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Research article

The sensor kinase MprB is required for *Rhodococcus equi* virulence

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ABSTRACT

Rhodococcus equi is a soil bacterium and, like *Mycobacterium tuberculosis*, a member of the mycolata. Through possession of a virulence plasmid, it has the ability to infect the alveolar macrophages of foals, resulting in pyogranulomatous bronchopneumonia. The virulence plasmid has an orphan two-component system (TCS) regulatory gene, *orf8*, mutation of which completely attenuates virulence. This study attempted to find the cognate sensor kinase (SK) of *orf8*. Annotation of the *R. equi* strain 103 genome identified 23 TCSs encoded on the chromosome, which were used in a DNA microarray to compare TCS gene transcription in murine macrophage-like cells to growth *in vitro*. This identified six SKs as significantly up-regulated during growth in macrophages. Mutants of these SKs were constructed and their ability to persist in macrophages was determined with one SK, MprB, found to be required for intracellular survival. The attenuation of the *mprB*− mutant, and its complementation, was confirmed in a mouse virulence assay. *In silico* analysis of the *R. equi* genome sequence identified an MprA binding box motif homologous to that of *M. tuberculosis*, on *mprA*, *pepD*, *sigB* and *sigE*. The results of this study also show that *R. equi* responds to the macrophage environment differently from *M. tuberculosis*. MprB is the first SK identified as required for *R. equi* virulence and intracellular survival.

1. Introduction

The genus *Rhodococcus* contains 44 species of soil-living Gram-positive actinomycetes including one important animal pathogen, *Rhodococcus equi*. *R. equi* infection causes pyogranulomatous pneumonia in foals and immunocompromised humans, and cervical lymphadenitis in swine (Meijer and Prescott, 2004). Clinical isolates from foals possess virulence plasmids with a pathogenicity island (PAI) (Fig. 1) (Takai et al., 2000; Letek et al., 2008). This PAI encodes virulence-associated protein A (VapA), which is essential for *R. equi* survival and growth in macrophages (Giguère et al., 1999; Jain et al., 2003), and arrest of phagosomal maturation (Fernandez-Mora et al., 2005; Von Bargen et al., 2009).

Two transcriptional regulators are also present in the PAI: *virR*, a LysR-family transcriptional regulator, and *orf8*, an orphan response regulator (RR) which may be part of a two-component system (TCS) of which the cognate sensor kinase (SK) is assumed to be encoded on the *R. equi* chromosome. Mutation of either *virR* or *orf8* completely attenuates virulence (Ren and Prescott, 2004), because both are members of the VirR operon that controls the *vapA* operon, and therefore *vapA* expression (Byrne et al., 2007). Although the relationship between *orf8* and the regulation

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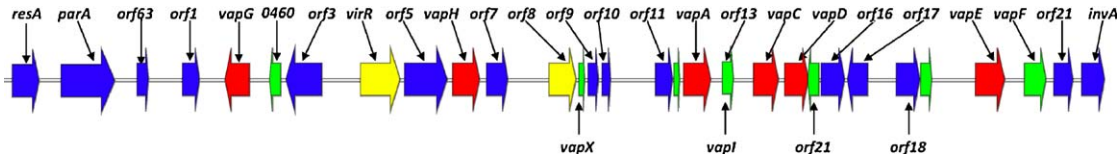

Fig. 1. Schematic of the *R. equi* pathogenicity island with the VapA encoding gene in red, the transcriptional regulator encoding genes *orf8* and *virR* in yellow and pseudogenes in green (based on Letek et al., 2008).

of VapA is known (Russell et al., 2004; Byrne et al., 2007), the relationship between *orf8* and the chromosomally encoded TCSs have not been determined. The *R. equi* genome sequence encodes 24 SKs and 26 RRs of which 23 are encoded in pairs. To investigate the role of TCSs in the regulation of virulence of *R. equi*, and as an approach to identifying the SK of *orf8*, we used a DNA microarray to measure the expression of each TCS in macrophages and assessed the virulence of 6 SK mutants in macrophages and in mice.

2. Materials and methods

2.1. Bacterial strains, plasmids and propagation

Bacterial strains used in this study are listed in Table 1. *R. equi* 103^+ (virulence plasmid-positive) was the wild-type strain used in all experiments. The plasmid-cured strain *R. equi* 103^- was used as a negative (avirulent) control in the infection studies (Ren and Prescott, 2004). *R. equi* strains were grown in brain-heart infusion (BHI) or Luria-Bertani (LB) broth (Difco, Detroit, MI, USA) at pH 7.0, at 37 °C or 30 °C. *Escherichia coli* cultures were grown on LB at 37 °C for 16 h. To assess growth of *R. equi* TCS mutants, bacterial growth was measured in 10 mL of BHI at 37 °C shaking at 200 rpm in test tubes. Cultures were inoculated from overnight cultures to give a starting $OD_{540\ nm}$ of 0.1.

The plasmid pNBV1 was used as a complementation vector (Howard et al., 1995) and the suicide plasmid vector pUCApr was used for single crossover mutagenesis (Pei et al., 2007). The pGEM-T Easy vector (Promega, Madison, WI, USA) was used for cloning. Plasmid DNA was purified using a Qiagen DNA purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Apramycin, hygromycin and/or kanamycin (Sigma, St. Louis, MO, USA) was added as appropriate. The final concentration of apramycin was 50 μg/ml, of hygromycin was 200 μg/ml, and of kanamycin was 200 μg/ml for *R. equi* and 50 μg/ml for *E. coli*. For blue-white screening of colonies containing recombinant plasmids, 50 μg/ml of X-gal (Sigma) was spread onto LB agar.

2.2. Polymerase chain reaction (PCR)

Genomic DNA template was made using InstaGene Matrix (BioRad, Hercules CA, USA) according to the manufacturer's instructions. A 1 μl aliquot of purified DNA was used in each 25 μl PCR reaction. Each reaction contained genomic DNA template, buffer as per the manufacturer's specification, dNTPs (25 μM each), 0.5 μM of each primer, and 2.5 units of Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA). PCR reactions were carried out using a touchdown PCR program with denaturing at 95 °C for 3 min, 25 cycles of 95 °C for 45 s, annealing temperatures ranging from 65 to 50 °C for 45 s (the annealing temperature is decreased by 1 °C every cycle until 50 °C), 72 °C for 1 min, and a final elongation time of 1 min at 72 °C. The sequences of primers used in this study are shown in Table 2.

2.3. Mutagenesis of selected two-component sensor kinases

Target genes were amplified by PCR and PCR products were cloned into pGEM-T Easy (Promega). The approximately 600 bp amplicon that corresponded to the target gene was subcloned in the suicide vector pUCApr, the construct denatured (Navas et al., 2001; Ren and Prescott, 2004) and introduced into wild-type *R. equi* 103^+ by electroporation at 1.8 KV/cm using 1 μg of DNA. The

Table 1
Bacterial strains used.

Strains[a]	Description	Source
E. coli DH5α	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	Invitrogen
R. equi 103^+	Wild-type virulent strain, contains virulence plasmid	Laboratory strain
R. equi 103^-	Virulence plasmid minus strain	Laboratory strain
	Recombinant strains	
9230-	Mutant of 9230 SK gene	This work
prrB-	Mutant of 9960 (*prrB*) SK gene	This work
10530-	Mutant of 10530 SK gene	This work
devS-	Mutant of 11030 (*devS*) SK gene	This work
mprB-	Mutant of 11650 (*mprB*) SK gene	This work
45230-	Mutant of 45230 SK gene	This work
mprB(11650c2)	*mprB*-mutant containing pNBV1-::11650c2	This work

[a] Numbers (e.g. *9230*) refer to gene number assigned in annotated genome of 103^+; gene named assigned if homologous to named SKs in other genomes.