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

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ABSTRACT. *Rhodococcus equi* is a Gram-positive facultative intracellular bacterium that causes pyogranulomatous pneumonia in foals and immunocompromised people. In the present study, signature-tagged transposon mutagenesis was applied for the negative selection of *R. equi* mutants that cannot survive *in vivo*. Twenty-five distinguishable plasmid-transposon (plasposon) vectors by polymerase chain reaction (PCR), each containing a unique oligonucleotide tag, were constructed and used to select the transposon mutants that have *in vivo* fitness defects using a mouse systemic infection model. Of the 4,560 transposon mutants, 102 mutants were isolated via a real-time PCR-based screening as the mutants were unable to survive in the mouse model. Finally, 50 single transposon insertion sites were determined via the self-cloning strategy. The insertion of the transposon was seen on the virulence plasmid in 15 of the 50 mutants, whereas the remaining 35 mutants had the insertion of transposon on the chromosome. The chromosomal mutants contained transposon insertions in genes involved in cellular metabolism, DNA repair and recombination, gene regulation, non-ribosomal peptide synthesis, and unknown functions. Additionally, seven of the chromosomal mutants showed a reduced ability to multiply in the macrophages *in vitro*. In this study, we have identified several biosynthetic pathways as fitness factors associated with the growth within macrophages and survival in mice.

KEY WORDS: bacterial infection, macrophage, *Rhodococcus equi*

Rhodococcus equi is a Gram-positive coccobacillus that frequently causes bronchopneumonia in 1- to 3-month-old foals [32]. *R. equi* also infects various vertebrates, including pigs, wild boars, camels, cattle, goats, cats, and dogs [22, 23, 30, 34, 44, 48, 49]. This bacterium parasitizes macrophages in the hosts and replicates within the phagosomes. A large virulence plasmid confers the ability to survive within the macrophages and cause disease in the animals [11, 12, 46, 47, 55]. Equine isolates express virulence-associated protein A (VapA) that is encoded in 80 to 90 kb virulence plasmids. VapA is known to be a major virulence determinant of *R. equi*, and the deletion of this single gene reduces the pathogenicity to an avirulent level [14].

In a previous study, the intraphagocytic environment mediated by VapA enables *R. equi* to survive and multiply inside the macrophages but still restrict the growth of bacteria, such as *Escherichia coli* [40]. These observations suggested that *R. equi* might possess the ability to adapt to harsh environments under bactericidal activity and nutritional depletion. However, knowledge about the genetic determinants required for its growth and fitness in the macrophages and the animal host is rather limited. Systemic infection of mice by intravenous injection with *R. equi* has been often used as a model of experimental animal infection. Although this model does not produce typical pulmonary lesions with abscessation seen in infected foals, the persistence of the bacteira in the liver and spleen is thought to be consistent with pathogenicity and has been used to identify the virulence factors of *R. equi* along with *in vitro* macrophage infection. Several genes, including isocitrate lyase (*aceA*), catalase (*katA*), chorismate mutase (*aroQ*), bifunctional anthranilate synthase (*REQ_23850*), β-ketoacyl-(acyl carrier protein)-synthase A (*kasA*), rhequichelin synthase (*rhbC*), peptidase D (*pepD*), sensor histidine kinase (*mprB*), peptidase S1 (*pepS1*), nitrate reductase (*narG*), mycoredoxins (*mrx*), and coenzyme A transferase (*ipdA* and *ipdB*), are involved in the ability of *R. equi* to survive in macrophages and/or ability to infect animals [4, 18, 21, 27, 29, 31, 45, 50, 52, 54, 56].

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Transposon-based mutagenesis is a powerful molecular technique essential for investigating bacterial virulence and identifying virulence determinants in various bacteria [6]. Signature-tagged transposon mutagenesis (STM) is a negative selection strategy used to identify virulence factors of many bacteria [10, 13, 36]. STM allows the screening of a large number of mutants using fewer animals. Nevertheless, the molecular tool for STM has not been available in the study of *R. equi*.

In the present study, we developed an STM procedure to isolate and identify fitness genes of *R. equi* required for the *in vivo* survival. The procedure included the use of a real-time polymerase chain reaction (PCR)-based screening step for detecting each tag in the input and output pools. A plasposon pTNR-KA containing *E. coli* plasmid replication origin was used to facilitate the identification of flanking regions of the transposon insertion site.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Wild-type *R. equi* strain ATCC 33701 was used as the genetic background for all experiments reported in this study. All *R. equi* strains were grown in brain heart infusion (BHI) broth at 30°C or 37°C. Apramycin (80 µg/ml) or kanamycin (200 µg/ml) was added to BHI agar to select for *R. equi* growth when necessary. *E. coli* DH5α was used and routinely cultured in Luria-Bertani (LB) broth at 37°C. Ampicillin (100 µg/ml) or kanamycin (20 µg/ml) was added to select the transformants.

Construction of a plasposon carrying unique oligonucleotide tags

The plasposon-based vector pTNR-KA of *Rhodococcus erythropolis* was kindly gifted by Dr. Tomohiro Tamura (Bioproduction Research Institute, National Institute of Advanced Industrial Sciences and Technology, Sapporo, Japan). The pTNR-KA carries transposon containing a kanamycin-resistant gene, replication origin of *E. coli*, and multiple cloning sites (MCS) that contain 10 different enzyme sites [40]. Twenty-five different oligonucleotides were annealed to complementary molecules to yield double-stranded oligonucleotides (Supplementry Table 1). Each tag was separately introduced into the *Hin*dIII and *Nsi*I sites of MCS of the pTNR-KA plasmid to generate 25 plasposons (pTNR-KA-RE tag1–tag25). To confirm if the proper construct was made, nucleotide sequencing was conducted using Tag-seq primer (Supplementry Table 2).

Transposon mutagenesis

The pTNR-KA plasmid containing each unique tag (pTNR-KA-RE tag1–tag25) was electroporated into the *R. equi* strain. The competent cells of *R. equi* were made according to the method described by Sekizaki *et al*. [42]. Frozen electrocompetent cells (100 µl) were thawed on ice and gently mixed with 5 µl pTNR-KA-RE tag1 to tag25, respectively. Electroporation was performed in pre-chilled sterile electroporation cuvette (2 mm electrode gap; Bio-Rad, Hercules, CA, USA) at 2.5 kV, 400 Ω, and 25 µF. BHI broth (900 µl) containing 20 mM Mg^{2+} was added, and cells were incubated at 30°C for 2 hr. Then, bacteria were plated onto LB agar supplemented with kanamycin (200 µg/ml) and cultured for 2 days at 30°C.

Preparation of input pool

A set of transposon insertion mutants (tag1–tag25) was sub-cultured on a BHI agar plate supplemented with kanamycin (200 µg/ml). After incubation at 30°C for 48 hr, each *R. equi* mutant was inoculated into 3 ml 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 m*l* of the bacterial pool using DNeasy Blood and Tissue Kit (Qiagen, Hilden, German) according to the manufacturer's instruction and used as the template for the analysis of the input pool. The bacterial number of the pool was estimated by measuring the optical density (OD) at 600 nm (culture showing OD_{600}=1 contains ~5 × 10^8 bacteria). The bacterial pool was diluted to a final concentration of 10^7 CFU/ml and used as an inoculum for mouse infection.

Animal infection

Female ddY mice (4 weeks old) were used for the infection of the transposon mutants. The animal experimentation protocol was approved by the president of Kitasato University based on the judgment of the Institutional Animal Care and Use Committee of Kitasato University (Approval No. 15-016). Bacteria (~10^6) were intravenously injected into the 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 phosphate-buffered saline (PBS). The homogenate was adjusted to a concentration of 100 mg liver/1 ml, poured into a 15 ml centrifuge tube, and allowed to stand for 10 min. The supernatant (100 µl) was plated onto LB agar supplemented with kanamycin (200 µg/ml) and incubated for 48 hr at 30°C. The bacterial colonies on the surface of the agar were harvested by scraping with 6 ml sterile PBS. The bacterial suspension was diluted and adjusted to 1.0 of the OD_{600} value. DNA was extracted from 1 ml of the bacterial suspension using the DNeasy Blood and Tissue Kit.

Semiquantification of each tag in the input and output pools by real-time PCR

In the preliminary experiments, the direct use of DNA extracted from the output pool disturbed amplification in real-time PCR. Thus, DNA fragments were amplified using a primer pair of Realtime-F primer and Pre-PCR-R and used for real-time PCR. The amplification cycle of the first PCR 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 PCR product was visualized on a 0.8% (w/v) agarose gel, and the amplified fragments were extracted

from the gel using the MinElute gel extraction kit (Qiagen). Subsequently, 1 µl extracted PCR product was mixed with 999 µl sterile distilled water and used as a real-time PCR template. Real-time PCR was conducted using the SYBR® Select Master Mix kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. The forward primer used was Realtime-F in combination with the tag-specific primer listed in Supplementary Table 3. The reaction was conducted under the following conditions: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. In each experiment, the cycle threshold values for each tag were obtained and used to calculate the number of copies per sample using a standard curve of known amounts of the DNA target. These data were used to calculate the ratio of each tag to all tags in the sample. The ratio of each tag in the input pool was also calculated by the same method. Finally, when the ratio of the output to the ratio of the input was less than one-fifth, it was selected as a mutant strain with a fitness defect.

Determination of the transposon insertion site

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 digest was self-ligated in a 50 µl ligation reaction, including T4 DNA ligase. The reaction mixture was kept at 4°C overnight, 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 kanamycin (200 µg/ml). Plasmid DNA was extracted using a mini-prep plasmid purification kit. The sequencing was performed by the primer pair pTNR1 and pTNR2. The sequence obtained was compared with the GenBank database by BLAST to determine the transposon insertion site.

Western blot analysis

The *R. equi* mutants were cultured in BHI broth (pH 6.5) for 48 hr at 37°C. This culture condition was shown to be optimal for VapA expression [15]. The pellet was suspended with 1× sample buffer, boiled for 10 min, and centrifuged briefly to remove the undissolved material. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was conducted with 15% polyacrylamide gel. Each sample (10 µl) was loaded onto each lane and electrophoresed at 100 V for 2 hr. The protein was electrophoretically transferred to a nitrocellulose membrane (GE healthcare, Chicago, IL, USA). The nitrocellulose membranes were incubated with Block Ace at 4°C overnight. The membranes were incubated for 2 hr with monoclonal antibodies against VapA (10G5) [15]. 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). The membranes were washed again, and the antibody bound to protein was visualized with diaminobenzoic acid.

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 were analyzed by Southern blotting. The genomic DNA was digested with *Apa*I, *Eco*RV, *Sac*II, or *Xma*I, which does not have a restriction site within the transposon. 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 (GE healthcare) using a pump with 40 mbar of vacuum pressure for 1 hr. During the transfer, agarose gel was always soaked with 0.4 M NaOH. The membrane was washed with 2× SSC buffer with shaking for 30 min and baked at 80°C for 2 hr. Southern hybridization was conducted using DIG High Prime DNA Labeling and Detection Starter Kit 1 according to the manufacturer's instruction (Roche, Basel, Switzerland). To prepare a probe, the kanamycin resistance marker was amplified by a pair of primers KanR-SBF and Tag1p using pTNR-KA-RE tag1 as the template.

Macrophage infection

To facilitate the observation of bacteria growing in macrophages, the plasmid pINT::P_{aphII}-*egfp* was electroporated into *R. equi* ATCC 33701 and transposon insertion mutants. Transformants were recovered on LB agar containing apramycin (80 µg/ml). The *R. equi* strains were cultured in BHI broth at 30°C with vigorous shaking for 48 hr. Macrophages (J774A.1) were infected with wild-type or *R. equi* transposon insertion mutants at a multiplicity of infection of 10 for 1 hr. Extracellular bacteria were removed from wells by washing three times with warm 1× PBS. Fresh Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 20 µg/ml amikacin was placed. After incubation for 2 and 24 hr in 5% CO_2 at 37°C, cells were washed three times with warm 1× PBS. The glass coverslips were mounted on glass slides by the Gold antifade reagent (Thermo Fisher Scientific). The monolayers of macrophages on glass coverslips were observed using a fluorescence microscope BX 51 (Olympus, Tokyo, Japan). The number of infected macrophages was determined by counting 200 macrophages over several fields on a glass coverslip. The percentages of macrophages containing one to nine bacteria, 10 or more bacteria per macrophage, and uninfected macrophages were calculated.

RESULTS

Verification of the efficiency of the STM strategy in the mouse infection model

To check if the negative screening method developed in this study actually detects the mutants defective in mouse infection, preliminary experiments were conducted. The pTNR-KA-RE tag1 was electroporated into a virulence plasmid-cured 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. The output DNA was prepared from bacteria collected from the liver of the mouse on day 5 post-infection. Real-time PCR analysis of output DNA showed that the ratio of tag1 to the other tags was very low in all three mice (Fig. 1). Furthermore, the ratio of the other tags was consistent among the three mice. These results suggested that the STM developed in this study is useful for detecting the *R. equi* strain defective in mouse infection.

Screening of signature-tagged transposon mutant library

To identify the 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 used to examine the survival in mice. The mutants whose relative abundance was dramatically 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 inoculated to a mouse again. The mutants that showed reduced ability to infect at least two independent screenings were finally selected as fitness defect mutants. According to these criteria, 102 of the 4,650 mutants were selected as fitness defect mutants.

Identification of the transposon insertion sites

To confirm the insertion of the transposon in 102 mutants, Southern blot analysis was conducted. Figure 2 shows a representative example of Southern blot analysis. Ninety-one *R. equi* mutants contained a single transposon insertion, whereas 11 mutants had more than two insertions. The plasposons were successively rescued in 73 of 91 mutants. The nucleotide sequence of the DNA flanking the site of the transposon insertion was used to search the GenBank database. The transposon insertion site was identified in 50 of 73 plasposons. Table 1 shows the results. The insertion of the transposon was seen on the virulence plasmid in 15 of the 50 mutants. Most of these mutants showed no or reduced expression level of VapA (Table 1). The remaining 35 mutants had the insertion of transposon on the chromosome. The genes were assigned to 1 of 14 functional categories: amino acid transport and metabolism; nucleotide biosynthesis and metabolism; fatty acid and phospholipid metabolism; biosynthesis of cofactors, prosthetic groups, and carriers; transcription, RNA processing, and degradation; DNA replication, recombination, modification, and repair; transcriptional regulator; transport of small molecules; cell wall, Lipopolysaccaride, capsule; membrane proteins; energy metabolism; non-ribosomal peptide synthesis; and putative enzyme. The mutants in which the transposon was located in the intergenic non-coding region were assigned to the category of hypothetical, unclassified, or unknown.

Growth defects of transposon insertion mutants in J774A.1 macrophages

The ability of *R. equi* to replicate within macrophages is critical in the survival of the host. Thus, the attenuated mutants selected in STM might show a survival defect in macrophages. To test this, murine macrophage-like cell

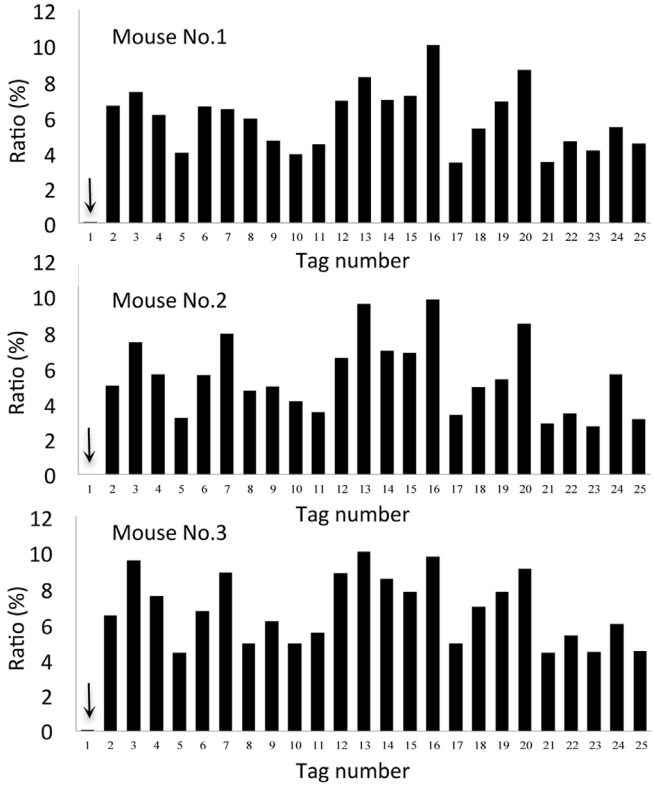

Fig. 1. Three mice were intravenously injected with an input pool containing transposon mutants (tag2–tag25) together with an avirulent *R. equi* strain marked with tag1. After 5 days of infection, the semiquantitative analysis of each mutant in an output pool was conducted via real-time PCR, and the ratio of each tag was indicated. An arrow indicates a decreased ratio of tag1.

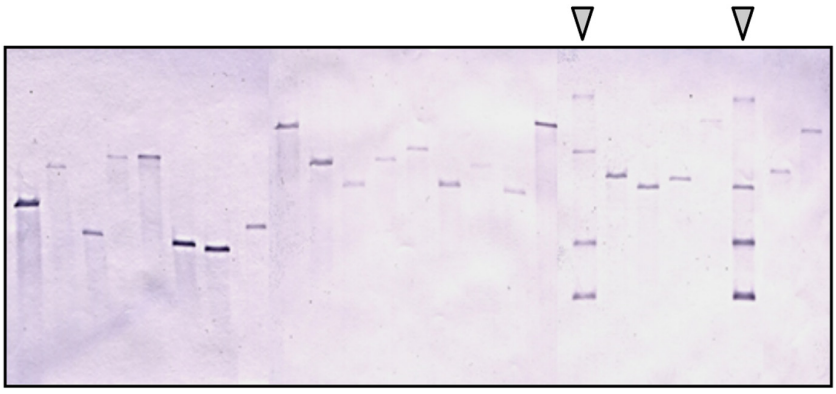

Fig. 2. Southern blot analysis of 25 randomly chosen transposon insertion mutants. *Xma*I- or *Apa*I-digested chromosomal DNA was electrophoresed and transferred on a nylon membrane. The DNA fragments containing the transposon were detected with the DIG-labeled kanamycin resistance gene. The multiple bands were detected on two lanes indicated by an arrowhead, and these mutants were eliminated from this study.

Table 1. Identification of the genes with transposons in the survival-defective mutants of *Rhodococcus equi*

Class	Mutant	Location	Known or putative function	VapA expression
Amino acid transport and metabolism	M95-15	REQ_29700	Gamma-glutamyl kinase-GP-reductase multienzyme complex ProA	Normal
Nucleotide biosynthesis and metabolism	M79-5	REQ_39910	Orotate phosphoribosyltransferase PyrE	Normal
Fatty acid and phospholipid metabolism	M77-6	REQ_25310	Acyl-CoA thioesterase II	Normal
Biosynthesis of cofactors, prosthetic groups, and carriers	M73-2	REQ_17830	Putative pyrazinamidase	Normal
Transcription, RNA processing and degradation	M121-1	REQ_05430	Putative DEAD/DEAH box helicase	Normal
DNA replication, recombination, modification, and repair	M134-15	REQ_00040	DNA replication and repair protein RecF	Normal
	M178-25	REQ_19710	Putative Rec X regulatory protein	Normal
Transcriptional regulator	M165-8	REQ_29340	Heat-inducible transcriptional repressor HrcA	Normal
	R118-3	REQ_26260	Putative MarR family transcriptional regulator	Reduced
	M79-17	REQ_06490	TetR family transcriptional regulator	Normal
Transport of small molecules	M165-11	REQ_25710	Putative MFS transporter	Normal
Cell wall, LPS, and capsule	M134-23	REQ_45210	Putative D-alanyl-D-alanine carboxypeptidase metabolism	Normal
Membrane proteins	M74-13	REQ_00210	Putative membrane protein	Normal
	M154-10	REQ_14130	Putative integral membrane protein	Normal
Energy metabolism	M77-23	REQ_32930	Putative nitrite reductase large subunit NirB1	Normal
Non-ribosomal peptide synthesis	M60-10	REQ_23810	Putative non-ribosomal peptide synthetase	Normal
	M70-16	REQ_23810	Putative non-ribosomal peptide synthetase	Normal
	M87-15	REQ_23810	Putative non-ribosomal peptide synthetase	Normal
	M122-3	REQ_23810	Putative non-ribosomal peptide synthetase	Normal
	M162-21	REQ_23810	Putative non-ribosomal peptide synthetase	Normal
	M158-13	REQ_23810	Putative non-ribosomal peptide synthetase	Normal
	M56-20	REQ_35940	Putative non-ribosomal peptide synthetase	Normal
Putative enzyme	M45-4	REQ_00880	Putative short chain dehydrogenase	Normal
	M43-12	REQ_28270	Putative carotenoid oxygenase	Normal
	M124-23	REQ_41020	Short chain dehydrogenase	Normal
	M151-1	REQ_15620	Short chain dehydrogenase	Normal
	M161-16	REQ_10430	Oxidoreductase	Normal
Hypothetical, unclassified, or unknown	M6-19	REQ_26170	Hypothetical protein	Normal
	M82-16	REQ_26170	Hypothetical protein	Normal
	M129-14	REQ_38700	Conserved hypothetical protein	Normal
	M130-19	REQ_38850	Conserved hypothetical protein	Normal
	M153-13*	REQ_27330/ REQ_ 27340	Putative HNH endonuclease/ putative Fis family transcriptional regulator	Normal
	M162-10*	REQ_12930/ REQ_12940	Hypothetical protein/ putative secreted esterase	Normal
	M168-10	REQ_23910	Hypothetical protein	Normal
	M182-17	REQ_38870	Hypothetical 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
	M162-10*		IcgA/VapH	Reduced
	M175-8		VirR	Reduced
	M112-17		VirS	No
	M114-17		VirS	No
	M120-9		VirS	No
	M57-20		Orf25	Normal
	M115-3		Putative conjugative transfer TraG/TraD family protein	Normal
	M168-5*		ResA/ParB	Reduced
	M169-21*		VapG/Orf3	Normal

*These mutants had an transposon inserted in the intergenic region between two genes indicaded.

lines J774A.1 were infected with the transposon mutants to determine their intracellular growth abilities. Only the strains with transposon insertions in genes on the chromosome were examined. At 24 hr post-infection, the proportion of macrophages containing more than 10 bacteria were significantly (P<0.01) lower in the mutants whose transposon was inserted in putative DEAD/DEAH box helicase (REQ_05430), oxidoreductase (REQ_10430), acyl-CoA thioesterase II (REQ_25310), hypothetical protein (REQ_26170), putative carotenoid oxygenase (REQ_28270), putative nitrite reductase large subunit NirB1 (REQ_32930), and the intergenic region between putative HNH endonuclease (REQ_27330) and putative Fis family transcriptional regulator (REQ_27340; Fig. 3). Other mutants did not show a noticeable decrease in intramacrophage proliferation.

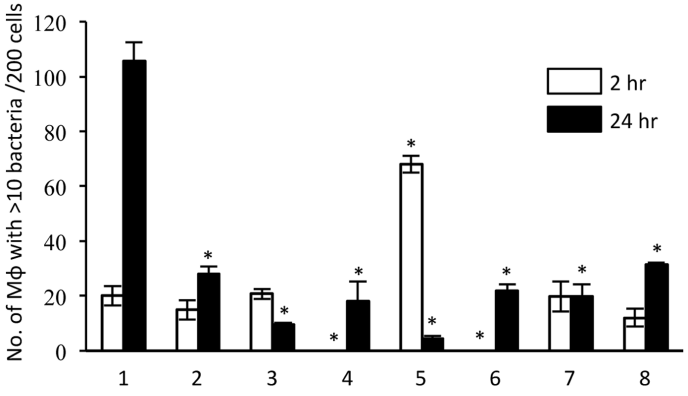

Fig. 3. Multiplication of transposon mutants in macrophage J774.1. The number of macrophages with more than 10 bacteria was indicated in 200 macrophages at 2 and 24 hr post-infection. 1, wild-type; 2, putative DEAD/DEAH box helicase (REQ_05430) mutant; 3, oxidoreductase (REQ_10430) mutant; 4, acyl-CoA thioesterase II (REQ_25310) mutant; 5, hypothetical protein (REQ_26170) mutant; 6, putative HNH endonuclease (REQ_27330)/ putative Fis family transcriptional regulator (REQ_27340) mutant; 7, putative carotenoid oxygenase (REQ_28270) mutant; 8, putative nitrite reductase large subunit NirB1 (REQ_32930) mutant. Data represent the means and standard deviations from three independent experiments and are analyzed by the two-tailed unpaired Student's *t* test (*P<0.01 vs. wild-type).

DISCUSSION

The STM is a powerful tool for identifying the novel virulence factor of microbial pathogens and has been applied to many bacterial infection models. In *R. equi*, several transposon-based mutagenesis events have been reported, but there is no research using STM yet [3]. In this study, a plasposon vector for STM was developed by introducing a tag sequence that can be identified by PCR into a pTNR-KA, which was used in *R. erythropolis* [37]. The vector was used to identify the gene involved in establishing infection with *R. equi* in a mouse infection model. Of the 4,560 transposon mutants, 102 mutants were selected as strains with a reduced ability to infect mice; finally, transposon insertion sites were determined in 50 mutants. Of these, 15 strains had the transposon inserted into a virulence plasmid, which confers the ability for *R. equi* to proliferate in macrophages. This shows the validity of the STM screening performed in this study. However, whereas multiple mutants having transposon in genes, such as icgA and virS were selected, no strain in which the transposon was inserted into *vapA*, which is a central virulence determinant of *R. equi*, was selected, suggesting that the transposon used in this study has a bias in the insertion site. Alternatively, considering the genome size (5.0 Mb) [21], insufficient library size was considered to be the reason why the *vapA* mutant strain could not be obtained.

VapA was released from bacteria in the vacuole of macrophages, and an intraphagosomal environment that allows bacterial growth is created by the exclusion of the host's proton pump from the phagosome membrane and by proton permeabilization [54]. The VapA expression level of the mutants in which a transposon was inserted into a virulence plasmid was examined, and VapA expression was abolished in three of the 15 strains (Table 1). In these strains, the transposon was inserted into a *virS* gene. This is consistent with the result of a previous study in which a significant decrease in the transcriptional activity of the *vapA* promoter was observed in the deletion mutant of *virS* [15]. In addition, the obvious decrease in VapA expression was observed in nine of the remaining 12 strains. These mutants have the transposon within virR, which is another transcriptional regulator involved in the transcriptional regulation of virS, and icgA, which is located upstream of virS and polycistronically transcribed [7, 35]. The reduction of VapA expression in icgA mutants was likely to have resulted from the reduced level of VirS caused by a polar effect. The efficient selection of these mutants by our screening, although they express VapA, suggests that amount of VapA expressed is crucially important in mouse infection. Bargen *et al.* also reported that a lower expression level of VapA did not rescue the vapA mutant with respect to growth in the macrophages [53].

A *pyrE* gene encoding an orotate phosphoribosyltransferase was identified as *in vivo* fitness gene of *R. equi*; PyrE is an enzyme that works in the fifth stage of the pyrimidine biosynthesis pathway and catalyzes the production of orotidine 5′-monophosphate from orotic acid and phosphoribosyl pyrophosphate [5]. The deletion of *pyrE* results in pyrimidine auxotroph in *Listeria monocytogenes* and *Salmonella* Typhimurium [16, 58]. The transcription of *pyrE* in *L. monocytogenes* has been reported to increase at the onset of macrophage infection, suggesting a low pyrimidine concentration in the phagosome. In *S.* Typhimurium, strains showing purine or pyrimidine auxotroph could not survive in macrophages and were attenuated in mice.

REQ_28270 encodes a putative carotenoid oxygenase. The ortholog of *Mycobacterium tuberculosis* (MtCCO) has been shown to convert some carotenoids and apocarotenoids *in vitro*, suggesting that *M. tuberculosis* may utilize carotenoids to interfere with the host's retinoid metabolism [41]. Low vitamin A levels in the circulation have been epidemiologically shown to be associated with increased susceptibility to *Mycobacterium* infection [1]. Vitamin A derivatives were involved in the antibacterial activity of macrophages *in vitro* [2, 8, 9, 57]. In addition, *Leishmania donovani* infection was shown to reduce the transcription of the gene encoding retinoic acid synthase in J774.1 macrophages [51]. Because the REQ_28270 mutant showed reduced growth in J774.1, this gene product might enhance the ability to survive within macrophages by interfering with retinoid metabolism in macrophages.

The pyrazinamidase (REQ_17830) plays a role in nicotinamide adenine dinucleotide (NAD^+) biosynthesis. NAD^+ is a central molecule in cellular metabolism and is implicated in hundreds of biological reactions. Organisms synthesize NAD^+ using the *de novo* and salvage pathway. The pyrazinamidase is involved in the salvage pathway and converts nicotinamide to nicotinate. In the *de novo* NAD^+ biosynthesis pathway, NadA, NadB, and NadC synthesize nicotinic acid mononucleotide from aspartate to give NAD^+ as a final product. The deletion of the first two genes involved in the *de novo* biosynthesis pathway does not affect the *in vivo* growth in *M. tuberculosis*, suggesting that *M. tuberculosis* can obtain NAD^+ required for growth using the salvage pathway in the host [52]. As the *REQ_17830* mutant showed multiplication comparable with that of the wild-type strain in macrophages, it is believed that the mutant in vacuole obtains NAD^+ via the *de novo* synthesis system. However, the reduced survival of this mutant during mouse infection may result from the competition with other mutants, showing that the lack of the salvage pathway gives it enough disadvantage to lose the competition in survival *in vivo*.

REQ_32930 encodes a putative nitrite reductase large subunit (NirB1). NirB1 is part of the nitrite reductase complex NirBD that catalyzes nitrite reduction to ammonium in nitrate assimilation [24]. *R. equi* also has narGHJI-encoded nitrate reductase, and the *narG* mutant of *R. equi* was reported to be completely attenuated in mice [31]. In this study, the *nirB1* mutant showed a reduced ability to multiply in macrophage and survive in mice. This result suggested that *R. equi* might utilize nitrate and nitrite as a sole source of nitrogen by their assimilation during infection.

Non-ribosomal peptide synthetases (NRPSs) are biosynthetic enzymes that generate secondary peptide metabolites with a wide range of bioactivities, such as antibiotics, siderophores, immunosuppressors, and surfactants [28, 43]. The *R. equi* 103S strain has 11 NRPS genes on the genome [18]. Three NRPSs (REQ_08160, REQ_23810, and REQ_07630) are involved in the synthesis of the siderophores [25–27]. IupS (REQ_08160) and IupU (REQ_23810) are responsible for the synthesis of a diffusible catecholate-containing siderophore (rhequibactin) and a nonsoluble siderophore, respectively [26]. The RhbC (REQ_07630) is involved in the synthesis of a hydroxamate siderophore (rhequichelin) [37]. The deletion mutant of *rhbCD* cannot grow at low concentrations of free iron and showed reduced ability to multiply in the macrophages and survive in mice [27]. The disruption of *iupS* and *iupU* prevents growth under low-iron conditions but is not attenuated in a mouse infection model [26]. In our *in vivo* screening, six independent transposon mutants of *iupU* were selected. As an explanation for the discrepancy, this study might find the defect that could not be detected by a single infection because of competition with other mutants.

The *REQ_05430* mutant could not grow as much as the wild-type strain in the vacuole of macrophages and was also attenuated in mice. This gene encodes a putative DEAD/DEAH-box RNA helicase. RNA helicases of these families in bacteria are involved in ribosome biogenesis, RNA turnover, and translation initiation [33]. REQ_05430 could be a DEAH-box RNA helicase, as it contains the D-E-C-H at the N terminal that corresponds to motif II (DEXH) of this family [33]. In *E. coli*, the mRNA of the *daaA-E* operon encoding a fimbrial adhesin is cleaved by a DEAH-box RNA helicase, HrpA [17, 19, 20]. Splitting the *daaA-E* transcript in two results in the degradation of the upstream fragment containing the genes encoding the accessory proteins during the biogenesis of fimbriae, whereas the downstream fragment containing the gene of the structural protein of the fimbriae remains stable, which provides a suitable stoichiometry of different fimbrial proteins. In the spirochete *Borrelia burgdorferi*, the causative agent of the tick-borne Lyme disease, the *hrpA* mutant was not transmitted to mice via tick bite and could not infect mice even by needle inoculation [38, 39]. At least 180 proteins were expressed at the different levels in the *hrpA* mutant relative to the wild-type strain, although there were no dramatic changes in the mRNA levels in microarray profiling, suggesting that the differences in the protein expression levels occurred at the post-transcriptional level. The *R. equi REQ_05430* mutant expressed an amount of VapA protein comparable with that of the wild-type strain. Therefore, the *REQ_05430* product may be involved in the post-transcriptional regulation of the other genes required for *R. equi* to proliferate in the vacuole of macrophages.

In summary, STM was used to determine the genes in *R. equi* that contribute to fitness in a mouse model of systemic infection. As an intracellular parasite, *R. equi* survives by adapting to the harsh environment in the vacuole of macrophages. The fitness gene unraveled in this study is believed to be a clue for revealing the environment that *R. equi* in the vacuole encounters. Further characterization of these fitness genes is necessary to elucidate the infection mechanism of *R. equi*.

CONFLICT OF INTEREST. The authors have nothing to disclose.

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