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NOTE

Bacteriology

Rescue of an intracellular avirulent *Rhodococcus equi* replication defect by the extracellular addition of virulence-associated protein A

Nuttapone SANGKANJANAVANICH[1)], Masanori KAWAI[1)], Tsutomu KAKUDA[1)]* and Shinji TAKAI[1)]

[1)]Laboratory of Animal Hygiene, School of Veterinary Medicine, Kitasato University, Higashi 23-35-1, Towada, Aomori 034-8628, Japan

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ABSTRACT. *Rhodococcus equi* is a facultative intracellular bacterium that can escape from bactericidal mechanisms associated with phagocytosis. Virulence-associated protein A (VapA), encoded on a virulence-associated plasmid, is essential for intracellular survival in macrophages, but its function is not known. Here, we show that the extracellular addition of recombinant glutathione S-transferase (GST)-VapA fusion protein rescued the intracellular replication defect of a mutant lacking the *vapA* gene. Furthermore, the virulence-plasmid-cured strain could also 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*.

KEY WORDS: intracellular growth, *Rhodococcus equi*, VapA

Rhodococcus equi is a Gram-positive coccobacillus that can cause suppurative pneumonia in foals less than 6 months of age and in immuno-compromised humans, especially individuals with AIDS [11, 14].

R. equi is a facultative intracellular pathogen that can replicate in compartments known as *R. equi*-containing vacuoles (RCVs) within infected macrophages [4]. The ability of *R. equi* to survive and multiply inside macrophages is regulated by a virulence-associated plasmid [5]. The plasmid-cured *R. equi* strains are unable to survive in macrophages and are avirulent in a foal or murine infection model [3, 4].

Virulent strains of *R. equi* express a 15–17 kDa bacterial surface protein named virulence-associated protein A (VapA), which is encoded by the virulence-associated plasmid [15]. VapA is an essential virulence determinant and plasmid-cured derivatives expressing wild-type levels of VapA could not survive in macrophages and remained avirulent in both mice and foals [5]. Besides VapA, a recent study revealed that two transcriptional regulators encoded on the virulence-associated plasmid, VirR and VirS, were required and were sufficient for intracellular growth [3].

Phagosomes containing virulent *R. equi* pass normally thorough the early phase of phagosome maturation but are arrested in between the early and late maturation stage [4]. The resulting RCVs possess some late endocytic markers, such as Rab7, BMP, LAMP 1 and 2, but acquire neither cathepsin D nor v-ATPase. Recently, it was reported that VapA was taken into endocytic compartments 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 Lab7 [10]. Here, we show that the extracellular addition of VapA can exert an influence on the growth of intraphagocytic *R. equi*.

R. equi strain ATCC33701, originally isolated from a pneumonic foal, was used in this study [13]. The isogenic but avirulent *R. equi* strain ATCC33701P^{-} is a derivative in which the virulence-associated plasmid has been cured. TKR255 is an *R. equi* ATCC33701 derivative in which *lacZ* replaces *vapA* [9]. These strains were routinely grown in brain–heart infusion (BHI) broth with vigorous shaking at 30°C. *Escherichia 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 / 15% glycerol (vol/vol).

The plasmid pGEX-4T-1 (GE Healthcare Life Sciences, Piscataway, NJ, U.S.A.) was used for the expression of *R. equi* VapA as a GST fusion protein. The *vapA* gene was amplified by PCR with the primers vapA-BamF (GGATCCACCGTTCTTGATTCCGGTAG) and vapA-MfeR (CAATTGCGCAGCCTGCATGTTTCTGG). The amplified gene

*Correspondence to: Kakuda, T.: kakuda@vmas.kitasato-u.ac.jp

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fragment (618 bp) was digested with *Bam*HI and *Mfe*R and then ligated into *Bam*HI and *Eco*RI double-digested pGEX-4T-1 to create pGEX-4T-1::*vapA*. Then, BL21 cells were transformed with pGEX-4T-1::*vapA*. Bacteria were grown in LB broth with ampicillin (50 μg/m*l*) at 37°C. The expression of GST-VapA fusion protein was induced by the addition of 1 mM final concentration of IPTG for 5 hr at 30°C. Bacteria were harvested and then disrupted by sonication. The GST-VapA fusion protein was purified using Glutathione Sepharose 4B (GE Healthcare Life Sciences, Piscataway, NJ, U.S.A.) from the soluble fraction obtained after centrifugation of the bacterial lysate according to the manufacturer's instructions.

The *aphII* promoter (P_{aphII}) region was amplified using the primers Pkan2-termF (GGATCCGCACCGGCC CCGGAGGACCACCGCGTCCTCCGG GGCCGATTCAGCTTCACGCTGCCGCAAGC) and Pkan2-nTR (ACTAGTCAA TTGGATATCCATATGAAACGATCCTCATCCTG). The PCR product (421 bp) was digested with *Bam*HI and *Spe*I and cloned into the *Streptomyces* φC31 integrase-based integration vector pINT [6, 9] digested with *Bam*HI and *Spe*I to create pINT::P_{aphII}. The *egfp* ORF was amplified using the primers egfp-F (CATATGGTGAGCAAGGGCGAGGA) and egfp-R (CAATTGTTACTTGTACAG CTCGTCCATGC). The PCR product (729 bp) was digested with *Nde*I and *Mfe*I and cloned into pINT::P_{aphII} digested with *Nde*I and *Mfe*I to create pINT::P_{aphII}-*egfp*. pINT::P_{aphII}-*egfp* was electroporated into *R. equi* ATCC33701, ATCC33701P$^-$, TKR255 and *E. coli* DH5α. Transformants were recovered on LB agar containing 60 μg/m*l* apramycin.

DNA amplification was carried out using *Taq* DNA polymerase according to the manufacture's instructions (Greiner Bio-One International GmbH, Kremsmünster, Austria). The PCR conditions used routinely were (step1) 94°C, 2 min; (step2, 30 cycles) 94°C, 30 sec; 55°C, 1 min; 72°C, 1 min; (step3) 72°C, 4 min.

J774A.1 murine macrophage-like cell line was obtained from the RIKEN Bioresource Center (RIKEN BRC, Ibaraki, Japan). J774A.1 cells were seeded into 24-well culture dishes (Greiner Bio-One International GmbH, Kremsmünster, Austria) containing sterile glass cover slips at 1.5 $\times 10^5$ cells per well and were grown overnight in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific K.K., Kanagawa, Japan) containing 10% fetal calf serum in a humidified 5% CO_2 atmosphere at 37°C. Cells were infected with bacteria at MOI 10. After incubating the cells at 37°C for 1 hr, the cells were washed three times with PBS to remove extracellular bacteria. Then, 1 m*l* of medium containing amikacin at 20 μg/m*l* and the recombinant protein at 100 μg/m*l* was added and the cells were incubated for the indicated period in 5% CO_2 at 37°C. The cells were then washed three times with PBS and observed using a fluorescence microscope BX51 (Olympus, Tokyo, Japan).

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 [7, 16]. Therefore, we constructed an EGFP-expressing strain of *R. equi* to observe the intracellular growth of *R. equi* by fluorescent microscopy without the need for staining.

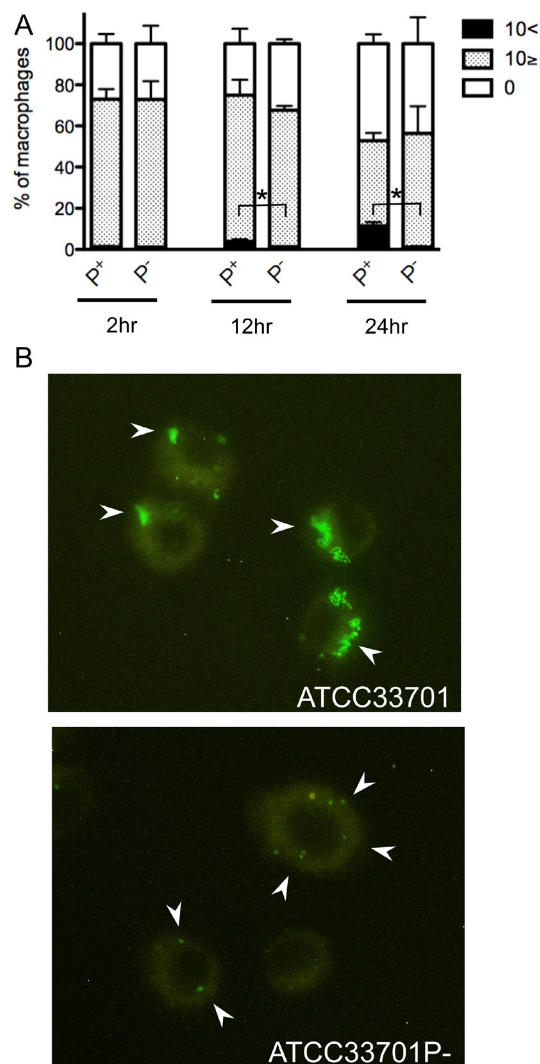

Fig. 1. Intramacrophage growth of EGFP-expressing *R. equi*. J774A.1 macrophages were infected with *R. equi* ATCC33701 and P$^-$ strains. A: The number of macrophages with 0, ≤10, >10 bacteria was recorded at various times throughout the infection. The data are expressed as the percentage of macrophages 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 of data). The significance of differences in frequencies of *R.equi*-infected macrophages was calculated using the Chi-square tests (*, $P<0.01$). B: Macrophages were observed by fluorescence microscopy at 24 hr post-infection. *R. equi* cells are indicated by the arrowheads.

Macrophage J774A.1 cells were infected with strain ATCC33701 and the ATCC33701P$^-$ strain expressing EGFP and were observed by fluorescence microscopy by 24 hr post-infection. The macrophages were observed by fluorescence microscopy at different time points and the number of macrophages containing no bacteria, 10 or less and more than 10 bacteria, was counted. The percentage of macrophages containing more than 10 bacteria increased in strain ATCC33701 strain over time, but no such increase was observed in strain ATCC33701P$^-$ (Fig. 1A). At 24 hr post-infection, a number of macrophages containing the bacterial clusters were observed when cells were infected with strain ATCC33701 (Fig. 1B). By contrast, no growth in clusters was observed in macrophages infected with ATCC33701P$^-$ although a few EGFP-expressing bacteria were still observed in many of the macrophages.

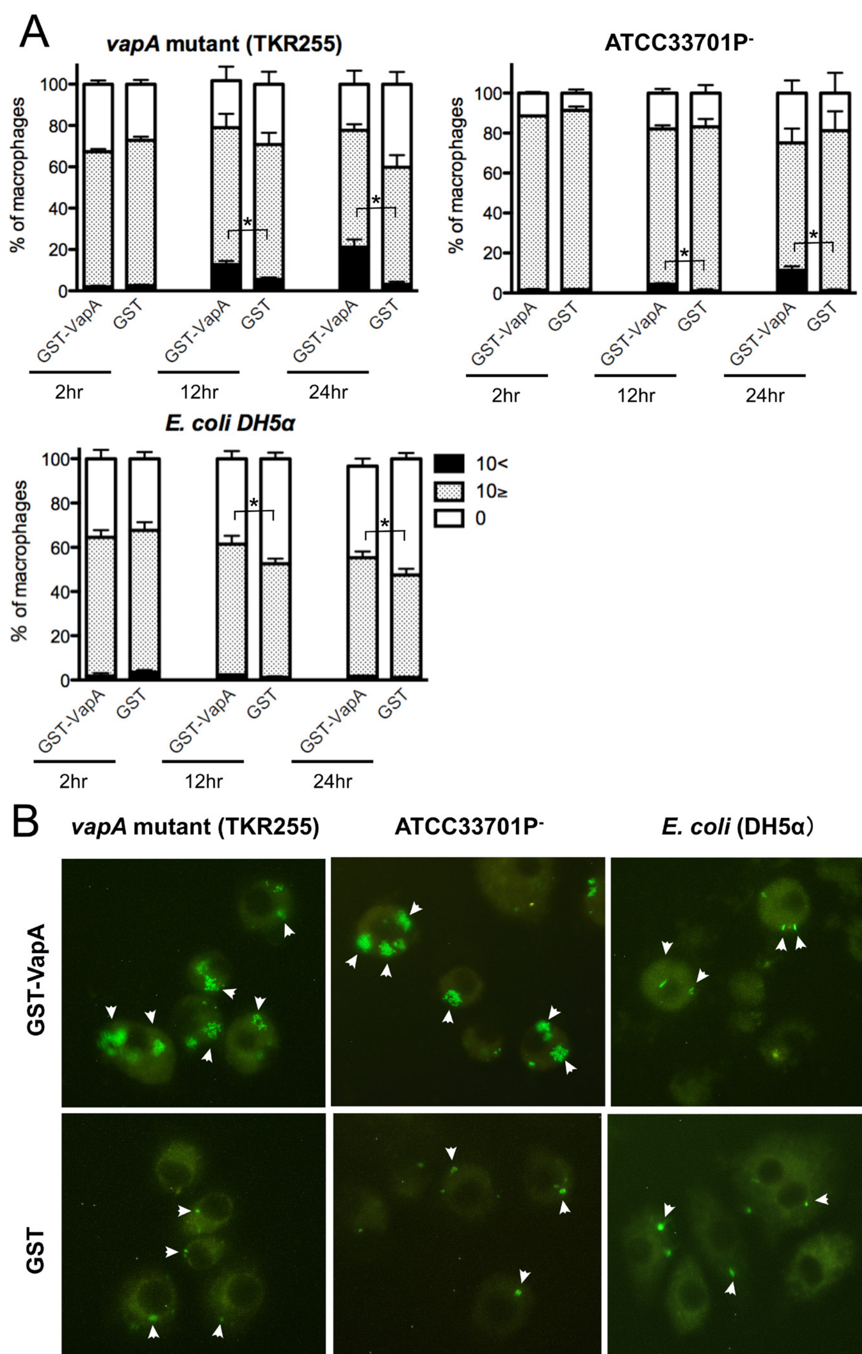

Fig. 2. Influence of the extracellular addition of VapA on intramacrophage growth of EGFP-expressing *R. equi* strains. J774A.1 macrophages were infected with the *R. equi vapA* mutant (TKR255), the P⁻ strain and *E. coli* DH5α in the presence of GST-VapA or GST. A: The number of macrophages with 0, ≤10, >10 bacteria was recorded at various times throughout the 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 of data). The significance of differences in frequencies of *R.equi*-infected macrophages was calculated using the Chi-square tests (*, $P<0.01$). B: Macrophages were observed by fluorescence microscopy at 24 hr post-infection. *R. equi* or *E. coli* cells are indicated by the arrowheads.

It was reported that the *R. equi* strain lacking the *vapA* gene exhibited a growth defect in macrophages [8, 16]. To examine whether the extracellular addition of VapA rescued the growth defect of the *vapA* mutant in macrophages, J774A.1 macrophages were infected with the *vapA* mutant and were observed at 24 hr post-infection. Little growth of the *vapA* mutant was observed in accordance with previously published data [8, 16] (Data not shown). When infected macrophages were co-cultured with GST-VapA, the number of macrophages containing more than 10 bacteria increased and bacterial growth was observed at 24 hr post-infection (Fig. 2A and 2B). By contrast, the addition of GST alone did not result in growth of the *vapA* mutant.

It has been reported that a plasmid-cured strain expressing VapA at the same level as virulent *R. equi* failed to grow in macrophages [5]. To confirm whether the same result would be obtained following the extracellular addition of VapA, macrophages were infected with strain ATCC33701P⁻ in the presence of VapA. Contrary to expectations, the percentage of macrophages containing more than 10 bacteria increased over time and strain ATCC33701P⁻ multiplied in clusters at 24 hr post-infection (Fig. 2A and 2B).

These results suggested that the *vapA* mutant and plasmid-cured strain multiplied in vacuoles whose antibacterial activities were reduced by the addition of VapA. To examine whether addition of VapA allowed the growth of bacteria that cannot normally multiply in macrophages, an *E. coli* strain DH5α was employed. No obvious growth of *E. coli* was observed by 24 hr post-infection although the frequencies of macrophages containing 10 or less than 10 bacteria were significantly higher in the presence of GST-VapA than of GST at 12 and 24 hr post-infection (Fig. 2A and 2B). This suggested that the vacuoles containing *E. coli* were still inhospitable for the growth of *E. coli* despite the addition of VapA to the culture medium.

The present study demonstrated that the extracellular addition of VapA rescued the intracellular growth defect of the plasmid-cured strain. Rofe *et al*. 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 Lab7, suggesting the loss of functional endolysosomes [10]. These features are similar to those found in RCVs [4]. Our findings also demonstrated that *E. coli* could not multiply in 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 might possess the ability to overcome some of the VapA-mediated growth limitations in phagosomes.

Giguère *et al*. demonstrated that a plasmid-cured derivative expressing wild-type levels of VapA could not replicate in murine macrophages [5]. Coulson *et al*. revealed that expression of VapA from a constitutive promoter failed to restore the intracellular growth defect of the strain lacking the pathogenicity island locus containing the *vapA* gene [2]. 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*, besides *vapA* were required for intracellular survival of *R. equi* [3]. It has been proposed that these transcriptional regulators confer enhanced fitness of the intracellular environments for *R. equi* through the transcriptional regulation of many genes encoded on the chromosome. The present data revealed that a plasmid-cured strain that did not possess *virS* and *virR* could multiply in macrophages in the presence of VapA similarly to 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 [12]. Also, VapA is not secreted into the extracellular space and is therefore restricted on the surface of bacteria at least during *in vitro* growth [1]. Therefore, differences in the quantity, timing of expression and localization of VapA may be responsible for this discrepancy.

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