A *Riemerella anatipestifer* Metallophosphoesterase That Displays Phosphatase Activity and Is Associated with Virulence

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ABSTRACT *Riemerella anatipestifer* is an important pathogen of waterfowl, causing septicemic and exudative diseases. In our previous study, we demonstrated that bacterial virulence and secretion proteins of the type IX secretion system (T9SS) mutant strains Yb2Δ*gldK* and Yb2Δ*gldM* were significantly reduced, in comparison to those of wild-type strain Yb2. In this study, the T9SS secretion protein AS87_RS00980, which is absent from the secretion proteins of Yb2Δ*gldK* and Yb2Δ*gldM*, was investigated by construction of gene mutation and complementation strains. The virulence assessment showed >1,000-fold attenuated virulence and significantly reduced bacterial loads in the blood of ducks infected with Yb2Δ*00980*, the AS87_RS00980 gene deletion mutant strain. Bacterial virulence was recovered in complementation strain cYb2Δ*00980*. Further study indicated that the T9SS secretion protein AS87_RS00980 is a metallophosphoesterase (MPPE), which displayed phosphatase activity and was cytomembrane localized. Moreover, the optimal reactive pH and temperature were determined to be 7.0 and 60°C, respectively, and the K_m and V_{max} were determined to be 3.53 mM and 198.1 U/mg. The rMPPE activity was activated by Zn^{2+} and Cu^{2+} but inhibited by Fe^{3+} , Fe^{2+} , and EDTA. There are five conserved sites, namely, N267, H268, H351, H389, and H391, in the metallophosphatase domain. Mutant proteins Y267-rMPPE and Y268-rMPPE retained 29.30% and 19.81% relative activity, respectively, and mutant proteins Y351-rMPPE, Y389-rMPPE, and Y391-rMPPE lost almost all MPPE activity. Taken together, these results indicate that the *R. anatipestifer* AS87_RS00980 gene encodes an MPPE that is a secretion protein of T9SS that plays an important role in bacterial virulence.

IMPORTANCE *Riemerella anatipestifer* T9SS was recently discovered to be associated with bacterial gliding motility and secretion of virulence factors. Several T9SS genes have been identified, but no effector has been reported in *R. anatipestifer* to date. In this study, we identified the T9SS secretion protein AS87_RS00980 as an MPPE that displays phosphatase activity and is associated with bacterial virulence. The enzymatic activity of the rMPPE was determined, and the K_m and V_{max} were 3.53 mM and 198.1 U/mg, respectively. Five conserved sites were also identified. The AS87_RS00980 gene deletion mutant strain was attenuated >1,000-fold, indicating that MPPE is an important virulence factor. In summary, we identified that the *R. anatipestifer* AS87_RS00980 gene encodes an important T9SS effector, MPPE, which plays an important role in bacterial virulence.

KEYWORDS *Riemerella anatipestifer*, T9SS, secretion protein, virulence, MPPE

R *iemerella anatipestifer* is a Gram-negative, non-spore-forming, rod-shaped bacterium (1). *R. anatipestifer* is reported worldwide as the cause of epizootic infectious

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polyserositis of domestic ducks; it is also pathogenic for turkeys and has been isolated from chickens, pheasants, and waterfowl (2). It belongs to the family *Flavobacteriaceae* in rRNA superfamily V, based on 16S rRNA gene sequence analyses (3). Infected ducks show clinical signs of lethargy, diarrhea, and respiratory and nervous symptoms, all of which cause serious economic losses in the duck industry (4). An increasing number of virulence factors, i.e., CAMP cohemolysin, OmpA, nicotinamidase PncA, iron acquisition protein SprA, and glycosyltransferase VapD, etc., have been identified in *R. anatispestifer* (5–12).

The type IX secretion system (T9SS) or Por secretion system has been found in members of the phylum *Bacteroidetes* (13). The T9SS was recently reported in *R. anatispestifer*, which is a member of the phylum *Bacteroidetes* (14). The T9SS is associated with gliding motility and the secretion of virulence factors. The proteins secreted by T9SSs have a typical N-terminal signal peptide and traverse the cytoplasmic membrane into the periplasm via the general secretion (Sec) system. The proteins also typically have conserved C-terminal domains (CTDs) that target them to the T9SS for secretion across the outer membrane (15). The T9SS is functional in *R. anatispestifer* and contributes to its virulence by exporting key proteins (16). The T9SS component GldM is required for bacterial gliding motility and the secretion of the cell surface motility adhesins SprB and RemA in *Flavobacterium johnsoniae* (17).

In our previous study, 49 virulence genes were identified in *R. anatispestifer* strain Yb2 by using random transposon mutagenesis (18). The virulence of mutant strain RA256, where the *AS87_08785* gene was deleted, was reduced >7,000-fold. We also found that *R. anatispestifer* genes *AS87_08785* and *AS87_RS08465* encode the type IX secretion system components GldK and GldM, which function in bacterial gliding motility, protein secretion, and bacterial virulence. Liquid chromatography-tandem mass spectrometry analysis revealed that the putative metallophosphoesterase (MPPE; encoded by the *R. anatispestifer* *AS87_RS00980* gene) was absent in cultures of T9SS mutant strains Yb2Δ*gldK* and Yb2Δ*gldM*, suggesting that MPPE is a secretion protein of T9SS (19, 20).

According to bioinformatics analyses from the NCBI Conserved Domain Database (CDD), MPPE contains a purple acid phosphatase, an N-terminal domain, a Por secretion system C-terminal sorting domain, and a metallophosphatase domain. There are five conserved sites in MPPE (N267, H268, H351, H389, and H391) (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The homology protein of MPPE belongs to a diverse family of binuclear metallohydrolases that have been identified and characterized in plants, animals, and fungi (21). The structures of several bacterial and eukaryotic MPPEs have been described and reveal a conserved core α/β -sandwich-fold. Metal ions, typically two cations such as $\text{Fe}^{3+}/\text{Fe}^{2+}$ or Mn^{2+} , are coordinated in the active site of these enzymes by conserved His, Asp, and Asn residues, mutation of which results in markedly reduced catalytic activity (22–25). Despite highly conserved core catalytic domains, members of the superfamily perform diverse and crucial functions, ranging from nucleotide and nucleic acid metabolism to phospholipid hydrolysis (26). In this study, we demonstrated that the *R. anatispestifer* *AS87_RS00980* gene encodes an MPPE that is a T9SS secretion protein involved in bacterial virulence. MPPE from the wild-type strain Yb2 displays the phosphatase activity.

RESULTS

Characterization of Yb2Δ*00980* mutant strain and cYb2Δ*00980* complementation strain. PCR amplifications using primers AS87_RS00980-F/AS87_RS00980-R, 16S rRNA-F/16S rRNA-R, and Erm-F/Erm-R were performed to determine the mutant and complementation strains. As shown in Fig. 1A, 1,818-bp *AS87_RS00980* and 460-bp 16S rRNA fragments were amplified from the wild-type strain Yb2 (lane 1), 460-bp 16S rRNA and 801-bp Erm fragments, but no 1,818-bp *AS87_RS00980* fragment, were amplified from the mutant strain (lane 2), and 460-bp 16S rRNA, 801-bp Erm, and 1,818-bp *AS87_RS00980* fragments were all displayed in the complementation strain cYb2Δ*00980* (lane 3), demonstrating that the *AS87_RS00980* gene deletion mutant

FIG 1 Identification of mutant strain Yb2Δ00980 and complementation strain cYb2Δ00980. (A) PCR amplification. Primer pairs AS87_RS00980-F/AS87_RS00980-R, 16S rRNA-F/16S rRNA-R, and Erm-F/Erm-R were used to amplify the *R. anatipestifer* AS87_RS00980 gene, 16S rRNA, and Erm fragment, respectively. Lane M, DL2000 DNA marker (Vazyme, Nanjing, China). Lane 1, the *R. anatipestifer* AS87_RS00980 gene and 16S rRNA were amplified from wild-type strain Yb2; lane 2, RA 16S rRNA and Erm fragment were amplified, but no 1,818-bp fragment of the *R. anatipestifer* AS87_RS00980 gene was amplified, from mutant strain Yb2Δ00980; lane 3, the *R. anatipestifer* AS87_RS00980 gene, 16S rRNA, and Erm fragment were amplified from complementation strain cYb2Δ00980; lane 4, distilled water was used as a nonstrain control. (B) Identification of complementation strain cYb2Δ00980 by a Western blotting assay. Lane M, PageRuler prestained protein ladder (Thermo Scientific, Waltham, MA, USA); lane 1, whole-cell proteins from the wild-type strain Yb2; lane 2, whole-cell proteins from mutant strain Yb2Δ00980; lane 3, whole-cell proteins from complementation strain cYb2Δ00980. A second antibody directed against the TonB-dependent receptor of *R. anatipestifer* was used to control for protein loading in the assay.

strain Yb2Δ00980 and the complementation strain cYb2Δ00980 were constructed successfully. Distilled water was used as a nonstrain control (lane 4).

Western blotting was further used to confirm the presence of AS87_RS00980 in the strains. As shown in Fig. 1B, a 68-kDa AS87_RS00980 protein was shown in whole protein extracts of wild-type strain Yb2 (lane 1) but not in mutant strain Yb2Δ00980 (lane 2). Complementation strain cYb2Δ00980 displayed the 68-kDa band (lane 3), indicating that expression of AS87_RS00980 was defective in mutant strain Yb2Δ00980 but was rescued in complementation strain cYb2Δ00980. A second antibody directed against the TonB-dependent receptor of *R. anatipestifer* was used to control for protein loading in the assay.

Determination of bacterial growth and virulence. The growth rates of wild-type strain Yb2, mutant strain Yb2Δ00980, and complementation strain cYb2Δ00980 showed no significant difference in tryptic soy broth medium (TSB) culture, as determined by values of optical density at 600 nm (OD₆₀₀) (Fig. 2A). To investigate whether the AS87_RS00980 gene is associated with bacterial virulence, the bacterial median lethal dose (LD₅₀) and loads in the blood were determined in Cherry Valley ducklings. The LD₅₀ was determined to be 2.21×10^8 CFU for mutant strain Yb2Δ00980, which was >1,000-fold attenuated compared to that of wild-type strain Yb2 (2.2×10^5 CFU), suggesting that the AS87_RS00980 gene is associated with bacterial virulence.

We further measured the bacterial loads in the blood of ducks infected with wild-

FIG 2 Determination of bacterial growth curves and bacterial loads. (A) No significant difference was detected in bacterial growth rate among *R. anatipestifer* wild-type strain Yb2, mutant strain Yb2Δ00980, and complementation strain cYb2Δ00980. (B) Bacterial loads in the blood of ducks infected with *R. anatipestifer* Yb2, Yb2Δ00980, and cYb2Δ00980 were determined at 6, 12, 24, and 36 hpi. Bacterial CFU are presented as the means $\pm$ standard deviations of results for 10 infected ducks and were analyzed using a two-tailed independent Student *t* test. Asterisks indicate statistically significant differences between groups (****, $P < 0.0001$; ***, $P < 0.001$; nonsignificant [ns], $P > 0.05$).

FIG 3 Bacterial adherence (A) and invasion (B) assays. The assays were performed on HD11 cells with the infection dose at an MOI of 100. The data are the counts of bacteria bound to or having invaded HD11 cells in each well of a 12-well plate. Error bars represent the standard deviations calculated from three independent experiments performed in triplicate (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; ns, $P > 0.05$).

type strain Yb2, mutant strain Yb2Δ00980, and complementation strain cYb2Δ00980. As shown in Fig. 2B, the bacterial loads in infected duck blood were 8.45×10^3 CFU/ml, 1.26×10^4 CFU/ml, 8.25×10^4 CFU/ml, and 8.05×10^4 CFU/ml at 6, 12, 24, and 36 hpi, respectively, for mutant strain Yb2Δ00980, significant less than the bacterial loads of 8.0×10^4 CFU/ml, 8.36×10^4 CFU/ml, 5.7×10^5 CFU/ml, and 1.57×10^6 CFU/ml at 6, 12, 24, and 36 hpi, respectively, for wild-type strain Yb2. The complementation strain cYb2Δ00980 recovered the bacterial loads.

Deletion of the AS87_RS00980 gene reduced bacterial adherence and invasion capacities. HD11 cells were used to determine bacterial adherence and invasion abilities. When cells were infected with *R. anatipestifer* at a multiplicity of infection (MOI) of 100, the number of cell-adherent Yb2Δ00980 bacteria was 7.08×10^4 CFU/well, which was significantly lower than that of cells infected with Yb2 (8.2×10^5 CFU/well) or cYb2Δ00980 (4.96×10^5 CFU/well). When the cell-invasive bacteria were counted, the number of Yb2Δ00980 bacteria was 5×10^3 CFU/well, lower than the number of Yb2 (2.35×10^5 CFU/well) or cYb2Δ00980 (8.13×10^4 CFU/well) bacteria. The adherence and invasion capacities of mutant strain Yb2Δ00980 were significantly reduced, compared with that of wild-type strain Yb2, indicating that the AS87_RS00980 gene plays a critical role in bacterial adherence and invasion. The complementation strain cYb2Δ00980 restored partial adherence and invasion capacities (Fig. 3A and B).

The *R. anatipestifer* AS87_RS00980 gene encodes a secretion protein located in the cytomembrane. The proteins in the cell-free culture fluids of strains Yb2, Yb2Δ00980, and cYb2Δ00980 were identified by Western blotting. As shown in Fig. 4A, a 61-kDa band was detected in wild-type strain Yb2 (lane 1) but not in mutant strain Yb2Δ00980 (lane 2). Complementation strain cYb2Δ00980 recovered the band (lane 3). This result indicates that AS87_RS00980 is a secretion protein.

The bacterial cytoplasmic and membrane proteins were extracted and analyzed by Western blotting. As shown in Fig. 4B, a 68-kDa band was obviously displayed in whole-cell proteins (lane 1). No band was found in cytoplasmic proteins (lane 2), but the band was found in purified membrane proteins from Yb2 (lane 3), indicating that AS87_RS00980 is expressed in the cytomembrane of *R. anatipestifer*. Because the CTD of AS87_RS00980 was cut when secreted by the T9SS, a <68-kDa band was displayed in secretory proteins (lane 4).

The AS87_RS00980 gene encodes a metallophosphoesterase that displays phosphatases activity. The Pfam domains and conserved sites of AS87_RS00980 were searched in the NCBI Conserved Domain Database (CDD). As shown in Fig. 5A, protein AS87_RS00980 contains a purple acid phosphatase, an N-terminal domain, a metallophosphatase domain, and a Por secretion system C-terminal sorting domain.

FIG 4 Western blotting assay. (A) Extracellular proteins. Lane M, PageRuler prestained protein ladder; lane 1, wild-type strain Yb2; lane 2, mutant strain Yb2Δ00980; lane 3, complementation strain cYb2Δ00980; lane 4, recombinant AS87_RS00980. (B) Subcellular localization of AS87_RS00980 in *R. anatipestifer*. Lane 1, whole-cell proteins from wild-type strain Yb2; lane 2, extract of cytoplasmic proteins from Yb2; lane 3, extract of membrane proteins from Yb2; lane 4, extract of secretory proteins from Yb2.

AS87_RS00980 has five conserved sites (N267, H268, H351, H389, and H391) in its metallophosphatase domain (Fig. 5B). To investigate the enzymatic activity, the glutathione S-transferase (GST)-tagged recombinant protein AS87_RS00980 was efficiently expressed in *Escherichia coli* BL21 (DE3). As shown in Fig. 6A, *E. coli* BL21(DE3) transformed with pGEX-4T-1 displayed no AS87_RS00980 (91-kDa) band (lane 1), and *E. coli* BL21(DE3) transformed with pGEX-4T-1-AS87_RS00980 displayed a 91-kDa band in a Coomassie blue-stained SDS-PAGE gel (lane 2). After purification with BeaverBeads GSH, the recombinant AS87_RS00980 was identified in the gel (lanes 3 and 4). The activity of the recombinant AS87_RS00980 was measured using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate. As shown in Fig. 6B, the recombinant AS87_RS00980 showed high-level release of *p*-nitrophenol (*p*-NP) (lane 1), whereas bovine serum albumin (BSA) as a negative control

FIG 5 Bioinformatics analysis of AS87_RS00980. (A) The full length of the gene comprises 1,818bp and encodes a polypeptide of 605 amino acid residues containing a purple acid phosphatase, an N-terminal domain, a metallophosphatase superfamily, a metallophosphatase domain, and a Por secretion system C-terminal sorting domain. (B) The five conserved residues (N267, H268, H351, H389, and H391) of the MPPE were all identified in the amino acid sequence.

FIG 6 Expression and enzymatic activity assay of *R. anatipestifer* AS87_RS00980. (A) SDS-PAGE analysis of recombinant AS87_RS00980 expression. Lane M, PageRuler prestained protein ladder; lane 1, *E. coli* BL21(DE3) transformed with pGEX-4T-1 with IPTG induction; lane 2, supernatant from *E. coli* BL21(DE3) transformed with pGEX-4T-1-AS87_RS00980, with IPTG induction; lane 3, purified recombinant AS87_RS00980; lane 4, the second-time-purified recombinant AS87_RS00980. Lane 1, the second-time-purified recombinant AS87_RS00980; lane 2, BSA as a negative control. (B) Enzymatic reaction of recombinant AS87_RS00980. Lane 1, the second-time-purified recombinant AS87_RS00980; lane 2, BSA as a negative control. (C) Enzymatic activity of secretory proteins of bacteria. The activity of wild-type strain Yb2 toward p-NPP is taken to be 100%, while mutant strain Yb2Δ00980 showed only 13.55% relative activity compared to wild-type strain Yb2. Complementation of AS87_RS00980 recovered the enzymatic activity. The error bars represent the standard deviations of data from three independent experiments (****, $P < 0.0001$).

showed no release of p-NP (lane 2), indicating that the *R. anatipestifer* AS87_RS00980 gene encodes an MPPE that displays phosphatase activity.

The activity of the proteins in cell-free culture fluids is shown in Fig. 6C. The proteins in the cell-free culture fluids of strains Yb2, Yb2Δ00980, and cYb2Δ00980 were identified. Phosphatase activity analysis showed high-level production of phosphatase activity for wild-type strain Yb2 and complementation strain cYb2Δ00980, while mutant strain Yb2Δ00980 showed only 13.55% relative activity, compared to wild-type strain Yb2.

Determination of optimal pH, temperature, and enzymatic K_m and V_{max} . As shown in Fig. 7A, the relative activity reached a peak at pH 7.0 and retained over 80% activity between pH 6.5 and 9.0. A pH lower than 6.5 inhibited the enzymatic activity.

The effect of temperature on the activity is shown in Fig. 7B. The optimal temperature of recombinant MPPE (rMPPE) activity was 60°C, and the reactive temperature range was comparatively broad. rMPPE showed >60% relative activity at temperatures ranging from 50°C to 80°C, suggesting that the supporting matrix increases the thermal tolerance of the enzyme by absorbing some amount of heat and making some conformational changes in it through covalent bond formation between matrix and enzyme. K_m and V_{max} were determined under the optimal pH 7.0 and a temperature of 60°C. From a Lineweaver-Burk plot, K_m and V_{max} values for rMPPE were calculated using p-NPP as a substrate (Fig. 7C). The K_m and V_{max} of the enzyme were found to be 3.53 mM and 198.1 U/mg, respectively.

Effect of metal cations and EDTA on rMPPE activity. rMPPE activity was tested using p-NPP as a substrate in the experiment. The influence of metal ions on the purified rMPPE is shown in Table 1. The enzymatic activity of purified rMPPE was inhibited by Fe^{3+} , Fe^{2+} , and EDTA in a dose-dependent pattern, while the activity was significantly enhanced by Cu^{2+} and Zn^{2+} . The activity was not significantly influenced by Mg^{2+} , Mn^{2+} , or Ca^{2+} .

Positions 351, 389, and 391 are necessary for the enzymatic activity. GST-tagged mutant proteins were efficiently expressed in *E. coli* BL21(DE3). *E. coli* BL21(DE3) transformed with pGEX-4T-1 displayed no MPPE (91-kDa) band (Fig. 8A, lane 1); *E. coli* BL21(DE3) transformed with pGEX-4T-1-AS87_RS00980 displayed a 91-kDa band in a Coomassie blue-stained SDS-PAGE gel (lanes 2, 4, and 6). After purification with BeaverBeads GSH, Y267-rMPPE (lane 3), Y268-rMPPE (lane 5), and Y351-rMPPE (lane 7)

FIG 7 Effect of pH and temperature on activity of rMPPE. (A) pH optimization. The optimal pH was measured at 37°C for 10 min in sodium acetate buffer or Tris-HCl buffer. The relative activity reached its highest level at pH 7.0. (B) Temperature optimization. The optimal temperature was measured by incubating rMPPE with p-NPP in Tris-HCl buffer (pH 7.0) for 10 min at a temperature increasing from 30.0°C to 90.0°C at 10°C intervals. The optimal activity of rMPPE was found to be at 60.0°C. The error bars represent the standard deviations of data from three independent experiments. (C) Lineweaver-Burk plot for catalytic reaction of rMPPE. Values are means of results of three independent experiments.

were identified in the gel (Fig. 8A). *E. coli* BL21(DE3) transformed with pGEX-4T-1-AS87_RS00980 displayed a 91-kDa band in a Coomassie blue-stained SDS-PAGE gel (lanes 1 and 3). After purification, Y389-rMPPE (lane 2) and Y391-rMPPE (lane 4) were identified in the gel (Fig. 8B).

By *in vitro* site-directed mutagenesis, we generated Y267, Y268, Y351, Y389, and Y391 mutants and expressed GST fusion mutant proteins in *E. coli* BL21(DE3). The influences of various site-directed mutageneses on the protein are presented in Table 2. The mutant proteins Y267-rMPPE (lane 2) and Y268-rMPPE (lane 3) retained 29.30% and 19.81% relative activity, respectively, compared to rMPPE (lane 1). Almost no activity was detected in mutant proteins Y351-rMPPE (lane 4), Y389-rMPPE (lane 5), and Y391-rMPPE (lane 6), indicating that H351, H389, and H391 are vitally important for MPPE. BSA was used as a negative control (Fig. 8C, lane 7).

DISCUSSION

Protein secretion systems are vital for prokaryotic life because they allow bacteria to acquire nutrients, communicate with other species, defend themselves against biological and chemical agents, and facilitate disease through the delivery of virulence factors (27). T9SS or the Por secretion system associated with the secretion of virulence factors was discovered in many species of *Bacteroidetes* (14), which secretes proteins either as large com-

TABLE 1 Metal cation effects on enzymatic activity of rMPPE

Metal cation	Concn (mM)	Relative activity (%)
None		100
Fe ³⁺	1	16.52
	0.1	80.30
Fe ²⁺	1	83.94
	0.1	95.08
Mn ²⁺	1	104.39
	0.1	91.21
Ca ²⁺	1	97.20
	0.1	93.18
Zn ²⁺	1	179.17
	0.1	178.03
Cu ²⁺	1	147.73
	0.1	160.38
Mg ²⁺	1	96.52
	0.1	93.18
EDTA	10	82.26
	30	40.65
	50	14.31

FIG 8 Expression and enzymatic activity assay of the five mutant proteins. (A) SDS-PAGE analysis of expression of Y267, Y268, and Y351 mutants. Lane M, PageRuler prestained protein ladder; lane 1, *E. coli* BL21(DE3) transformed with pGEX-4T-1 with IPTG induction; lane 2, supernatant from *E. coli* BL21(DE3) transformed with mutant Y267-pGEX-4T-1 with IPTG induction; lane 3, purified Y267-rMPPE; lane 4, supernatant from *E. coli* BL21(DE3) transformed with mutant Y268-pGEX-4T-1 with IPTG induction; lane 5, purified Y268-rMPPE; lane 6, supernatant from *E. coli* BL21(DE3) transformed with mutant Y351-pGEX-4T-1 with IPTG induction; lane 7, purified Y351-rMPPE. (B) SDS-PAGE analysis of the expression of Y389 and Y391 mutants. Lane 1, supernatant from *E. coli* BL21(DE3) transformed with mutant Y389-pGEX-4T-1 with IPTG induction; lane 2, purified Y389-rMPPE; lane 3, supernatant from *E. coli* BL21(DE3) transformed with mutant Y391-pGEX-4T-1 with IPTG induction; lane 4, purified Y391-rMPPE. (C) Enzymatic activity of the mutant proteins. Lane 1, purified rMPPE; lane 2, purified Y267-rMPPE; lane 3, purified Y268-rMPPE; lane 4, purified Y351-rMPPE; lane 5, purified Y389-rMPPE; lane 6, purified Y391-rMPPE; lane 7, BSA as a negative control.

plexes on the bacterial cell surface or into the extracellular milieu (28). T9SS secreting virulence factors have been studied in the motile *Flavobacterium columnare* as well as in the nonmotile *Porphyromonas gingivalis* and *Tannerella forsythia*. *Flavobacterium columnare* is a common fish pathogen that causes columnaris disease. Enzymes secreted through the type IX secretion system, such as proteases and chondroitin sulfate lyases, have been suggested as possible *F. columnare* virulence factors (29). *P. gingivalis* is regarded as a keystone pathogen in chronic periodontitis. The gingipains (RgpA, RgpB, and Kgp) and cell surface adhesins are important virulence factors and have the ability to degrade many important host proteins and to dysregulate the host immune response that is directed to the cell surface via the type IX secretion system (30). *T. forsythia* plays a role in the etiology of several chronic diseases of humans (31). It can secrete a variety of virulence factors, and the Slayer glycoproteins TfsA, TfsB, and BspA were confirmed and related to its pathogenicity (32). In this study, we characterized the *R. anatispestifer* AS87_RS00980 gene encoding T9SS effector MPPE, which plays an important role in bacterial virulence. In addition, since the bacterial loads in the blood of ducks infected with a AS87_RS00980 gene deletion mutant were significantly reduced, it is entirely possible that MPPE promotes extracellular survival and spread *in vivo*.

MPPE has an activity preference toward *p*-nitrophenyl phosphate (p-NPP). At its optimal pH of 7.0 and optimum temperature of 60°C, rMPPE showed the highest activ-

TABLE 2 MPPE activity of the mutant proteins

Protein	Relative activity (%)
rMPPE	100
Y267-rMPPE	29.30
Y268-rMPPE	19.81
Y351-rMPPE	0.49
Y389-rMPPE	1.26
Y391-rMPPE	0.15

ity. By analogy, immobilized acid phosphatase, a novel acid phosphatase (AcP101), and *Burkholderia cenocepacia* J2315 PAP-like protein (BcPAP) have activity toward p-NPP as well. An optimal temperature and optimal pH similar to those of MPPE have been reported for immobilized acid phosphatase. Different results were seen for soluble acid phosphatase and BcPAP (21, 33, 34). The kinetic parameters K_m and V_{max} for rMPPE were determined to be 3.53 mM and 198.1 U/mg, respectively. It can be activated by Zn^{2+} or Cu^{2+} but is inhibited by Fe^{3+} , Fe^{2+} , and EDTA. However, for PlcP, Mn^{2+} gives the highest activity, followed by Zn^{2+} and Fe^{2+} (35). On the other hand, Zn and Fe have also been found in several proteins of this family (36). MPPE has five conserved sites in the amino acid sequence of its metallophosphatase domain (N267, H268, H351, H389, and H391). All members of this group carry seven invariant amino acid residues, distributed on five conserved motifs (GDXX/GDXXY/GNH[D/E]/XXXH/GHXH), which are involved in coordinating the dimetallic center in the active site (37). Mutant proteins Y267-rMPPE and Y268-rMPPE retained 29.30% and 19.81% relative activity, respectively, compared to rMPPE. Activity was almost abolished in mutant proteins Y351-rMPPE, Y389-rMPPE, and Y391-rMPPE.

MPPE was suggested to be involved in bone resorption and immune function in mammals (38). A novel member of the MPPE superfamily from *Streptococcus pneumoniae*, namely, SapH, which catalyzes the hydrolysis of phosphodiester and ATP, has been reported (39). CpdA was originally identified in *Escherichia coli* as a regulator of 3'5'-cyclic AMP (cAMP) levels, an important second messenger molecule that mediates catabolite repression (40). Homologues of CpdA have been identified from several bacteria, such as *Pseudomonas aeruginosa* (41), *Haemophilus influenzae* (42), and *Mycobacterium tuberculosis* (referred to as Rv0805) (43). LpxH-like enzymes act on UDP-2,3-diacylglucosamine (UDP-DAGn) to produce lipid X (44). Lipid X, in turn, is required for production of lipid A, an important component of Gram-negative bacterial cell walls. Thus, deletion of the *lpxH* gene from *E. coli* results in the accumulation of UDP-DAGn in the bacterium (45).

M. tuberculosis resides in the highly oxidative environment of human macrophages (46). Both reactive oxygen and reactive nitrogen intermediates are antimycobacterial effector molecules (47). A recombinant peroxynitritase was shown to prevent oxidative damage to the bacterium by removing reactive oxygen and nitrogen species (48). By analogy, purple acid phosphatases (PAPs) can remove reactive oxygen intermediates in a Fenton-type reaction (49). Hence, in *M. tuberculosis*, a PAP enzyme could assist in pathogen survival by reducing the respiratory burst of its host or removing potentially lethal free radicals. The phylogeny of PAP was not entirely clear; the presence of this enzyme in a limited number of microorganisms is an indication of its role in specialized metabolic pathways. So, it is important to advance our knowledge of the functions of bacterial PAPs. In this study, the adhesion and invasion capacities of mutant strain Yb2Δ00980 were significantly reduced, indicating that MPPE may play an important role in removing reactive oxygen intermediates or reducing the respiratory burst of its host. It may be a major secreted factor when the bacteria are exposed to host cells or tissue. Thus, the MPPE that we have uncovered, as well as other dependent factors, may be quite relevant for the pathogenesis of *R. anatipestifer*.

In conclusion, we showed that the *R. anatipestifer* AS87_ RS00980 gene encodes MPPE, which is responsible for bacterial virulence. MPPE displays acid phosphatases activity. It may be closely related to extracellular survival, spread *in vivo*, or cell infection. The molecular mechanisms by which this protein acts in bacterial virulence require further clarification, which should facilitate the development of effective strategies to control *R. anatipestifer* infection.

MATERIALS AND METHODS

Ethics statement. One-day-old Cherry Valley ducks were purchased from JinHu Duck Farm (Jiangsu, China) and housed in cages at a controlled temperature of 28 to 30°C under biosafety conditions, with water and food provided *ad libitum*. The study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the Institutional Animal Care and Use

TABLE 3 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description ^a
Strains and plasmids	
Yb2	<i>Riemerella anatipestifer</i> serotype 2 strain
Yb2Δ00980	AS87_RS00980 gene mutant of <i>R. anatipestifer</i> Yb2
cYb2Δ00980	Mutant Yb2Δ00980 carrying plasmid pCP29-AS87_RS00980
pCP29	ColE1 ori (pCP1 ori), Ap ^r (Em ^r); <i>E. coli</i> - <i>F. johnsoniae</i> shuttle plasmid
pCP29-AS87_RS00980	pCP29 containing <i>ompA</i> promoter and AS87_RS00980 ORF, <i>cfxA</i> (Ap ^r)
Primers	
AS87_RS0098-F	5'-GATAATGGACATTCTTAAGAAATGG-3'
AS87_RS0098-R	5'-TCATTTTGATACAGATAGCGAGGAG-3'
AS87_RS00980 P1-F	5'-TAATATGCTGGTAACCTCAAATGTG-3'
AS87_RS00980 P1-R	5'-GAACGGGCAATTTCTTTTTGTGATATTCACCTTAGTTAATTTTACGGT-3'
Erm-P-F	5'-ACCGTAAATTAACCTAAGGTGAATATGACAAAAAGAAATTGCCCGTT-3'
Erm-P-R	5'-TTCATTTGTTAGAAATAGACTATTTCTACGAAGGATGAAATTTTTCAGGG-3'
AS87_RS00980 P2-F	5'-CCCTGAAAAATTCATCCTTCGTAGAAATAGTCTATTTCTAACAAATGAA-3'
AS87_RS00980 P2-R	5'-CTAGTATCCCTGCTGCCGAAGGAAT-3'
Erm-F	5'-ATGACAAAAAGAAATTGCCCGTT-3'
Erm-R	5'-CTACGAAGGATGAAATTTTTCAGGG-3'
16S rRNA-F	5'-GAGCGGTAGAGTATCTTCGGATACT-3'
16S rRNA-R	5'-TATTACCGCGCTGCTGGCA-3'
AS87_RS00980-orf-F	5'-CCGCTCGAGATGAAAAAATTAAATTACTATTGAG-3' (XhoI site underlined)
AS87_RS00980-orf-R	5'-CATGCATGCTTATTTTACAATTATCTTATGG-3' (SphI site underlined)
AS87_RS00980-4T-F	5'-GAAAAATATTCTGTACCTTCTG-3'
AS87_RS00980-4T-R	5'-CTTTTACAATTATCTTATGTTTG-3'
AS87_RS00980-F-25(4T)	5'-GGATCTGGTCCGCGTGGATCCGAAAAATATTCTGTACCTTCTG-3'
AS87_RS00980-N267-R	5'-TCTACTATACTTACATCATGGCTTCCCAACACAGGTACTA-3'
AS87_RS00980-N267-F	5'-TAGTACCTGTGTTGGGAAGCCATGATGTAAGTATAGTAGA-3'
AS87_RS00980-R-605(4T)	5'-ATGCGCGCGCTCGAGTCGACCTTTTACAATTATCTTATGTTTG-3'
AS87_RS00980-H268-R	5'-TATCTACTATACTTACATCAGCATTCCCAACACAGGTACTAT-3'
AS87_RS00980-H268-F	5'-ATAGTACTGTGTTGGGAAATGCTGATGTAAGTATAGTAGATA-3'
AS87_RS00980-H351-R	5'-ACCTGTGTAATATTTCTAGCCGAAGCTAAAAACGCCATT-3'
AS87_RS00980-H351-F	5'-AATGGCGTTTTTTCAGCTTCGGCTAGAAATATTTACACAGGT-3'
AS87_RS00980-H389-R	5'-ACTTCATAAACATGTCGCGCCCTTGAAAAATAGAAATCAATA-3'
AS87_RS00980-H389-F	5'-TATTGATTCTATTTTCAAGGGGCCGACCATGTTTATGAAGT-3'
AS87_RS00980-H391-R	5'-GGACCTATAACTTCATAAACAGCGTCGTGCCCTTGAAAAATAG-3'
AS87_RS00980-H391-F	5'-CTATTTTCAAGGGCACGACGCTGTTTATGAAGTTATAGGTCC-3'

^aORF, open reading frame; *cfxA*, cefoxitin resistance gene.

Committee (IACUC) guidelines set by Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (CAAS). The study protocol was approved by the Institutional Animal Care and Use Committee of the Shanghai Veterinary Research Institute, CAAS (approval no. SHVRI-SZ-20200619-03). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 3. *R. anatipestifer* strain Yb2 is the wild-type strain used in this study. The AS87_RS00980 gene deletion mutant strain Yb2Δ00980 was constructed using the natural transformation method, and the complementation strain cYb2Δ00980 was established using plasmid pCP29 and Yb2Δ00980. All *R. anatipestifer* strains were grown in tryptic soy broth medium (TSB; Difco, NJ, USA) or on solid tryptic soy agar (TSA) medium containing 1.5% agar at 37°C with 5% CO₂. The *Escherichia coli* strains were grown at 37°C on Luria-Bertani (LB) plates or in LB broth. The *Escherichia coli*-*F. johnsoniae* shuttle plasmid pCP29 and *E. coli* strain S17-1 were kindly provided by Mark J. McBride (University of Wisconsin–Milwaukee, Milwaukee, WI, USA). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; erythromycin, 1 μg/ml; kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; and cefoxitin, 5 μg/ml.

Construction of mutant strain Yb2Δ00980 and complementation strain cYb2Δ00980. Mutant strain Yb2Δ00980 was constructed using the natural transformation method (50). Briefly, the upstream sequence (~800 bp) and the downstream sequence (~800 bp) of the AS87_RS00980 gene were amplified using primer pairs AS87_RS00980 P1-F/AS87_RS00980 P1-R and AS87_RS00980 P2-F/AS87_RS00980 P2-R, respectively (Table 3). The erythromycin resistance (Erm) gene was amplified using primers Erm-P-F and Erm-P-R (Table 3). The resulting PCR fragments were ligated by overlap PCR using primers AS87_RS00980 P1-F and AS87_RS00980 P2-R (51) and then purified and introduced into wild-type strain Yb2 by natural transformation. The transformants were selected on TSA plates supplemented with erythromycin.

The shuttle plasmid pCP29 carrying the *ompA* promoter of *R. anatipestifer* was used to construct

complementation strain cYb2Δ00980 as described previously (52). Briefly, the open reading frame (ORF) of AS87_RS00980 was amplified from *R. anatipestifer* Yb2 genomic DNA with primers AS87_RS00980-orf-F/AS87_RS00980-orf-R, digested with SphI and XhoI, and ligated into pCP29 that had been digested with the same enzymes, generating the pCP29-AS87_RS00980 plasmid. The plasmid was then transferred into mutant strain Yb2Δ00980 by conjugation to construct complementation strain cYb2Δ00980.

The AS87_RS00980 gene mutation or complementation strain was identified by PCR amplification of the AS87_RS00980 gene, *R. anatipestifer* 16S rRNA, and the *Erm* gene by using primer pairs AS87_RS00980-F/AS87_RS00980-R, 16S rRNA-F/16S rRNA-R, and *Erm*-F/*Erm*-R, respectively (Table 3). The presence of AS87_RS00980 in the bacteria was identified by Western blotting using mouse anti-AS87_RS00980 polyclonal antibody (generated in our laboratory) as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG polyclonal antibody (Bio-Rad Laboratories, Hercules, CA, USA) as the second antibody. The specific bands were developed with the basic luminol chemiluminescent kit (S-Wb001), visualized using a Tanon 5200 automatic chemiluminescence image analysis system (Tanon, Shanghai, China). A rabbit anti-TonB-dependent receptor antibody was used as a protein loading control.

Determination of bacterial growth and median lethal dose. The growth curves of wild-type strain Yb2, mutant strain Yb2Δ00980, and complementation strain cYb2Δ00980 were measured as described previously (53). After growth in TSB at 37°C for 8 h with shaking, equal amounts of each bacterial culture were transferred into fresh TSB at a ratio of 1:100 (vol/vol) and incubated at 37°C with shaking at 220 rpm. Bacterial growth was measured by measuring the OD₆₀₀ value at 2-h intervals for 18 h.

To determine whether the AS87_RS00980 gene had an influence on the virulence of *R. anatipestifer*, the bacterial median lethal doses (LD₅₀s) of wild-type strain Yb2 and mutant strain Yb2Δ00980 were determined using 14-day-old Cherry Valley ducks as described previously (54). Forty ducks for each strain were divided randomly into 5 groups (8 ducks per group). Ducks in groups 1 to 5 were injected with 10⁵, 10⁶, 10⁷, 10⁸, and 10⁹ CFU of mutant strain Yb2Δ00980, and ducks in groups 6 to 10 were injected with 10⁴, 10⁵, 10⁶, 10⁷, and 10⁸ CFU of wild-type strain Yb2, respectively. The infected ducks were clinically observed for a period of 7 days. Dead ducks were subjected to *R. anatipestifer* identification. The LD₅₀ value was calculated by the improved Karber method (55).

Determination of bacterial loads in infected duck blood. To determine bacterial survival in infected duck blood, 30 21-day-old ducks were divided randomly into three groups and inoculated intramuscularly with wild-type strain Yb2, mutant strain Yb2Δ00980, and complementation strain cYb2Δ00980 at a dose of 2.5×10^7 CFU. Blood samples were collected at 6, 12, 24, and 36 h postinfection (hpi), diluted appropriately, and plated on TSA for bacterial counting (8). The plates were incubated at 37°C with 5% CO₂ for 36 h for bacterial counting.

Bacterial adherence and invasion assays. Adhesion and invasion assays were performed using HD11 cells as described previously (7). Briefly, each well in a 12-well tissue culture plate was seeded with 2.5×10^5 cells in Dulbecco's modified Eagle medium (DMEM) (Biowest, France) containing 10% fetal bovine serum. Bacteria were collected, washed twice with phosphate-buffered saline (PBS), and diluted in fresh cell culture medium without antibiotics to 2.5×10^7 CFU/ml. After incubation at 37°C with 5% CO₂, the cell monolayer was rinsed with PBS and infected with bacteria at a multiplicity of infection (MOI) of 100 by centrifugation at $1,000 \times g$ for 10 min at room temperature. The infected cells were incubated at 37°C under 5% CO₂ for 1.5 h, washed three times with sterile PBS, and lysed with 0.1% trypsin (150 μl/well). The cell suspensions were serially 10-fold diluted and plated onto TSA plates to determine the number of viable bacterial cells. For the invasion assay, the extracellular bacteria were killed by incubating the monolayer with DMEM supplemented with 100 μg/ml gentamicin for an additional 1 h. The bacteria were then incubated and washed three times with PBS, and the number of intracellular bacteria was determined. All of these assays were performed in triplicate and replicated three times.

Secretion, subcellular localization, and bioinformatics analyses of AS87_RS00980. The secretion and subcellular localization of the *R. anatipestifer* AS87_RS00980-encoded protein were identified by Western blotting. Briefly, *R. anatipestifer* wild-type strain Yb2, mutant strain Yb2Δ00980, and complementation strain cYb2Δ00980 were grown in 200 ml of animal-derived component-free (ADCF) medium at 37°C with shaking until the OD₆₀₀ reached 0.8. The cultures were centrifuged at $8,000 \times g$ for 15 min, and the supernatants were purified by passage through 0.22-μm-pore-size polyvinylidene difluoride filters. The secretion proteins in the supernatants were collected with 3-kDa Amicon Ultra centrifugal filter units (Sigma), and the concentrations were determined with a bicinchoninic acid (BCA) protein assay kit, with BSA as the standard (Beyotime, Shanghai, China).

The membrane and cytoplasmic proteins from wild-type strain Yb2 were fractionated with a bacterial membrane protein extraction kit (BestBio, Shanghai, China) according to the manufacturer's protocol. The protein concentrations were determined with a BCA protein assay kit (Beyotime, Shanghai, China).

The secretion proteins and subcellular fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) for Western blotting using mouse anti-AS87_RS00980 polyclonal antibody as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG polyclonal antibody as the second antibody.

The amino acid sequence of AS87_RS00980 was retrieved from Universal Protein Resource (UniProt). The Pfam domains and conserved amino acids of AS87_RS00980 were predicted by searching the Conserved Domain Database of NCBI.

Recombinant protein expression and enzymatic activity analysis. The AS87_RS00980 gene was amplified from *R. anatipestifer* strain Yb2 by using primers AS87_RS00980-4T-F and AS87_RS00980-4T-R

(Table 3). After purification, the amplicon was inserted into the expression vector pGEX-4T-1 to construct pGEX-4T-1-AS87_RS00980 plasmid, and *E. coli* strain BL21(DE3) was subsequently used for transformation. The insertion of pGEX-4T-1-AS87_RS00980 plasmid was confirmed by DNA sequencing. Expression of GST-tagged recombinant AS87_RS0098 was induced in *E. coli* strain BL21(DE3) cells by treatment with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 24 h at 18°C with shaking. The cells were harvested by centrifugation at $10,000 \times g$ for 5 min at 4°C, resuspended in buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), and purified with BeaverBeads GSH (Beaver, Boston, USA) according to the manufacturer's protocol. The purified fractions were confirmed by SDS-PAGE, followed by Coomassie brilliant blue staining.

AS87_RS00980 was predicted as a metallophosphoesterase (MPPE) with acid phosphatase activity (20). The enzymatic activity of the recombinant AS87_RS0098 was measured by release of *p*-nitrophenol (p-NP) from *p*-nitrophenyl phosphate (p-NPP) according to the method of Ueki and Sato (56). The reaction mixture (5.0 ml) contained 5 mM p-NPP, 200 mM sodium acetate buffer, 10 mM MgSO₄, and 20 μ g of recombinant AS87_RS0098. The reaction proceeded at 37°C for 10 min and was terminated by the addition of 2.5 ml saturated 200 mM NaOH solution. BSA was used as a negative control.

Secretory proteins of wild-type strain Yb2, mutant strain Yb2 Δ 00980, and complementation strain cYb2 Δ 00980 were extracted and quantified as described above. The enzymatic activity was measured by using 100 μ g of secretory proteins, and the reaction was conducted at 37°C for 2 h. The absorbance at OD₄₀₅ was measured to estimate liberated *p*-nitrophenol. Data were collected in triplicate.

Optimization of the reactive pH and temperature for enzymatic activity analysis. The optimal pH was determined by measuring the activity of recombinant AS87_RS0098 in 200 mM sodium acetate buffer at pH 4.0, 5.0, 6.0, and 6.5 and in 100 mM Tris-HCl buffer at pH 7.0, 8.0, 9.0, and 10.0. The effect of temperature on the phosphatase activity of recombinant AS87_RS0098 was assessed at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C. The reaction mixtures containing 5 mM p-NPP, 10 mM MgSO₄, and 20 μ g purified recombinant AS87_RS0098 in the respective buffers were incubated at the respective temperatures for 10 min, and the reaction was quenched by the addition of 200 mM NaOH. The absorbance at OD₄₀₅ was measured to estimate liberated *p*-nitrophenol. Data were collected in triplicate.

Determination of K_m and V_{max} values for recombinant AS87_RS0098. The apparent K_m and V_{max} values of recombinant AS87_RS0098 for catalytic p-NPP were determined by measuring the rate of dephosphorylation at substrate concentrations of 5 mM, 10 mM, 15 mM, 20 mM, and 30 mM. The reaction mixture (5.0 ml) contained 100 mM Tris-HCl buffer, 10 mM MgSO₄, and 4 μ g of recombinant AS87_RS0098. The reaction was conducted under the optimal pH of 7.0 and optimal temperature of 60°C. The kinetic parameters K_m and V_{max} were evaluated based on the Lineweaver-Burk plot, which was generated by the formula $V = V_{max}[S]/(K_m + [S])$, where S is substrate and brackets indicate concentration.

Metal cation effects on enzymatic activity of recombinant AS87_RS0098. Metal cations of Fe³⁺, Fe²⁺, Mn²⁺, Ca²⁺, Zn²⁺, Cu²⁺, Mg²⁺, and EDTA were examined for their effects on the phosphatase activity of recombinant AS87_RS0098. The reactions were carried out using 5 mM p-NPP as the substrate and 4 μ g of purified recombinant AS87_RS0098 incubated at 60°C for 10 min in 100 mM Tris-HCl buffers (pH 7.0) containing the respective metal ion or EDTA. Released p-NP was detected by measuring the OD₄₀₅. Distilled water was used as a negative control. Data were collected in triplicate.

Site-directed mutagenesis of recombinant AS87_RS00980. According to the bioinformatics analysis, the protein AS87_RS00980 has five conserved sites at positions 267, 268, 351, 389, and 391. To investigate their function in enzymatic activity, missense mutations were introduced into the AS87_RS00980 gene using the two-stage PCR-based overlap extension method (57). Complementary oligodeoxynucleotide primers AS87_RS00980-F-25(4T), AS87_RS00980-R-605(4T), AS87_RS00980-N267-R/AS87_RS00980-N267-F, AS87_RS00980-H268-R/AS87_RS00980-H268-F, AS87_RS00980-H351-R/AS87_RS00980-H351-F, AS87_RS00980-H389-R/AS87_RS00980-H389-F, and AS87_RS00980-H391-R/AS87_RS00980-H391-F (Table 3) were used to generate every two DNA fragments having overlapping ends. These fragments were combined in a subsequent "fusion" reaction in which the overlapping ends anneal, allowing the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. The resulting fusion product was amplified further by PCR. Restriction fragments of the mutated second-stage PCR products were inserted into the vector pGEX-4T-1. The inserts of the resulting plasmids were sequenced to confirm the presence of the desired mutations and the absence of any unwanted coding changes.

Five mutant pGEX-4T-1-AS87_RS00980 plasmids were transformed into *E. coli* BL21(DE3). Recombinant mutant protein production was induced by adjusting exponentially growing cultures to 1 mM IPTG and then incubating them at 18°C for 24 h with shaking. The GST-tagged recombinant AS87_RS00980 mutant proteins were purified with BeaverBeads GSH, according to the manufacturer's protocol. The purified fractions were confirmed by SDS-PAGE, followed by Coomassie brilliant blue staining.

Determination of enzymatic activity for mutant proteins. The activity of five mutant proteins was measured. The reaction mixture contained 5 mM p-NPP, 100 mM Tris-HCl buffer, and 4 μ g of recombinant AS87_RS00980 mutant protein at 60°C for 10 min. Recombinant AS87_RS00980 was used as a positive control, and BSA was used as a negative control.

Statistical analysis. Statistical analyses were conducted using GraphPad Software version 6.0 (La Jolla, CA, USA). One-way analysis of variance (ANOVA) was used for analyses of growth curves, adhesion and invasion data, and enzymatic activity. Two-tailed independent Student's *t* test was used for analyses of the bacterial loads in blood. *P* values of <0.05 were considered significant.

Data availability. All the data and materials in this study are available from S. Yu by request.

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We declare that we have no competing interests.

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P.N., Z.C., X.R., W.H., and R.S. performed the experiments. P.N. analyzed the data and prepared the manuscript. H.D. and C.D. contributed reagents, materials, and analysis tools. S.Y. and S.Z. designed the study and revised the manuscript. All authors read and approved the final manuscript.

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