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# Identification of a gene in *Riemerella anatipestifer* CH-1 (B739-2187) that contributes to resistance to polymyxin B and evaluation of its mutant as a live attenuated vaccine

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ABSTRACT

*Riemerella anatipestifer* (*R. anatipestifer*) causes severe perihepatitis, pericarditis, airsacculitis and meningitis in the duck, leading to great economic losses worldwide. Given the increased prevalence of drug-resistance strains, vaccination is the best strategy to prevent *R. anatipestifer* infection in ducklings. In this study, we identified a gene in *R. anatipestifer* (B739-2187) that can restore the resistance of the *Salmonella phoP* mutant to polymyxin B using genetic complementation. Furthermore, the deletion of B739-2187 in *R. anatipestifer* resulted in a mutant exhibiting increased sensitivity to polymyxin B. The *R. anatipestifer* B739-2187 mutant did not exhibit phenotypic defects, as indicated by its growth curve, lipopolysaccharide and outer membrane protein profiles, and attachment and invasion of duck embryo fibroblast cells. The duck animal experiments demonstrated that the deletion of B739-2187 significantly decreased the virulence of *R. anatipestifer*, and the B739-2187 mutant provided 100% protection against challenge with wild-type *R. anatipestifer*, exhibiting the characteristics of an ideal live vaccine.



## 1. Introduction

Infectious serositis is caused by *Riemerella anatipestifer* (*R. anatipestifer*) in ducks and is a major disease that leads to great economic losses in the farmed duck industry worldwide, especially in China and South-Eastern Asia [1,2]. The clinical symptoms of this disease include perihepatitis, pericarditis, airsacculitis and meningitis [1,3]. Antibiotics have been widely used to prevent and control *R. anatipestifer* infection on duck farms, but the increasing prevalence of drug-resistant strains is posing serious challenge to antibiotic treatment [4–6]. Therefore, the vaccination of ducklings provides an optimal option to prevent ducklings from being infected with *R. anatipestifer*. Several inactivated vaccines have been developed for current clinical use, but these vaccines display poor cross-protection among the 21 serotypes [7,8]. Because attenuated live vaccines can induce long-term immunity and confer good protection, these vaccines would be advantageous over vaccines based on the subunit or dead bacteria [9–11].

Several virulence factors, including outer membrane protein A (OmpA) [12], CAMP cohemolysin [13], VapD [14] and the putative genes associated with lipopolysaccharide (LPS) synthesis, have been identified [15,16], and some of these virulence factors were used to develop subunit or live attenuated vaccine candidates [15,16]. Global regulator genes may be preferable for live vaccine

*Abbreviations: R. anatipestifer, Riemerella anatipestifer; E. coli, Escherichia coli; S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; TSB, tryptic soybean broth; LB, Luria–Bertani broth; DAP, Diaminopimelic acid; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; $LD_{50}$, 50% lethal dose; ELISA, Enzyme-linked immunosorbent assay; AP, alkaline phosphatase; i.m., intramuscularly; LPS, lipopolysaccharide; OMP, outer membrane protein; CFU, colony-forming unit.

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development over these single virulence factors because global regulators mediate and control a cascade of genes that are essential for bacterial virulence, and their mutation in vaccine development could improve the safety and immunogenicity of vaccines compared with single virulent gene-based vaccines [17,18]. For example, the PhoP/Q global regulator is a vital two-component system in bacterial invasion and survival in the host. This regulator can positively and negatively regulate more than 40 proteins expression in *Salmonella* and other enteric bacteria [19]. PhoQ is a membrane-bound kinase that senses environmental signals, such as low $Mg^{2+}$ levels, low pH levels or sub-lethal antimicrobial peptides, and delivers phosphate to PhoP to activate or repress genes [20]. The *phoPQ* mutant of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) has been shown to be significantly attenuated in BALB/c mice and sensitive to antimicrobial cationic peptides, such as polymyxin B [21], while remaining capable of inducing strong systematic and mucosal immune responses in these mice [22].

Although the detailed infection and survival mechanism of *R. anatipestifer* in the host is not clear, *R. anatipestifer* has been shown to infect ducks and other avian hosts via the respiratory tract, digestive tract or cuts on the feet, leading to systemic distribution in the duck host [2]. During infection, *R. anatipestifer* encounters a wide range of adverse environments, including dramatic shifts in nutrient abundance and pH as well as innate immune molecules, such as reactive oxygen, antimicrobial peptides and peptides found in immune cells. *R. anatipestifer* also encounters low metal ions concentrations, such as $Fe^{2+}$, $Mg^{2+}$, and $Mn^{2+}$, in the host during infection, and the bacteria must acquire these essential ions from their hosts for their own use via specific mechanisms [23]. Methods to overcome these limitations are essential for establishing an *R. anatipestifer* infection in the host. *R. anatipestifer* may exploit a mechanism similar to that of the two-component system in *Salmonella* to sense exterior signals. Accordingly, alkaline and acid phosphatase activities were observed in strain ATCC11845 [24], highlighting the possibility of a putative PhoPQ two-component system in *R. anatipestifer*.

*R. anatipestifer* CH-1 was isolated from the brains of sick ducks raised in Chengdu, Sichuan province, China and is a highly virulent strain classified as serotype 1; the most common serotypes in clinical samples have been classified into the five categories [25]. The genome of *R. anatipestifer* CH-1 is approximately 140 kb larger than that of the three other *R. anatipestifer* strains and carries 16 unique gene families [25]. Because of the complexity of its genome, its high virulence, its clinical prevalence and its close relationship with other strains according to an evolutionary analysis, *R. anatipestifer* CH-1 was selected to investigate the putative two-component system in this study.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, media and growth conditions

The bacterial strains, plasmids and their related characteristics are described in Table 1. The *R. anatipestifer* strains were cultured in tryptic soybean broth (TSB) or agar plates (Difco Laboratories, USA) at 37 °C. *S.* Typhimurium and *Escherichia coli* were grown in the Luria–Bertani broth (LB) or on agar plates (Difco Laboratories). Diaminopimelic acid (DAP) was added to the medium at a concentration of 50 μg/ml for the growth of Δ*asd* strains [26]. When needed, appropriate antibiotics were added to the broth at the following concentrations: chloramphenicol (Cm, 25 μg/ml), ampicillin (Amp, 100 μg/ml), spectinomycin (Spec, 50 μg/ml), and kanamycin (Kan, 50 μg/ml).

### 2.2. Plasmid construction

The primers used in this study are listed in Table 2. Three putative *phoP* genes, B739-0744 (*phoP1*), B739-2187 (*phoP2*) and B739-0215 (*phoP3*) in the genome of *R. anatipestifer* CH-1 were amplified with the primers *phoP*-1-F/*phoP*-1-R, *phoP*-2-F/*phoP*-2-R or *phoP*-3-F/*phoP*-3-R, respectively, and the purified PCR products were cut using the NcoI and HindIII enzymes and ligated into pQK664 plasmid derived from pYA3337 [27] and digested by the same enzymes. Then, three plasmids, pQK012, pQK013, and pQK014, carrying *phoP1*, *phoP2* and *phoP3*, respectively, were created.

The suicide plasmid used to create the *R. anatipestifer* CH-1 ΔB739-2187 mutant was based on T-vector pYA4278 [28], a derivative of pRE112 [29]. Briefly, the sequences 412 bp upstream and 399 bp downstream of B739-2187 were amplified by PCR using the primer pairs D*phoP*-1F/1R and D*phoP*-2F/2R, which contained SbfI and NotI sites. The two fragments were purified from an agarose gel and used as templates at a 1:1 M ratio for joining via crossover PCR with the primers D*phoP*-1F and D*phoP*-2R. The PCR product was then ligated into pYA4278 to yield an intermediate plasmid in χ7232 [30]. A spectinomycin resistance ($Spec^R$) cassette (1185 bp) amplified from plasmid pYES7 (Life Technology, USA) by PCR using the primers spec-F and spec-R was digested with the NotI and SbfI enzymes and ligated into the intermediate plasmid to yield plasmid pQK016, which was used to delete B739-2187.

### 2.3. Peptide susceptibility assays

Peptide susceptibility was assessed by modifying a previously described approach [31]. The plasmids pQK012, pQK013 and pQK014 were transformed into S412 (*S.* Typhimurium Δ*asd* Δ) to identify the protein(s) that can restore the resistance to the polymyxin B (Sigma, St. Louis, USA). Briefly, bacteria were statically grown in N-minimal medium containing 10 mM $MgCl_2$ at pH 7.4 overnight at 37 °C. On the second day, the cells were diluted 1:100 in fresh N-minimal medium and incubated for 4 h-5 h at 37 °C with aeration to a final $OD_{600}$ of 0.8. Next, the bacterial cell were harvested and washed three times with N-minimal medium containing 10 mM $MgCl_2$ at pH 7.4. Polymyxin B was dissolved in autoclaved distilled water at a concentration of 5 μg/ml. The bacteria were diluted to $10^5$–$10^6$ CFU/ml, and 90 μl of bacterial suspension was then mixed with 10 μl of polymyxin B solution to a final polymyxin B concentration of 0.5 μg/ml. After a 1 h incubation at 37 °C with aeration, the bacterial cells were diluted with N-minimal medium, and the appropriate diluted suspension was plated on an LB plate to determine the number of colony-forming units (CFU). A final polymyxin B concentration of 2 μg/ml was used to test the sensitivity of *R. anatipestifer* CH-1 and its ΔB739-2187 mutant to polymyxin B. The relative survival ratio of each strain was calculated by dividing the mean CFU of the polymyxin B-treated group by the mean CFU of the untreated group.

### 2.4. Construction of R. anatipestifer CH-1 ΔB739-2187 mutant

The *R. anatipestifer* CH-1 ΔB739-2187 mutant, named K10, was constructed via allelic exchange using the suicide plasmid pQK016. The donor strain χ [30] was transformed with pQK016 and grown overnight in LB medium supplemented with 50 μg/ml DAP, 50 μg/ml Spec and 15 μg/ml Cm. The recipient strain *Riemerella anatipestife* CH-1 was cultured in TSB medium to an $OD_{600}$ of 0.4–0.5. One milliliter of donor strain and 3 ml of receptor strain were centrifuged at 3000 rpm for 5 min, and the pellet was resuspended in 1 ml TSB. The mixed cultures were then incubated on a TSB plate supplemented with 50 μg/ml DAP at 37 °C for over 24 h, facilitating