Identification of genes required for the fitness of *Rhodococcus equi* during infection of mice by signature-tagged transposon mutagenesis

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シグネチャータグトランスポゾン変異導入法による
ロドコッカス・エクイの
マウス感染への適応に関与する遺伝子の同定

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Chapter 1 Introduction

The genus *Rhodococcus* belongs to the order *Actinomycetales*, the sub-order *Corynebacterineae* and the family *Nocardiaceae*. This family contains the genera *Corynebacterium*, *Mycobacterium* and *Nocardia*. *Rhodococcus* is a Gram-positive, variably acid-fast, nonmotile, mycolate-containing, nonsporulating, catalase positive and oxidase negative coccobacillus [3, 48]. This genus contains more than 40 species, several of which are used in industrial biotechnology as bioactive compound producers [37].

Rhodococcus equi is the only one that can cause diseases in animals among species of *Rhodococcus* [37]. *R. equi* was first isolated from the lung lesions of Swedish pneumonic foals in 1923 [23]. *R. equi* is one of the most serious bacterial pathogens causing suppurative pneumonia and enteritis associated with lymphadenitis in foals less than 6 months of age. *R. equi* infection causes high mortality in newborn foals. This species is also an opportunistic pathogen of other mammals including immunocompromised humans, especially individuals with AIDS [70, 73, 74, 86].

Disease caused by *R. equi* has serious economic impacts on horse breeding farms, and it has been isolated from the soil and feces at farms with or without a history of disease [37, 62, 70]. The foals are infected with *R. equi* by inhalation of contaminated soil dust. The main routes of infection are thought to be the respiratory and alimentary tracts. Clinical manifestations in the early stage of infection include pyrexia, cough and an increase of white blood cells. Infection can lead to pyogranulomatous pneumonia and the development of multiple lung abscesses. In addition, naturally infected foals with severe pneumonic lesions may have granulomatous mesenteric lymphadenitis and ulcerative colitis [23, 48, 86].

R. equi is known as an intracellular bacteria that can survive and multiply inside macrophages. However, not all strains of *R. equi* possess the ability to resist death by macrophages. Only the strains bearing the virulence plasmid can multiply in macrophages and

exhibit virulence in animals [70]. After the virulent strains of *R. equi* are phagocytosed, they remain inside phagosome and grow up within it [24]. The phagosomes containing the virulent *R. equi*, termed *R. equi*-containing vacuoles (RCVs), normally acquire early endosome markers, but are arrested between the early and late maturation stages of endosomes. RCVs do not acquire hydrolytic enzymes, such as the cathepsin family, and are not acidified. The intraendocytic environment mediated by virulent *R. equi* is thought to be permissive for growth of *R. equi*, but the mechanism involved in this phenomenon is not fully known [15, 52, 80].

R. equi has three different types of virulence plasmids, pVAPA, pVAPB and pVAPN [76]. *R. equi* strains bearing pVAPA are mainly isolated from the horses and their environment. pVAPA encodes the gene for virulence-associated protein A (VapA) whose molecular weight is 15-17 kDa [70, 73]. The strains possessing pVAPB are isolated from the submaxillary lymph nodes of pigs, wild bores and their environment. This type of plasmid encodes the gene for VapB whose molecular weight is 20 kDa [63, 64, 71]. pVAPN is a linear plasmid that was recently discovered in the *R. equi* isolates derived from lymph nodes of cattle, and the gene *vapN* is encoded on this plasmid [61, 76]. These three plasmids distribute separately, but all three types were found among the strains isolated from humans infected with *R. equi* [51]. The nucleotide sequences of all virulence plasmids have been determined. pVapA contained 73 open reading frames (ORFs), which were divided into four major regions consisting of the virulence-related gene region, the plasmid replication and partitioning-related gene region, the conjugation-related gene region and the region containing genes with unknown function [65, 75]. The virulence-related gene region is a crucial part of the plasmid for bacterial virulence and is designated as a pathogenicity island (PAI). The size of the PAI is 21.3 kb and its G+C content is 60.8%. This locus contains six *vap* genes (*vapA, vapC, vapD, vapE, vapG* and *vapH*) and three *vap* pseudogenes (*vapI*, *vapF* and *vapX*) [38]. The expression of *vapA* is

transcriptionally regulated by at least two regulators in the PAI, LysR-type regulator VirR and two-component regulator VirS [31, 32, 54].

VapA is a necessary factor for survival of *R. equi* in mice and intracellular growth in macrophages. However, it was reported that a plasmid-cured derivative expressing wild-type levels of VapA could not replicate in murine macrophages and was not pathogenic in the infected foals [23, 69]. Coulson *et al*. [12] reported that two transcriptional regulators, *virS* and *virR*, in addition to *vapA* were required for intracellular survival of *R. equi*. Therefore, these transcriptional regulators may confer enhanced fitness to *R. equi* in the intracellular environments through the transcriptional regulation of many genes encoded on the chromosome.

The genome sequence of the 103S strain of *R. equi* isolated from a sick foal was previously determined [37]. The circular chromosome is 5,043,170 bp in length and encodes 4,525 predicted genes. Analysis of the *R. equi* chromosome revealed that half of the genes were involved in metabolism, secretion and extracellular proteins. The regulatory genes occupy 10.3% of chromosomal genes. However, approximately one-third of the gene functions are unknown. *R. equi* possesses an average-size genome among actinobacteria, but the percentages of the regulome and secretome are larger than in the other actinobacteria. This may be an evolutional consequence of adaptation to stress conditions that occurred during the change from extra- to intrahost environments [37].

Several chromosomal genes of *R. equi* have already been identified as virulence factors. Catalase encoded by *katA* plays an important role in the defense mechanism against hydrogen peroxide (H_2O_2) derived from macrophages [4, 5]. Deletion of *katA* resulted in inability to survive in macrophages [4, 5].

Nitrate reductase (*narG*) has been reported to be important for the virulence of *R. equi* [47]. The product of this gene is involved in respiration under the hypoxic conditions [21].

R. equi forms necrotic pyogranulomatous lesions in which a microaerobic environment is typically found [60]. The *narG* mutant of *R. equi* results in full attenuation of growth in mice [47].

The mutation of the *aceA* gene encoding isocitrate lyase, the first enzyme of the glyoxylate bypass, resulted in attenuation in mice and disability to replicate in macrophages [82]. When 3-week-old foals were intrabronchially infected with the *aceA* mutant, they did not develop pneumonic disease [82].

Both α- (*ipdA*) and β-subunits (*ipdB*) of heterodimeric coenzyme A transferase are essential for the steroid catabolic pathway. The mutant with deletion of *ipdAB* had partially attenuated replication in macrophages [77].

It has been reported that high-temperature requirement A (*htrA*) and peptidase D (*pepD*) are required for virulence of *R. equi* [47]. The *pepD* gene that is found downstream of the *mprAB* genes is regulated by sensor kinase (*mprA*) and its response regulator (*mprB*). The *mprB* mutation exhibited defects in bacterial growth in macrophages and mouse infection. The *mprAB* genes are considered to be involved in response to stress conditions after macrophage phagocytosis [40].

Rhequichelin biosynthesis gene cluster (*rhbABCDE*), which is involved in biosynthesis of the hydroxamate siderophore rhequichelin, is related with iron uptake under iron limitation. Iron is an essential micronutrient for bacterial growth. The transcriptional level of *rhbC* in *R. equi* was highly up-regulated during growth under iron-depleted conditions [42]. Deletion of *rhbCD* resulted in the inability to grow in macrophages and *in vivo*, indicating that iron acquisition systems are important for virulence of *R. equi* [42].

The comparative genomic analysis of *Mycobacterium tuberculosis* and *Rhodococcus* sp. revealed that they were quite similar in structure and gene function; 60% of *Mycobacterium tuberculosis* H37Rv genes are conserved in *Rhodococcus* sp. strain RHA1 [78]. However, only

a few virulence factors of *R.equi* (see above) have been identified, whereas more than 100 genes have been identified in *Mycobacterium tuberculosis* complex [17]. Therefore, the main purpose of this thesis was to identify new virulence genes of *R. equi* to understand the mechanisms underlying *R. equi*. infection.

VapA is necessary for virulence of *R. equi*, but VapA alone is not sufficient to confer full virulence [19]. Recently, it was found that extracellular VapA influenced the endocytic maturation of tissue culture cells, including macrophages [52]. In chapter 2, whether the extracellular addition of VapA could rescue the growth defect of the *vapA* mutant and a plasmid-cured strain in macrophages was examined. When infected macrophages were co-cultured with VapA, the number of macrophages containing proliferating bacteria increased. On the other hand, an *Escherichia coli* strain that cannot normally grow in macrophages did not proliferate in macrophages under the same conditions. These data suggested that the intraphagocytic environment mediated by VapA still restricted the growth of bacteria. Furthermore, *R. equi* lacking the virulence plasmid may possess the ability to overcome some of the growth limitations in VapA-mediated phagosomal environments [57].

The results in chapter 2 raised the possibility that the *R. equi* chromosome possesses unique genes for adaption and survival in the intraphagosomal environment. In chapter 3, the essential genes encoded on the chromosome for *R. equi* infection were identified by signature-tagged transposon mutagenesis (STM). A total of 4,650 *R. equi* transposon mutants were examined for bacterial survival during mouse infection. As a result, 35 mutants that had transposon insertions in their chromosomes demonstrated a reduced ability to infect mice. The transposon insertions were found in genes involved in amino acid metabolism, lipid metabolism, vitamin synthesis, DNA repair / recombination, peptidoglycan biosynthesis / degradation, energy metabolism, non-ribosomal peptide synthesis (NRPS) and unknown protein function.

In chapter 4, the ability of mutants selected by STM to survive and replicate within macrophages was examined. Mutation of DEAD/DEAH helicase (REQ05430), oxidoreductase (REQ10430), acyl-CoA thioesterase II (REQ25310), hypothetical protein (REQ26170) with transposon insertion between the putative HNH endonuclease (REQ27330) and putative Fis family transcriptional regulator (REQ27340), carotenoid oxygenase (REQ28270) and putative nitrite reductase large subunit NirB1 (REQ32930) resulted in a reduced ability to grow in macrophages. On the other hand, an NRPS (REQ35940) mutant had an enhanced ability to multiply in macrophages. In this thesis, the *R. equi* chromosome was revealed to encode factors contributing to the encouragement of bacterial growth in macrophages during infection that are not found in *E. coli*.

Chapter 2 Rescue of intracellular avirulent *R. equi* replication defect by the extracellular addition of virulence-associated protein A

2-1 Introduction to the chapter

R. equi is a facultative intracellular pathogen that can replicate in compartments known as RCVs within infected macrophages [15]. The ability of *R. equi* to survive and multiply inside macrophages is regulated by a virulence-associated plasmid [19]. The plasmid-cured *R. equi* strains are unable to survive in macrophages and are avirulent in foal or murine infection models [12, 15]. Deletion of VapA resulted in the loss of bacterial persistence in mouse as it abolished proliferation in macrophages. VapA alone restored full virulence in *vapA* and *vap* locus (*vapA*, -*C*, -*D*, -*E*, and –*F*) deletion mutants, whereas VapC, -D and –E did not [27]. However, the complementation of the plasmid-cured strain with *vapA* did not recover the ability for intracellular replication in murine macrophages [19].

Recently, it was reported that VapA was taken into endocytic compartments of the macrophages cultured *in vitro* when it was added to the culture media. Then, the cells fed VapA formed swollen endolysosome organelles with reduced cathepsin B activity and accumulation of LBPA, LC3 and Rab7 [52]. These results suggest that VapA causes disorder of endolysosomal function, which may lead to tolerance of bacterial growth in macrophages. It was reported that the *R. equi* strain lacking the *vapA* gene exhibited growth defects in the macrophages [27, 80].

In this study, it was therefore examined whether the extracellular addition of VapA could rescue the growth defects of the *vapA* mutant. Furthermore, to clarify the characteristics of the endocytic environment mediated by VapA, a plasmid-cured strain and *E. coli* that cannot normally grow in macrophages were also used.

2-2 Materials and Methods

2-2-1 Bacterial strains and plasmids

R.equi strain ATCC 33701, originally isolated from a pneumonic foal, was used in this study [70, 72]. The isogenic but avirulent *R. equi* strain ATCC 33701P$^-$ is a derivative in which the virulence-associated plasmid has been cured. TKR 255 is an *R. equi* ATCC 33701 derivative in which lacZ replaces *vapA* [31]. These strains were routinely grown in brain-heart infusion (BHI) broth with vigorous shaking at 30˚C. *E. coli* DH5α and BL21 strains were grown in Luria-Bertani (LB) broth. All *R. equi* and *E. coli* strains were stored at -80˚C in 85% broth and 15% glycerol solution.

Reagents, solution and equipment

1) Culture medium

- Brain-heart infusion broth (BBL)
- Luria-Bertani agar (Invitrogen)
- Luria-Bertani broth (GibcoBBL)

2) 80% Glycerol

- 80 ml of glycerol (KANTO CHEMICAL CO., INC.)
- Make up to 100 ml by distilled water

2-2-2 Construction of the bacteria strains

For preparation of the GST-VapA fusion protein, the plasmid pGEX-4T-1 (GE Healthcare Life Sciences, Piscataway, NJ, U.S.A.) was used to express *R. equi* VapA as a GST fusion protein. The *vapA* gene was amplified by polymerase chain reaction (PCR) with the primers vapA-BamF and vapA-MfeR (Table 2-1). The amplified gene fragment (618 bp) was cloned into the pGEM-T vector. pGEM-*vapA* was digested with *Bam*HI and *Eco*RI, and then

ligated into *Bam*HI and *Eco*RI double-digested pGEX-4T-1 to create pGEX-4T-1::*vapA*. BL21 cells were then transformed with pGEX-4T-1::*vapA*. Bacteria were grown in LB broth with ampicillin (50 μg/ml) at 37˚C.

The *aphII* promoter (P_{aphII}) region was amplified using the primers P_{kan2}-temF and P_{kan2}-nTR (Table. 2-1). The PCR product (421 bp) was digested with *Bam*HI and *Spe*I, and cloned into the *Streptomyces* φC31 integrase-based integration vector pINT [25, 31] that was digested with *Bam*HI and *Spe*I to create pINT::P_{aphII}. The enhanced green fluorescent protein (*EGFP*) ORF was amplified using the primers EGFP-F and EGFP-R (Table 2-1). The PCR product (729 bp) was digested with *Nde*I and *Mfe*I, and cloned into pINT::P_{aphII} that was digested with *Nde*I and *Mfe*I to create to pINT::P_{aphII}-*EGFP*. pINT::P_{aphII}-*EGFP was* electroporated into *R. equi* ATCC 33701, ATCC 33701P$^-$, TKR 255 and *E. coli* DH5α. Transformants were recovered on LB agar containing 60 μg/ml of apramycin.

Reagents, solution and equipment

1) Culture medium

- Brain-heart infusion broth
- Luria-Bertani agar
- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of ampicillin soluble in distilled water (SIGMA)
- 80 mg/ml of apramycin soluble in distilled water (SIGMA)

3) pGEM-T Easy vector (Promega)

4) Plasmid pGEX-4T-1 (GE Healthcare Life Sciences, Piscataway, NJ, U.S.A.)

5) DNA extraction kit (DNeasy® Blood and Tissue Kit, QIAGEN)

6) Plasmid purification kit mini (QIAprep® Spin Miniprep Kit, QIAGEN)

7) Gel Extraction kit (MinElute® Gel Extraction Kit, QIAGEN)

8) PCR Purification kit (QIAquick® PCR Purification Kit, QIAGEN)

2-2-3 Preparation of GST-VapA fusion protein

The *E. coli* strain BL21 was transformed with pGEX-4T-1::*vapA*. Bacteria were grown in LB broth with ampicillin (50 μg/ml) at 37˚C with shaking. The expression of GST-VapA fusion protein was induced by the addition of IPTG at a final concentration of 1 mM for 5 hr at 30˚C. The bacteria were pelleted at 4,500 rpm for 15 min at 4˚C. The cell pellet was resuspended in 20 ml of bacterial lysis buffer (1% (v/v) Triton X-100 and 20 mg of lysozyme in 1x PBS) and placed on ice for 30 min. The cells were lysed by sonication (10-second short pulse intervals) on ice for 10 min. The lysed cells were centrifuged at 12,000 rpm for 30 min. Ten ml of supernatant were incubated with 1 ml of Glutathione Sepharose 4B beads for 30 min with gentle rotation. The beads were centrifuged at 500 × g for 5 min and washed with 1x PBS 3 times. To elute GST or GST-VapA fusion protein, 1 ml of elution buffer was added, gently rotated at room temperature for 10 min and then centrifuged at 500 × g for 5 min. The eluted proteins were dialyzed against 1x PBS for 1 hr. The protein concentration was measured with the Quick Start Bradford protein assay kit (BIO-RAD) according to the manufacturer's instructions.

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar
- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of ampicillin soluble in distilled water

3) Plasmid pGEX-4T-1 (GE Healthcare Life Sciences, Piscataway, NJ, U.S.A.)

4) Isopropyl-*β*-D(-)-thiogalactopyranoside (IPTG) (Wako pure Chemical Industries, Ltd.)

5) 1x Phosphate buffer saline (1x PBS)

- 80 g of NaCl (KANTO CHEMICAL CO., INC.)
- 2 g of KCl (KANTO CHEMICAL CO., INC.)
- 29 g of $Na_2HPO_4 \cdot 12\ H_2O$ (KANTO CHEMICAL CO., INC.)
- 2 g of KH_2PO_4 (KANTO CHEMICAL CO., INC.)
- 800 ml of distilled water, adjust pH 7
- Make up to 1,000 ml by distilled water
- Dilute 1:10 with distilled water before use

6) Octylphenoxypolyethoxyethanol (Triton X-100) (SIGMA)

7) Lysozyme from chicken egg white (SIGMA)

8) Glutathione Sepharose™ 4B (GE Healthcare)

9) Elution buffer (50 mM Tris-HCl pH 8.0, 10 mM reduced glutathione)

- 121.1 g of Tris (Trizma® base, Sigma Life Science)
- 800 ml of distilled water, adjust pH 8.0 using concentrated HCl
- Make up to 1,000 ml by distilled water
- Autoclave
- 1 ml of 1 M Tris-HCl pH 8.0 with 19 ml of distilled water
- Add 61 mg of L-Glutathione reduced minimum 99% (SIGMA)
- Store -30˚C

10) Molecularporous membrane tubing (Spectra/Por®)

11) Column 1, 5 ml (Halbmikro)

12) Quick Start Bradford Dye Reagent (BIO-RAD)

13) Quick Start Bradford protein assay kit 2 (BIO-RAD)

2-2-4 Macrophage cell culture

Murine macrophage-like J774A.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum and 0.1% (v/v) gentamicin sulfate in a humidified 5% CO_2 atmosphere at 37˚C. The cells were passaged by harvesting with 0.25% Trypsin-EDTA in Hank's Balanced Salt Solution. A total of 1.5×10^5 cells per ml was seeded onto glass coverslips in 24-well plates and cultured without antibiotic for 24 hr.

Reagents, solution and equipment

1) Culture medium

- Dulbecco's modified Eagle's medium (DMEM [gibco®])
- Fetal bovine serum (CCT)

2) Antibiotics stock solution

- Gentamicin sulfate solution (Wako)

3) Enzymatic cell dissociation reagent (0.25% Trypsin-EDTA in Hank's Balanced Salt Solution)

- 1.25 g of Trypsin (MERCK)
- 0.186 g of EDTA.2Na (DOJINDO)
- Make up to 450 ml by distilled water
- 50 ml of Hank's Balanced Salt Solution (HBSS [gibco®])

4) Cell culture flask, 250 ml (Greiner bio-one)

5) Micro cover glass (MATSUNAMI)

6) 24 well cell culture plate sterile, with lid (CELLSTAR®)

2-2-5 *R. equi* and *E. coli* infection

R. equi strains were grown in BHI broth at 30˚C with vigorous shaking for 48 hr. *E. coli* was cultured overnight in LB broth with shaking at 37˚C. Macrophages were infected with bacteria at a multiplicity of infection (MOI) of 10 for 1 hr, and then the monolayers were washed 3 times with warm 1x PBS. Fresh DMEM medium with 10% fetal bovine serum containing amikacin (20 μg/ml) and the recombinant protein at 100 μg/ml was added. The cells were incubated for the indicated period in 5% CO_2 at 37˚C. After incubation for 2, 12 or 24 hr, the cells were washed 3 times with warm 1x PBS and observed using a fluorescence microscope BX 51. The number of infected macrophages was determined by counting 200 macrophages on the glass coverslip. The percentage of macrophages containing 1-9 bacteria, 10 or more bacteria per macrophage and uninfected macrophages was calculated.

Reagents, solution and equipment

1) Culture medium

- Brain-heart infusion broth
- Luria-Bertani agar
- Luria-Bertani broth
- DMEM
- Fetal bovine serum

2) Antibiotics stock solution

- 20 mg/ml of amikacin disulfate salt soluble in distilled water (SIGMA)

3) 1x PBS

4) Micro slide glass

5) Gold antifade reagent (SlowFade®)

6) Fluorescence microscope BX 51 (Olympus, Tokyo, Japan)

2-3 Results

2-3-1 The replicative ability of *R. equi* within macrophages

To examine the influence of the extracellular addition of VapA on the intracellular growth of *R. equi*, determination of the bacterial numbers inside cells is required. However, a traditional colony forming unit (CFU) assay may not be sufficiently accurate because *R. equi* multiples within macrophages in robust clusters [24]. Therefore, we constructed an EGFP-expressing strain of *R. equi* to observe the intracellular growth of *R. equi* by fluorescence microscopy without the need for staining.

The macrophage J774A.1 cells were infected with strains ATCC 33701 and ATCC 33701P$^-$ expressing EGFP, and were observed by fluorescence microscopy at 2, 12 and 24 hr post-infection. The number of macrophages containing no bacteria, 10 or less and more than 10 bacteria were counted. The percentage of macrophages containing more than 10 bacteria increased in strain ATCC 33701 over time, but no such increase was observed in strain ATCC 33701P$^-$. The percentage of macrophages containing more than 10 bacteria for strain ATCC 33701 was significantly (P<0.01) higher than that for strain ATCC 33701P$^-$ at 12 and 24 hr post-infection (Fig. 2-1). At 24 hr post-infection, a number of macrophages containing the bacterial clusters were observed when cells were infected with strain ATCC 33701 (Fig. 2-1). In contrast, no growth in clusters was observed in the macrophages infected with ATCC 33701P$^-$, although a few EGFP-expressing bacteria were still observed in many of the macrophages.

2-3-2 The extracellular addition of GST-VapA rescued the growth defect of the *vapA* mutant and ATCC 33701P$^-$ in macrophages

The J774A.1 macrophages were infected with the *vapA* mutant in the presence of GST-VapA (100 μg/ml). The percentage of macrophages containing more than 10 bacteria in the

vapA mutant significantly (P<0.01) increased and bacterial growth in clusters was observed at 12 and 24 hr post-infection (Fig. 2-2A and B). In contrast, the addition of GST alone did not result in growth of the *vapA* mutant.

In macrophages infected with ATCC 33701P$^-$ and fed GST-VapA, the number of macrophages containing more than 10 bacteria increased over time, and bacteria inside vacuoles multiplied in clusters at 24 hr post-infection (Fig. 2-2A and B).

2-3-3 The effects of the extracellular addition of GST-VapA in the macrophages infected with *E. coli* DH5α

To examine whether the endocytic environment mediated by VapA restricts the growth of bacteria that cannot normally grow in macrophages, *E. coli* DH5α was used. No obvious growth of *E. coli* was observed by 24 hr post-infection, although the frequencies of macrophages containing 10 or less bacteria were significantly higher in the presence of GST-VapA than with GST at 12 and 24 hr post-infection (Fig. 2-2A and B).

2-4 Discussion

The ability of *R. equi* to survive and multiply inside macrophages is conferred by a virulence plasmid encoding VapA [24].VapA is a known major virulence factor, but its function is still unclear [80]. It has been reported that *R. equi* strains bearing the virulence plasmid can replicate inside macrophages, whereas its plasmid-cured strain cannot [24]. The present study clearly reconfirmed the importance of the virulence plasmid in intra-macrophage growth of *R. equi* by using bacteria expressing green fluorescent protein.

In this study, the extracellular addition of VapA rescued the intracellular growth defect of the plasmid-cured strain. Rofe *et al.* [52] reported that the extracellular addition of VapA resulted in the formation of swollen endolysosome organelles with reduced cathepsin B activity and accumulation of late endocytic markers, such as LBPA and Rab7, suggesting the loss of functional endolysosomes. These features are similar with those found in RCVs [15]. Our findings also demonstrated that *E. coli* could not multiply in the macrophages in the presence of VapA. This suggested that the intraphagocytic environment can still restrict the growth of bacteria. Conversely, *R. equi* lacking the virulence plasmid may possess the ability to overcome some of the VapA-mediated growth limitations in phagosomes.

Giguère *et al*. [19] reported that a plasmid-cured derivative expressing wild-type levels of VapA could not replicate in murine macrophages. Coulson *et al*. [11] revealed that expression of VapA from a constitutive promoter failed to restore the intracellular growth defect of a strain lacking the pathogenicity island locus containing the *vapA* gene. These authors suggested that the virulence plasmid encodes additional genes required for growth in macrophages. Thereafter, it was demonstrated that two transcriptional regulators, *virS* and *virR*, in addition to *vapA* were required for the intracellular survival of *R. equi* [12]. It has been proposed that these transcriptional regulators confer enhanced fitness of the intracellular environment to *R. equi* through the transcriptional regulation of many genes encoded on the

chromosome. In the present study, a plasmid-cured strain that lacked *virS* and *virR* was able to multiply in macrophages in the presence of VapA, similar with virulent *R. equi*. This suggests that the vacuoles resulting from the extracellular addition of VapA may have different characteristics from those of RCVs formed by the infection of virulent *R. equi*. The expression of VapA is strictly regulated by pH and temperature [67]. Furthermore, VapA is not secreted into the extracellular space and is therefore restricted to the surface of bacteria, at least during *in vitro* growth [8]. Thus, differences in the quantity, timing of expression and localization of VapA may be responsible for this discrepancy.

2-5 Summary

The ability of *R. equi* to survive and multiply inside macrophages is linked to possession of the virulence plasmid encoding VapA. Intracellular growth within macrophages is critical for *R. equi* to survive in mice [24]. It was also reported that the *R. equi* ability to grow within macrophages is correlated with virulence in foals [19, 24]. Recently, VapA was found to be taken into endocytic compartments of macrophages cultured *in vitro* when it was added to the culture media [52]. Therefore, the purpose of this study was to examine the effects of extracellular addition of GST-VapA during bacterial infection of macrophages.

In this study, the integration vector containing the *EGFP* gene was introduced into *R. equi* ATCC 33701, the plasmid-cured strain (ATCC 33701P$^-$), a *vapA* mutant and *E. coli* DH5α. J774A.1 macrophages were infected with these bacteria. The numbers of bacteria were determined by counting 200 macrophages at 2, 12 and 24 hr post-infection. Macrophages were infected with the plasmid-cured strain or *vapA* mutant and were co-cultured with GST-VapA, which was added extracellularly. The number of macrophages containing proliferating bacteria increased when both strains infected macrophages in the presence of GST-VapA at 24 hr post-infection. On the other hand, the addition of GST alone did not result in growth of the plasmid-cured strain or *vapA* mutant. The *E. coli* strain DH5α was chosen because it is unable to multiply within macrophages. The percentage of macrophages containing more than 10 bacteria was not significantly different between 2 and 24 hr post-infection in *E. coli* even when GST-VapA was added to the culture medium. However, the virulence-plasmid-cured strain was able to multiply to nearly wild-type levels by the addition of GST-VapA. The present data suggest that VapA can alter the intraphagocytic environment, thereby affecting its suitability for the growth of *R. equi*. Although the addition of GST-VapA rescued intracellular survival of *R. equi*, *E. coli* survival was not rescued. Thus, *R. equi* chromosomes may possess unique genes for adaption and survival in the intraphagosomal environment.

Table 2-1. Sequences of forward and reverse primers for PCR

Gene	**Primer (5′-3′)**
vapA-BamF	GGATCCACCGTTCTTGATTCCGGTAG
vapA-MfeR	CAATTGCGCAGCCTGCATGTTTCTGG
Pkan2-termF	GGATCCGCACCGGCCCCGGAGGACCACCGCGTCCTCCGG
Pkan2-nTR	ACTAGTCAATTGGATATCCATATGAAACGATCCTCATCCTG
EGFP-F	CATATGGTGAGCAAGGGCGAGGA
EGFP-R	CAATTGTTACTTGTACAGCTCGTCCATGC

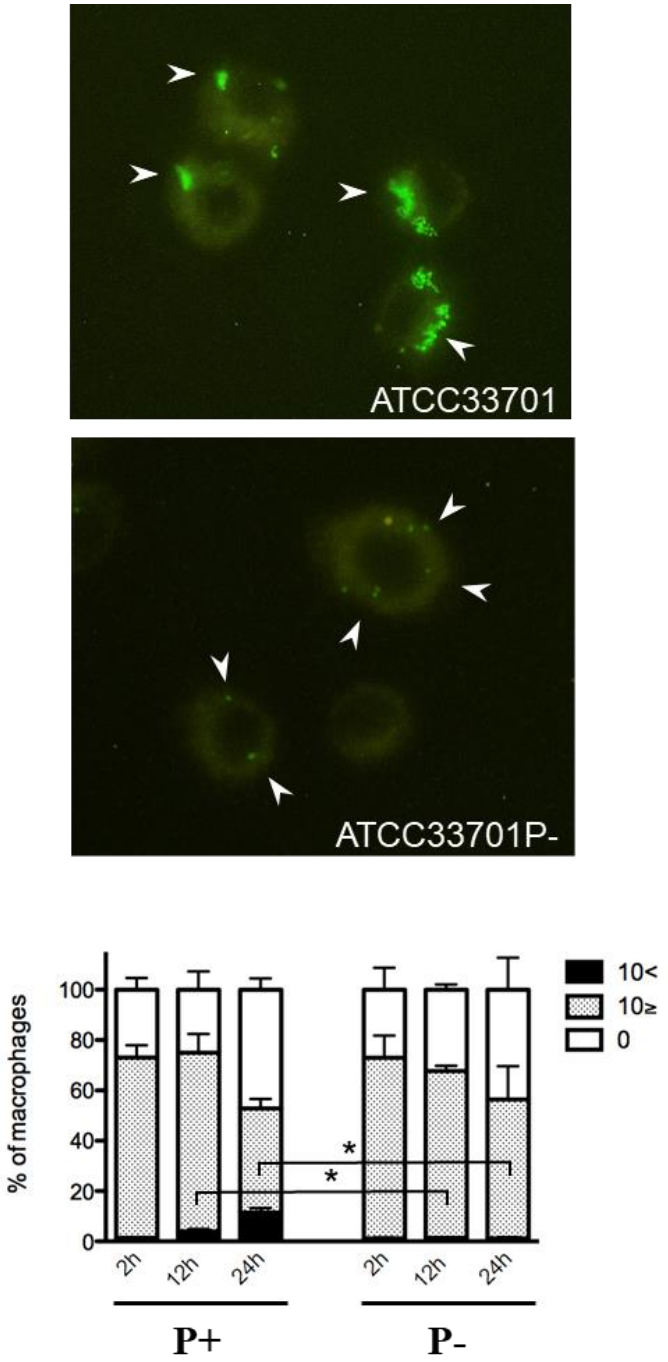


Fig. 2-1. Intramacrophage growth of EGFP-expressing *R. equi*. J774A.1 macrophages were infected with *R. equi* ATCC 33701 and P^- strains. The numbers of macrophages with 0, ≤10 or >10 bacteria were recorded at several times throughout infection. The data are expressed as the percentage of macrophages containing the bacterial numbers indicated. Each error bar

represents the standard deviation for the mean of three data sets (200 macrophages were examined per set). The significance of differences in frequencies of *R. equi*-infected macrophages was calculated using the Chi-square test (*, $P<0.01$). Macrophages were observed by fluorescence microscopy at 24 hr post-infection. *R. equi* cells are indicated by arrowheads.

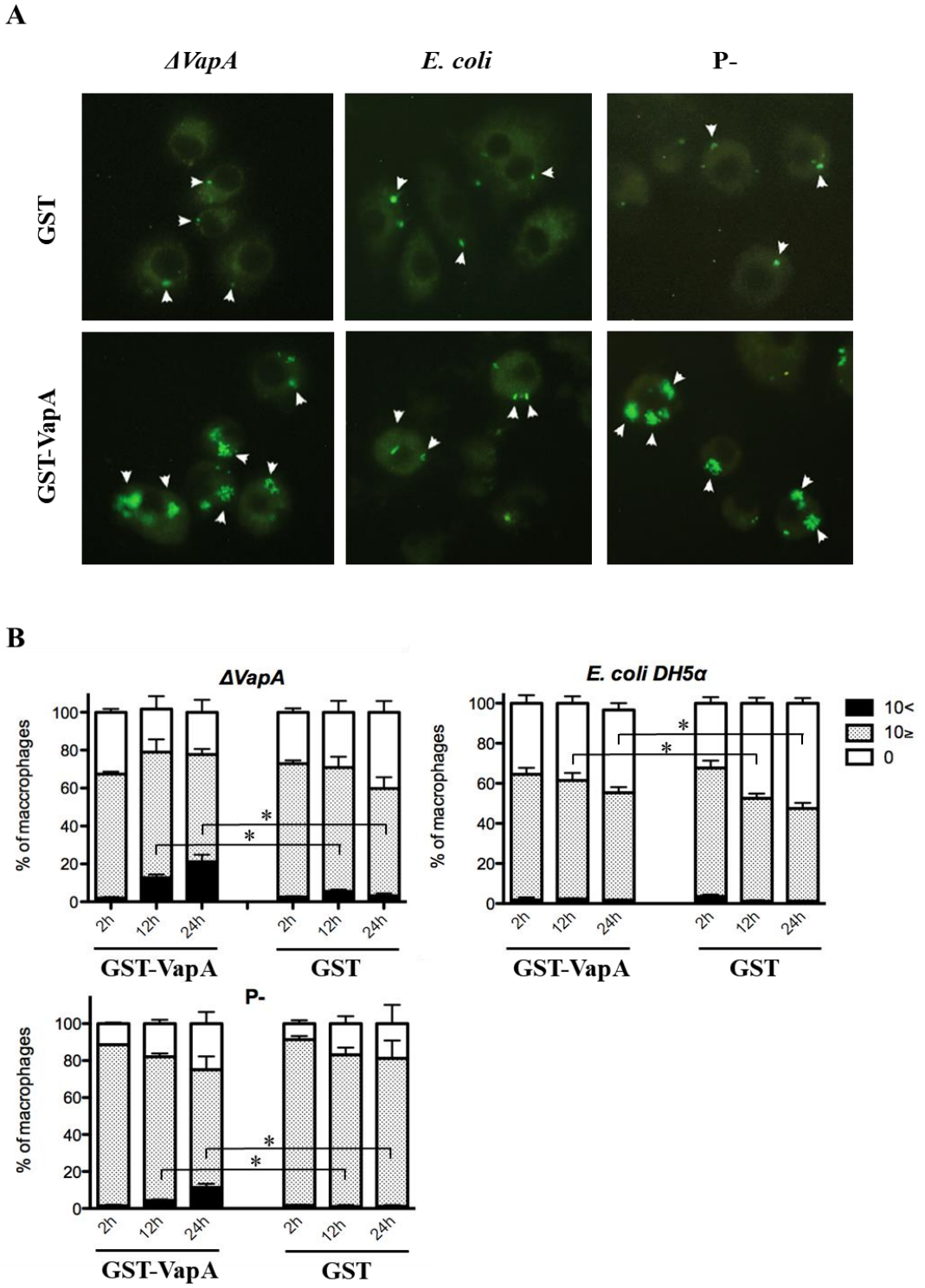


Fig. 2-2. Influence of the extracellular addition of VapA on intramacrophage growth of EGFP-expressing *R. equi* strains. J774A.1 macrophages were infected with the *R. equi vapA*

mutant (TKR 255), the P^- strain or *E. coli* DH5α in the presence of GST-VapA or GST. A: Macrophages were observed by fluorescence microscopy at 24 hr post-infection. *R. equi* or *E. coli* cells are indicated by arrowheads. B: The numbers of macrophages with 0, ≤10 or >10 bacteria were recorded at several times throughout infection. The data are expressed as the percentage of macrophages containing the bacterial numbers indicated. Each error bar represents the standard deviation for the mean of three data sets (200 macrophages were examined per set). The significance of differences in frequencies of *R. equi*-infected macrophages was calculated using the Chi-square test (*, $P<0.01$).

Chapter 3 Identification of *R. equi* fitness genes during infection by signature-tagged transposon mutagenesis

3-1 Introduction to the chapter

In the previous chapter, the intraphagocytic environment mediated by VapA was found to enable *R. equi* to survive and multiply inside the macrophages but still restrict growth of bacteria such as *E. coli*. These observations suggested that *R. equi* may possess the ability to adapt to harsh environments with bactericidal activity and nutritional depletion. However, little is known about the genetic determinants required for growth and fitness in the macrophages and animal host. It has been reported that several genes, including isocitrate lyase (*aceA*), catalase *(katA*), AroQ chorismate mutase (REQ23860) / TrpEG-like bifunctional anthranilate synthase (REQ23850) and rhequichelin biosynthesis (*rhbC*), are related with the ability of *R. equi* to survive in macrophages, and that the genes encoding peptidase D (*pepD*), sensor kinase (*mprAB*), high-temperature requirement A (*htrA*), nitrate reductase (*narG*) and α-/ β-subunit of heterodimeric coenzyme A transferase (*ipdAB*) are involved in the ability to infect mice [5, 37, 40, 42, 47, 77, 82].

Transposon-based mutagenesis is a powerful molecular technique that is necessary for investigating bacterial virulence and identifying virulence determinants in a variety of bacteria [2, 18, 50]. STM is a negative selection strategy that has been used for identification of virulence factors in many bacteria [13, 14, 26, 28]. Although STM allows for the screening of a large number of mutants by using fewer animals [55], molecular tools for STM are not available for *R. equi*.

Therefore, in the present study, a STM procedure to isolate and identify fitness genes of *R. equi* required for the *in vivo* survival was developed. The procedure included the use of a real-time PCR-based screening step for detecting each tag in the pool. In addition, a plasposon

containing the *E. coli* plasmid replication origin was used to facilitate the identification of flanking regions of the transposon insertion site.

3-2 Materials and Methods

3-2-1 Bacterial strains and plasmids

The wild-type *R. equi* strain ATCC 33701 and its plasmid-cured derivative ATCC 33701P$^-$ were used. All *R. equi* strains were grown in BHI broth with vigorous shaking at 30˚C. *E. coli* DH5α was used and routinely cultured in LB broth with shaking overnight at 37˚C. The transformants were selected by the addition of 100 μg/ml of ampicillin or 20 μg/ml of kanamycin for *E. coli* and 200 μg/ml of kanamycin for *R. equi* to the culture media. Competent *R. equi* cells were made according to the method described by Sekizaki *et al*. [58]. To prepare electrocompetent cells, *R. equi* was grown in 100 ml of BHI broth supplemented with 0.4% glucose, 1% glycerol, 0.2% Tween 80 and 2% glycine with shaking (150 rpm) for 24 hr at 38˚C. The culture was heated at 50˚C for 9 min and was immediately cooled in an ice water bath for 4 min. The cells were always kept on ice to maintain a high efficiency of transformation until electroporation was accomplished. The bacteria were pelleted by centrifugation (10,000 rpm, 10 min) at 4˚C and resuspended in 30 ml of ice-cold electroporation buffer. The cells were washed three times with ice-cold electroporation buffer and finally resuspended in 2 ml of ice-cold electroporation buffer. Aliquots of 100 μl were then stored at -80˚C. Electroporation was performed in pre-chilled sterile electroporation cuvettes (2 mm electrode gap, Bio-Rad) at 2.5 kV, 400 Ω and 25 μF with 100 μl of thawed cells. The electroporated cells were allowed to recover for 2 hr at 30˚C in 900 μl of BHI broth containing 20 mM $MgCl_2$. The cells were spread onto LB agar with appropriate antibiotics.

Reagents, solution and equipment

1) Culture medium

- Brain-heart infusion broth

- Luria-Bertani agar

- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of ampicillin soluble in distilled water

- 50 mg/ml of kanamycin soluble in distilled water (SIGMA)

3) Glucose (KANTO CHEMICAL CO., INC.)

4) Glycerol

5) Tween 80 (KANTO CHEMICAL CO., INC.)

6) Glycine (KANTO CHEMICAL CO., INC.)

7) 500 mM $MgCl_2$

- 10.165 g of $MgCl_2$ (KANTO CHEMICAL CO., INC.)

- Make up to 100 ml by distilled water

- Autoclave

8) Compatible cuvettes 2 mm electrode gap (Bio-Rad Gene Pulser®)

9) BIO-RAD Gene Pulser™ and BIO-RAD Pulse controller

10) Electroporation buffer

- 50 g of sucrose (KANTO CHEMICAL CO., INC.)

- 5 ml of 200 mM phosphate-K (pH 8.37)

- 50 ml of glycerol

- Make up to 500 ml by distilled water

- Autoclave

3-2-2 Construction of a plasposon carrying unique oligonucleotide tags

The transposon-based vector pTNR-KA of *Rhodococcus erythropolis* was kindly gifted by Dr. Tomohiro Tamura (Research Institute of Genome-based Biofactory National Institute of Advanced Industrial Sciences and Technology). The pTNR-KA carries a transposon containing

a kanamycin resistance gene, replication origin of *E. coli* and multiple cloning site (MCS) that encodes 10 different enzyme sites [56]. Twenty-eight different oligonucleotides were annealed to complementary molecules to yield double-stranded oligonucleotides (Table 3-1). Each tag was separately introduced into the *Hind*III and *Nsi*I sites of the MCS of pTNR-KA to generate twenty-eight plasposons (pTNR-KA-RE tag1 through 28). To confirm if proper constructs were made, nucleotide sequencing was carried out using the Tag-seq primer (Table 3-3).

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar
- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of ampicillin soluble in distilled water

3) 2x Rapid ligation buffer (Promega)

4) T4 DNA ligase (Promega)

5) Plasmid purification kit mini

6) PCR solution

- 10x buffer	5 µl
- dNTP	4 µl
- $MgCl_2$	3 µl
- Forward primer	2.5 µl
- Reverse primer	2.5 µl
- DNA template	1 µl
- Distilled water	31.5 µl
- *Taq* polymerase	0.5 µl
Total	50 µl

7) 0.8% (w/v) agarose gel

- 1.6 g of agarose (GE Healthcare)
- 200 ml of 1x TAE buffer
- 20 µl of ethidium bromide

8) Purification kit for sequencing (BigDye® Xterminator™)

3-2-3 Transposon mutagenesis

The pTNR-KA plasmid containing each unique tag (pTNR-KA-RE tag 1 through 28) was electroporated into *R. equi*. One hundred microliters of frozen electrocompetent cells was thawed on ice and gently mixed with 5 µl of pTNR-KA-RE tag 1 to 28, respectively. Then, 900 µl of BHI broth containing $MgCl_2$ was added and the cells were incubated at 30˚C for 2 hr. The bacteria were next plated onto LB agar supplemented with 200 µg/ml of kanamycin and cultured for 2 days at 30˚C.

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar
- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of kanamycin soluble in distilled water

3) Compatible cuvettes 2 mm electrode gap

4) BIO-RAD Gene Pulser™ and BIO-RAD Pulse controller

5) Plasmid purification kit mini

6) Enzymatic lysis buffer

- 2 ml of 1 M Tris-HCl (pH 8.0)
- 0.4 ml of 0.5 M EDTA

- 12 ml of 10% Triton-X 100
- Make up to 100 ml by distilled water
- Immediately before use, add lysozyme from chicken egg white to final concentration of 20 mg/ml

6) DNA extraction kit

- The pellet of culture cells were incubated with 180 µl of enzymatic lysis buffer for 30 min at 37˚C
- Add 25 µl of proteinase K and 200 µl of lysis buffer incubate for 30 min at 56˚C
- The sample is washed by 99% ethanol and followed by Quick-Start protocol
- Finally, elute DNA by adding 100 µl of elution buffer

3-2-4 Preparation of the input pool

To create the master plate, a set of transposon insertion mutants (tag1~25) was sub-cultured on LB agar plates supplemented with 200 µg/ml of kanamycin. After incubating at 30˚C for 48 hr, each *R. equi* mutant was inoculated into 3 ml of BHI broth and cultured with shaking at 30˚C for 48 hr. A 400-µl aliquot of culture containing each mutant was pooled in one tube. DNA was extracted from 3 ml of the bacterial pool and used as the template for analysis of the input pool. The bacterial number in the pool was estimated by measuring the optimal density (OD) at 600 nm (culture with OD_{600} = 1 contains bacteria). The bacterial pool was diluted to a final concentration of 10^7 CFU and used as inoculum to infect mice.

Reagents, solution and equipment

1) Culture medium

- Brain-heart infusion broth
- Luria-Bertani agar
- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of kanamycin soluble in distilled water

3) Sterile toothpicks

5) Photo meter (mini photo 518R TAITEC)

6) DNA extraction kit

- Proteinase K and enzymatic lysis buffer

3-2-5 Animal infection

Female Slc:ddY mice (16-18 g; Kumagai-shigeyasu Co., Ltd) were used for each infection pool of 25 *R. equi* transposon mutants. Approximately 10^6 Bacteria were intravenously (IV) injected into each mouse through the lateral tail vein. Mice were kept under standard conditions of animal welfare. On day 5 post-infection, mice were euthanized. The liver was aseptically removed and homogenized by manual grinding in sterile 1x PBS. The homogenate was adjusted to a concentration of 100 mg of the liver per 1 ml, poured into a 15-ml centrifuge tube and left to stand for 10 min. One hundred microliters of the supernatant was plated onto LB agar supplemented with 200 μg/ml of kanamycin and incubated for 48 hr at 30˚C. The bacterial colonies on the surface of the agar were harvested by scraping with 6 ml of sterile 1x PBS. The bacterial suspension was diluted and adjusted to an OD_{600} value of 1.0. DNA was extracted using the DNeasy blood and tissue kit according to manufacturer's instructions.

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar

- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of kanamycin soluble in distilled water

3) Photo meter

4) 1 ml syringe (NIPRO)

5) 27-gauge needle (TERUMO)

6) Diethyl Ether (KANTO CHEMICAL CO., INC.)

7) Sterile operating dissecting scissors sharp blunt and forceps

8) Sterile mortars and pestles

9) Sterile sea sand (KANTO CHEMICAL CO., INC.)

10) 1x PBS

11) Hot water bath

12) 50 ml centrifuge tube

13) 15 ml centrifuge tube

14) 70% ethanol

15) 99% ethanol

3-2-6 Semi-quantification of each tag in the input and output pools by real-time PCR

The DNA sample of the output was diluted 10-times with distilled water and used as a DNA template for PCR. The sample of DNA for real-time PCR was amplified from DNA extracted from the output pool using the primer pair of Realtime-F primer and Pre PCR-R (Table 3-3) because direct use of DNA extracted from the output pool disturbed amplification in real-time PCR. The amplification cycle was as follows: 2 min at 94˚C followed by 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 1 min. The product was visualized on a 0.8% (w/v) agarose gel and purified using the gel extraction method. Subsequently, 1 µl of extracted PCR product was mixed with 999 µl of sterile distilled water and used as a template for real-time PCR. Real-time PCR was carried out using the SYBR® Select Master Mix kit according to the manufacturer's instructions. The forward primer used was Realtime-F in combination with the Tag-specific primer listed in Table 3-2. The reaction was carried out under the following

conditions: 50˚C for 2 min, 95˚C for 2 min, followed by 40 cycles of 95˚C for 3 sec, 60˚C for 30 sec.

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar

2) Antibiotics stock solution

- 50 mg/ml of kanamycin soluble in distilled water

3) 1x PBS

4) Photo meter

5) DNA extraction kit

- Proteinase K and enzymatic lysis buffer

6) PCR solution

7) 0.8% (w/v) agarose gel

8) Gel Extraction kit

9) SYBR® Select Master Mix kit (Applied biosystems®)

10) Real-time PCR solution

- Select Master Mix	10 µl
- Forward primer (10 pmol/µl)	0.4 µl
- Reverse primer (10 pmol/µl)	0.4 µl
- DNA template	2 µl
- Distilled water	7.2 µl
Total	20 µl

3-2-7 Identification and analysis of transposon insertion

The nucleotide sequences surrounding the transposon insertion site were identified using the plasmid rescue procedure. The genomic DNA of each mutant was digested with *Apa*I, *Eco*RV, *Xho*I or *Xma*I. The digests were ligated in a 50-μl ligation reaction including T4 DNA ligase. The reaction mixture was left overnight at 4˚C, followed by phenol-chloroform extraction and ethanol precipitation. *E. coli* DH5α was transformed with the self-ligated plasmid. Transformants were selected on LB agar supplemented with 20 μg/ml of kanamycin. Plasmid DNA was extracted using the mini-prep plasmid purification kit. Sequencing was performed with the primer pair pTNR 1 and pTNR 2 (Table 3-3).

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar
- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of kanamycin soluble in distilled water

3) DNA extraction kit

- Proteinase K and enzymatic lysis buffer

4) Restriction enzyme (BioLabs®)

5) 2x Rapid ligation buffer

- Genomic DNA	20 μl
- Ligation buffer	25 μl
- Distilled water	3 μl
- Ligase	2 μ
Total	50 μl

6) 3M NaOAC

- 24.61 g of sodium acetate (KANTO CHEMICAL CO., INC.)
- Make up to 100 ml by distilled water

7) Phenol-chloroform extraction technique

- Phenol-chloroform (KANTO CHEMICAL CO., INC.)
- 3 M NaOAC
- Isopropanol (KANTO CHEMICAL CO., INC.)
- 70% ethanol
- Elution buffer

8) 0.8% (w/v) agarose gel

9) Plasmid purification kit mini

10) Purification kit for sequencing

3-2-8 Southern blot analysis

To confirm the insertion of a single transposon in the *R. equi* chromosome, the chromosomal DNA from each of the selected mutants was analyzed by Southern blotting. The genomic DNA was digested with *Apa*I, *Eco*RV, *Sac*II or *Xma*I, for which there are no restriction sites within the transposon [56]. The DNA fragments were loaded and resolved on a 0.7% agarose gel by running at 80 V for 2 hr. The agarose gel was soaked in 0.25 N HCl for 8 min at room temperature. DNA was then transferred to a Hybond-N^+ membrane using a pump with 40 mbar of vacuum pressure for 1 hr. The agarose gel was always soaked with 0.4 M NaOH during the transfer. The membrane was washed with 2x SSC buffer with shaking for 30 min and baked at 80˚C for 2 hr. Southern hybridization was carried out using DIG High Prime DNA Labeling and Detection Starter Kit 1 according to the manufacturer's instructions. To prepare a probe, the kanamycin resistance marker was amplified by a pair of primers KanR-SBF and Tag1p (Table 3-3) using pTNR-KA-RE tag1 as the template. Next, 8 µl of gel-purified PCR

product was diluted with 8 µl of distilled water. Denaturation of the PCR product was performed by placing it in a boiling water bath for 5 min, followed by placing it on ice for 15 min to prevent reannealing of the strands. Four microliters of DIG-High Prime was added to the denatured PCR product and incubated at 37˚C for overnight. After incubation, 2 µl of 0.2 M EDTA was added to stop the reaction and heat-inactivated at 65˚C for 10 min. One microliter of the probe was added to 19 µl of distilled water and boiled for 5 min, then chilled on ice for 15 min before use. Twenty microliters of denatured probe was added to 10 ml of pre-hybridization solution (DIG Easy Hyb Granules) and then incubated with the transfer membrane overnight at 42˚C. The membrane was washed with 2x SSC containing 0.1% sodium dodecyl sulfate (SDS) for 5 min and then twice with 0.5x SSC containing 0.1% SDS at 68˚C for 15 min. Blocking was performed using blocking buffer containing maleic acid for 30 min. Ten milliliters of blocking buffer containing anti-digoxigenin AP conjugate was poured onto the membrane, incubated for 30 min and washed twice with washing buffer. Visualization of the hybridized probe was carried out in color substrate solution. TE buffer was used to stop the reaction.

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar
- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of kanamycin soluble in distilled water

3) DNA extraction kit

- Proteinase K and enzymatic lysis buffer

4) Restriction enzyme

5) 0.7% (w/v) agarose gel

- 0.35 g of agarose

- 50 ml of 1x TAE buffer

6) 2 N HCl

- 164 ml of distilled water
- 36 ml of HCl (KANTO CHEMICAL CO., INC.)

7) 0.4 M NaOH

- 0.8 g of NaOH (KANTO CHEMICAL CO., INC.)
- Make up to 100 ml by distilled water

8) Hybond-N^+ membrane (GE Healthcare Amersham Hybond™-N^+)

9) Vacuum pump (Pharmacia)

10) 20x SSC

- 87.66 g of NaCl
- 44.115 g of trisodium (KANTO CHEMICAL CO., INC.)
- Make up to 500 ml by distilled water
- Autoclave

11) 2x SSC

- 5 ml of 20x SSC
- Make up to 50 ml by distilled water

12) 10% SDS

- 10 g of SDS (Wako Pure Chemical Industries, Ltd.)
- Make up to 100 ml by distilled water

13) 2x SSC containing 0.1% SDS

- 9.9 ml of 20x SSC
- 1 ml of 10% SDS
- Make up to 100 ml by distilled water

14) 0.5x SSC containing 0.1% SDS

- 4.95 ml of 20x SSC
- 2 ml 10% SDS
- Make up to 100 ml by distilled water

15) DIG-High Prime DNA Labeling and Detection Starter Kit 1 (Roche)

16) DIG Wash and Block Buffer Set (Roche)

- 10ml of pre-hybridization solution (DIG Easy Hyb Granules)
- Washing buffer
    - 30 ml of washing buffer
    - Make up to 100 ml by distilled water
- Blocking buffer (Blocking buffer with Maleic acid buffer)
    - 1 ml of blocking buffer
    - 10 ml of maleic acid buffer
    - Make up to 100 ml by distilled water
- 20 ml of blocking buffer (Blocking buffer with Maleic acid buffer) with detection buffer with 4 µl of DIG High Prime DNA Labeling and Detection Starter Kit 1 number 4
- Detection buffer
    - 3 ml of detection buffer
    - Make up to 30 ml by distilled water
    - 10 ml of detection buffer with 200 µl of DIG High Prime DNA Labeling and Detection Starter Kit 1 number 5
- 4 µl of DIG High Prime DNA Labeling and Detection Starter Kit 1 number 1

17) TE buffer

- 10 ml of 1 M Tris-HCl
- 2 ml of 0.5 M EDTA (pH 8.0)

- Make up to 1000 ml by distilled water

18) PCR solution

19) 0.2 M EDTA

- 5.84 g of EDTA

- Make up to 100 ml by distilled water

3-2-9 Western blot analysis

The *R. equi* mutants were cultured in BHI broth (pH 6.5) for 48 hr at 37˚C. The pellet was suspended with 1x sample buffer, boiled for 10 min and centrifuged briefly to remove undissolved material. Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the discontinuous method of Laemmli [35] with a slab gel of 15% polyacrylamide and a stacking gel of 45% polyacrylamide. Ten microliters of each sample was loaded into each lane and electrophoresed at 100 V with 50 mA for 2 hr [68]. The protein-containing bands were electrophoretically transferred to nitrocellulose sheets at 500 V for 1.3 hr. The nitrocellulose membranes were incubated with Block Ace overnight at 4˚C. The membranes were incubated for 2 hr with monoclonal antibodies against VapA diluted at 1:10,000 in Block Ace solution [66]. After washing with 0.05% Tween 20 in Tris-buffered saline (pH 7.4), the membranes were incubated for 1 hr with peroxidase-conjugated goat anti-mouse immunoglobulin G (Cappel Laboratories) diluted at 1:1,000 in Block Ace solution. The membranes were washed again, and antibody signal was visualized with diaminobenzoic acid.

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar

- Luria-Bertani broth (pH 6.5)

2) Antibiotics stock solution

- 50 mg/ml of kanamycin soluble in distilled water

3) 2x Sample buffer solution

- 2 ml of 0.5 M Tris-HCl, 0.4 g of SDS (pH 6.8)
- 2 ml of 99% glycerol
- 1.2 ml of β-mercaptoethanol (Bio-Rad Laboratories)
- 1 ml of 0.1% bromophenol blue (Bio-Rad Laboratories)
- Make up to 10 ml by distilled water

4) 30% Acrylamide/Bis solution

- 29.2 g of acrylamide (PlusOne Acrylamide IEF, Amersham Biosciences)
- 0.8 g of N, N'-Methylenebis-acrylamide (PlusOne Methylenebis-acrylamide GE Healthcare)
- Make up to 100 ml by distilled water

5) Running gel buffer

- 18.2 g of Tris
- 0.4 g of SDS
- Make up to 100 ml by distilled water, adjust pH 8.8 using concentrated HCl

6) 10% Ammonium persulfate (APS)

- 0.2 g of APS (Bio-Rad Laboratories)
- Make up to 2 ml by distilled water

7) TEMED (BIO-RAD)

8) Stucking gel buffer

- 6.1 g of Tris
- 0.4 g of SDS
- Make up to 100 ml by distilled water, adjust pH 6.8

9) Running gel

- 3,250 µl of distilled water
- 4,150 µl of 30% acrylamide/Bis solution
- 2,500 µl of running gel buffer
- 50 µl of 10% Ammonium persulfate
- 6.5 µl of TEMED

10) Diluted butanol

- 50 ml of butanol (KANTO CHEMICAL CO., INC.)
- Make up to 100 ml by distilled water
- Keep at room temperature for overnight

11) Stucking gel

- 1,680 µl of distilled water
- 550 µl of 30% acrylamide/Bis solution
- 750 µl of stucking gel buffer
- 12.5 µl of 10% Ammonium persulfate
- 3.5 µl of TEMED

12) 10 M Tris-glycine buffer

- 30 g of Tris
- 144 g of glycine
- Make up to 1000 ml by distilled water, adjust pH 8.3 using 2 N HCl

13) Running buffer

- 100 ml of 10 M Tris-glycine buffer
- 5 ml of 10% SDS
- Make up to 1000 ml by distilled water

14) Nitrocellulose blotting membrane 0.2 µm (GE Healthcare Life science)

15) 3 MM papers

16) SDS-PAGE transport buffer

- 100 ml of 10 M Tris-glycine buffer
- 200 ml of 99% methanol
- Make up to 1000 ml by distilled water
- Autoclave

17) Block Ace solution

- 4 g of Block Ace powder (Block Ace® powder)
- Make up to 100 ml by distilled water

18) Monoclonal antibodies against antigens of *R. equi*

19) Peroxidase-conjugated goat anti-mouse immunoglobulin G (Cappel Laboratories)

20) SDS washing buffer

- 10 ml of 1 M Tris-HCl (pH 7.5)
- 100 ml of 5 M NaCl
- 10 ml of 5% Tween20
- Make up to 1000 ml by distilled water

21) 3, 3'-Diaminobenzidine Tetrahydrochloride (nacalai tesque)

22) 1 M Tris-HCl (pH 7.5)

- 6.05 g of Tris
- Make up to 50 ml by distilled water, adjust pH 7.5 using concentrated HCl

23) Diluted hydrogen peroxide

- 60 µl of distilled water
- 20 µl of hydrogen peroxide (KANTO CHEMICAL CO., INC.)

24) Diaminobenzoic acid and hydrogen peroxide reaction

- 0.008g of 3, 3'-Diaminobenzidine Tetrahydrochloride
- 3 ml of 1 M Tris-HCl (pH 7.5)

- Make up to 27 ml by distilled water

- 40 µl of diluted hydrogen peroxide

3-2-10 Growth of *R. equi* under low-iron conditions

The growth of the *R. equi* mutant under low-iron conditions was assessed by growth inhibition assays on LB agar containing 0, 100, 160, 200 or 220 µM of 2, 2'-Dipyridyl. The bacteria was streaked over the agar surface and incubated for 48 hr at 30˚C.

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar

2) 2, 2'-Dipyridyl (SIGMA)

3-3 Results

3-3-1 Examination of cross-reaction among tags

To check if DNA fragments containing each signature-tag were specifically amplified only with the corresponding primers, each tag-specific primer was used in real-time PCR in which only the corresponding tag was eliminated from the reaction mixture. After all the primers were examined, cross-reaction among the 28 tags was determined. As three of 28 tags (tag 26, 27, and 28) demonstrated strong cross-reaction with the other tags, these tags were thereafter excluded in this study.

After each round of PCR amplification, a melting curve was generated for each run of 25 samples. The melting curve of all reactions exhibited a peak at approximately 79.6˚C, indicating that all unique tags in this STM were amplified specifically.

3-3-2 Verification of efficiency of the STM strategy in the mouse infection model

To assess if the negative screening method developed in this study was able to detect the mutants defective in mouse infection, preliminary experiments were carried out. The pTNR-KA-RE tag 1 was electroporated into an avirulent *R. equi* strain. The resultant avirulent strain bearing tag1 was mixed with 24 mutants containing the other tags and intravenously injected into three mice. Output DNA was prepared from bacteria collected from the liver of the mice at 5 days post-infection. Real-time PCR analysis of output DNA revealed that the proportion of tag 1 to the other tags was very low in all three mice (Fig. 3-1). Furthermore, the proportion of the other tags was consistent among the three mice. These results suggested that the STM developed in this study is useful for detection of *R. equi* strains defective in mouse infection.

Theoretically, if all the transposon mutants in an input pool have the same level of ability to infect, the proportion of each tag in the output pool becomes 4%. In the preliminary

experiment using an avirulent strain, the proportion of tags other than tag 1 were different, although they were consistent among the three mice. This difference may be due to differences in amplification efficiency among the tags. To test this hypothesis, the average proportion of each tag was calculated from the results of 16 independent experiments. As a result, the average proportion of each tag was approximately 4% and not significantly different among the tags (Fig. 3-2). Therefore, the amplification efficiency of tags used in this study was not different.

In this experiment, the bacterial concentration of transposon mutants was estimated from turbidity based on optimal density measured by spectrophotometer when the input pool was prepared. To verify the accuracy of input-pool preparation, DNA extracted from the input pool in several experiments was analyzed by real-time PCR. The proportion of tags was not identical even though all of the transposon mutants were equally added based on concentrations estimated by optimal density. These input pools were further inoculated into mice. Comparison between input and output pools revealed that the proportion of each tag was correlated between the input and output pools (Fig. 3-3). These results demonstrated that numerical estimation by optimal density is not accurate. Thus, the data from the output pool were corrected with data from the input pool in the screening step.

3-3-3 Screening of signature-tagged transposon mutant library

To identify fitness genes important for the survival of *R. equi* in mice, a total of 4,650 *R. equi* transposon mutants were arranged in pools of 25 unique mutants and were used to examine the survival in mice. The mutants whose relative abundance was markedly reduced after infection (>5-fold reduction) were selected as defective mutants in infection. To eliminate potential false positives, the mutants attained from the first screening were added to the new input pool and were inoculated again. The mutants that with reduced ability to infect in at least two independent screenings were finally selected as fitness defect mutants. According to these criteria, 102 out of the 4,650 mutants were selected as fitness defect mutants.

3-3-4 Identification of the transposon insertion sites

To confirm insertion of transposon in 102 mutants, Southern blot analysis was carried out. Figure 3-4 shows a representative example of Southern blot analysis. Ninety-one *R. equi* mutants contained a single transposon insertion, while 11 mutants had more than two insertions. The plasposons were successively rescued in 73 out of 91 mutants. The nucleotide sequence of the DNA flanking the site of transposon insertion was searched using the GenBank database. The transposon insertion site was identified in 50 out of 73 plasposons. The results are shown in Table 3-4.

Insertion of transposon was seen in the virulence plasmid in 15 out of the 50 mutants. Most of these mutants exhibited no or reduced VapA expression. The remaining 35 mutants had insertion of the transposon in the chromosome. The genes were assigned to one of ten functional categories: amino acid metabolism, nucleotide metabolism, lipid metabolism, DNA repair/recombination, regulation, peptideglycan biosynthesis/degradation, energy metabolism, transport, or NRPS. Insertions in genes encoding proteins homologous to proteins or predicted proteins of unknown function were categorized as unknown. The mutants in which the transposon was located in the intergenic non-coding region were also assigned to this category. Nine chromosomal mutants expressed VapA at low levels, but remaining the 26 mutants had the same expression level of VapA as wild-type (Fig. 3-5).

3-3-5 Growth of *R. equi* mutants under low-iron conditions

Seven mutants had the transposon within the gene encoding NRPS. It has been reported that NRPS may be involved in siderophore biosynthesis [43]. To examine if these genes are involved in iron acquisition of *R. equi*, wild-type and mutant strains were cultured on iron-chelated agar with 2, 2'-dipyridyl. However, no obvious growth defect was observed.

3-4 Discussion

STM is a powerful genetic technique to identify novel virulence genes [55]. The STM technique has been applied for several pathogens, but not for *R. equi* [13, 14, 26, 28]. Previously, pTNR-KA was used for random mutagenesis in *Rhodococcus erythropolis* [56]. In this study, a new plasmid that could be used for STM was developed by insertion of distinguishable unique oligonucleotides into a plasposon of pTNR-KA. Out of 4,650 transposon mutants, 102 mutants were selected as mutants defective in mouse infection. Although 52 mutants were eliminated from this study because of inability to determine transposon-insertion sites, the genetic locus with insertion of a transposon was identified in the remaining 50 mutants. Sequence analysis revealed that 15 mutants had the transposon in the virulence plasmid. Most of them exhibited reduced VapA expression. It was reported that reduced expression of VapA resulted in reduced ability to survive within macrophages. Therefore, it is reasonable to think that this phenotype is responsible for the defective growth of the mutants in mice. Frequency of virulence-plasmid mutants in the selection demonstrated that the screening method used in this study was sensitive enough to detect mutants defective in mouse infection.

One of mutants possessed a disrupted gamma-glutamyl kinase-GP-reductase multi-enzyme complex ProA gene. This gene encodes multiple enzymes for the conversion of glutamate to L-glutamate 5-semialdehyde, which is involved in the proline catabolism pathway [59]. Although proline is a non-essential amino acid, some pathogens require proline catabolism for virulence [9, 39, 59]. It was demonstrated that the proline utilization system of *Brucella abortus* was critical for the chronic infection of mice [9]. *R. equi* possess *de novo* synthesis pathways for all essential amino acids. Thus, *R. equi* can grow in amino acid-deficient conditions [37]. However, survival defect of the *proA* mutant revealed that the proline catabolism pathway is critical for *R. equi* survival during infection.

Acyl-CoA thioesterase II (REQ25310) and short chain dehydrogenase (REQ41020) may be involved in lipid metabolism. As *R. equi* utilizes only organic acids and fatty acids as carbon sources, the *R. equi* genome encodes many proteins involved in lipid metabolism [37]. Deletion of a gene encoding glyoxylate shunt enzyme isocitrate lyase resulted in attenuation in mice [82]. The present study suggested that lipids may be major growth substrates for *R. equi* during infection. It should be noted that a downstream gene of REQ25310 encodes a putative ABC transporter ATPase subunit that may be involved in transport of vitamin B_{12}. It was reported that the transport of vitamin B_{12} was implicated in chronic infection of *Mycobacterium tuberculosis* [20]. The defect of REQ25310 mutant may be a polar effect of transposon insertion in this gene.

Two mutants had an insertion in genes encoding a putative pyrazinamidase and putative carotenoid oxygenase, respectively. These genes are involved in vitamin synthesis, essential nutrients for organisms [36]. It was reported that disruption of the riboflavin (vitamin B_2) biosynthesis gene affected bacterial replication *in vivo* [2]. In addition, *R. equi* cannot grow in medium lacking thiamine (vitamin B_1) supplementation [37]. Pyrazinamidase plays a role in nicotinamide adenine dinucleotide (NAD^+) biosynthesis [81]. NAD^+ is a central molecule in cellular metabolism and implicated in hundreds of biological reactions. The organisms synthesize NAD^+ using *de novo* and salvage pathways. Pyrazinamidase converts nicotinamide (vitamin B_3) to nicotinate and then transfers a phosphoribosyl group to give nicotinic acid mononucleotide (NAMN). The original product of NAMN is recycled to synthesize NAD^+ by the salvage pathway. On the other hand, NAMN is synthesized from aspartate by *nadB*, *nadA* and *nadC* genes to give NAD^+ as a final product in the *de novo* pathway. It was reported that the *nadABC* mutant of *Mycobacterium bovis* fails to grow in mice, whereas *Mycobacterium tuberculosis* grows normally [79, 87]. The present study revealed that impediment of the salvage pathway in *R. equi* causes reduced infectivity to mice.

Several mutants with insertions in the genes related with DNA replication and repair were selected. REQ05430 encodes a putative DEAD/DEAH box helicase. DEAD/DEAH families participate in many different aspects of RNA metabolism, including RNA synthesis, RNA folding, RNA-RNA interactions, RNA localization and RNA degradation. A DEAD box RNA helicase gene of *Staphylococcus aureus* regulates *agr* mRNA abundance. It was reported that inactivation of the DEAD box helicase gene resulted in dysregulation of biofilm formation and hemolysis, which are virulence factors for this bacterium [45]. The deletion of the DEAD box RNA helicase gene in *Cryptococcus neoformans* induced a marked reduction in virulence in a mouse model [46].

Four attenuated mutants had an insertion in genes encoding putative short chain dehydrogenase, short chain dehydrogenase, oxidoreductase, and putative nitrite reductase large subunit NirB1, respectively. These genes may be involved in electron transfer, which plays an essential role in a broad range of energy conversion processes, including respiration [22]. In anaerobic environments, electrons are transferred to nitrate, sulfate and carbonate [7, 29]. *R. equi* can cause necrotic pyogranulomatous lesions. Microaerobic environments are typically found under such conditions [84]. The putative nitrite reductase large subunit NirB1 gene is involved in the denitrification process [37]. This enzyme presumably regenerates NAD^+ and detoxifies nitrite that accumulates as a result of nitrate respiration [7]. In this study, the *nirB1* mutant grew slowly in the absence of oxygen in the preliminary experiment. It was reported that *narGHIJ* genes of *R. equi* are required for mouse infection [47]. However, whether nitrate assimilation is required for *R. equi* survival *in vivo* remains unknown.

The most frequently identified gene was REQ23810. This gene encodes NRPS. It was reported that the REQ23810 gene may be involved in siderophore biosynthesis [43]. Siderophores are small compounds that bacteria secret to scavenge iron from the environment. Bacteria require iron as an essential element for growth [42]. Miranda-CasoLuengo *et al*. [43]

reported that the mutant with disruption of REQ23810 was unable to grow under iron-depleted conditions. However, in this study, REQ23810 mutants did not exhibit any defect in growth on iron-depleted agar. The basis for this discrepancy is not clear. In a previous report, the REQ23810 mutant was not attenuated in mice; however, the mutant was individually inoculated in the previous study but was inoculated with the other transposon mutants in this study. Therefore, competition with the other mutants may have caused the survival defect of this mutant.

3-5 Summary

This is the first report of successful establishment of STM for *R. equi*. The purpose of this study was to identify *R. equi* genes involved in bacterial survival during infection. To carry out transposon-based random mutagenesis in *R. equi*, pTNR-KA, which is used for random mutagenesis of *Rhodococcus erythropolis*, was used. The Southern blot analysis of DNA extracted from the mutants revealed that transposons were randomly inserted into *R. equi* chromosomal DNA. To establish an STM system to study *R. equi*, tags with a unique oligonucleotide sequence were inserted into pTNR-KA. *R. equi* was randomly mutagenized by each of the 25 different signature-tagged transposons and 25 mutants with different tags were pooled as a set. A two-step STM screening method based on quantitative real-time PCR using the tag-specific primers was employed. To verify the usefulness of the screening, an avirulent mutant with one signature-tagged transposon was mixed with 24 mutants containing the other tags and injected into mice. DNA was extracted from the livers of infected mice and used for quantitative real-time PCR. The avirulent mutant was not detected from outputs, indicating that the screening method employed in this study was effective.

A total of 4,650 *R. equi* transposon mutants was examined for ability of bacterial survival during infection. The mutants that could not survive in mice were identified by quantifying the copy number of each tag in the output sample. Mutants whose relative abundance was markedly reduced after infection (>5-fold reduction) were selected as defective mutants in infection. To eliminate potential false positives, the mutants selected in the first screen were used to make a new input pool and were inoculated into mice again. The mutants that exhibited reduced ability to infect on both occasions were finally selected as fitness defect mutants. As a result, 102 mutants or approximately 2.15% of the total mutants examined were selected. Among them, transposon insertion sites were successively determined in 50 transposon insertion mutants. Insertion of transposons was seen in the genes encoded on the virulence

plasmid in 15 out of the 50 mutants. Most of these mutants had reduced expression of VapA, which was likely responsible for their reduced ability to infect mice. The remaining 35 mutants had insertion of transposons in the genes encoded on the chromosome. Nine chromosomal mutants expressed VapA at low levels, but the remaining 26 mutants had the same VapA expression level as wild-type. The genes were assigned to one of nine functional categories: amino acid metabolism, nucleotide metabolism, lipid metabolism, DNA repair/recombination, regulation, peptideglycan biosynthesis/degradation, energy metabolism, transport or NRPS. Eight mutants had insertion in genes for proteins of unknown function. The most frequently identified gene was REQ23810, which encodes the NRPS protein. It was reported that REQ23810 was responsible for the synthesis of siderophores required for growth under iron-depleted conditions. However, no growth defect of REQ23810 mutants in iron-restricted media was observed in this study.

Table 3-1. Nucleotide sequences of tags for STM

Tag no.	Sequence
tag1	**5′-AGCTC**TGAACGT**TTAAGCGCGTGCA-3′**
tag2	**5′-AGCTC**AGGCTAT**TTAAGCGCGTGCA-3′**
tag3	**5′-AGCTC**GAGACTT**TTAAGCGCGTGCA-3′**
tag4	**5′-AGCTC**TACCAGT**TTAAGCGCGTGCA-3′**
tag5	**5′-AGCTC**AACATGC**TTAAGCGCGTGCA-3′**
tag6	**5′-AGCTC**GGTTACA**TTAAGCGCGTGCA-3′**
tag7	**5′-AGCTC**TTGCCTA**TTAAGCGCGTGCA-3′**
tag8	**5′-AGCTC**ACGATTG**TTAAGCGCGTGCA-3′**
tag9	**5′-AGCTC**GTCTTGA**TTAAGCGCGTGCA-3′**
tag10	**5′-AGCTC**CATGTCA**TTAAGCGCGTGCA-3′**
tag11	**5′-AGCTC**TCCAGTA**TTAAGCGCGTGCA-3′**
tag12	**5′-AGCTC**AGTGAAC**TTAAGCGCGTGCA-3′**
tag13	**5′-AGCTC**TCTGCAT**TTAAGCGCGTGCA-3′**
tag14	**5′-AGCTC**AGCTATG**TTAAGCGCGTGCA-3′**
tag15	**5′-AGCTC**CTCTACT **TTAAGCGCGTGCA-3′**
tag16	**5′-AGCTC**TAGGTAC**TTAAGCGCGTGCA-3′**
tag17	**5′-AGCTC**ACAGTAG**TTAAGCGCGTGCA-3′**
tag18	**5′-AGCTC**GAGTTCA**TTAAGCGCGTGCA-3′**
tag19	**5′-AGCTC**TGATACC**TTAAGCGCGTGCA-3′**
tag20	**5′-AGCTC**TAGTTCG**TTAAGCGCGTGCA-3′**
tag21	**5′-AGCTC**TTCACAG**TTAAGCGCGTGCA-3′**
tag22	**5′-AGCTC**GATTCGA**TTAAGCGCGTGCA-3′**
tag23	**5′-AGCTC**CGATATC**TTAAGCGCGTGCA-3′**
tag24	**5′-AGCTC**ACATACG**TTAAGCGCGTGCA-3′**
tag25	**5′-AGCTC**GCGATAT**TTAAGCGCGTGCA-3′**
tag26*	**5′-AGCTC**TATCACG**TTAAGCGCGTGCA-3′**
tag27*	**5′-AGCTC**TCAACTG**TTAAGCGCGTGCA-3′**
tag28*	**5′-AGCTC**GTGATAC**TTAAGCGCGTGCA-3′**

* Tags that cross-reacted with other tags were excluded from the study

Table 3-2. Sequences of reverse primers for tag specific oligonucleotides

Gene	Primer (5′-3′)
Tag1p	TGCACGCGCTTAAACGTTCAG
Tag2p	TGCACGCGCTTAAATAGCCTG
Tag3p	TGCACGCGCTTAAAAGTCTCG
Tag4p	TGCACGCGCTTAAACTGGTAG
Tag5p	TGCACGCGCTTAAGCATGTTG
Tag6p	TGCACGCGCTTAATGTAACCG
Tag7p	TGCACGCGCTTAATAGGCAAG
Tag8p	TGCACGCGCTTAACAATCGTG
Tag9p	TGCACGCGCTTAATCAAGACG
Tag10p	TGCACGCGCTTAATGACATGG
Tag11p	TGCACGCGCTTAATACTGGAG
Tag12p	TGCACGCGCTTAAGTTCACTG
Tag13p	TGCACGCGCTTAAATGCAGAG
Tag14p	TGCACGCGCTTAACATAGCTG
Tag15p	TGCACGCGCTTAAAGTAGAGG
Tag16p	TGCACGCGCTTAAGTACCTAG
Tag17p	TGCACGCGCTTAACTACTGTG
Tag18p	TGCACGCGCTTAATGAACTCG
Tag19p	TGCACGCGCTTAAGGTATCAG
Tag20p	TGCACGCGCTTAACGAACTAG
Tag21p	TGCACGCGCTTAACTGTGAAG
Tag22p	TGCACGCGCTTAATCGAATCG
Tag23p	TGCACGCGCTTAAGATATCGG
Tag24p	TGCACGCGCTTAACGTATGTG
Tag25p	TGCACGCGCTTAAATATCGCG

Table 3-3. Sequences of forward and reverse primers

Gene	**Primer (5′-3′)**
Tag-seq	GACTAGACAGTCGGCACAAG
Realtime-F	TCACCTGGAGAGCAACTAAC
Pre PCR-R	CTCGTATGTTGTGTGGAATTGTG
KanR-SBF	GGATTGCACGCAGGTTCTCC
pTNR-seq1	TGAGTGCTTGCGGCAGCGTCTAG
pTNR-seq2	GATCCTTTGATCTTTTCTACGGGG

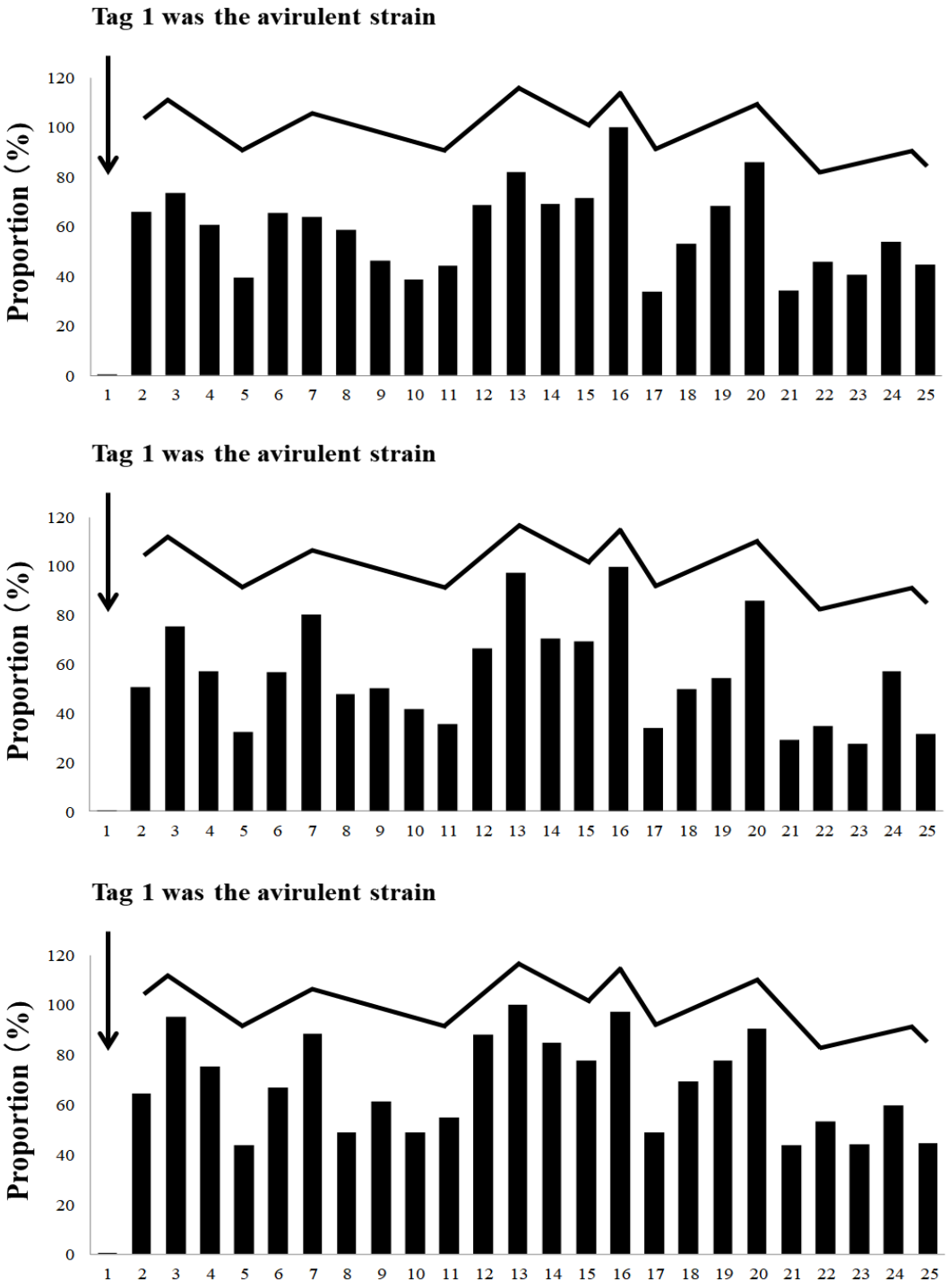


Fig. 3-1. Three mice were injected with the mutant pool containing avirulent *R. equi* marked with tag 1. Five days after infection, the proportion of mutants with each tag in the output pool was determined by real-time PCR.

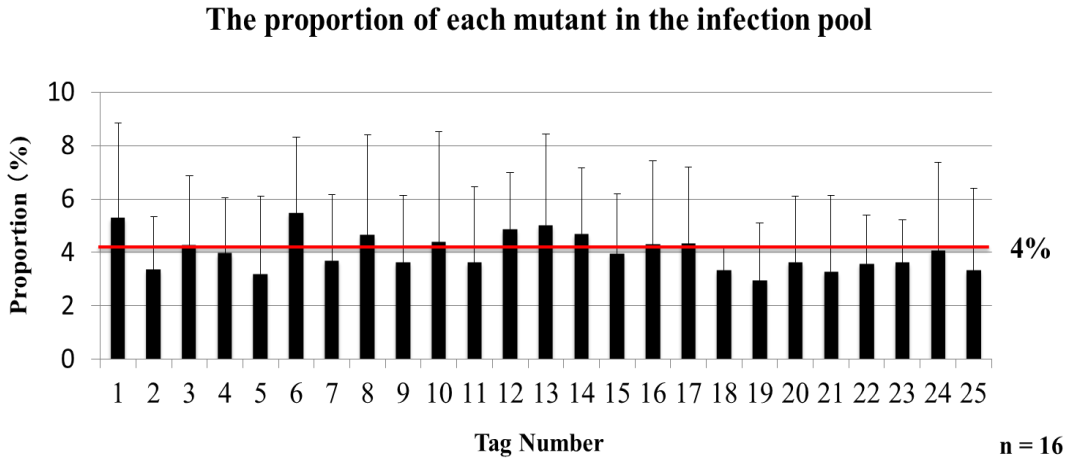


Fig. 3-2. Real-time PCR amplification of input pool sample. The average proportion of each tag was calculated from 16 independent infections.

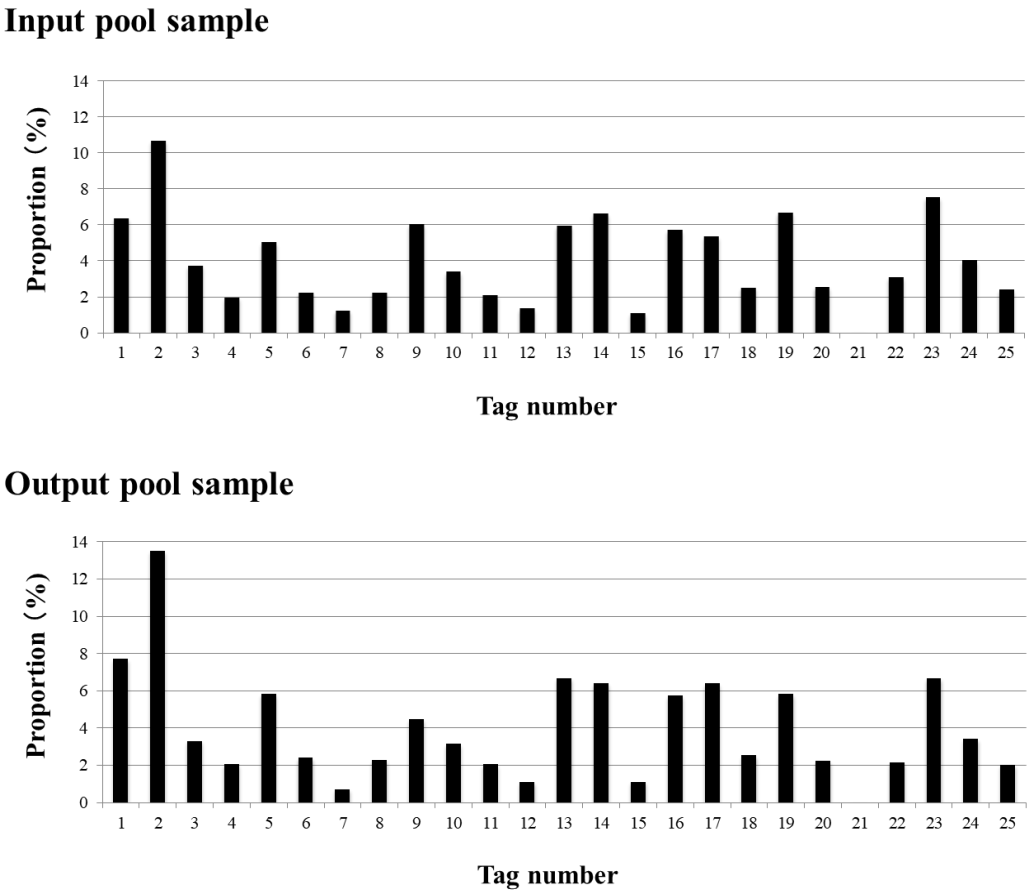


Fig. 3-3. Real-time PCR amplification of input and output pool samples. Twenty-five mutants of *R. equi* were cultured in BHI broth for 48 hr at 30˚C. The mutants were pooled and diluted to 10^7 cell per ml. Mice were injected with 10^6 bacteria containing 25 *R. equi* transposon mutants. DNA was extracted from the input pool and used as the template for input data analysis. The liver was removed on day 5 post-infection and homogenized. The tissue suspension was plated on LB agar containing 200 μg/ml of kanamycin. The DNA was extracted from bacterial colonies and used as the output sample.

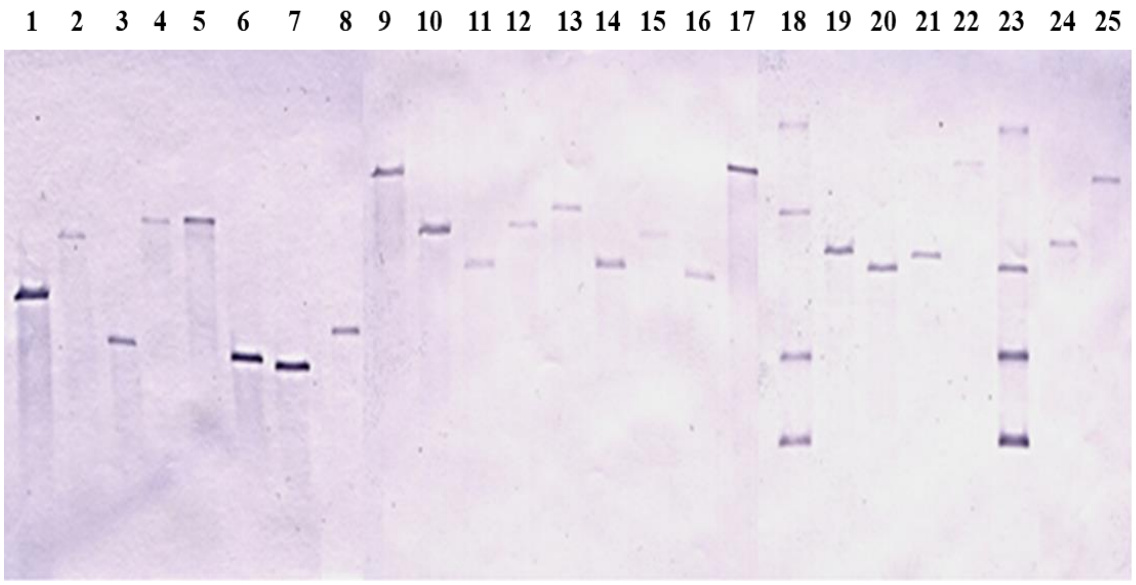


Fig. 3-4. Southern blot analysis of 25 randomly chosen transposon insertion mutants. *Xma*I or *Apa*I-digested chromosomal DNA was hybridized to the DIG-labelled kanamycin resistance gene. *R. equi* transposon mutants on lanes 18 and 23 contained multiple transposon insertions in chromosome, and were eliminated from this study.

Table 3-4. Identification of genes with transposon insertion in *R. equi*

Class	Mutant	Gene location	Known or putative function	Expression of VapA
Amino acid metabolism	M95-15	REQ29700	Gamma-glutamyl kinase-GP-reductase multi-enzyme complex ProA	Normal
Nucleotide metabolism	M79-5	REQ39910	Orotate phosphoribosyltransferase PyrE	Reduced
Lipid metabolism	M77-6	REQ25310	Acyl-CoA thioesterase II	Reduced
	M124-23	REQ41020	Short chain dehydrogenase	Reduced
Vitamin synthesis	M73-2	REQ17830	Putative pyrazinamidase	Reduced
	M43-12	REQ28270	Putative carotenoid oxygenase	Normal
DNA repair / recombination	M134-15	REQ00040	DNA replication and repair protein RecF	Normal
	M121-1	REQ05430	Putative DEAD/DEAH box helicase	Normal
	M178-25	REQ19710	Putative Rec X regulatory protein	Normal
Regulatory	M165-8	REQ29340	Heat-inducible transcriptional repressor HrcA	Reduced
	M118-3	REQ26260	Putative MarR family transcriptional regulator	Reduced
	M79-17	REQ06490	TetR family transcriptional regulator	Reduced
Transport	M165-11	REQ25710	Putative MFS transporter	Normal
Peptidoglycan biosynthesis / degradation	M134-23	REQ45210	Putative D-alanyl-D-alanine carboxypeptidase metabolism	Normal
Energy metabolism	M45-4	REQ00880	Putative short chain dehydrogenase	Normal
	M151-1	REQ15620	Short chain dehydrogenase	Normal
	M161-16	REQ10430	Oxidoreductase	Normal
	M77-23	REQ32930	Putative nitrite reductase large subunit NirB1	Normal
Non-ribosomal peptide synthesis	M60-10	REQ23810	Putative non-ribosomal peptide synthetase	Normal
	M70-16	REQ23810	Putative non-ribosomal peptide synthetase	Normal
	M87-15	REQ23810	Putative non-ribosomal peptide synthetase	Normal

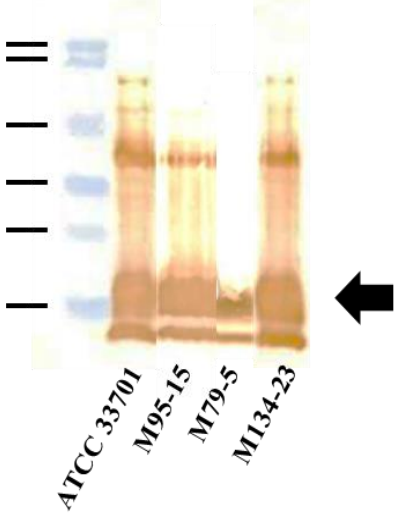
Table 3-4. (continued)

	M122-3	REQ23810	Putative non-ribosomal peptide synthetase	Normal
	M162-21	REQ23810	Putative non-ribosomal peptide synthetase	Normal
	M158-13	REQ23810	Putative non-ribosomal peptide synthetase	Normal
	M56-20	REQ35940	Putative non-ribosomal peptide synthetase	Normal
Unknown	M162-10*	REQ12930/12940	Hypothetical protein/putative secreted esterase	Normal
	M6-19	REQ26170	Hypothetical protein	Normal
	M82-16	REQ26170	Hypothetical protein	Normal
	M153-13*	REQ27330/27340	Putative HNH endonuclease/putative Fis family transcriptional regulator	Normal
	M129-14	REQ38700	Conserved hypothetical protein	Normal
	M130-19	REQ38850	Conserved hypothetical protein	Reduced
	M182-17	REQ38870	Hypothetical protein	Normal
	M168-10	REQ23910	Hypothetical protein	Reduced
	M74-13	REQ00210	Putative membrane protein	Normal
	M154-10	REQ14130	Putative integral membrane protein	Normal
Virulence plasmid	M58-1		*IcgA*	Reduced
	M85-1		*IcgA*	Reduced
	M96-2		*IcgA*	Reduced
	M142-10		*IcgA*	Reduced
	M173-14		*IcgA*	Reduced
	M175-7		*IcgA*	Reduced
	M57-20		*Orf 25*	Normal
	M112-17		*VirS*	None
	M114-17		*VirS*	None
	M120-9		*VirS*	None
	M115-3		Putative conjugative transfer TraG/TraD family protein	Normal
	M168-5*		*ResA/ParB*	Reduced
	M169-21*		*VapG/orf3*	Normal
	M175-8		*VirR*	Reduced
	M62-10*		*IcgA/vapH*	Reduced

*** Transposon was located in the intergenic region**

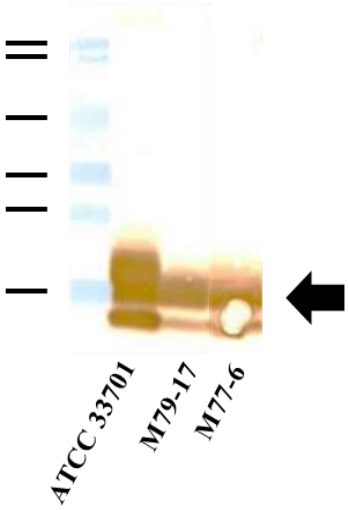
A

Amino acid metabolism



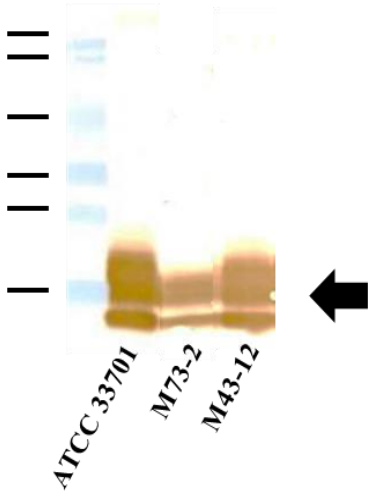
B

Lipid metabolism



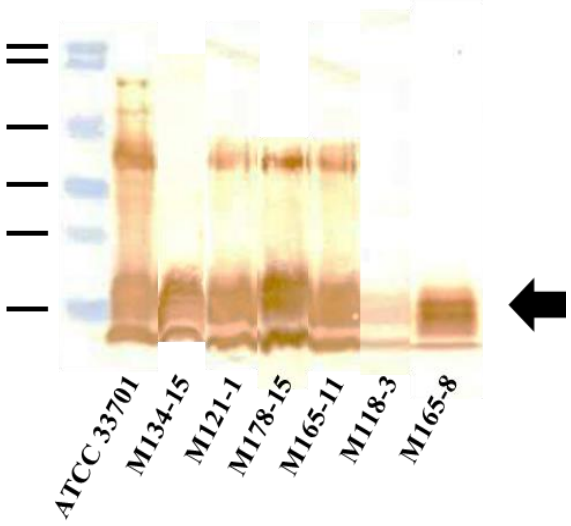
C

Vitamin synthesis



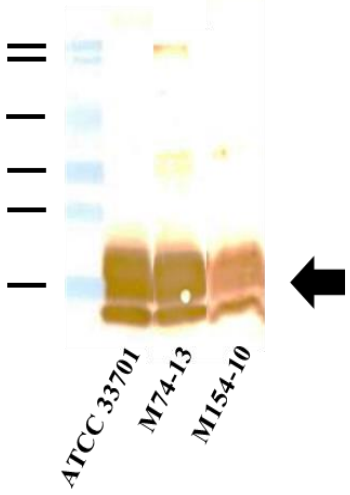
D

DNA repair / recombination



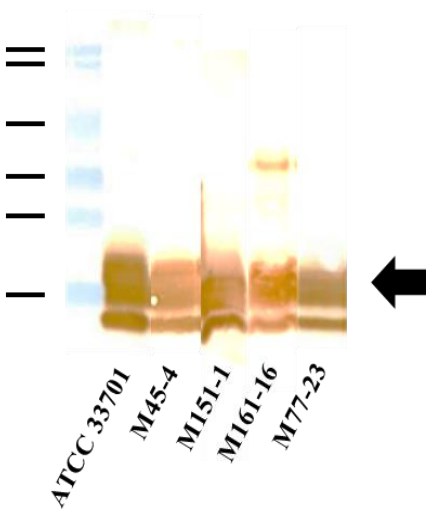
E

Peptidoglycan biosynthesis / degradation



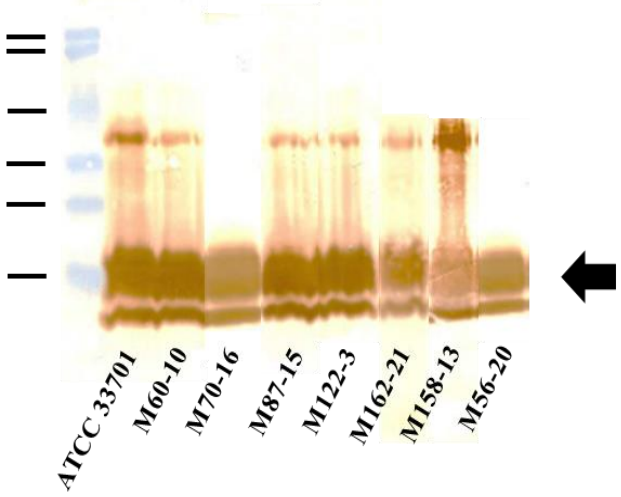
F

Energy metabolism



G

Non-ribosomal peptide synthesis



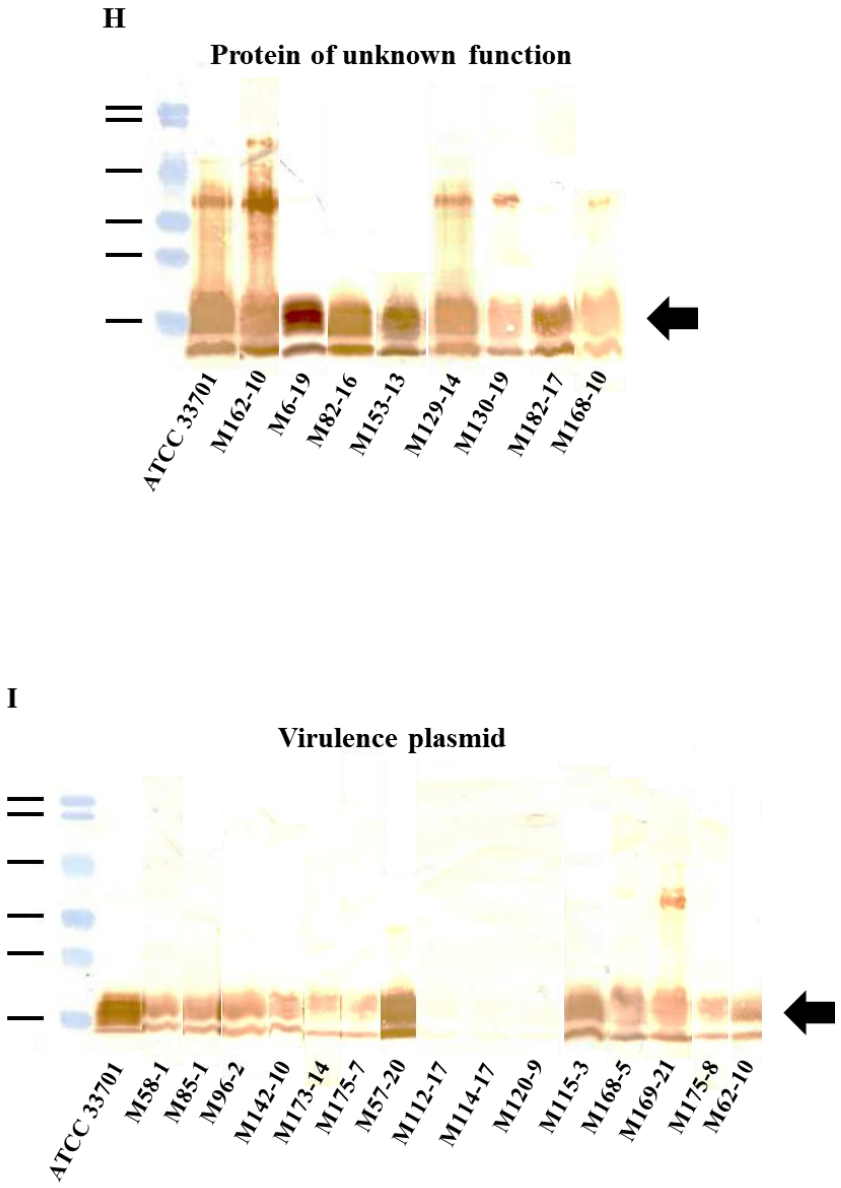


Fig. 3-5. Western immunoblot profiles of *R. equi* transposon insertion mutants. Whole-cell preparations of *R. equi* mutants with insertions compromising genes involved in amino acid metabolism (A), lipid metabolism (B), vitamin synthesis (C), DNA repair/recombination (D), peptidoglycan biosynthesis/degradation (E), energy metabolism (F), NRPS (G), protein of unknown function (H) or virulence plasmid (I) were analyzed by immunoblotting using monoclonal antibodies against the virulence-associated antigens. The molecular weight markers

phosphorylase b (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500) and lysozyme (14,400) are indicated by bars on the left side. The black arrow on the right indicates the band for the 15- to 17 kDa antigens.

Chapter 4 Intracellular growth of transposon-insertion mutants of *R. equi* in mouse macrophages

4-1 Introduction to the chapter

In chapter 3, 35 mutants had attenuated growth due to transposon insertion in the genes encoded on the chromosome. The transposon insertions were seen in the genes involved in amino acid metabolism, nucleotide metabolism, lipid metabolism, DNA repair/recombination, regulation, peptideglycan biosynthesis/degradation, energy metabolism, transport and NRPS. The virulence of *R. equi* depends on its ability to resist and multiply in macrophages, and the growth within macrophages is critical for *R. equi* to survive in mice [24, 27]. Therefore, some of the mutants identified by the screening using STM may have some defect in ability to grow inside macrophages. To test this hypothesis, macrophage J774A.1 cells were infected with attenuated transposon mutants. The plasmids encoding EGFP were electroporated into these mutants. The intracellular growth of the mutants was observed by fluorescence microscopy at 24 hr post-infection.

4-2 Materials and Methods

4-2-1 Bacterial strains and plasmids

R. equi ATCC 33701 and its plasmid-cured derivative ATTCC 33701P$^-$ were used. *R. equi* transposon insertion mutants were created as described in the previous chapter. The wild-type strain and all transposon mutants were grown on LB agar at 30˚C. The mutant strains were grown on the agar plate with 200 μg/ml of kanamycin. BHI broth was routinely used for liquid culture. Bacterial cultures were incubated with shaking at 100 rpm at 30˚C for 48 hr. The *R. equi* integration plasmid, pINT, was electroporated into *R. equi* ATCC 33701 to confer apramycin resistance.

The rifampicin-resistant *R. equi* strain was created by spontaneous mutation. *R. equi* ATTC 33701 was plated onto LB agar supplemented with 100 μg of rifampicin per ml. After 48-hr incubation at 30˚C, a colony was picked up and used for further experiments.

Reagents and solution

1) Culture medium

- Brain-heart infusion broth
- Luria-Bertani agar
- Luria-Bertani broth

2) Antibiotics stock solution

- 80 mg/ml of apramycin soluble in distilled water
- 50 mg/ml of kanamycin soluble in distilled water
- 1 mg/ml of rifampicin soluble in dimethyl Sulfoxide (Wako)

3) Dimethyl Sulfoxide (KANTO CHEMICAL CO., INC.)

4-2-2 Construction of *R. equi* transposon insertion mutants expressing EGFP

The plasmid pINT::P_{aphII}-*EGFP* was electroporated into *R. equi* ATCC 33701, ATCC 33701P$^-$ and transposon insertion mutants. Transformants were recovered on LB agar containing 80 μg/ml of apramycin.

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar

2) Antibiotics stock solution

- 80 mg/ml of apramycin soluble in distilled water

3) Compatible cuvettes 2 mm electrode gap

4) BIO-RAD Gene Pulser™ and BIO-RAD Pulse controller

4-2-3 Macrophage cell culture

Preparation of cell cultures is described in chapter 2. The mouse macrophage-like J774A.1 cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum and 0.1% (v/v) gentamicin sulfate in a humidified 5% CO_2 atmosphere at 37˚C. For infection assays, J774A.1 cells were passaged by harvesting with 0.25% Trypsin-EDTA in Hank's Balanced Salt Solution. A total of 1.5×10^5 cells per ml was seeded onto 13-mm diameter glass coverslips in 24-well plates and cultured without antibiotic. The macrophage cells were allowed to adhere to the coverslip for 24 hr.

Reagents, solution and equipment

1) Culture medium

- DMEM

- Fetal bovine serum

2) Antibiotics stock solution

- Gentamicin sulfate solution

3) Enzymatic cell dissociation reagent (0.25% Trypsin-EDTA in Hank's Balanced Salt Solution)

- 1.25 g of Trypsin
- 0.186 g of EDTA.2Na
- Make up to 450 ml by distilled water
- 50 ml Hank's Balanced Salt Solution

4) Cell culture flask, 250 ml

5) Micro cover glass

6) 24 well cell culture plate sterile, with lid

4-2-4 The macrophage infection

R. equi strains were cultured in BHI broth at 30˚C with vigorous shaking for 48 hr. Macrophages were infected with wild-type or *R. equi* transposon insertion mutants at a MOI of 10 for 1 hr. The extracellular bacteria were removed from wells by washing 3 times with warm 1x PBS. Fresh DMEM medium containing 10% fetal bovine serum and 20 µg/ml of amikacin was then added. The cells were incubated for the indicated period in 5% CO_2 at 37˚C. After incubation for 2 or 24 hr, the cells were washed 3 times with warm 1x PBS. The glass coverslips were mounted on glass slides with Gold antifade reagent. Monolayers of macrophages on glass coverslips were observed using the fluorescence microscope BX 51. The number of infected macrophages was determined by counting 200 macrophages over several fields on glass coverslips. The percentage of macrophages containing 1-9 bacteria, 10 or more bacteria per macrophage and uninfected macrophages was calculated.

Reagents, solution and equipment

1) Culture medium

- Brain-heart infusion broth
- Luria-Bertani agar
- Luria-Bertani broth
- DMEM
- Fetal bovine serum

2) Antibiotics stock solution

- 20 mg/ml of amikacin disulfate salt soluble in distilled water

3) 1x Phosphate buffer saline

4) Micro slide glass

5) Gold antifade reagent

6) Fluorescence microscope BX 51

4-2-5 Complementation of disrupted oxidoreductase, hypothetical protein (REQ26170) and NRPS (REQ35940) mutants

To complement the oxidoreductase mutant, the wild-type oxidoreductase gene was amplified by PCR using primers REQ10430F and REQ10430R (Table 4-1). The 1,263-bp PCR product was ligated into the pGEM-T vector. The sequence of the insert was confirmed by DNA sequencing with primers SP6 and T7 (Table 4-1). pGEM-REQ10430 was digested with *Not*I and then ligated into *Not*I-digested pINT to generate pINT::REQ10430. Then, pINT::REQ10430 was electroporated into the oxidoreductase mutant, and the transformants were selected on LB agar supplemented with apramycin (80 μg/ml) at 30˚C.

To complement the hypothetical protein (REQ26170) mutant, the REQ26170 gene with its 185-bp upstream region was amplified using the primer pair of REQ26160F and

REQ26160R (Table 4-1). The 1,263-bp amplicon was cloned into the pGEM-T vector. The clones were grown on LB agar supplemented with ampicillin (100 μg/ml). The insert sequence was confirmed by DNA sequencing with SP6 and T7 primers (Table 4-1). pGEM-REQ26170 was digested with *Not*I, and REQ26170 was cloned into *Not*I-digested pINT to generate pINT::REQ26170. The sequence of pINT::REQ26170 was confirmed using REQ26160F and REQ26170R primers. pINT::REQ26170 was electroporated into the hypothetical protein mutant cells. The transformants were incubated on LB agar supplemented with apramycin (80 μg/ml) for 48 hr at 30˚C.

To complement the NRPS (REQ35940) mutant, the first half of the NRPS gene was amplified by hot start PCR using REQ35940F and REQ35940H-SpeR primers (Table 4-1). PCR was carried out as follows: 2 min at 94˚C, five cycles of 98˚C for 10 s, 74˚C for 6 min, five cycles of 98˚C for 10 s, 72˚C for 6 min, five cycles of 98˚C for 10 s, 70˚C for 6 min, and then 25 cycles of 98˚C for 10 s, 68˚C for 6 min, the final step was at 68˚C for 7 min. The PCR product was cloned into the pGEM-T vector. pGEM-first half of REQ35940 was digested by *Nde*I and *Spe*I. The fragment was ligated into pINT::P_{kan2} to generate pINT P_{kan2}-first half of REQ35940. The latter part of the REQ35940 gene was generated with KOD DNA polymerase using REQ35940half-F and REQ35940R primers (Table 4-1). The PCR product acquired an A overhang at the 3′-end by adding A-attachment mix and incubating at 60˚C for 30 min. It was then cloned into the pGEM-T vector to generate pGEM-latter part of REQ35940. pGEM-latter part of REQ35940 was cleaved with *SnaB*I and *Xba*I and ligated into the *SnaB*I and *Spe*I site of pINT P_{kan2}-first half of REQ35940. Resulting plasmids were electroporated into *E. coli* DH5α. The REQ35940 gene sequence was confirmed by DNA sequencing with the primers REQ35940-colF and REQ35940-colR (Table 4-1).

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar

- Luria-Bertani broth

2) Antibiotics stock solution

- 50 mg/ml of ampicillin soluble in distilled water

- 80 mg/ml of apramycin soluble in distilled water

3) T4 DNA ligase

4) IPTG

5) 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside (X-Gal)

- 20 mg of X-Gal (TAKARA BIO INC.)

- 1 ml of N, N-Dimethylformamide (KANTO CHEMICAL CO., INC.)

6) pGEM-T Easy vector

7) Plasmid purification kit mini

8) Gel Extraction kit

9) Restriction enzyme

10) PCR solution

11) Colony PCR solution

- 10x buffer	5 µl
- dNTP	4 µl
- MgCl2	2 µl
- Forward primer	2.5 µl
- Reverse primer	2.5 µl
- DNA template	1 µl
- Distilled water	30 µl
- *Taq* stabilizer	2.5 µl
- *Taq* polymerase	0.5 µl

Total	50 µl

12) Hot start PCR solution

- 10x AccuPrime™ PCR buffer II	10 µl
- Forward primer	2 µl
- Reverse primer	2 µl
- DNA template	2 µl
- Distilled water	82 µl
- AccuPrime™ *Taq* polymerase	2 µl
Total	100 µl

13) KOD DNA polymerase PCR solution

- 10x PCR buffer for KOD	5 µl
- 2 mM dNTPs	5 µl
- 25 mM $MgSO_4$	3 µ
- Forward primer	1 µl
- Reverse primer	1 µl
- DNA template	2 µl
- Distilled water	32 µl
- KOD DNA polymerase	1 µl
Total	50 µl

14) A-attachment mix (Target Clone™)

15) 0.8% (w/v) agarose gel

16) Alkaline Phosphatase, Calf Intestinal (BioLabs®)

17) Purification kit for sequencing

18) Compatible cuvettes 2 mm electrode gap

19) BIO-RAD Gene Pulser™ and BIO-RAD Pulse controller

4-2-6 Staining of *R. equi* with SYTO13 live-cell nucleic acid stains

Non-immunohistochemistry staining was used for observing intracellular replication of *R. equi* strains. J774A.1 macrophages were infected with *R. equi* at a MOI of 10 for 1 hr at 37˚C. Infection of macrophages with *R. equi* was carried out as described above. After incubation for 2 or 24 hr, the cells were rinsed with warm 1x PBS twice, and fixed with 3% formaldehyde in 1x PBS for 5 min. Cells were washed 2 times with warm 1x PBS and incubated with SYTO13 in 1x PBS (1: 7,500) for 10 min. The glass coverslips were mounted onto glass slides for fluorescence microscopy analysis.

Reagents, solution and equipment

1) Culture medium

- DMEM
- Fetal bovine serum

2) Antibiotics stock solution

- 20 mg/ml of amikacin disulfate salt soluble in distilled water

3) 1x PBS

4) Micro slide glass

5) SYTO13 green fluorescent nucleic acid stain (SYTO™ invitrogen)

6) 3% formaldehyde solution

- 3 ml of formaldehyde solution (KANTO CHEMICAL CO., INC.)
- Make up to 100 ml by distilled water

7) Gold antifade reagent

8) Fluorescence microscope BX 51

4-2-7 Competitive infection assay in mice

R. equi strains were cultured in 10 ml of BHI broth with vigorous shaking for 48 hr at 30˚C. Glycerol was added at a final concentration of 15% and stored at -80˚C. Frozen aliquots of the *R. equi* strains were thawed and serially diluted with sterile 1x PBS, and then plated on LB agar with appropriate antibiotics. Bacterial CFU of the infection pool was counted after a 48-hr incubation to determine the bacterial concentration. Slc:ddY mice were inoculated intravenously with 10^6 bacteria consisting of mutant or complemented strain and wild-type in equal proportions. Infected mice were sacrificed on day 5 post-infection. The liver was aseptically removed and homogenized by manual grinding in sterile 1x PBS, and serially diluted 10-fold. One hundred microliters of bacterial solution was plated onto LB agar supplemented with 200 μg/ml of kanamycin for *R. equi* mutants, 80 μg/ml of apramycin for *R. equi* wild-type strain or *R. equi* complemented strains, and 100 μg/ml of rifampicin for rifampicin-resistant *R. equi* wild-type in some experiments. The plates were incubated at 30˚C for 48 hr. Competitive index (CI) values were determined by dividing the number of mutant or complemented strain cells by the number of wild-type strain cells in the sample.

Reagents, solution and equipment

1) Culture medium

- Luria-Bertani agar
- Luria-Bertani broth

2) Antibiotics stock solution

- 80 mg/ml of apramycin soluble in distilled water
- 50 mg/ml of kanamycin soluble in distilled water
- 1 mg/ml of rifampicin soluble in dimethyl Sulfoxide

3) 80% glycerol

4) 1 ml syringe

5) 27-gauge needle

6) Diethyl Ether

7) Sterile operating dissecting scissors sharp blunt and forceps

8) Sterile mortars and pestles

9) Sterile sea sand

10) 1x PBS

11) Hot water bath

12) 70% ethanol

13) 99% ethanol

4-3 Results

4-3-1 Growth defects of transposon insertion mutants in J774A.1

The ability of *R. equi* to replicate within macrophages is critical for survival in the host. Therefore, the attenuated mutants selected in STM may exhibit survival defects in macrophages. To test this, murine macrophage-like J774A.1 cells were infected with the transposon mutants to determine their ability of intracellular growth. At 24 hr post-infection, the proportion of macrophages containing more than 10 bacteria was significantly ($P<0.01$) lower with the putative DEAD/DEAH helicase mutant, oxidoreductase mutant, acyl-CoA thioesterase II mutant, hypothetical protein (REQ26170) mutant, carotenoid oxygenase mutant, putative nitrite reductase large subunit NirB1 mutant and REQ27330 / REQ27340 mutant (Fig. 4-1A and B).

In contrast, the proportion of macrophages containing 10 or more bacteria with the NRPS (REQ35940) mutant was higher than with the virulent *R. equi* strain ATCC 33701 (Fig. 4-2A and B). Furthermore, large numbers of bacteria formed bacterial clumps and were released by disruption of macrophages during extended culture (Fig. 4-3).

4-3-2 Growth of complemented strains in macrophages

To examine if the growth defects resulted from dysfunction of the genes disrupted by the transposon, each mutant was complemented with an integration plasmid containing its respective functional gene. For this purpose, oxidoreductase and hypothetical protein (REQ26170) mutants were chosen because these genes were likely transcribed from their own promoters based on the gene map and complemented easily. The integration plasmid was introduced into each mutant strain, and J774A.1 macrophages were infected with transformants. SYTO 13 green fluorescent nucleic acid stain was used to stain bacteria and count the number of bacteria within macrophages. At 24 hr post-infection, both complemented strains had

restored intracellular growth to wild-type levels (Fig. 4-4). These results demonstrated that oxidoredactase and REQ26170 are essential fitness genes for macrophage infection.

4-3-3 The oxidoreductase and hypothetical protein (REQ26170) genes are required for *R. equi* survival *in vivo*

To examine if complementation of oxidoreductase and hypothetical protein (REQ26170) genes restore *in vivo* survival, competitive infection experiments were performed. The mice were injected with the bacterial pool containing mutant or complemented strains and wild-type *R. equi* in equal proportions. On the 5^{th} day from injection, the liver was collected and homogenate was plated onto LB agar supplemented with the appropriate antibiotic for selecting *R. equi* strains. The bacterial number was counted and used to calculate the CI values. CI values of the oxidoreductase mutant and hypothetical protein mutant (REQ26170) were 0.286 and 0.306, respectively. On the other hand, CI values were 1.02 in the complemented oxidoreductase mutant and 4.88 in the complemented hypothetical protein (REQ26170) mutant (Fig. 4-5). These results confirmed that oxidoreductase and hypothetical protein (REQ26170) are required for full virulence of *R. equi* in mice.

4-3-4 Complementation of the NRPS (REQ35940) mutant

The NRPS (REQ35940) mutant exhibited overgrowth in macrophages. To determine whether disruption of this gene was responsible for this phenotype, the REQ35940 mutant was complemented with the integration plasmid expressing REQ35940 with the promoter for the kanamycin resistance gene (P_{kan2}). The complemented mutant overgrew in macrophages compared with wild-type.

4-4 Discussion

Intracellular growth within macrophages is essential for the virulence of *R. equi*. Although VapA is indispensable for intracellular growth in macrophages, it is not sufficient for virulence because full expression of VapA in a plasmid-cured derivative does not restore virulence in mice and foals [19]. Furthermore, the intravacuolar environment mediated by VapA did not allow disordered growth of bacteria, as shown in chapter 2. This suggests that *R. equi* possesses unknown virulence factors other than VapA.

In the previous chapter, 35 transposon mutants were selected as mutants deficient in mouse infection. It was hypothesized that some of these mutants had decreased ability to grow in macrophages. As expected, seven mutants could not multiply to the level of the wild-type in macrophages. All mutants had no detectable growth defects in nutrient broth. Furthermore, they expressed the same level of VapA as the wild-type, except for the acyl-coA thioesterase II mutant. The nature of the intraphagocytic environment mediated by VapA has not been fully elucidated, but information about the function of the genes interrupted by transposons may indirectly provide clues.

DEAD/DEAH box helicases play critical roles in RNA processing. They have been demonstrated to participate in bacterial responses to stress, such as low pH and oxidative bursting, which *R. equi* may encounter in phagocytes [4, 80]. *R. equi* possesses an extensive lipid metabolic system, but has only 2 unique genes encoding acyl-CoA thioesterase II (REQ20820, REQ25310). Acyl-CoA thioesterase II plays a role in fatty acid synthesis, especially in the synthesis of palmitic acid. *R. equi* utilizes only organic acids and fatty acids as carbon sources. In the previous study, palmitate was an important substrate for *R. equi* growth in minimal media [37]; therefore, fatty acids may be important for *R. equi* in the intraphagocytic environment. Mutation in *crtB*, *crtYc*, and *crtYd* genes involved in the carotenoid biosynthesis pathway of *Mycobacterium marinum* resulted in poor growth in murine macrophages [18].

Carotenoid oxygenase is an enzyme involved in the cleavage of carotenoids to produce vitamin A, suggesting that exogenous vitamin A cannot be easily obtained by *R. equi* in phagocytes [34]. One of the host defense mechanisms in macrophages is thought to be oxygen-limitation [44, 85]. The genus *Rhodococcus* comprises more than 40 species, but only *R. equi* possesses nitrate reductase (*narGHIJ*) and nitric oxide reductase (REQ03280) [37]. Pei *et al.* [47] confirmed that the *narG* mutant was fully attenuated in a mouse model. In the present study, a putative nitrite reductase large subunit NirB1 mutant was unable to multiply efficiently in macrophages. This enzyme presumably regenerates NAD^+ and detoxifies nitrite that accumulates as a result of nitrite respiration. Therefore, nitrate assimilation by NirB1 may be an important pathway during infection of *R. equi* in macrophages.

One of the mutants identified in this study had a transposon insertion at the intergenic region between a putative HNH endonuclease and putative Fis family transcriptional regulator. Wang *et al*. [83] reported that the Fis regulator controlled the virulence mechanism of *salmonella* pathogenicity islands. Deletion of the *fis* gene in *Salmonella enterica* serovar typhimurium resulted in reduced intracellular replication in macrophages [10, 33]. The transposon insertion at the intergenic region may affect the promoter activity for the *fis* gene and result in reduced replication of this mutant in macrophages.

The results of this study demonstrated that oxidoreductase and hypothetical protein (REQ26170) genes are required for *R. equi* intracellular survival and virulence *in vivo*. Complementation with the respective functional gene restored the virulence of the oxidoreductase and hypothetical protein (REQ26170) mutants. Oxidoreductases are a class of enzymes that catalyze biological oxidation-reduction reactions [41]. These enzymes play an important role in electron transportation and biological functions such as ferric iron reduction, oxygen activation and ribonucleotide reductase [16]. The product of this oxidoreductase gene has a domain that is found in pyridine nucleotide-disulphide oxidoreductase, which acts on

sulfur groups. The oxidoreductases acting on sulfur groups have been reported to be important during infection of several virulent pathogens [1, 6, 16, 30, 49, 53]. Hypothetical protein (REQ26170) is not homologous with any proteins whose functions have been elucidated. It may be an intracellular protein as it has no signal sequence or transmembrane region. This protein may have a unique function that enables *R. equi t*o survive in macrophages.

The genome of *R. equi* encodes 11 NRPS genes. Among them, three genes were reported to be involved in siderophore synthesis [37, 43]. In the present study, the transposon mutant of a gene encoding NRPS (REQ35940) exhibited a fitness defect in mouse infection. On the other hand, the REQ35940 mutant exhibited marked intracellular growth and high cellular lethality in macrophages. One possible explanation for this discrepancy is that *R. equi* escapes from the host immunity by hiding inside macrophages. REQ35940 may be involved in controlling bacterial population density as the loss of function of REQ35940 causes bacterial overgrowth and disruption of macrophages. The released bacteria eventually encounter mature phagocytes, such as neutrophils, and are killed. Further study is necessary to clarify the function of REQ35940.

4-5 Summary

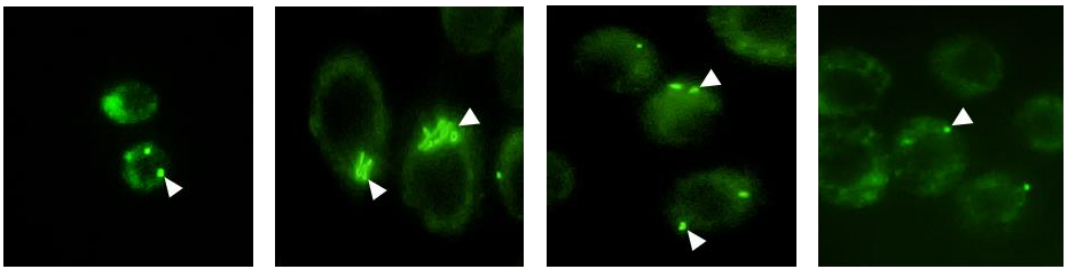
In this study, macrophage J774A.1 cells were infected with attenuated mutants. The plasmids expressing EGFP were electroporated into these mutants. The intracellular growth of the mutants was observed by fluorescence microscopy at 24 hr post-infection. The numbers of macrophages containing more than 10 bacteria were significantly lower in the putative DEAD/DEAH helicase mutant, oxidoreductase mutant, acyl-CoA thioesterase II mutant, hypothetical protein mutant (REQ26170), carotenoid oxygenase mutant, putative nitrite reductase large subunit NirB1 mutant, and the mutant with an insertion at the intergenic region between the putative HNH endonuclease and putative Fis family transcriptional regulator. Interestingly, the proportion of macrophages containing 10 or more bacteria was higher with the putative NRPS (REQ35940) mutant than the virulent *R. equi* strain. Complementation with oxidoreductase and hypothetical protein (REQ26170) genes restored the ability to survive and replicate in macrophages and to infect mice.

The NRPS (REQ35940) mutant demonstrated enhanced ability to multiply in macrophages, but had lower mouse infection capability. One possible explanation for this discrepancy is that *R. equi* escapes from the host immunity by hiding in the macrophages. The bacterial population density is suitably controlled by REQ35940. The loss of function of REQ35940 causes bacterial overgrowth and disruption of macrophages. The released bacteria eventually encounter the host immune system and are killed by neutrophils.

Table 4-1. Sequences of forward and reverse primers

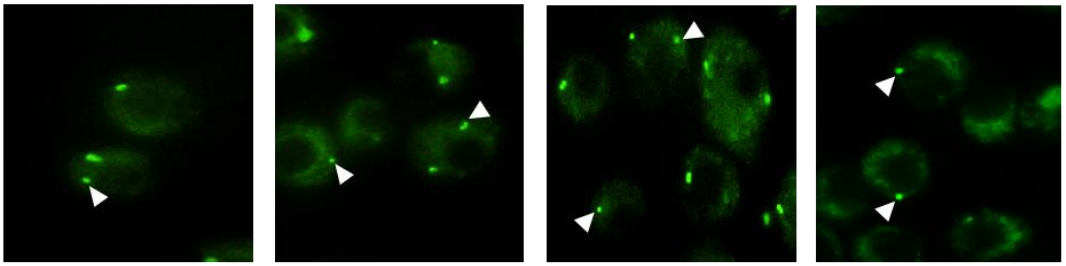
Gene	**Primer (5′-3′)**
REQ10430F	CGGCTGGTCCCGATCATCATGTG
REQ10430R	ATCCGCGTCACTGCTTCAGG
REQ26160F	CGTGCGTCTCCACCAAAGCC
REQ26160R	GCTGTCATGTCCCGTGTGGC
SP6	CATACGATTTAGGTGACACTATAG
T7	TAATACGACTCACTATAGGG
REQ35940F	GGGCATATGAGTGAGCTGGGTCGGGAGCGGGGAACTGACGGCAACG
REQ35940H-SpeR	GGGACTAGTTACGTACTCGCGGGAGGGCAACGCGAAAGCTCTTGCACGG
REQ35940half-F	TGCAAGAGCTTTCGCGTTGCCCTCCCGCGAGTACGTACTC
REQ35940R	GGGTCTAGATCAGCGACGCAGGTACCGGTCGAGTGCCGGTCCGACCACT
REQ35940-colF	AGGAGCTGCATGCTCTGCTC
REQ35940-colR	AGGTACAGCTCACCGGCGAC

A



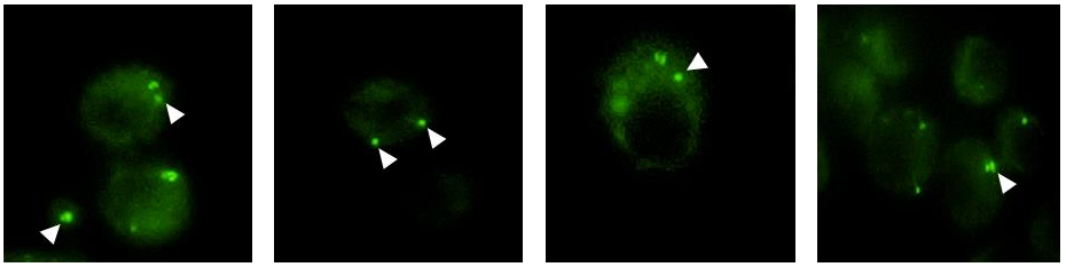
2 hr 24 hr — *R. equi* ATCC 33701

2 hr 24 hr — Putative DEAD/DEAH helicase mutant



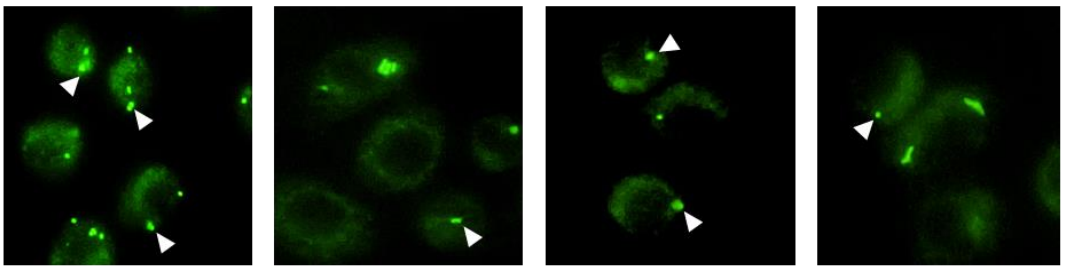
2 hr 24 hr — Oxidoreductase mutant

2 hr 24 hr — Acyl-CoA thioesteraseII mutant



2 hr 24 hr — Hypothetical protein mutant (REQ26170)

2 hr 24 hr — Intergenic region between putative HNH endonuclease and putative Fis family transcriptional regulator mutant



2 hr 24 hr — Carotenoid oxygenase mutant

2 hr 24 hr — Putative nitrite reductase large subunit NirB1 mutant

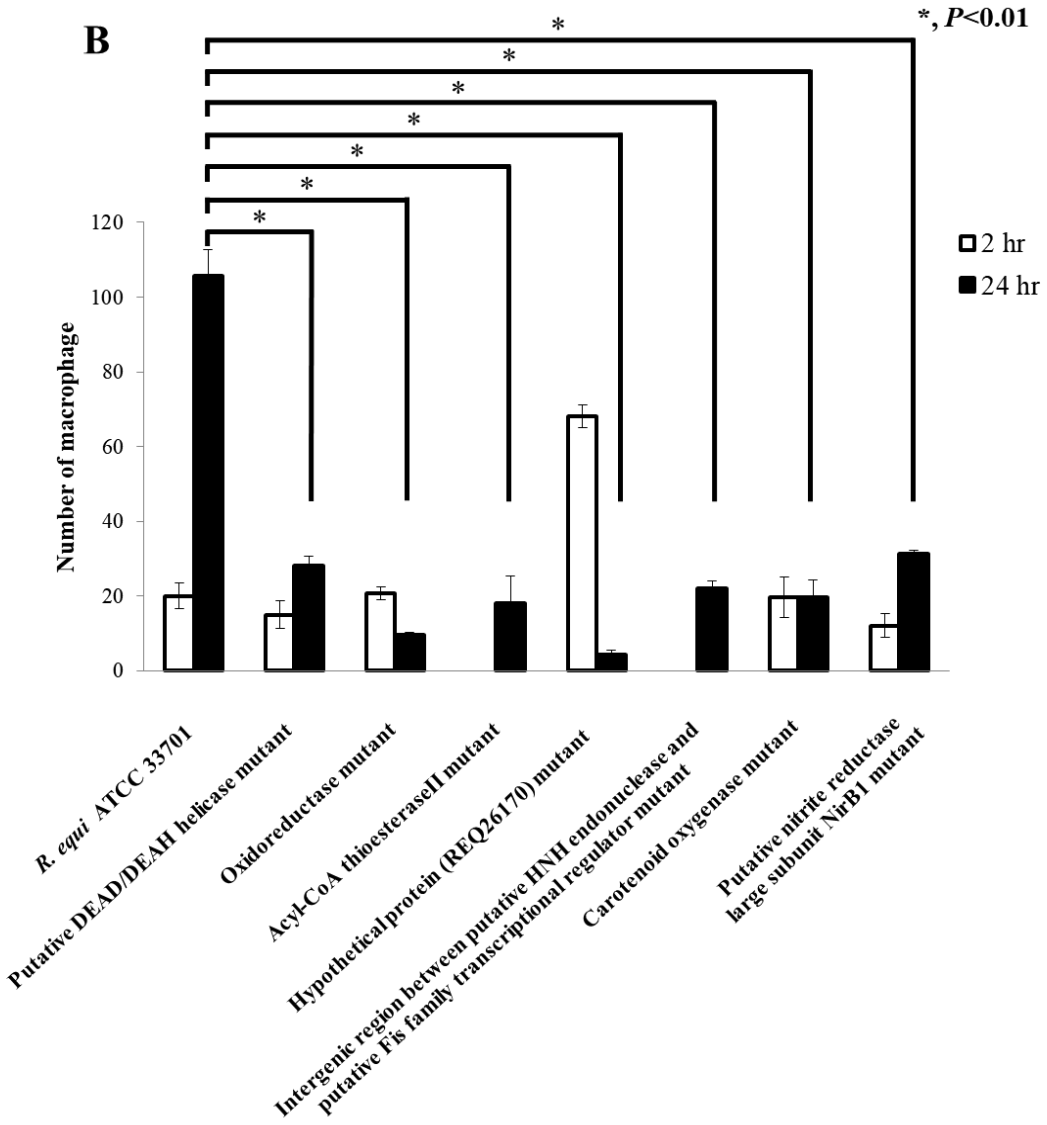


Fig. 4-1. Intramacrophage growth of EGFP-expressing *R. equi* mutants. J774A.1 macrophages were infected with *R. equi* ATCC 33701, the putative DEAD/DEAH helicase mutant, oxidoreductase mutant, acyl-CoA thioesteraseII mutant, hypothetical protein mutant (REQ26170), mutant with transposon insertion at intergenic region between putative HNH endonuclease and putative Fis family transcriptional regulator, carotenoid oxygenase mutant or the putative nitrite reductase large subunit NirB1 mutant. A: Macrophages were observed by fluorescence microscopy at 2 and 24 hr post-infection. *R. equi* cells are indicated by arrowheads. B: The number of macrophages with more than 10 bacteria was recorded at 2 and

24 hr post-infection. The data are expressed as the number of macrophages containing the bacterial numbers indicated. Each error bar represents the standard deviation for the mean of three data sets (200 macrophages were examined per set). The significance of differences in frequencies of *R. equi*-infected macrophages was calculated using the t-test (*, $P<0.01$).

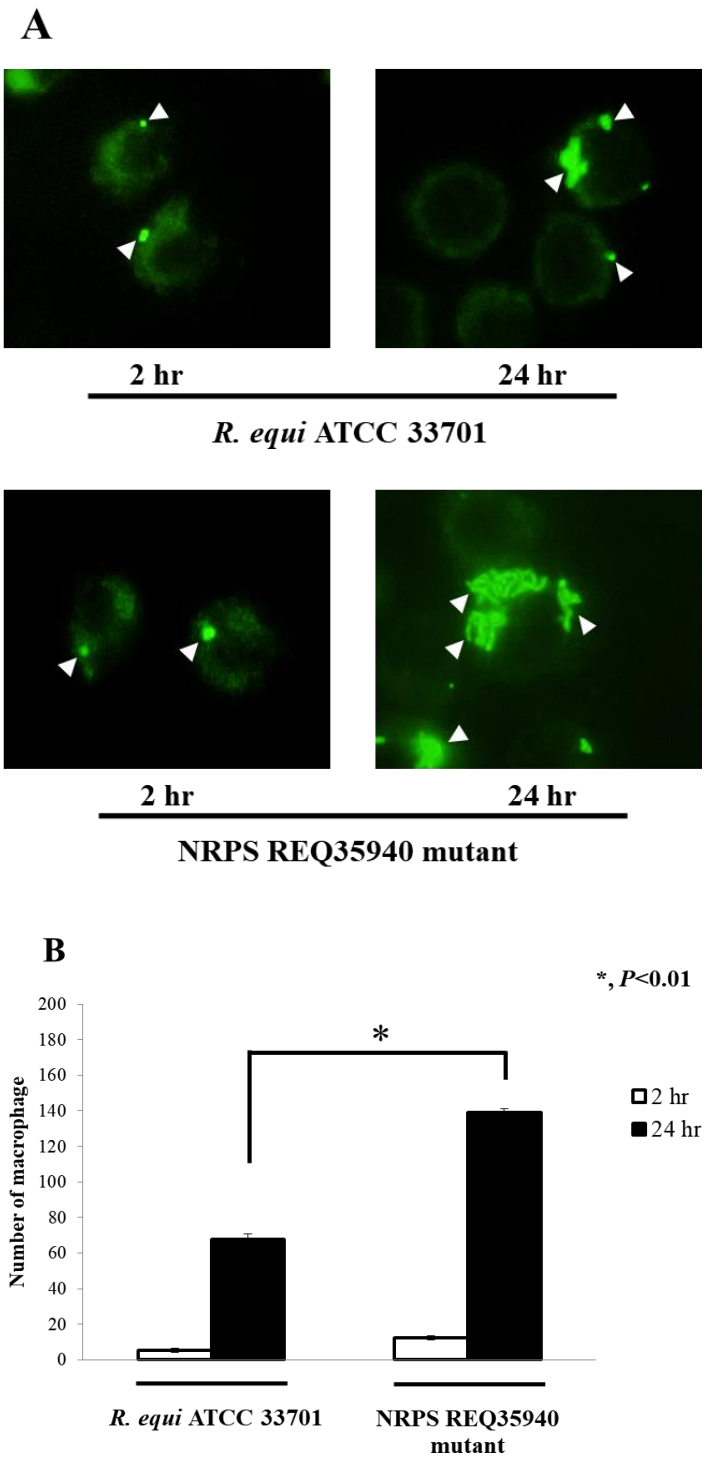


Fig. 4-2. Intramacrophage growth of the EGFP-expressing *R. equi* mutant. J774A.1 macrophages were infected with *R. equi* ATCC 33701 or NRPS (REQ35940) mutant. A: Macrophages were observed by fluorescence microscopy at 2 and 24 hr post-infection. *R. equi*

cells are indicated by arrowheads. B: The number of macrophages with more than 10 bacteria was recorded at 2 and 24 hr post-infection. The data are expressed as the number of macrophages containing the bacterial numbers indicated. Each error bar represents the standard deviation for the mean of three data sets (200 macrophages were examined per set). The significance of differences in frequencies of *R. equi*-infected macrophages was calculated using the t-test (*, $P<0.01$).

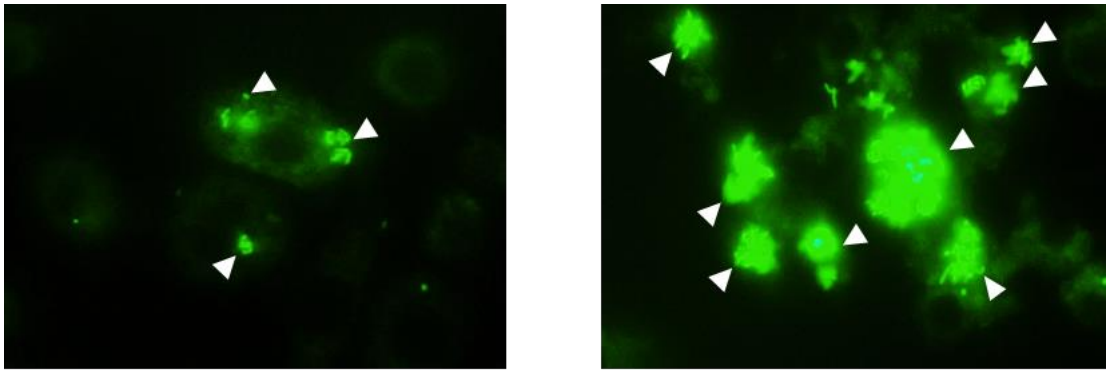


Fig. 4-3. Intramacrophage growth of the EGFP-expressing *R. equi* NRPS (REQ35940) mutant. J774A.1 macrophages were infected with *R. equi* ATCC 33701 or NRPS (REQ35940) mutant. Macrophages were observed by fluorescence microscopy at 24 hr post-infection. The NRPS (REQ35940) mutant grew rapidly inside macrophages, leading to massive macrophage damage. *R. equi* cells are indicated by arrowheads.

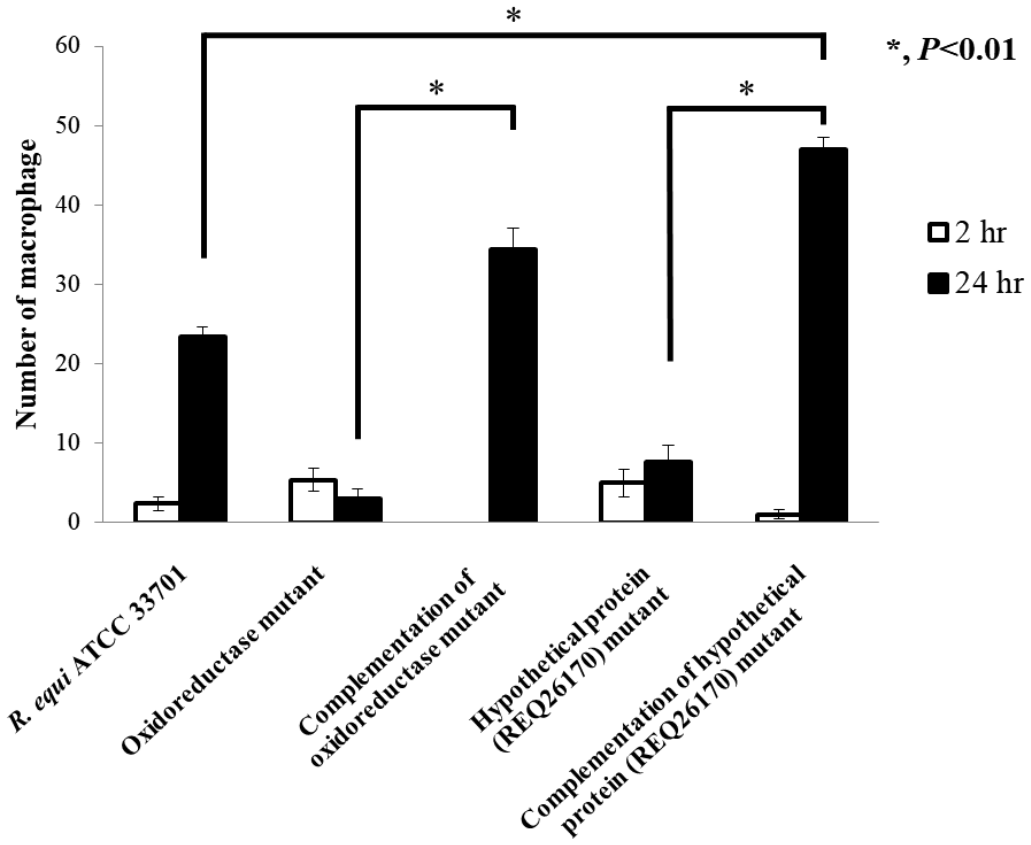


Fig. 4-4. Survival of complemented strains in macrophages. J774A.1 macrophages were infected with *R. equi* ATCC 33701, the oxidoreductase mutant or the hypothetical protein (REQ26170) mutant. After incubation, *R. equi* was stained by SYTO13 and observed under fluorescence microscopy. The number of macrophages with more than 10 bacteria was recorded at 2 and 24 hr post-infection. The data are expressed as the number of macrophages containing the bacterial numbers indicated. Each error bar represents the standard deviation for the mean of three data sets (200 macrophages were examined per set). The significance of differences in frequencies of *R. equi*-infected macrophages was calculated using the t-test (*, $P<0.01$).

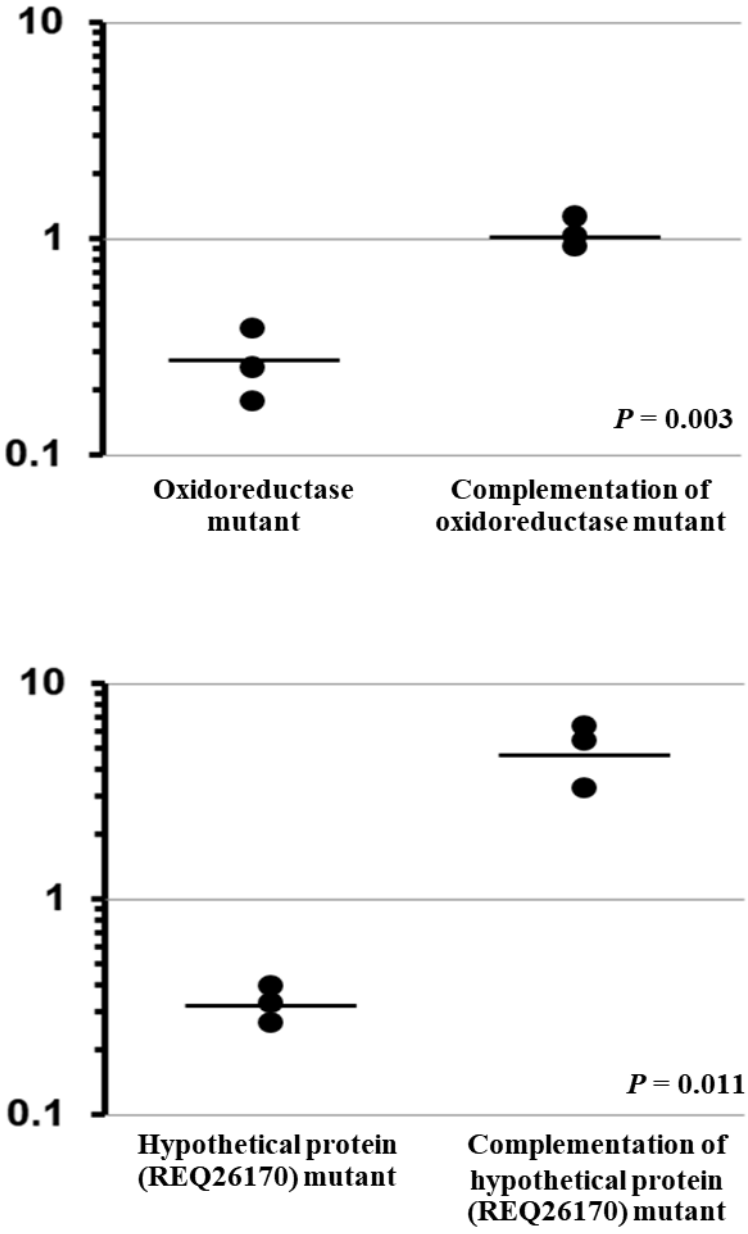


Fig. 4-5. CI analysis of mutants and complemented strains. Three mice were infected with 1:1 mutant or complemented strain and wild-type strain. Mice were sacrificed on day 5 post-infection. A CI of >1 indicates increased virulence, whereas a CI of <1 indicates reduced virulence. CIs of the complemented oxidoreductase mutant and complemented hypothetical protein mutant were significantly higher in the liver than their mutants, with *P*-values of 0.003

for the complemented oxidoreductase mutant and 0.011 for the complemented hypothetical protein mutant.

Chapter 5 Summary

R. equi is one of the most serious bacterial pathogens that causes suppurative pneumonia and enteritis associated with lymphadenitis in foals less than 6 months of age. *R. equi* is a facultative intracellular pathogen that can replicate within macrophages. The ability of *R. equi* to survive and multiply inside macrophages is conferred by a virulence-associated plasmid. The plasmid-cured strains of *R. equi* are unable to survive in macrophages and are avirulent in foal or murine infection model. Virulent strains of *R. equi* express a 15-17 kDa bacterial surface protein named VapA, which is encoded by the virulence-associated plasmid. However, the role of VapA remains enigmatic and the factors required for infecting animal hosts are poorly understood.

VapA is essential for intracellular survival in macrophages, but its function is still unclear. Phagosomes containing virulent *R. equi* pass normally thorough the early phase of maturation, but are arrested between the early and late maturation stages. Recently, it was reported that VapA was taken into endocytic compartments of macrophages cultured in vitro when it was added to the culture media, and that cells fed VapA formed swollen endolysosome organelles with reduced activity of cathepsin B and accumulation of LBPA, LC3 and Rab7. These results indicate that VapA causes defects in endolysosomal function, which may lead to tolerance for bacterial growth in macrophages. It was reported that the *R. equi* strain lacking the *vapA* gene exhibited growth defects in macrophages. Therefore, in this study, I examined whether the extracellular addition of VapA could rescue the growth defect of the *vapA* mutant and plasmid-cured strain in macrophages.

J774A.1 macrophages were infected with the plasmid-cured strain or *vapA* mutant and were co-cultured with VapA that was added extracellularly. The number of macrophages containing proliferating bacteria increased when both strains infected macrophages in the presence of VapA at 24 hr post-infection. These results suggested that these strains multiplied in

vacuoles whose antibacterial activities were reduced. To examine whether the addition of VapA allowed the growth of bacteria that cannot normally grow in macrophages, DH5α *E. coli* were employed. No obvious growth of *E. coli* was observed at 24 hr post-infection. The present study demonstrated that the extracellular addition of VapA rescued the intracellular growth defect of the plasmid-cured strain, but could not rescue that of *E. coli*. These data also suggested that the intraphagocytic environment can still restrict the growth of bacteria. Conversely, *R. equi* lacking the virulence plasmid may possess the ability to overcome some of the growth limitations in VapA-mediated phagosomal environments.

As the avirulent strain of *R. equi* was able to multiply in macrophages in the presence of VapA but *E. coli* could not, I thought that *R. equi* chromosomes may possess unique genes for adaption and survival in the intraphagosomal environment. Other than the *vapA* gene, only a few virulence factors of *R. equi* have been identified to date. Therefore, I tried to identify the essential genes encoded on the chromosome of *R. equi* for infection.

To carry out transposon-based random mutagenesis in *R. equi*, I used pTNR-KA, which is used for random mutagenesis of *Rhodococcus erythropolis*. pTNR-KA was electroporated into competent *R. equi* cells and transposon mutants were selected by antibiotics. Southern blot analysis of DNA extracted from randomly chosen mutants revealed that transposons were randomly inserted into *R. equi* chromosomal DNA. To establish an STM system to study *R. equi*, tags with a unique oligonucleotide sequence were inserted into pTNR-KA. *R. equi* was randomly mutagenized by each of the 25 different signature-tagged transposons, and 25 mutants with different tags were pooled as a set. A two-step STM screening method based on quantitative real-time PCR using the tag-specific primers was employed. To verify the usefulness of my screening method, an avirulent mutant with one signature-tagged transposon was mixed with 24 mutants containing the other tags and injected into mice. DNA was extracted from the livers of infected mice and used for quantitative real-time PCR. The avirulent mutant

was not detected in the output, indicating that the screening method employed in this study is useful.

A total of 4,650 *R. equi* transposon mutants were examined for ability of bacterial survival during infection. The mutants that could not survive in mice were identified by quantifying the copy number of the tag in the output. The mutants whose relative abundance was markedly reduced after infection (>5-fold reduction) were selected as defective mutants. To eliminate potential false positives, the mutants selected in the first screen were used to make a new input pool and were inoculated into mice again. The mutants with a reduced ability to infect in both screens were finally selected as fitness defect mutants. As a result, 102 mutants, equivalent to approximately 2.15% of the total number of the mutants examined, were selected. Among them, transposon insertion sites were successively determined in 50 transposon insertion mutants. Insertion of transposons was seen in the genes encoded on the virulence plasmid in 15 of the 50 mutants. Most of these mutants had no or reduced VapA expression, which may have been responsible for their reduced ability to infect mice. The remaining 35 mutants had insertion sites in the genes encoded on the chromosome. Nine chromosomal mutants expressed VapA at low levels, but the remaining 26 mutants had the same VapA expression level as wild-type. The transposon insertions were seen in the genes involved in amino acid metabolism, lipid metabolism, vitamin synthesis, DNA repair / recombination, peptidoglycan biosynthesis/degradation, energy metabolism and NRPS. Eight mutants had insertions in genes for proteins of unknown function. The most frequently identified gene was REQ23810, which encodes the NRPS protein. It was reported that REQ23810 was responsible for the synthesis of siderophores required for growth in low iron concentrations. However, growth defects of REQ23810 mutants in iron-restricted media were not observed in my study.

Intracellular growth within macrophages is critical for *R. equi* to survive in mice. Therefore, some of the mutants identified in my screening using STM may have some defect in

ability to grow inside macrophages. To test this hypothesis, J774A.1 cells were infected with attenuated mutants. The plasmids expressing EGFP were electroporated into these mutants. The intracellular growth of the mutants was observed by fluorescence microscopy at 24 hr post-infection. The numbers of macrophages containing more than 10 bacteria were significantly lower with the putative DEAD/DEAH helicase mutant, oxidoreductase mutant, acyl-CoA thioesterase mutant, hypothetical protein mutant (REQ26170), mutant with transposon insertion between the putative HNH endonuclease and putative Fis family transcriptional regulator, carotenoid oxygenase mutant and the putative nitrite reductase large subunit NirB1 mutant. Interestingly, the proportion of macrophages containing 10 or more bacteria was higher with the NRPS (REQ35940) mutant than with the virulent *R. equi* strain ATCC 33701. Complementation with the hypothetical protein (REQ26170) and oxidoreductase genes restored the ability to survive and replicate in the macrophages and to infect mice. These results demonstrated that the hypothetical protein (REQ26170) and oxidoreductase were necessary for *R. equi* virulence and intracellular survival.

The NRPS (REQ35940) mutant demonstrated enhanced ability to multiply in macrophages, but was attenuated in mouse infection. One possible explanation for this discrepancy is that *R. equi* escapes from the host immunity by hiding in the macrophages. The bacterial population density is suitably controlled by NRPS (REQ35940) because loss of function of NRPS (REQ35940) causes bacterial overgrowth and disruption of macrophages. The released bacteria eventually encounter the host immune system and are killed by neutrophils.

This is the first report of the successful establishment of STM for *R. equi*. Several novel *R. equi* fitness genes for infection were identified in this study. The *R. equi* chromosome encodes factors contributing to bacterial growth in macrophages during infection that are not found in *E. coli*.

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