5_1275AF/orf5_1275AR (*orf5*); orf6_1291AF/ orf6_1291AR (*orf6*) or orf7_1289AF/orf7_1289AR (*orf7*), respectively. Likewise, a ~1.3 kb DNA fragment downstream of the gene of interest was also amplified using primer pair orf5_1282BF/orf5_1282BR (*orf5*), orf6_1287BF/orf6_1287BR (*orf6*) or orf7_1289BF/ orf7_1289BR (*orf7*), respectively. Following purification of the upstream and downstream fragments, fragments were ligated together to yield a ~2.6 kb DNA fragment containing a shortened, *in frame*, version of the gene. These fragments were subsequently cloned into the relevant restriction sites (*orf5*: *EcoRI*/*XbaI*; *orf6*: *BamHI/NotI*; *orf7*: *XmaI*/*SpeI*) of pSelAct producing constructs pOrf5Δ, pOrf6Δ and pOrf7Δ (**Table 1**).

To generate the suicide plasmid used to construct the multiple-deletion mutant (MDM), a 715 bp region of DNA upstream of *vapI* was amplified using primers vapI-up-F and vapI-up-R and digested with *BamHI* and *EagI*. The digested product was then cloned into the plasmid pSelAct which had previously been digested with the same enzymes, yielding pGBC17. Subsequently, a 709 bp fragment of DNA downstream of *vapF* was amplified using primer pair vapF-up-F and vapF-up-R, and digested with *BamHI* and *EcoRI*. This fragment was then ligated

into pGBC17 which had been digested with *BamHI* and *EcoRI*. The resulting plasmid, pGBC18, was used to delete *vapI* through *vapF* on the PAI.

To create the complementing plasmid for the Δ*orf5-7* mutation, a ~3 kb fragment containing *orf5*, *orf6* and *orf7* and the P_{orf5} promoter was PCR amplified using primers *orf5*-7F2 and *orf7R*, followed by restriction digestion with *PciI*. The resulting product was cloned into pSET152.vapG (Coulson *et al*., 2010), which had been previously digested with *PciI*, giving rise to pGBC19.1. RT-PCR was used to confirm the expression of *orf5, 6* and *7* in the complemented strain.

To generate the complementing plasmid for the Δ*orf5* mutant, the complete *virR* operon and its upstream promoter sequence were cloned into pSET152, generating pPAI38, which was sequenced to confirm the integrity of the plasmid sequence. The *orf5* complementation construct pPAI35 (8534 bp) was obtained by digestion of pPAI38 with *PciI* and *PvuII*, followed by Klenow fill-in and re-ligation with T4 DNA Ligase.

To generate the 103- strain expressing only *orf4*, *orf8* and *vapA*, genomic DNA from *R. equi* Δ*vapG*Δ*orf5-7* carrying an in-frame deletion of *orf5-7* was used as a template for a PCR reaction amplifying a DNA fragment of 2890 bp starting at the terminal part of *orf3* and continuing until the stop codon of *orf8*. Primers used (PCR PAI38d12_2890F and Orf8 InterSeq_918R) introduced *BclI* and *PciI* restriction sites which facilitated cloning of the resulting PCR product into the respective *BamHI/PciI* sites of pSET152. The *vapA* gene with its native promoter was amplified (using primer pair vapA_*PciI* F/vapA_*PciI* R) and inserted into the *PciI* site of the aforementioned plasmid yielding the plasmid pPAI38d57p12.

Construction of *R. equi* mutants. To generate the required mutants of *R. equi*, the respective suicide vectors were electroporated into *R. equi* and transformants were selected as

previously described (van der Geize *et al*., 2008). Briefly, *R. equi* was transformed by electroporation and single crossovers were selected based on apramycin resistance and sensitivity to 5-Fluorocytosine. Double crossovers were generated during overnight growth in LB media without antibiotic selective pressure and were subsequently selected for by their insensitivity to 5-Fluorocytosine and sensitivity to apramycin. Candidates were screened by PCR to confirm the respective deletion. The absence of amplification products with internal primers and the production of shortened amplicons with external primers indicated the presence of the appropriate deletion. Total RNA was isolated from these candidates and endpoint RT-PCR targeting the internal regions of the deletion targets was employed as additional confirmation of the specific mutations.

Electroporation of *R. equi*. *R. equi* strains were grown in 200ml BHI broth to mid-logarithmic phase (OD_{600nm} of 0.8). Bacteria were pelleted and washed twice with an equal volume of cold distilled water, before finally being resuspended in 10ml of cold 10% glycerol made in dH_20. Aliquots (400µl) of the cells were stored at -80°C. For electroporation, an aliquot of cells were mixed with 0.5µg of plasmid DNA and placed in a prechilled 0.2cm electroporation cuvette. Electroporation was performed using a Gene Pulser (Bio-Rad) set at 2.5kV, 25µF, 1000Ω and single pulse. Immediately after electroporation, 1ml of BHI broth supplemented with 0.5M sucrose was added to the cuvette. Bacteria were then incubated for 1hr at 30°C and subsequently plated on BHI agar supplemented with the appropriate antibiotics.

RNA isolation. 5ml bacterial cultures were grown to mid-logarithmic phase (OD_{600nm} of 0.8), after which 1ml of culture (~1 x 10^8 bacteria) was removed and incubated with 2 volumes RNAprotect® Bacteria Reagent (Qiagen; Valencia, CA) and bacteria were subsequently harvested by centrifugation at 4000 X g for 10 min. The cells were resuspended in 700µl RLT

buffer (RNeasy® mini kit; Qiagen) and added to 0.1 mm diameter acid-washed zirconia/silica beads (BioSpec; Bartlesville, OK). The bacterial samples were lysed by 5 min exposure to a Mini BeadBeater-1 (BioSpec) cell disruptor set to maximum speed. Total RNA was subsequently isolated using the RNeasy® Mini Kit (Qiagen) according to the manufacturers' instruction.

Reverse-Transcriptase (RT) PCR. Total RNA in each sample was quantified by UV spectrophotometry and cDNA was synthesized using the ThermoScript™ RT-PCR system (Invitrogen; Carlsbad, CA) from equal amounts (1µg) of RNA template using 50ng of the supplied random hexamer primers and 15 U of ThermoScript™ RT enzyme according to manufacturers' instructions. One-tenth of the cDNA reaction mixture (2µl) was then used as template for standard PCR amplification with *Pfu* DNA polymerase (Stratagene; La Jolla, CA) using primer pairs specific for the gene of interest as listed in **Table 2**.

Real-Time Quantitative PCR. RNA concentration was determined in a NanoDrop spectrophotometer. Reverse transcription was carried out with Improm II reverse transcriptase using 100 ng of RNA as template and hexameric random primers as per manufacturer's directions (Promega). qPCR was performed in quartz capillaries using the LightCycler FastStart DNA Master SYBR Green I in a LightCycler 1.0 as recommended by the manufacturer (Roche). 2 µl of cDNA were used as template in 20 µl reactions. Reactions were subjected to 40 cycles of 95 °C for 15s, 60 °C for 30s and 72 °C for 30s. Temperature transition rates were set to 20°C/sec. Melting curves were obtained at the end of the amplification cycles to ensure the specificity of the fluorescence signal. Transcript levels of the genes of interest were normalized to the levels of 16S rRNA and fold changes obtained against the transcript levels of the *R. equi* 103+. No

reverse transcriptase controls were routinely performed to rule out the presence of contaminating genomic DNA.

Macrophages. Femurs and tibias of BALB/c mice (Charles River, Wilmington, MA) aged 6–8 weeks were flushed with 5 ml each of cold 1X PBS (without CaCl2 and MgCl2) supplemented with penicillin-streptomycin (P/S; 10 units/ml penicillin and 10 mg/ml streptomycin) and cells were collected in 50 ml conical tubes. The cells were spun for 10 min at 1100 rpm. The supernatant was discarded and the cell pellet was resuspended in 24 ml per mouse of DMEM, 10% FCS, 10% CSF-1 conditioned supernatant and 2 mM glutamine. The cells were plated in 6-well non-tissue culture treated plates (4 ml/well) and incubated at 37°C with 5% CO_2. On day 3, a further 4 ml/well media was added to the cells which were incubated for a further three days. On day 6, non-adherent cells were removed by aspiration of the media, and adherent cells were washed once with 4 ml 1X PBS. Adherent cells were then resuspended by addition of 8 ml/well cold 1X PBS and incubation of plates at 4°C for 15 min. After incubation, any remaining adherent cells were gently resuspended using a sterile cell scraper. Cells were then transferred to sterile 50 ml tubes and centrifuged at 1100 rpm for 10 min. The resulting pellet was suspended in medium composed of DMEM, 10% FCS, 10% CSF-1 conditioned supernatant and 2 mM glutamine. The total cell number was determined by using a haemocytometer and the cell concentration was adjusted to obtain 2 x 10^6 cells/ml. These cells were then used directly for macrophage assays or frozen in 90% FCS containing 10% DMSO in liquid nitrogen until further use. For intracellular growth assays, macrophages were seeded in 24-well tissue culture plates at $2x10^5$ cells per well, and incubated at 37°C overnight.

Intracellular growth of *R. equi* in macrophages. Overnight broth cultures of bacteria at an OD_{600nm} of 1.0 (~$2x10^8$ CFU/ml) were centrifuged and the resulting pellet washed once with

PBS and resuspended in PBS. Macrophage monolayers were washed once with warm DMEM, and the medium was replaced with fresh DMEM supplemented with 10% FCS, 10% CSF-1 conditioned supernatant and 2mM glutamine. Bacteria were added at a multiplicity of infection (MOI) of ~10 bacteria per macrophage. Bacterial incubation with macrophages proceeded for 45 min at 37°C, and then the monolayers were washed repeatedly with pre-warmed DMEM to remove unbound bacteria. After an additional 15 min incubation period to allow bound bacteria to be internalized, the monolayers were washed again, and the medium was replaced with complete DMEM supplemented with 20µg/ml of amikacin sulfate. At various times postinfection, macrophage monolayers were washed repeatedly, and 500µl sterile water was then added to lyse the macrophages upon further incubation at 37°C for 20min. Bacterial growth was determined by dilution-plating of lysates onto BHI agar plates. CFU were enumerated after 48 h incubation at 37°C.

***R. equi* gene expression in macrophages.** Analysis of *R. equi* gene expression inside macrophage-like cells J774A.1 was performed as previously described (Miranda-CasoLuengo, *In Press*). Briefly, macrophages were seeded at 6 x 10^5 cells/ml and cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids and 1 mM of L-Glutamine and incubated overnight at 37°C in 5% CO_2. Monolayers were washed once with the same media and infected with *R. equi* at a multiplicity of infection of 20. Internalization was synchronized by centrifugation (3 min at 160 x g). After 1h incubation at 37^{o}C in 5% CO_2, monolayers were washed three times and then supplemented with 5 µg/ml vancomycin. This point was designated time t = 0. Monolayers were harvested after 24h post-infection and eukaryotic cells lysed in guanidine thiocyanate buffer (4M guanidine thiocyanate, 0.5% [w/v] sodium N-lauryl sarcosine, 25 mM sodium citrate, and 0.1M β-mercaptoethanol). Macrophage DNA was shredded by

repeatedly passing samples through a needle until the viscosity was reduced. Bacterial pellets were recovered by centrifugation, RNA isolated, and transcript levels of *orf8* and *vapA* assessed by RT-qPCR using 16S rRNA as internal control. Normalized transcript levels of individual observations were calibrated against the geometric mean of the transcript levels of *R. equi* 103+.

Statistical analysis. Statistical analyses were performed using the SPSS Inc. statistical package (SPSS version 17.0.1, Chicago, IL). Normal distribution and equal variance of the data were assessed using the Shapiro-Wilk and Levene test, respectively. Comparison of the means in fold replication of intracellular bacterial numbers between bacterial strains was assessed using a one-way Analysis of Variance (ANOVA). When appropriate, multiple pairwise comparisons were done using Tukey's HSD test. Significance was set at a *P* value of <0.05.

RESULTS

Construction of the Δ*vapG*Δ*orf5-7* deletion mutant. The findings that *vapA* is crucial yet insufficient for virulence of *R. equi* (Giguere *et al*., 1999; Jain *et al*., 2003) and that expression of wildtype levels of *vapA* does not restore the virulence phenotype to a PAI-deletion mutant (Coulson *et al*., 2010), suggested that additional virulence determinants reside within the PAI region of the virulence plasmid. An obvious candidate virulence determinant was believed to be the highly similar VapG protein, yet surprisingly, deletion of the *vapG* gene produced no observable phenotype in macrophages or mice (Coulson *et al*., 2010). These findings prompted the question whether any of the other *vap* family member genes were needed for intramacrophage growth or whether that requirement was an exclusive property of *vapA*. As a first step to address that question, an unmarked in-frame deletion mutation of *R. equi* was constructed on the *R. equi* 103+ Δ*vapG* background in which *vapH* and the flanking genes *orf5*

and *orf7* (which are in the same operon as *vapH*) were collectively removed. We used the wildtype-like Δ*vapG* mutant strain as the background because the hygromycin resistance cassette marking the *vapG* deletion allowed the continuance of antibiotic pressure, ensuring virulence plasmid maintenance throughout the two-step allelic exchange strategy with 5'-FC counter-selection as described in the Materials and Methods section. We learned thorough experience that without direct pressure for maintenance of the virulence plasmid, many of the arising 5'-FC-resistant clones were not the desired mutant but rather were bacteria which had lost the entire virulence plasmid. We took care to leave the first and last genes of this operon intact, specifically the regulators encoded by *orf4* (*virR*) and *orf8* respectively, because they are critical for optimal *vapA* expression. To confirm the absence of transcripts from the targeted genes and the continued expression of *vapA* in the Δ*orf5-7* mutant, RT-PCR analysis using primer pairs (**Table 1**) that would specifically and individually amplify cDNA of *orf5*, *orf6*, *orf7* and *vapA* was performed (**Fig 1A**). Similar to the negative results obtained with cDNA from the virulence plasmid-cured strain, no amplicon was obtained when primer pairs designed to amplify *orf5*, *vapH* (*orf6*) and *orf7* respectively were used, thus confirming their absence in the Δ*orf5-7* mutant (**Fig 1A, lane 3**). In contrast, all primer pairs yielded an amplicon of the expected size when wildtype 103+ cDNA was used as control template (**Fig 1A, lane 1**). Importantly, the expression of *vapA* was maintained in the Δ*orf5-7* mutant (**Fig 1A, lane 3**), as was wildtype levels of VapA protein (data not shown).

Combined deletion of *orf5-7* leads to a hypervirulent phenotype in macrophages. To determine whether deletion of *vapH* (*orf6*) and the flanking genes *orf5* and *orf7* had any effect on the ability of *R. equi* to replicate intracellularly in macrophages, primary murine bone marrow-derived macrophages (BMDMs) were infected with the Δ*orf5-7* mutant and its intracellular

growth capacity relative to that of wildtype *R. equi* strain 103+ and the virulence plasmid-cured strain 103- was assessed (**Fig 2A** and **2B**). While the virulent strain 103+ multiplied 10-fold over a 48 h period, the plasmid-cured strain failed to replicate in the macrophages, as was expected. Surprisingly, the intracellular growth of the Δ*orf5-7* mutant significantly exceeded that of the wildtype strain ($p < 0.001$), with the mutant increasing 43-fold over the same period. This hypervirulent phenotype was reproducible, and importantly normal wildtype growth levels could be completely restored through complementation via integration of the plasmid pGBC19.1 (harboring a copy of *orf5-7*) on the chromosome.

***vapH* and *orf7* are dispensable for replication in macrophages while loss of *orf5* results in enhanced intracellular growth.** With the unexpected growth phenotype observed in the Δ*orf5-7* mutant, we sought to address which gene/s in the *orf5-7* region was responsible for the enhanced replication in macrophages. To achieve this, despite the aforementioned challenges, we generated individual unmarked, in-frame deletion mutants of *orf5*, *vapH* (*orf6*) and *orf7* on the wildtype 103+ background to assess the effects of the deletion of these genes individually. Confirmation of the mutations was achieved by RT-PCR analysis using primer pairs specific for each gene (**Table 1; Fig 1B**). As previously, the continued expression of *vapA* was confirmed in the individual mutants (**Fig 1B**). Results from the macrophage growth assays demonstrated that the deletion of *vapH* (**Fig 3B**) or *orf7* (**Fig 3C**) had no negative impact on bacterial growth as both mutants grew to similar levels as wildtype *R. equi*. In contrast, deletion of *orf5* led to significantly increased growth of the mutant relative to the wildtype strain (**Fig 3A**). Wildtype *R. equi* 103+ multiplied 18-fold over the 48 h course of infection, whereas the Δ*orf5* mutant multiplied 42-fold over the same time period ($p < 0.001$). This hypervirulent phenotype could be completely reversed to normal wildtype growth by complementation with pIA35 (**Fig. 3A**), an

integrating plasmid expressing wildtype levels of *orf5*. To exclude the possibility that deletion of *orf5* affected *orf8* and/or *vapA* expression, a possible explanation for the observed enhanced intracellular growth phenotype, we performed quantitative RT-PCR analysis on these two genes from bacteria isolated from infected macrophages. The results showed no difference in the expression of either *orf8* or *vapA* in the *orf5* mutant relative to wildtype strain *R. equi* 103+ (**Fig. 4**), suggesting that the hypervirulent phenotype observed in the Δ*orf5* mutant was specifically due to deletion of the gene, and not due to some downstream effect on *orf8* or *vapA* expression.

***vapA* is the only full-length *vap* gene required for intracellular replication.** Given a previous report indicating that *vapG* is not necessary for *R. equi* growth in macrophages (Coulson *et al*., 2010), and the current data suggesting that *vapH* is also dispensable for intramacrophage replication, we sought to determine whether any of the full-length *vap* genes aside from *vapA* were required for intracellular growth. To address this, we constructed a multiple deletion mutant (MDM) in which all the *vap* genes, excluding *vapA*, were removed (**Fig. 5A**). This mutant was constructed on the Δ*vapG*Δ*orf5-7* background in which both *vapG* and *vapH* were already deleted. On this background, a further deletion of ~7 kb fragment of DNA encompassing *vapC,-D, -E, -F,* and -*I*, and five genes of unknown function was performed. Deletion of each of the *vap* genes was confirmed by PCR analysis (**Fig. 5B**) using primer pairs specific for each gene (**Table 1**). As anticipated, an amplicon of the expected size was observed for *vapA* in both the wildtype 103+ strain (positive control) and the MDM mutant indicating the presence of this gene in both strains. However, no PCR product could be detected for any of the other *vap* genes (*vapC, -D, -E, -F, -G, -H* and *–I*) in the MDM mutant, whereas each could be detected in the wildtype strain *R. equi* 103+. To evaluate whether *vapA* is the only *vap* gene required for survival and growth in macrophages, we infected BMDMs with the MDM mutant

and assessed its replication compared to virulent *R. equi* 103+ and avirulent *R. equi* 103-. As expected, wildtype *R. equi* 103+ demonstrated a 10-fold increase in CFU over the 48 h time course of infection, whereas the plasmid-cured *R. equi* 103- did not replicate at all (**Fig. 6A**). In contrast, the MDM mutant reproducibly replicated significantly better than the wildtype strain ($p < 0.001$). The MDM mutant exhibited a 68-fold increase in CFU over the same time-frame, demonstrating that all other full-length *vap* genes aside for *vapA* are dispensable for growth in macrophages. To confirm that the hypervirulent phenotype displayed by the MDM mutant was due to the deletion of *orf5*, a complementation analysis was performed wherein the MDM mutant was transformed with pIA35, an integrating plasmid expressing wildtype levels of *orf5* transcript (**Fig. 6B**). Expression of wildtype levels of *orf5* resulted in full complementation of the MDM mutant, with the mutant no longer displaying the hypervirulent growth phenotype in macrophages (**Fig. 6A**).

***vapA* and its two regulators, *orf4* and *orf8*, constitute the minimal subset of genes required for intracellular growth in macrophages**. Since the data suggest that *vapA* is the only full-length *vap* family member crucial for *R. equi* replication in macrophages, we postulated that perhaps *vapA* and its two regulators (*virR* and *orf8*) were the only virulence determinants on the entire virulence plasmid necessary for intracellular growth. To test this hypothesis, we constructed an integrating plasmid pAI38d57p12 expressing *virR* and *orf8* from the native P_{virR} promoter and containing *vapA* with its native promoter sequence. The avirulent virulence-plasmid free *R. equi* strain 103- was transformed with pAI38d57p12 and expression of *virR*, *orf8* and *vapA* was confirmed in the transformed strain by RT-PCR analysis (**Fig. 7A)** As expected, no *orf5* transcripts were detected in the pAI38d57p12-transformed strain since the 103- background does not contain the virulence plasmid (**Fig 7A, lane 3**), whereas transcripts for

virR, *orf8* and *vapA* were readily detected by RT-PCR, demonstrating expression of these genes in the transformed strain (**Fig. 7A**). To determine whether these three genes constitute the minimal subset of plasmid-encoded genes required for intracellular replication, primary murine BMDMs were infected with the recombinant strain and bacterial growth relative to *R. equi* 103+ and *R. equi* 103- was assessed as described earlier. Wildtype *R. equi* 103+ increased in CFU approximately 8-fold over the 72 h period, while the plasmid-cured strain 103- did not replicate (**Fig. 7B**). Somewhat surprisingly, expression of *virR*, *orf8* and *vapA* in the *R. equi* 103- background allowed the bacteria to survive and multiply in macrophages, increasing in number approximately 13-fold in 72 h. Whereas *R. equi* 103- was incapable of replicating in macrophages, demonstrating a steady decline in CFU over the 72 h infection, the plasmid-cured strain expressing *virR*, *orf8* and *vapA* showed a consistent increase in CFU over the same 72 h period, with a total fold change in CFU at 72 h post-infection of 13-fold. The ability of this strain to replicate in macrophages suggests that these three genes alone are required for intracellular replication and imply that the regulators of *vapA* also are affecting expression of one or more chromosomal genes whose product(s) is/are necessary for survival and replication in the restrictive macrophage environment.

DISCUSSION

While it has been known for some time that the virulence plasmid of *R. equi* is absolutely essential for replication in macrophages and establishment of a chronic infection in mice and foals, the location and identity of all the genes within the plasmid required for virulence has largely remained unknown. The plasmid-encoded gene *vapA* was the first shown to be essential for intramacrophage replication and disease establishment in mice (Jain *et al*., 2003), but it is not

sufficient (Giguere *et al*., 1999) suggesting that other essential virulence factors are encoded by the virulence plasmid. In a recent report, Coulson and colleagues determined that expression of wildtype levels of VapA protein on the surface of a pathogenicity island (PAI) mutant could not restore virulence to the PAI mutant, suggesting that the additional virulence determinants reside within the PAI region (Coulson *et al*., 2010). Based on expression profiles in macrophages and in the lungs of foals infected with *R. equi,* the authors speculated in that report that *vapG* may play a role in virulence. Deletion of *vapG* however, had no effect on the ability of the mutant to replicate in macrophages or cause a chronic infection in mice, suggesting that *vapG* is dispensable for virulence. That finding prompted us to question whether any full-length *vap* gene aside from *vapA* was necessary for expression of the virulence phenotype. In this report, we assessed the contribution of the only remaining *vap* gene that had yet to be evaluated for a role in virulence, that being *vapH* (*orf6*). We did so by first constructing an in-frame unmarked deletion mutant of *vapH* along with the two genes flanking it, *orf5* and *orf7*, termed the Δo*rf5-7* deletion mutant. When the Δo*rf5-7* mutant was assessed for a growth defect in macrophages, we observed that the loss of these three genes did not impair the ability of the bacteria to replicate in macrophages, suggesting that *orf5*, *vapH* (*orf6*) and *orf7* are not needed for intracellular replication. Interestingly, not only could the Δo*rf5-7* mutant replicate efficiently in macrophages, it could do so significantly better than the wildtype *R. equi* strain 103+. This suggested that a gene, or genes, in the *orf5-7* region negatively influenced bacterial replication. This hypervirulent phenotype observed in the Δo*rf5-7* mutant could be completely reversed to wildtype growth levels upon complementation with a plasmid expressing wildtype levels of each of the deleted genes, indicating that the phenotype was specifically due to one of the genes deleted, and not due to downstream effects. In order to more directly assess the contribution of

each individual gene product to intracellular replication and to determine which gene/s in this region was responsible for the hypervirulent phenotype, we constructed individual in-frame deletion mutations of each of the genes (Δ*orf5*, Δ*orf6* and Δ*orf7*), respectively, and assessed each of these mutants for intracellular growth capability. The results from our analysis indicated that deletion of *orf5* alone was responsible for the hypervirulent phenotype, as the intracellular growth characteristics of the Δ*orf5* mutant mimicked closely the enhanced growth capacity observed with the Δ*orf5-7* mutant, each replicating significantly better than the wildtype strain. These data suggest the presence of the *orf5* gene product negatively impacts bacterial replication by some yet to be determined mechanism. Based on bioinformatic analysis, *orf5* is believed to encode a putative transporter protein of the major facilitator superfamily (MFS) of transporters. However, it is unknown what the substrate of the transporter might be, and determining such is currently under investigation but is beyond the scope of this work.

In contrast to the deletion of *orf5*, we observed no phenotype for the *vapH* (Δ*orf6*) and Δ*orf7* mutants, with both mutants replicating in macrophages indistinguishably from the wildtype. These data suggest both *vapH* and *orf7* are dispensable for growth in macrophages. Since we had recently found no role for *vapG* in *R. equi* virulence, we questioned whether any of the full-length *vap* genes, aside from *vapA*, were necessary for expression of the virulence phenotype. To address this question we constructed a multiple-deletion mutant (MDM) in which the full-length *vap* genes (*vapC*, *-D*, *-E*, *-G* and *–H*) and *vap* pseudogenes (*vapI* and *–F*) were deleted. The mutant also was deleted for seven other non-*vap* genes, including *orf5*, *orf7*, *orf16*, *orf17*, *orf18*, *orf680* and *orf740*), bringing the total number of deleted genes to 14 out of 26 (~55% of total PAI genetic content). When the MDM mutant was assessed in macrophage growth assays, we found that it was capable of efficient replication, demonstrating that *vapA* is

the only full-length *vap*-family member required for growth in macrophages. In fact, the MDM mutant replicated significantly better than the wildtype strain, a hypervirulent phenotype attributable to deletion of *orf5* since complementation of the MDM mutant with a plasmid expressing *orf5* reversed intracellular replication to wildtype levels.

Since the MDM mutant was fully capable of growth in macrophages, we were curious to know which of the remaining plasmid-encoded genes in this mutant were required for the virulent phenotype. In a previous report it was shown that *orf10* was dispensable for growth in mice (Ren & Prescott, 2004). Likewise, our unpublished data indicate that deletion mutants of *orf1* and *orf3* are not defective in macrophage replication (data not shown). Thus, the only genes aside from *vapA* and its two regulators, *virR* and *orf8*, that had not been assessed for a role in virulence to date were the pseudogene *vapX*, *orf9* (hypothetical protein), *orf11* (hypothetical protein), *orf600* (pseudogene) and *scm2* (putative chorismate mutase). Since none of these five genes appeared to be strong candidates for virulence determinants, we speculated that perhaps the only plasmid-encoded genes that were required for growth in macrophages were *vapA*, *virR* and *orf8*. The transcriptional regulators *virR* and *orf8* are required for optimal *vapA* expression (Byrne *et al.*, 2007; Ren & Prescott, 2004; Russell *et al.*, 2004). Deletion of either regulator is attenuating to the bacterium; a finding originally explained by the associated reduction or absence of expression of *vapA* (Ren & Prescott, 2004). Furthermore, we also know that the mere presence of VapA protein alone is not enough to produce a virulent phenotype and that additional PAI-encoded genes are required. Thus, if the expression of *virR*, *orf8* and *vapA* yield a virulent organism, then the most credible explanation for that result is that one or both regulators represent the additional PAI-encoded virulence determinants and their role(s) extends beyond the regulation of *vapA*. To test this, we expressed *vapA* and its regulators *virR* and *orf8* in virulence

plasmid-cured *R. equi* strain 103- and assessed growth capacity of the transformed strain in macrophages. While *R. equi* 103- was incapable of replicating in macrophages, the recombinant strain 103-/pAI38d57p12 (*R. equi* 103- expressing *vapA*, *virR* and *orf8*) could consistently grow in macrophages with similar kinetics to the virulent wildtype strain, *R. equi* 103+. Thus, based on our findings, we speculate that VirR, Orf8 or both mediate the regulation of chromosomal genes whose expression facilitates replication in macrophages. Consistent with this hypothesis is a recent report indicating the existence of cross-talk between the virulence plasmid and the *R. equi* chromosome. In that report it was found that the presence of the virulence plasmid in *R. equi* caused differential expression patterns of chromosomal genes (Letek *et al*., 2010). Letek and colleagues noted that *R. equi* chromosomal genes significantly upregulated in the presence of the virulence plasmid included a variety of transcriptional factors (*MarR*-, *MerR*-, *LasR*-like regulators), metabolic genes, iron acquisition genes (putative siderophores), deacidification enzymes (urease and histidine-ammonia lyase) and transporters (ABC and MFS transporter proteins) amongst others (Letek *et al*., 2010), many homologs of which have been shown to play important roles in pathogenesis in a number of other bacterial species (De Voss *et al*., 2000; Eaton *et al*., 1991; Heesemann *et al*., 1993; Ingavale *et al*., 2005; Jonsson *et al*., 2010; Kovacikova *et al*., 2004; Lamont *et al*., 2002; Ludwig *et al*., 1995; Sauer *et al*., 2005). Furthermore, while regulation of chromosomal genes by plasmid-encoded proteins is not often described, certain examples have been documented for a few bacterial pathogens (e.g. *E. coli*, *B. anthracis*, *P. aeruginosa* and *C. trachomatis*) (Aronson *et al.*, 2005; Chitlaru *et al.*, 2006; Gomez-Duarte & Kaper, 1995; O'Connell *et al.*, 2011; Shintani *et al.*, 2010). For instance, the global regulatory protein AtxA encoded on the pXO1 plasmid of *B. anthracis* has been shown to significantly alter the expression of both plasmid-encoded and chromosomal genes (Bourgogne

et al., 2003). AtxA, functioning as both an activator and repressor, was reported to alter expression of affected chromosomal genes 2- to 8-fold. Most of the chromosomal genes regulated by AtxA were predicted to be involved in either biosynthesis of branched-chain amino acids (e.g. *orf97* and *orf99*) and aromatic amino acids (e.g. *orf4962-orf4967*), or transport of oligopeptides (e.g. orf982, -4183 and -6016). While the role of amino acid biosynthetic genes and peptide transporters in pathogenesis is not known for *B. anthracis*, the oligopeptide permease Opp has been implicated in virulence gene expression in the closely related species, *B. thuringiensis* (Slamti & Lereclus, 2002).

This finding that *vapA* is the only full-length *vap* required for replication in macrophages is surprising given that virulent *R. equi* encode for multiple (six in total) full-length *vap* genes, all of which share considerable homology at their C-termini (Letek *et al*., 2008). The question, therefore, is why does virulent *R. equi* possess multiple *vap* genes, and what makes *vapA* functionally unique from the other *vap* genes? While gene duplication and amplification is a common occurrence in almost all bacterial species, duplicated genes are not thought to be fixed in the genome unless the selective pressure to maintain them is greater than the fitness cost required to keep them (Andersson & Hughes, 2009; Treangen & Rocha, 2011). Since amplification of *vap* genes is evident in both *vapA*-positive plasmids from equine isolates and *vapB*-positive plasmids from porcine isolates (Letek *et al*., 2008), it is suggestive that selective pressures driving the maintenance of the *vap* genes in plasmids of both types do exist. Amplification of the *vap* genes does not appear to represent a matter of gene dosage, as it is clear from our data, and previously published work (Jain *et al*., 2003), that the *vap* genes are not equivalent in function. While we could not find a role for any of the *vap* genes, aside from the requirement of *vapA* for bacterial replication in macrophages, we speculate that they have some

necessary function either in their natural environment (e.g. soil) or in their natural animal hosts. Furthermore, as the foal could be an incidental host for *R. equi,* the equine species may pose insufficient selective pressure on *R. equi* for the maintenance of the virulence plasmid and the encoded *vap* family of genes. Thus, we hypothesize that *vapA* and its homologues may be necessary for *R. equi* survival in an environmental niche, either in a free-living state or in relationship to an unknown host (e.g. amoeba or nematodes) as has been shown for other environmental intracellular bacterial species (e.g. *Legionella* and *Mycobacteria*) (Brassinga *et al.*, 2010; Greub & Raoult, 2004; Harb *et al.*, 2000; Harriff & Bermudez, 2009; Salah *et al.*, 2009; Steinert, 2011).

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Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Source or reference
Escherichia coli		
DH5 α	F-φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK- mK+) phoA supE44 thi-1 gyrA96 relA1, λ-	Zymo Research
Rhodococcus equi		
103+	Wildtype strain with 81kb virulence plasmid	Giguere *et al* 1999
103-	Plasmid-cured variant of strain 103+	Jain *et al* 2003
Δ*vapG*	Strain 103+ with deletion of the *vapG* gene	Coulson *et al* 2010
Δ*orf5*	Strain 103+ with deletion of the *orf5* gene	This study
Δ*orf5*/*orf5*	Strain Δ*orf5* complemented with pPAI35	This study
Δ*orf6*	Strain 103+ with deletion of the *orf6* gene	This study
Δ*orf7*	Strain 103+ with deletion of the *orf7* gene	This study
Δ*vapG*Δ*orf5-7*	Strain Δ*vapG* gene with deletion of genes *orf5*, *orf6* and *orf7*	This study
Δ*vapG*Δ*orf5-7*/*pGBC19.1*	Strain Δ*vapG*Δ*orf5-7* complemented with pGBC19.1	This study
ΔMDM	Strain Δ*vapG*Δ*orf5-7* with deletion *vapI-vapF*	This study
Plasmids		
pSelAct	Apr^R, *lacZ*, *codA*:*upp*	vd Geize *et al* 2008
pSET152.*vapG*	pSET152 containing *vapG* gene under expression of Hsp60 promoter	Coulson *et al* 2010
pGBC10	pSelAct containing 734 bp upstream flank of *orf5*	This study
pGBC11	pGBC10 containing 726 bp downstream flank of *orf7*	This study
pGBC17	pSelAct containing 751 bp upstream flank of *vapI*	This study
pGBC18	pGBC17 containing 709 bp downstream flank of *vapF*	This study
pGBC19.1	pSET152.*vapG* containing 3 kb fragment consisting of *orf5*, *orf6* and *orf7*	This study
pΔorf5	pSelAct containing a deleted version of orf5	This study
pΔorf6	pSelAct containing a deleted version of orf6	This study
pΔorf7	pSelAct containing a deleted version of orf7	This study
pOrf8	pSET152 containing a 918 bp fragment including *orf8* and its intergenic region with *orf7*.	This study
pPAI38	pOrf8 derivative containing a 5219 bp fragment including the 5' end of *orf3* and *virR* operon	This study
pPAI36	Derivative of pPAI38 obtained by deletion of *orf7* and *orf8* contained in a 1222bp PciI-AscI fragment.	This study
pPAI35	Derivative of pPAI38 obtained by deletion of *orf6* to *orf8* contained in a 1904bp PciI and PvuII fragment	This study
pPAI38	pOrf8 derivative containing a 5219 bp fragment including the 5' end of *orf3* and *virR* operon	This study
pPAI38d57p12	Derivative of pPAI38 carrying a deletion of *orf5-7* and containing a 1268 bp fragment containing P_{vapA} *vapA* downstream of *virR* operon	This study

Table 2
Primers used in this study

Primer name[a]	Sequence[b]	Reference
VapA-hsp60-F	5'- CGC *TGG CCA* CTC TTC ACA AGA CG-3'	Coulson *et al* 2010
VapA-hsp60-R	5'- CTA *TGG CCA* CTA GGC GTT GTG CCA-3'	Coulson *et al* 2010
vapC-int-F	5'- GGG TCG TCC ATC CAA ATC-3'	This study
vapC-int-R	5'- CAT TCC CAC CGC CTA TAC-3'	This study
VapD1	5'- ATA TAT *TCT AGA* ATG GGT CGG AAG GTA AAC-3'	Jain *et al* 2003
VapD1c	5'- ATA TAT *GTT AAC* ACT TGT TCC TCA CGC AGC-3'	Jain *et al* 2003
VapE1	5'- ATA TAT *TCT AGA* GTC GCG CTT GAA GTG CGG-3'	Jain *et al* 2003
VapE1c	5'- ATA TAT *GTT AAC* CAG CTA TCG CCA GGC G-3'	Jain *et al* 2003
VapF1	5'- ATA TAT *TCT AGA* CTG ACG ATA GCT GGG CCT-3'	Jain *et al* 2003
VapF1c	5'- ATA TAT *GTT AAC* CAA TCA TTG CGC TAA CAC-3'	Jain *et al* 2003
vapG-int-F	5' - CCG CCA GAA TCA CCA GTA AAC-3'	Coulson *et al* 2010
vapG-int-R	5' - GCG AAC GCG GAA ACT TCA ATG-3'	Coulson *et al* 2010
vapI-int-F	5'- GCC AGT GCG CAA GAA ATG-3'	This study
vapI-int-R	5'- CCG TGT ACC GAT ACT GAT TCC-3'	This study
ORF5F-RTPCR	5'- TAG TAG GAC GAG TTG CAG AG-3'	This study
ORF5F-RTPCR	5'- CCG CAA ACG AGA ATC CAT AG-3'	This study
ORF6F-RTPCR	5'- AGC TGT GCC TGC AAC ATT CG-3'	Coulson *et al* 2010
ORF6R-RTPCR	5'- CTA CGC TAC ATC GCC TAT CC-3'	Coulson *et al* 2010
ORF7F-RTPCR	5'- CTA TCG GAC TTC CGC TCG AC-3'	This study
ORF7R-RTPCR	5'- GAT CGG CCA CCT TCT TCC AC-3'	This study
gyrB-qPCR-F	5' - GTC GAG CAG GGT CAC GTG TA-3'	Coulson *et al* 2010
gyrB-qPCR-R	5'- AGC TCC TTT GCG TTC ATC T-3'	Coulson *et al* 2010
orf5-up-F	5'- ACT A*GA ATT C*GC GAG GCC ATC GAA GC-3'	This study
orf5-up-R	5'- AAT A*GG ATC C*CG CAA CTC CGA TCA GG-3'	This study
orf7-down-F	5'- GTA T*GG ATC C*TT TCC GCG ATG AGT TCA G-3'	This study
orf7-down-R	5'- TTA A*CG GCC G*AC CGT ATG ACC ATT CC-3'	This study
vapI-upstream-F	5'- AAT T*CG GCC G*TA ATG CGA CCG TTC TTG-3'	This study
vapI-upstream-R	5'- AAT T*GG ATC C*TG CAG TCA GCG CGA TTG-3'	This study
vapF-downstream-F	5'- AAG T*GG ATC C*TG GCA TTC TTG GGA GTG-3'	This study
vapF-downstream-R	5'- AAG T*GA ATT C*CG CGT TGC ATC AGG TGG-3'	This study
orf5_1275AF	5'- CGC C*GA ATT C*GC CCG CAT TGA ACG ACA GGT-3	This study
orf5_1275AR	5'- GCT A*AA GCT T*CA TCA TCC CCT CTG CAA CTC-3	This study
orf5_1282BF	5'- GCG G*AA GCT T*TA GAT AAC GCA GGA GGG ACC-3	This study
orf5_1282BR	5'- GGA T*TC TAG A*AG ATG AGC ATT GCC CTA ACC A-3	This study
orf6_1291AF	5'- GCT G*GG ATC C*TG TTT GCG ATT GGG CAG GAT-3	This study
orf6_1291AR	5'- GGC C*AA GCT T*CA TAA ATG CAC CCC TCT CGT-3	This study
orf6_1287BF	5'- CCT G*AA GCT T*GC GTA GTC CAA GCG AAG AAT-3	This study
orf6_1287BR	5'- GCG C*GC GGC CGC* CCC CAT ACC GTT TCG AT-3	This study

Table 2 (continued)
Primers used in this study

Primer name[a]	Sequence[b]	Reference
orf7_1289AF	5'- CGC C*CC CGG G*TTT GAG CCG TGA CCC TTT TAA-3	This study
orf7_1289AR	5'- GCC C*AA GCT T*CA CCT CAC AGA ATC GTT TAC T-3	This study
orf7_1289BF	5'- GCG G*AA GCT T*TG AGT TCA GCG ACA GGA GTG-3	This study
orf7_1289BR	5'- TGA A*AC TAG T*TA CGA AGT GCC GTC TAC CCA-3	This study
orf5_489IF	5'- CGG CTG TCG TAA TTC TGT TC-3'	This study
orf5_489IR	5'- AGC GTG TAA ATA GAC GAG GC-3'	This study
orf5_1125EF	5'- ACC TCG TCT GCG ACT CTG T-3'	This study
orf5_1125ER	5'- GTC TCG TCC ACT TTG GTT TC-3'	This study
orf6_291IF	5'- CAT TAC CAG ATG GTC CCA CA-3'	This study
orf6_291IR	5'- CGC TGT ATG TTG TCG GTG AA-3'	This study
orf6_687EF	5'- AAC CAA AGT GGA CGA GAC GA-3'	This study
orf6_687ER	5'- GAG CGG AAG TCC GAT AGT TT-3'	This study
orf7_274IF	5'- TTC CGC TCG ACC TGC TTT C-3'	This study
orf7_274IR	5'- GGC CAC CTT CTT CCA CTG A-3'	This study
orf7_614EF	5'- TAA TCG GTA TTG GCG GAG GT-3'	This study
orf7_614ER	5'- CTA ACC ATT ATC AGC GAG GC-3'	This study
16SrRNA F200	5'- ACG AAG CGA GAG TGA CGG TA-3'	Byrne *et al* 2007
16SrRNA R200	5'- ACT CAA GTC TGC CCG TATCG-3'	Byrne *et al* 2007
008F_RTPCR	5'- GAA CAA CTG GGA ATG GTG G T-3'	Byrne *et al* 2007
008R_RTPCR	5'- GTT CGC CGT TTC TAG ACG AA-3'	Byrne *et al* 2007
Orf8 InterSeq_918F	5'-AAT *GCG GCC GC*T GAG TTC AGC GAC AGG AGT G-3'	This study
Orf8 InterSeq_918R	5'-GCC G*AC ATG T*GC AGG TCC GGA ATA CAT GA-3'	This study
vapA_PciI F	5'- GCA *ACA TGT* AGA CCA ACA TCG TTC GCG-3'	This study
vapA_PciI R	5'-GAT C*AC ATG T*CT AGG CGT TGT GCC AGC TAC-3'	This study
PAI38d12_2890 F	5' -GTC *TGA TCA* CCT CAA GGG CGT CGA CCA AG-3'	This study

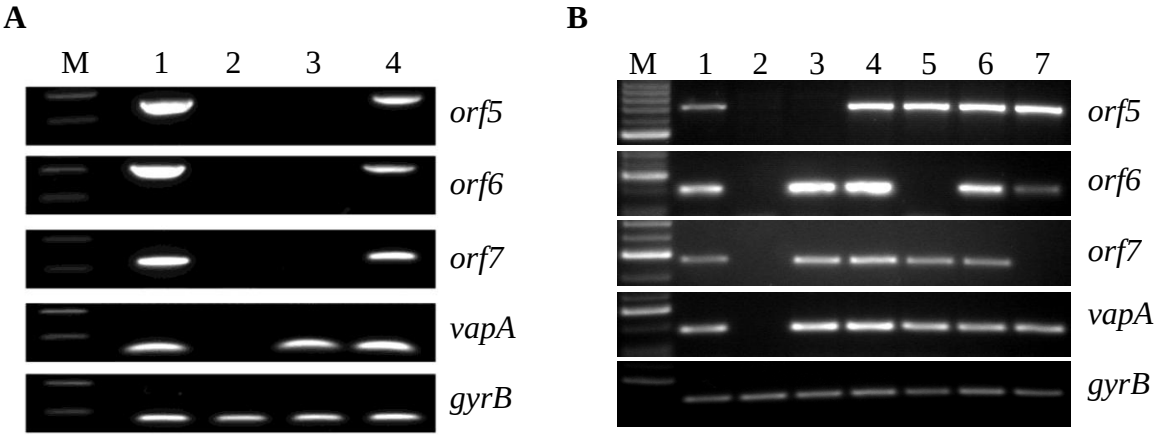


Figure 1. RT-PCR confirmation of the Δ*orf5-7* deletion mutation (A) and individual Δ*orf5*, Δ*orf6* and Δ*orf7* deletions. A) Total RNA was extracted from wildtype *R. equi* strain 103+ (lane 1), virulence plasmid-cured strain 103- (lane 2), Δ*vapG*Δ*orf5-7* (lane 3), and Δ*vapG*Δ*orf5-7*/pGBC19.1 (lane 4). cDNA was synthesized using equivalent concentrations of total RNA as the template. The presence of *orf5*, *orf6*, *orf7*, *vapA* and *gyrB* (internal control) was assessed by PCR using primer pairs specific for internal regions of each of the genes. B) Total RNA was extracted from *R. equi* strains 103+ (lane 1), 103- (lane 2), Δ*orf5* (lane 3), Δ*orf5*/pPAI35 (lane 4), Δ*orf6* (lane 5), Δ*orf6*/pPAI36 (lane 6) and Δ*orf7* (lane 7). cDNA was synthesized using equivalent concentrations of total RNA as the template. The presence of *orf5*, *orf6*, *orf7*, *vapA* and *gyrB* (internal control) was assessed by PCR using primer pairs specific for internal regions of each of the genes. Standard DNA markers are indicated on the left (M).

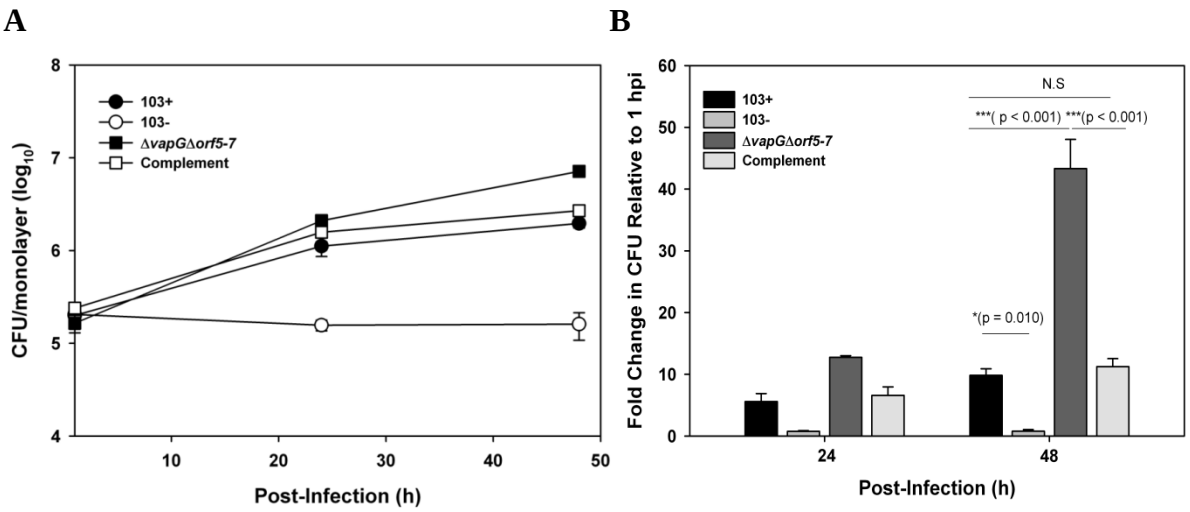


Figure 2. Deletion of *orf5-7* enhances growth in primary macrophages. (A) Intracellular replication of *R. equi* strains in BMDMs. Macrophage monolayers were infected with *R. equi* 103+, 103–, Δ*vapG*Δ*orf5-7* and Δ*vapG*Δ*orf5-7*/pGBC19.1 (complement) at an MOI of 10:1. Following uptake and repeated washing to remove unbound bacteria and the addition of antibiotic to kill any remaining extracellular bacteria, triplicate macrophage monolayers were lysed at 1 h, 24 h, and 48 h postinfection. Lysates were plated onto BHI agar plates to determine the associated CFU. The data are expressed as means ± standard deviations. (B) Fold change in the CFU of intracellular bacteria at 24 h and 48 h postinfection (hpi) relative to 1 hpi. The data shown here are representative of three independent experiments.

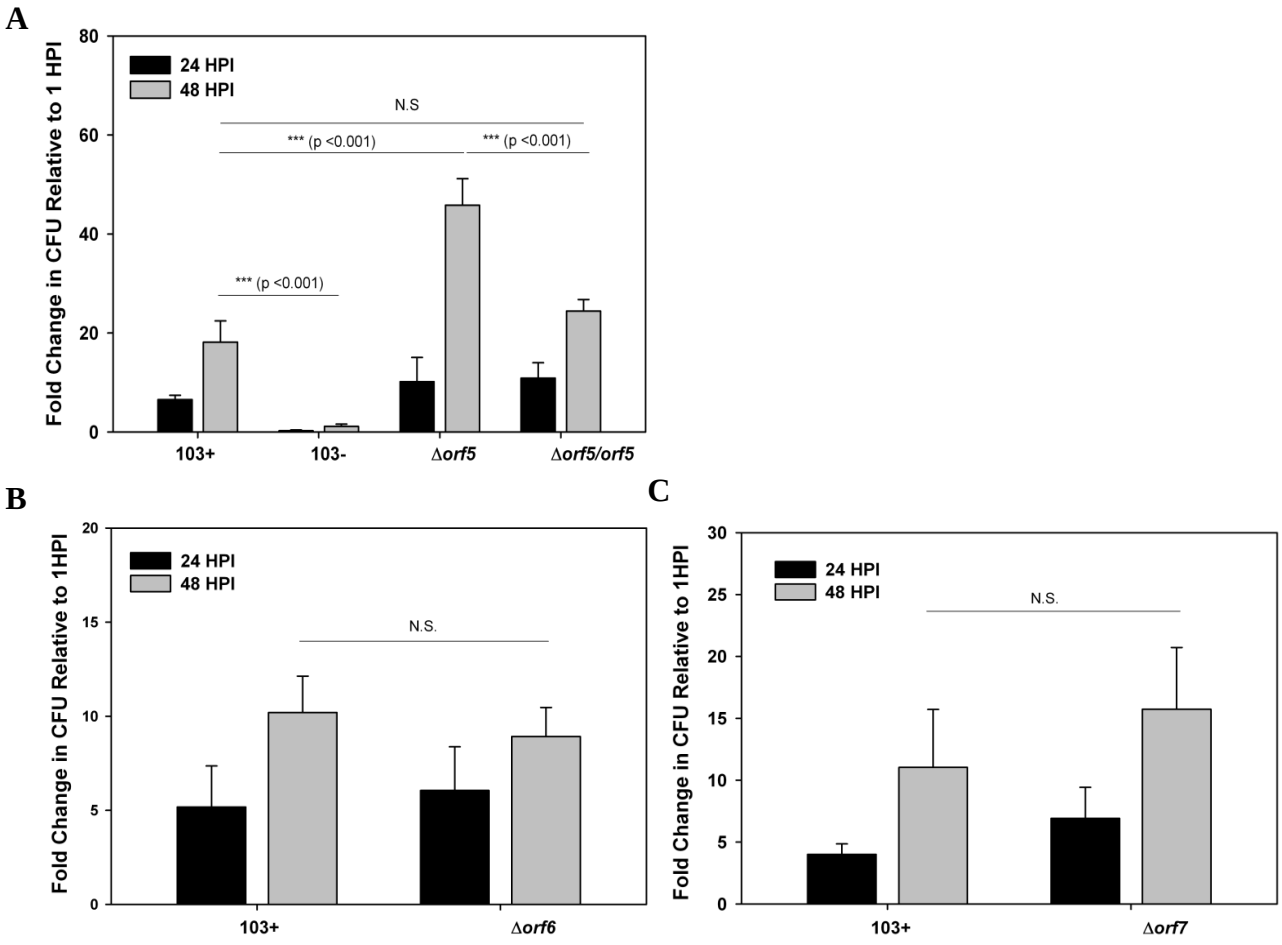


Figure 3. Deletion of *orf5* promotes intracellular growth whereas *orf6* and *orf7* are dispensable for growth in macrophages. Macrophage monolayers were infected with *R. equi* 103+, 103–, (A) Δ*orf5*, Δ*orf5*/*orf5*, (B) Δ*orf6* and (C) Δ*orf7*, and bacterial intracellular replication was followed as described in the legend to Figure 2. Values shown are the means ± standard deviations for monolayers assessed in triplicate. The data shown here are representative of three independent experiments.

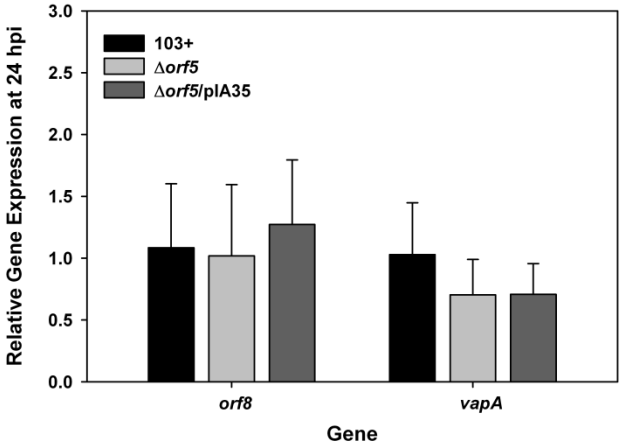
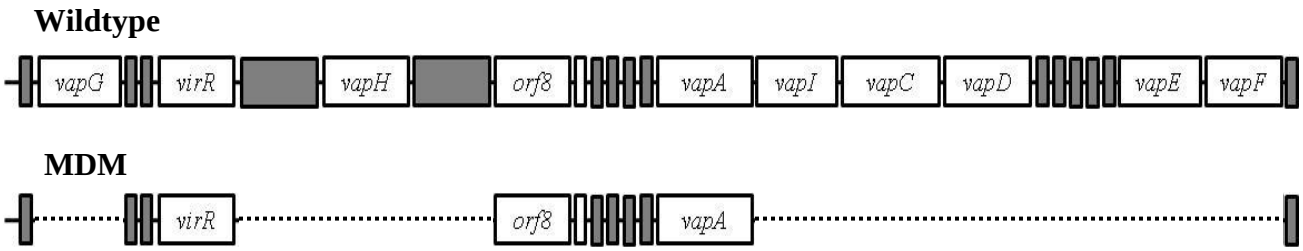


Figure 4. Enhanced replication of the Δ*orf5* mutant in macrophages is not due to increased expression of *vapA* and *orf8* expression. Murine J771.4 macrophages were infected with *R. equi* strains 103+, Δ*orf5* or Δ*orf5*/pIA35 for 24h. At 24 hpi bacterial RNA was isolated from lysed macrophages and cDNA synthesized from 100 ng of total RNA using random hexamer primers as described in the Materials and Methods. Transcript levels of *orf8, vapA* were assessed by qPCR using specific primers, normalized to the levels of expression of 16S rRNA and expressed as a relative fold changes in transcript level relative to *R. equi* 103+. Data shown are the average ± standard deviation of two independent experiments performed in triplicate.

A



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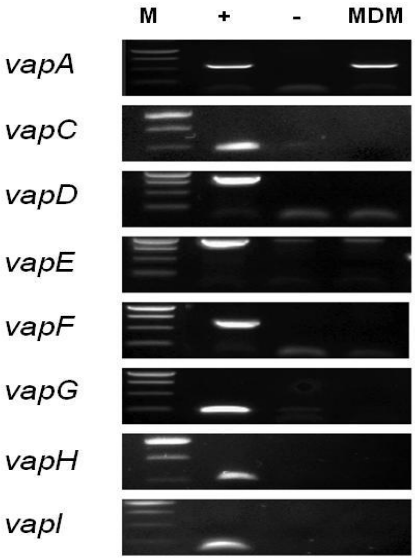

Figure 5. Construction and confirmation of multiple-deletion mutant (MDM). A) Schematic representation of the MDM mutant showing gene deletions. B) The presence of *vapA, vapC, vapD, vapE, vapF, vapG, vapH* and *vapI* in *R. equi* strains 103+ (+), 103- (-) and the MDM mutant was assessed by PCR using primer pairs specific for internal regions of each of the genes. Standard DNA markers are indicated on the left (M).

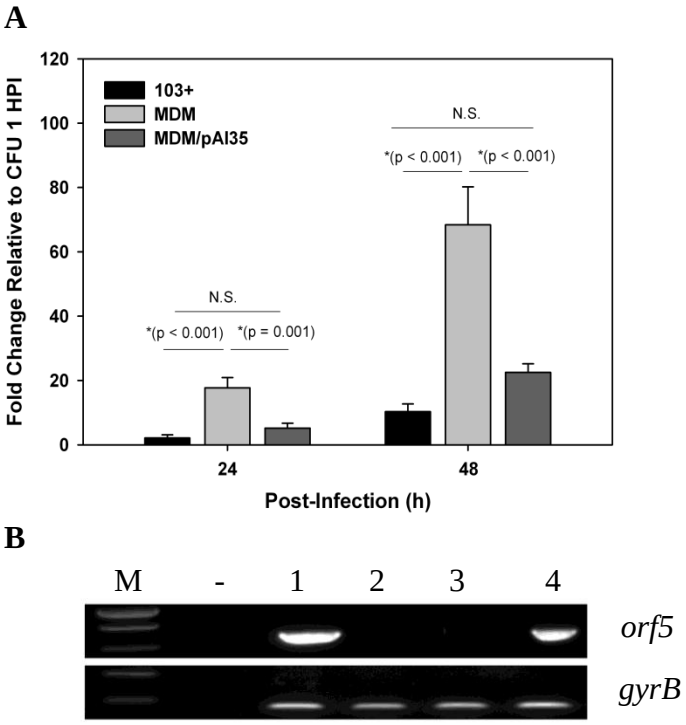

Figure 6. VapA is the only Vap-family member required for replication in macrophages. (A) Intracellular replication of *R. equi* strains in BMDMs. Macrophage monolayers were infected with *R. equi* 103+, the MDM mutant and the MDM mutant complemented with *orf5* (MDM/pAI35). Fold change in intracellular bacterial CFU among the various strains at 24 h and 48 h post-infection relative to 1 h post-infection is shown as the mean ± standard deviations for monolayers assessed in triplicate. The data shown here are representative of three independent experiments. B) RT-PCR confirmation of *orf5* expression. Total RNA was extracted from *R. equi* strains 103+ (lane 1), 103- (lane 2), MDM mutant (lane 3), and MDM/pIA35 (lane 4). cDNA was synthesized using equivalent concentrations of total RNA as the template. The presence of *orf5* and *gyrB* (internal control) was assessed by PCR using primer pairs specific for internal regions of each of the genes. Standard DNA markers are indicated on the left (M). No template control (-).

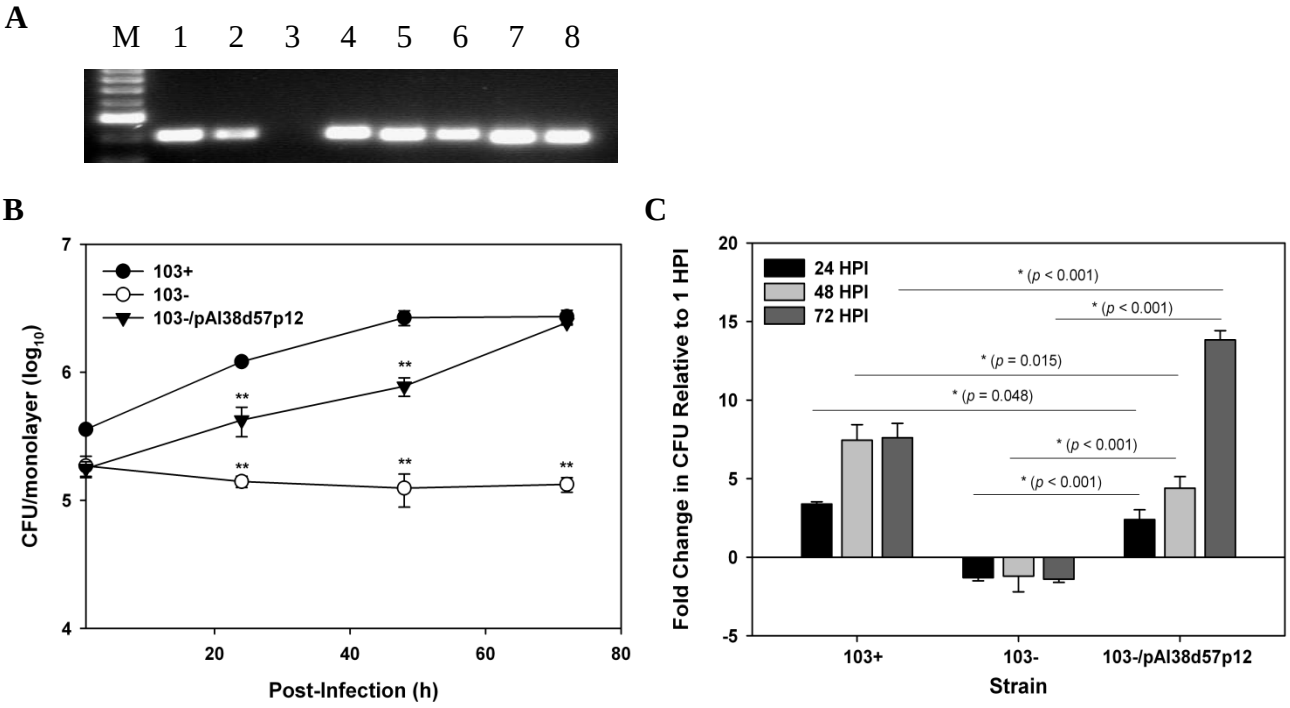

Figure 7. *vapA* and its regulators, *orf4* and *orf8*, are the only plasmid-encoded genes required for intramacrophage survival. A) RT-PCR confirmation of expression of *orf4*, *orf8* and *vapA* in *R. equi* 103- transformed with pPAI38d57p12. Total RNA was extracted from *R. equi* strains 103-/pPAI38d57p12 (lanes 1, 3, 5 and 7), and *R. equi* 103+ as a positive control (lanes 2, 4, 6 and 8). cDNA was synthesized using equivalent concentrations of total RNA as the template. The expression of *orf4* (lanes 1 and 2), *orf5* (lanes 3 and 4), *orf8* (lanes 5 and 6) and *vapA* (lanes 7 and 8) was assessed by PCR using primer pairs specific for internal regions of each of the genes. Standard DNA markers are indicated on the left (M) (B) Murine BMDM macrophage monolayers were infected with *R. equi* 103+, 103– and 103- expressing *orf4*, *orf8* and *vapA* (103-/pAI38d57p12), and bacterial intracellular replication was followed as described in the legend to Figure 2. (C) Fold change in intracellular bacterial CFU among the various strains at 24 h, 48 h and 72 h post-infection. Values shown are the means ± standard deviations for monolayers assessed in triplicate.

CHAPTER 5

CONCLUSION

The genus *Rhodococcus* is composed of a large number of genetically diverse and physiologically robust species of bacteria capable of inhabiting a large number of environmental niches (Larkin *et al*., 2005; Martinkova *et al*., 2009). Because of their metabolic flexibility, *Rhodococcus spp.* have been exploited for many years for their commercial potential in bioremediation and biodegradation (Bell *et al*., 1998; Martinkova *et al*., 2009). However, it is becoming increasingly recognized that *Rhodococcus spp*. are not only microbes of profound industrial interest, but some species are also important causes of disease in animals and humans (e.g. *R. equi*) and plants (e.g. *R. fascians*) (Brown & Hendler, 1989; Crespi *et al.*, 1992; Kedlaya *et al.*, 2001; Prescott, 1991). An intriguing question is what genetic factors distinguish the pathogenic *Rhodococcus spp.* from the non-pathogenic environmental species? The environmental members of the *Rhodococcus* genus have large plastic genomes (e.g. 9.7 Mb in *R. jostii* RH1; 8.2 Mb in *R. opacus* and 6.9 Mb in *R. erythropolis*); the result of extensive gene acquisition and amplification leading to great functional diversity and redundancy. In contrast, however, the pathogenic species, *R. equi*, has a relatively small genome of 5 Mb that appears to have undergone genome contraction rather than expansion (Letek *et al*., 2010); suggesting that the particular niche that *R. equi* inhabits is more stable than the natural niches that the environmental *Rhodococcus spp*. occupy and thus *R. equi* does not require as an diverse array of metabolic enzymes and pathways.

Although *R. equi* possesses a smaller genome compared to the environmental *Rhodococcus* spp, its genome still encodes a diverse array of virulence factors, most of which are located within horizontal gene transfer (HGT) islands on the chromosome and the virulence plasmid. Surprisingly, nearly 90% of the putative virulence determinants in *R. equi* are also found in the non-pathogenic *Rhodococcus* spp. (Letek *et al*., 2010). What truly separates *R. equi* from the non-pathogenic species is the acquisition of i) a chromosomal HGT island (*rpl* island) encoding genes for pilus biogenesis and assembly, which is believed to mediate attachment to macrophages and epithelial cells (Letek *et al*., 2010); and ii) a plasmid HGT island (pathogenicity island [PAI]). However, since both avirulent and virulent *R. equi* possess the chromosomally-encoded *rpl* island, it is believed that acquisition of the PAI is the defining evolutionary event that allowed *R. equi* to convert from a strict saprophyte to a facultative intracellular pathogen (Letek *et al*., 2008).

The *R. equi* pathogenicity island is a 21 kb fragment of foreign DNA likely acquired through an HGT event; although the identity of the organism from which this DNA originated is unknown. Since most of the chromosomal HGT genes probably originated from other *Actinobacteria*, it is possible that the PAI was acquired from a member of the same class of bacteria. However, since there are no homologs of many of the PAI-encoded genes (particularly the *vap* genes) in the bioinformatic databases, it is still unclear who is the ancestral parent of the PAI. The work of this dissertation has shown that the PAI is absolutely essential for the ability of *R. equi* to replicate in macrophages and establish of a chronic infection in the mouse model of *R. equi* infection (Coulson *et al*., 2010). Since the PAI-encoded gene *vapA* is a required virulence factor for intramacrophage replication (Jain *et al*., 2003), it is not surprising that deletion of the entire PAI led to complete attenuation of the organism. Since we could not

complement a PAI-deletion mutant with *vapA* alone, we hypothesized that there were additional virulence determinants within the PAI. Using a systematic gene deletion approach we were able to show that *vapA* was the only full-length *vap*-family member required for intracellular replication. Further gene deletion analyses revealed that only three genes on the entire PAI are required for replication of *R. equi* in macrophages (Coulson *et al*; unpublished data). These genes are *virR* (a LysR-type transcriptional regulator), *orf8* (an orphan response regulator component of a two-component type regulatory system) and *vapA*. This suggests that the acquisition of a novel functional gene and two regulators by *R. equi* was all that was required to allow a non-pathogenic *R. equi* ancestor to adapt to life within host macrophages.

While the function of VapA is still unclear, there is evidence that is consistent with a role for VapA in modulation of the *R. equi*-containing vacuole, preventing its acidification and subsequent fusion with lysosomes (von Bargen *et al*., 2009). Through manipulation of the endocytic pathway, virulent *R. equi* are capable of blocking their exposure to the harsh bactericidal conditions present within host lysosomes, thereby maintaining a favorable vacuolar environment that is beneficial to bacterial survival and replication (Fernandez-Mora *et al*., 2005). While *vapA* is necessary for intracellular growth, we and others have shown that it is not sufficient (Coulson *et al*., 2010; Giguere *et al*., 1999). In this report, we demonstrate that in addition to *vapA*, the activity of the PAI-encoded regulators, *virR* and *orf8* are also required for replication in macrophages. While both regulators are necessary for optimal *vapA* expression (Byrne *et al*., 2007; Russell *et al*., 2004), it is hypothesized that one or both additionally engage in cross-talk with the chromosome, resulting in altered gene expression of chromosomal genes. It has recently been shown via global transcription profiling and network expression analysis that a large number (n=88) of chromosomal genes are significantly differentially expressed in the

presence of the virulence plasmid under inducing conditions (37°C and pH= 6.5), suggestive of plasmid-chromosome crosstalk (Letek *et al*., 2010). Thus, it is speculated that the plasmid PAI has recruited housekeeping genes on the chromosome under its regulatory influence. In light of this recently published report and the data in this dissertation, we propose that acquisition of *vapA*, *virR* and *orf8* alone allowed the non-pathogenic saprophyte *R. equi* to parasitize macrophages of susceptible hosts.

The transcriptional regulators VirR and Orf8 drive optimal expression of the key virulence factor, *vapA*. Production of VapA and its localization at the bacterial cell surface presumably somehow permits the bacterium to persist in phagosomes by aiding in the resistance against acidification of bacteria-containing vacuoles and their fusion with host lysosomes. In addition to driving maximal *vapA* expression, the data suggest one or both of the regulators (VirR or Orf8) also engage in crosstalk with a select number of chromosomally-encoded genes, actively changing their expression profiles in a significant manner. That many of the chromosomal genes whose expression is positively influenced by the presence of the virulence plasmid include genes involved in various metabolic pathways implies that changing the expression of key metabolic enzymes in the nutrient-deprived environment of the phagocytic vacuole may be crucial to surviving in this harsh intracellular environment. Thus, it is the combined action of VapA at the host-pathogen interface and the activity of the plasmid-derived regulators altering gene expression of key chromosomally encoded metabolic enzymes that we speculate ultimately allowed *R. equi* survival and replication in host macrophages.

In conclusion, the research reported in this dissertation provides a conceptual framework for the evolutionary mechanism behind the conversion of an environmental organism, *R. equi*, into an intracellular pathogen. Through the acquisition of just a few genes via a horizontal gene

transfer event and their subsequent cooption of chromosomal genes into a complex regulatory network, the organism acquired pathogenic potential that led to its establishment as a major pathogen of horses and opportunistic pathogen of humans. While we have generated a preliminary working model for this process, several key questions remain that are currently under investigation in our laboratory. Namely, which of the regulators (VirR or Orf8, or both) are responsible for altered gene expression of chromosomal genes? Which of those chromosomal genes that are up- or down-regulated in the presence of the virulence plasmid-encoded regulators are responsible for promoting bacterial intracellular survival and replication? While *virR*, *orf8* and *vapA* promote bacterial multiplication in macrophages, are they sufficient to allow *R. equi* survival *in vivo* either in the mouse model of *R. equi* infection or in its natural host, the foal? A more thorough understanding of the answers to these questions and others will contribute to our knowledge of bacterial virulence of *R. equi* which is crucial to our ongoing efforts to develop the means to effectively prevent or control disease caused by this pathogen.

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