



In Silico Analysis and Immune Response of YaeT Protein Against *Riemerella anatipestifer* in Ducks

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Abstract

Riemerella anatipestifer (RA) is one of the most harmful bacterial pathogens in waterfowl and causes enormous economic loss worldwide. Due to weak cross-immunity protection against different serotypes of RA, inactivated and attenuated vaccines are only effective for RA of specific serotypes. In this paper, outer membrane protein YaeT in RA was analyzed through bioinformatics, in vivo, and in vitro assays. Homology, physicochemical and structural properties, transmembrane domains, and B-cell binding epitopes were investigated. The recombinant outer membrane protein YaeT was then inoculated into Cherry Valley ducks to analyze its immune protection against RA. Results showed that the protein was conservative in different RA strains and had sufficient B-cell binding epitopes. The immunized duck serum contains high-affinity antibodies that could activate complement and promote the opsonophagocytosis of RA by phagocytes. After RA challenge, the survival rate of the YaeT protein-immunized ducks was 80%.

Keywords RA · YaeT protein · Bioinformatics · Vaccine

Introduction

Riemerella anatipestifer (RA) is a Gram-negative member of the family Flavobacteriaceae that has no flagella or spores [18]. It produces a capsule and infects domestic birds such as ducks [6, 15], geese [9], and turkeys [27]. This widely distributed bacterium is currently a main pathogen affecting waterfowl breeding and is difficult to be eliminated from duck

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farms around the world. The *RA* infection is predominantly via respiratory system and skin contact (especially foot skin), causing acute or chronic septic bacterial infections [6, 26]. The prevalence of the disease can be as high as 90% or more. However, because the virulence of the various serotypes differs greatly, the mortality ranges from 5 to 75% [26, 34].

Long-term use of antibiotics has led to a significant increase in the resistance of pathogenic bacteria [16, 38]. The massive use of antibiotics enhances the resistance of pathogenic bacteria, which in turn further promotes the use of larger doses of antibiotics, resulting in a vicious cycle of stronger resistance of pathogenic bacteria. At the same time, the problem of antibiotic residues due to massive use is getting worse [19, 36]. These problems have become global and need to be addressed. In the long run, improving breeding conditions and vaccination are effective ways to reduce the abuse of antibiotics. Vaccines against *RA* mainly include attenuated vaccines [18], inactivated vaccines [17], and subunit vaccines [4]. Because of the weak cross-immunity protection among *RA* serotypes, inactivated vaccines and attenuated vaccines must be based on the local serotypes of *RA*. OmpA protein was used to prepare *RA* subunit vaccine, but its immune protection was inconsistent [32, 33, 35].

The YaeT protein, belonging to the Omp85 protein family [31], is an outer membrane protein that is ubiquitous among Gram-negative bacteria. The N-terminus, which is a periplasmic polypeptide-transport-associated (POTRA) domain, is located in the periplasmic space, and the C-terminus is a transmembrane region, comprising 16 discrete β -barrel transmembrane chains. As a part of the outer membrane assembly complex, YaeT is primarily involved in the proper assembly of outer membrane proteins [13]. Loss of the YaeT protein is fatal to *Escherichia coli* because it causes a series of outer membrane proteins (including TolC, OmpF, OmpC, and OmpA) to fail to assemble correctly on the outer membrane [3]. Reduced YaeT expression causes a reduction in the bacterial sensitivity to contact-dependent growth inhibitors and the downregulation of outer membrane protein synthesis [1]. The YaeT protein is also associated with cell adhesion. In previous studies, we showed that this protein is highly conserved in *E. coli*, *Salmonella*, and *Shigella*. When mice were immunized with a recombinant *E. coli* YaeT protein, the sera they produced had high affinity for *E. coli*, *Salmonella*, and *Shigella* and a strong immunoprotective effect on mice after *E. coli* challenge [8]. However, the immunoprotective effect of this protein against *RA* has not been studied.

Bioinformatic analysis is used to predict and analyze unknown protein structure, physical and chemical properties, functions, etc., and provides certain guidance for subsequent protein study. Our previous results also proved that the bioinformatic analysis of vaccine candidate proteins is helpful for vaccine development. In this study, the outer membrane protein YaeT of *RA* was analyzed with bioinformatics and the recombinant protein was expressed and purified. Its immunoprotection against different *RA* infections was determined to develop an ideal vaccine candidate against *RA*.

Materials and Methods

Experimental Animals and *RA* Strains

One-day-old Cherry Valley ducklings were obtained from the Hainan Tianyuan Industrial Co., Ltd. (Haikou, China). The ducks were housed in cages, and provided water and food

ad libitum during the study. The animal experiments were approved by Hainan University Institutional Animal Care and Use Committee.

RA strain ATCC 11,845 was purchased from Shanghai Yiyan Biological Technology Co. Ltd. (Shanghai, China). Serotype 1 *RA* strain CH-1 (*RA*-CH1) was isolated from sick ducks in Haikou, China, and grown in tryptic soy broth medium (TSB) at 37 °C. *E. coli* strain DH5 α and BL21 (DE3) strains were purchased from TransGen Biotech, Inc. (Beijing, China) and grown on Luria–Bertani (LB) agar at 37 °C.

Protein Sequence

The sequence of YaeT (UniProt Accession no. E4TA62) of *RA* was retrieved from the Universal Protein Resource (UniProt) at <http://www.uniprot.org> saved in FASTA format. The status of the protein belonged to the computer-annotated TrEMBL section up to now (unreviewed). The protein was from *RA* strain ATCC 11,845 (DSM 15,868/JCM 9532/NCTC 11,014).

Homology Analysis

Sequence alignments of YaeT were carried out using the National Centre for Biotechnology Information (NCBI) at <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. Representative sequences with identity higher than 50% were selected and phylogenetic tree was constructed by MEGA X with maximum likelihood method [14].

Physicochemical Property Analysis

Different physicochemical parameters of the YaeT protein, including number of amino acids, molecular weight, theoretical pI, amino acid (aa) composition, extinction coefficients, estimated half-life, instability index, aliphatic index, and hydrophobicity, were predicted using the ExPASy ProtParam online server (<https://web.expasy.org/protparam/>).

Transmembrane Domain Prediction

PRED-TMBB tool (<http://biophysics.biol.uoa.gr/PRED-TMBB/>) was used to predict transmembrane domains of YaeT with Viterbi method, N-best method, and posterior decoding method. Subcellular location of the YaeT was predicted by QuickGO online services (<https://www.ebi.ac.uk/QuickGO/>).

Structure Prediction

Homology modeling of the protein was carried out by the SWISS-MODEL server at <https://www.swissmodel.expasy.org/>. The best homology models were selected according to Global Model Quality Estimation (GMQE) and QMEAN statistical parameters. The predicted model was then analyzed by Swiss-PdbViewer 4.1 software. The 3D structure of YaeT was also predicted in AlphaFold Protein Structure Database (<https://alphafold.ebi.ac.uk/>), by the mean of AlphaFold 2 [11, 29]. The 3D structure predictions of the two methods were then evaluated and superposed by in TM-align tool (<https://zhanggroup.org/TM-align/>), to get root-mean-square deviation (RMSD) and template modeling (TM) scores.

B-Cell Binding Epitope Prediction

The B-cell (<http://tools.immuneepitope.org/bcell/>) server was applied for linear B-cell epitope prediction based on physicochemical properties such as hydrophilicity, flexibility, polarity, and exposed surface on a non-redundant dataset. Discontinuous epitope was predicted by ElliPro (<http://tools.iedb.org/ellipro/>) server, with minimum score of 0.5, and maximum distance of 6 Å and number of residues of more than 7.

Expression of YaeT Protein

The construction of recombinant plasmids, recombinant protein expression in *E. coli*, and purification of the recombinant protein were performed as described previously [7]. The primers, F-BamHI-YaeT: 5'-GGATCCGGAGATGGGCAAGTATTATCATT-3' and R-EcoRI-YaeT: 5'-GAATTCCTCTATTGTTCTATCAAATCCGTAAGCA-3', were used for amplification of YaeT gene of ATCC 11,845. Briefly, the amplified YaeT gene and pET-28a vector were digested and purified by BamHI and EcoRI, and then ligated by T4 DNA ligase. The recombinant YaeT-28a plasmid was transformed into *E. coli* BL21 strain, and then transformed into self-induction medium after activation, cultured at 37 °C for 24 h. Purification and refolding of the recombinant protein were performed based on the previous protocol [7]. The bacterial cells were lysed by ultrasonic lysis on ice with lysis buffer and the lysed cells were centrifuged at 14,000 g for 20 min, and the supernatant was removed. Each gram of inclusion bodies was incubated in 50 ml Tris-HCl, pH 7.9 with 1.5% (v/v) lauryldimethylamine oxide (LDAO), and shaken at 20 °C for 1 h. The inclusion bodies were precipitated by centrifugation at 16,000 g for 30 min. Inclusion bodies were dissolved in 50 mM, Tris-HCl solution, pH 7.9, and centrifuged at 16,000 g for 30 min, and the supernatant was removed. The sediment was added with denaturation buffer (10 mM Tris-HCl buffer, pH 7.5 containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 8 M urea), and then centrifuged at 14,000 g for 20 min. The supernatant was gradually added with the refolding solution (20 mM Tris-HCl buffer, pH 7.9 containing 1 M NaCl and 5% (v/v) LDAO) to 1:1 with quick stirring for 1 h. Then, the mixture was dialyzed in 4 L permeabilization buffer (20 mM Tris-HCl buffer, pH 7.9 containing 0.5 M NaCl and 0.1% (v/v) LDAO) for 6–8 h. The mixture was then purified by HisTrap column and desalted by HPrepTM26/10 desalting column. The purity of the YaeT protein was detected by SDS-PAGE electrophoresis, and then freeze dried and stored at –20 °C.

Animal Experiments

Thirty healthy 1-day-old Cherry Valley ducks were randomly divided into 3 groups of 10 ducks in each group. PBS group was immunized with PBS; PBS+adjuvant group was immunized with PBS and complete Freund's adjuvant (catalogue number: F5881); YaeT group was immunized with YaeT protein and complete Freund's adjuvant. The ducks were raised to 7-day-old to start the first immunization. The lyophilized YaeT protein was diluted with PBS and mixed thoroughly with complete Freund's adjuvant in a 1:1 ratio. Each duck was injected subcutaneously with 0.2 mg YaeT protein. The second immunization started at 14 days of age, and the diluted YaeT protein was mixed with the incomplete Freund's adjuvant (catalogue number: F5506) in a 1:1 ratio. Blood was collected at 6-day-old, 13-day-old, 20-day-old, 27-day-old, and 34-day-old, and the serum was stored at –20 °C.

Indirect Enzyme-Linked Immunosorbent Assay (iELISA)

The iELISA was performed as described previously [8]. In short, 100 μl of 2 $\mu\text{g/ml}$ YaeT protein and 10^8 CFU of *RA* strains were added to each well respectively, incubated overnight at 4 $^{\circ}\text{C}$, and washed 4 times with 100 μl PBST buffer (1 \times PBS [pH 7.4] and 0.5% Tween 20) washing solution, 5 min each time. The antigen was then blocked, and added with series dilution of duck serum and horseradish enzyme-labeled polyclonal rabbit anti-duck IgY (IgG). 1 \times TMB was added as chromogenic reagent for 30 min and then added with 2 M H_2SO_4 . The OD value of each well was measured at a wavelength of 450 nm. The ratio of the OD value of the test well to the negative control well (P/N) is greater than 2.1 as positive.

Serum Bactericidal Assay (SBA)

A single colony of *RA-CH1* strain was inoculated into TSB liquid medium and cultivated to OD 600=1.5. After washing, the cells were diluted with PBS to 10^8 CFU/ml. Duck serum was diluted with PBS to a series of concentrations of 100%, 50%, 25%, 12.5%, and 5%. Heat-inactivated 100% duck serum and PBS were used as controls. Ten microliters of diluted bacterial solution was mixed with 190 μl of duck serum of different concentrations, and incubated at 37 $^{\circ}\text{C}$ for 30 min. The survival rate of bacteria was counted by the plate counting method.

Opsonophagocytosis Assay

A single colony of *RA-CH1* strain was inoculated into TSB liquid medium and cultivated to OD 600=1.5. After washing, the cells were diluted with PBS to 5×10^8 CFU/ml. Twenty microliters of test serum and 20 μl of bacterial solution were added to a 96-well cell plate and incubated in a 5% carbon dioxide incubator at 37 $^{\circ}\text{C}$ for 15 min. Ten microliters of duck serum complement and 40 μl of whole duck blood were added to the cell plate, and incubated for 1 h at 37 $^{\circ}\text{C}$ in a 5% carbon dioxide incubator. The survival rate of bacteria was counted by the plate counting method.

Challenge Assay

The *RA-CH1* strain was inoculated on a blood plate and cultured in a CO_2 incubator at 37 $^{\circ}\text{C}$ for 12 h. Single colony was inoculated in 5-ml TSB medium and cultured with shaking at 37 $^{\circ}\text{C}$ for 18 h. The amount of bacteria was determined by a McDonald's turbidity tube. The bacterial suspension is diluted 10 times with PBS buffer. Forty-eight healthy 31-day-old Cherry Valley ducks were randomly divided into 8 groups with 6 ducks in each group. The dilutions of bacterial suspension were injected into leg muscles of different groups of ducks. The mortality rate was recorded and calculated, and lethal dose of 50% (LD_{50}) of *RA-CH1* strain was calculated by the modified Karber method.

At 14 days after the second immunization of the duck (31 days old), 0.5 ml of 10 times LD_{50} dose of *RA-CH1* bacterial suspension was injected intramuscularly in the leg. The mortality rate after the challenge was recorded.

Statistical Analysis

GraphPad Prism 8.0 was used to analyze the data of each experiment. ANOVA statistical method was used for the statistical analysis, where the symbol “*” indicates significant difference ($0.01 < P < 0.05$) and “***” indicates extremely significant difference ($P < 0.01$); $P > 0.05$ indicates no significant difference.

Results

Homology Analysis

The aa sequence of YaeT protein in RA was analyzed with a BLAST search in the NCBI database, which showed that it shares > 50% identity with homologues from *R. anatipestifer* (identity > 97%), *Chryseobacterium* spp. (identity 71–73%), *Elizabethkingia* sp. (identity 70%), and *Ornithobacterium rhinotracheale* (identity 71–73%). The aa sequences of the YaeT proteins were downloaded and a phylogenetic tree was constructed (Fig. 1). The tree showed that the YaeT protein is conserved across the different serotypes of RA.

Physicochemical Property Analysis

The physicochemical properties of the 850 aa of YaeT were analyzed with the ExPasy ProtParam online server, which showed that the initial 19 s constituted the signal peptide, and the molecular weight of the protein was 93,518.73 Da. The total number of negatively charged residues (Asp + Glu) was 84, and the total number of positively charged residues (Arg + Lys) was 105. The theoretical isoelectric point (pI) was

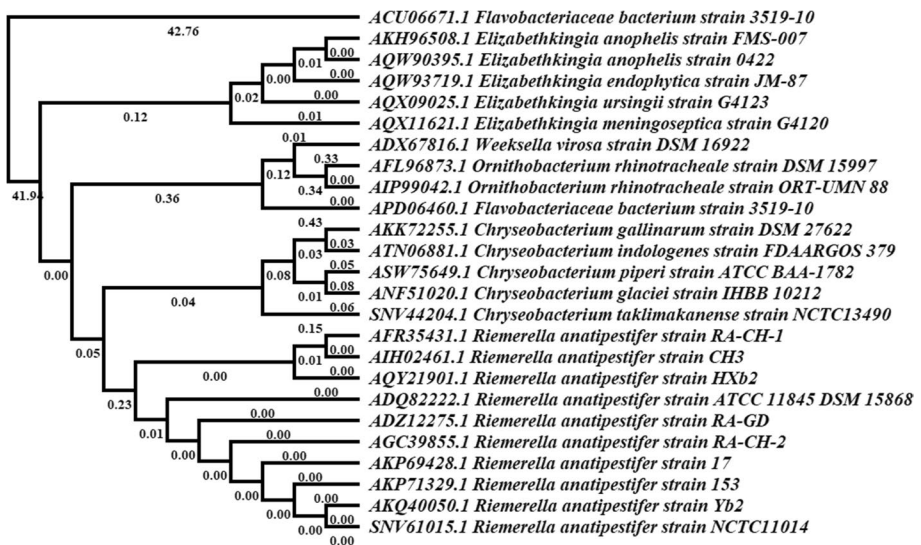


Fig. 1 Phylogenetic tree analysis of YaeT protein in different strains

9.35 and the estimated half-life in *E. coli* was 10 h. The extinction coefficient was 117,120. The instability index (II) was computed to be 27.95, which classifies the protein as stable. The aliphatic index of the protein was 76.68, and the grand average of hydropathicity (GRAVY) was -0.476 . The results of a hydrophobicity analysis performed with the Kyte–Doolittle method are shown in Fig. 2a.

Transmembrane Domains and Subcellular Localization Prediction

The transmembrane domains of YaeT were predicted with the Viterbi method, the N-best method, and the posterior decoding method, and are shown in Table 1. The Viterbi method and N-best method shared the same prediction in 1–828 aa domain, whereas the Viterbi method and the decoding method shared the same prediction in 829–847 aa domain. The domain predicted with the Viterbi method and visualized with the TMRPres2D tool is shown in Fig. 2b. The QuickGO program predicted that the subcellular location of YaeT is the outer membrane.

Fig. 2 Hydrophobicity and subcellular localization analysis of YaeT protein. **a** Hydrophobicity analysis of YaeT protein with the Kyte–Doolittle method. **b** Subcellular localization of YaeT protein by TMRPres2D

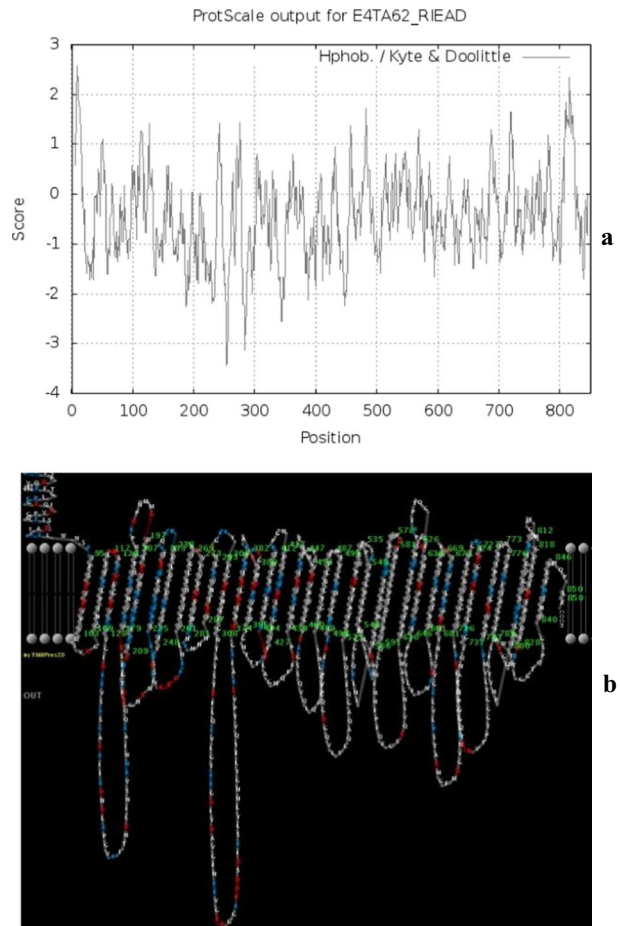


Table 1 Transmembrane domain prediction

Transmembrane domains	Position (Viterbi method)		Position (N-best method)		Posterior (decoding method)	
in	1	94	1	94	1	58
tm	95	103	95	103	59	69
out	104	108	104	108	70	94
tm	109	117	109	117	95	103
in	118	119	118	119	104	108
tm	120	128	120	128	109	117
out	129	178	129	178	118	178
tm	179	187	179	187	179	187
in	188	196	188	196	188	196
tm	197	209	197	209	197	209
out	210	224	210	224	210	222
tm	225	233	225	233	223	233
in	234	237	234	237	234	237
tm	238	248	238	248	238	248
out	249	260	249	260	249	266
tm	261	269	261	269	267	281
in	270	272	270	272	282	286
tm	273	281	273	281	287	293
out	282	286	282	286	294	438
tm	287	293	287	293	439	447
in	294	299	294	299	448	451
tm	300	308	300	308	452	460
out	309	373	309	373	461	524
tm	374	382	374	382	525	535
in	383	388	383	388	536	539
tm	389	395	389	395	540	546
out	396	403	396	403	547	565
tm	404	412	404	412	566	578
in	413	416	413	416	579	580
tm	417	427	417	427	581	591
out	428	438	428	438	592	615
tm	439	447	439	447	616	626
in	448	453	448	453	627	637
tm	454	460	454	460	638	646
out	461	478	461	478	647	762
tm	479	487	479	487	763	773
in	488	489	488	489	774	776
tm	490	498	490	498	777	784
out	499	524	499	524	785	799
tm	525	535	525	535	800	812
in	536	539	536	539	813	817
tm	540	546	540	546	818	828
out	547	565	547	565	829	839
tm	566	578	566	578	840	846

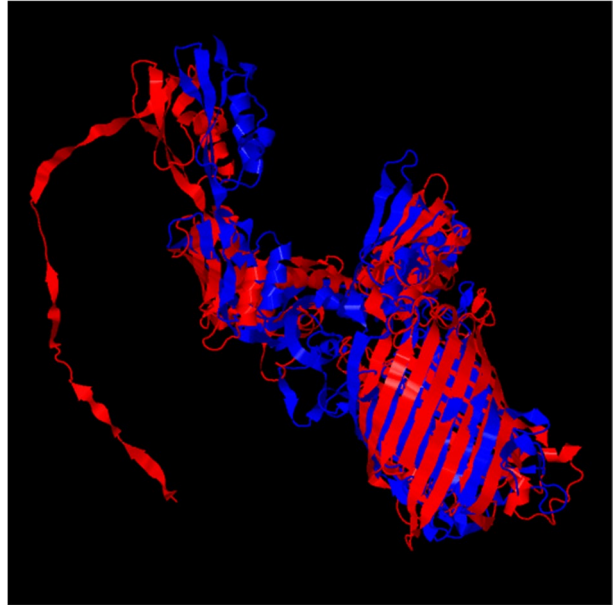
Table 1 (continued)

Transmembrane domains	Position (Viterbi method)		Position (N-best method)		Posterior (decoding method)	
in	579	580	579	580	847	850
tm	581	591	581	591		
out	592	615	592	615		
tm	616	626	616	626		
in	627	637	627	637		
tm	638	646	638	646		
out	647	660	647	660		
tm	661	669	661	669		
in	670	672	670	672		
tm	673	681	673	681		
out	682	715	682	715		
tm	716	724	716	724		
in	725	726	725	726		
tm	727	737	727	737		
out	738	762	738	762		
tm	763	773	763	773		
in	774	775	774	775		
tm	776	784	776	784		
out	785	799	785	799		
tm	800	812	800	812		
in	813	817	813	817		
tm	818	828	818	828		
out	829	839	829	850		
tm	840	846				
in	847	849				
tm	850	850				

Structure Prediction

The 3D structure was predicted by SWISS-MODEL [30] server and AlphaFold Protein Structure Database, and superposed by TM-align tool, which was shown in Fig. 3. The global model quality estimation (GMQE) and qualitative model energy analysis (QMEAN) values with SWISS-MODEL were 0.56 and -3.30 , respectively. The Ramachandran favored value was 92.02%. The predicted three-dimensional (3D) structure was also predicted in AlphaFold Protein Structure Database based on AlphaFold method. Except for 1–41, 329–242 (pLDDT < 50), 131–138, 238–245, 342–347, 475–477, 498–503, 723–725, 847–850 ($70 > \text{pLDDT} > 50$), model confidence of remaining amino acid residues in AlphaFold Protein Structure Database were high ($90 > \text{pLDDT} > 70$) and very high ($\text{pLDDT} > 90$). RMSD and TM score of the two predictions were 7.11 and 0.64. Superposition of the two predicted protein structures showed that the protein comprised α -helices, random coils, and β -sheets that form the β -barrel protein, a representative structure of the outer membrane proteins of Gram-negative bacteria.

Fig. 3 Superposition of 3D structure prediction of YaeT protein. Blue: prediction by SWISS-MODEL. Red: prediction by AlphaFold 2



Predicted B-Cell Binding Epitopes

Twenty-eight, 16, and 33 linear B-cell epitopes were predicted in the YaeT protein with Bepipred-2.0, a linear epitope predictor, Emini's surface accessibility prediction, and the Kolaskar and Tongaonkar [12] antigenicity method, respectively. When short sequences (less than 7 aa) were excluded, the three methods predicted 15 epitopes in the same sequence, 10 of which were located outside the cell membrane. The linear B-cell epitopes are listed in Table 2. Seven discontinuous epitopes were predicted, but only numbers 2–5 were outside the outer membrane. The residues and 3D structures of the epitopes are shown in Table 3 and Fig. 4, respectively.

Table 2 Combined of prediction linear epitopes

Start	End	Peptide	Length
129	140	VGIDKKVSKSKA	12
160	166	INSVKHN	7
279	287	VWRNKNNNY	9
341	356	DGGKEDSDIKSLYLN	16
360	370	LFSQVTPVEKA	11
461	473	VEKGSSQVQLQAG	13
506	520	QGDGQVLSLQAQAGQ	15
547	554	NNSIVKYA	8
651	660	SLFSNKDYST	10
683	693	NTVVGKLVLR	11

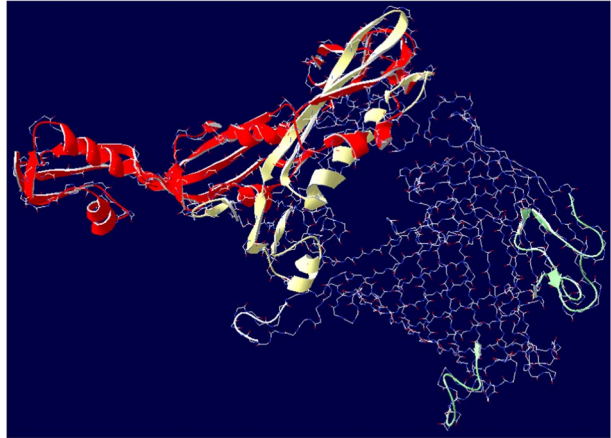
Table 3 Discontinuous epitope prediction of YaeT protein

No	Residues	Number of residues	Score
1	A:R562, A:N597, A:N598	3	0.801
2	A:Y46, A:I47, A:L48, A:K49, A:D50, A:I51, A:V52, A:V53, A:D54, A:G55, A:I56, A:K57, A:K58, A:Y59, A:S60, A:P61, A:D62, A:Q63, A:V64, A:L65, A:R66, A:F67, A:T68, A:G69, A:L70, A:R71, A:K72, A:G73, A:E74, A:Q75, A:I76, A:E77, A:V78, A:P79, A:G80, A:Q81, A:K82, A:I83, A:S84, A:E85, A:A86, A:I87, A:K88, A:K89, A:L90, A:W91, A:N92, A:S93, A:K94, A:Y95, A:F96, A:E98, A:V99, A:E100, A:V101, A:Y102, A:V103, A:Q104, A:S105, A:V106, A:D107, A:G108, A:Q109, A:N110, A:I111, A:V112, A:L113, A:R114, A:F115, A:L116, A:L117, A:E118, A:D119, A:L120, A:K121, A:D122, A:L123, A:G124, A:E125, A:V126, A:K127, A:V128, A:V129, A:G130, A:I131, A:D132, A:K133, A:K134, A:V135, A:S136, A:K150, A:P151, A:G152, A:V153, A:K154, A:I155, A:T156, A:N157, A:D158, A:I160, A:N161, A:K164, A:F176, A:S177, A:D178, A:A179, A:K180, A:V181, A:N182, A:V183, A:K184, A:N185, A:T186, A:A187, A:N188, A:P189, A:K190, A:D191, A:P192, A:N193, A:M194, A:M195, A:D196, A:W197, A:T198, A:I199, A:E200, A:V201, A:Q202, A:R203, A:G204, A:K205, A:I207, A:N284, A:N285, A:N286	137	0.796
3	A:D210, A:R211, A:T213, A:F214, A:E215, A:G216, A:N217, A:E218, A:N219, A:I220, A:S221, A:S222, A:K223, A:K224, A:K227, A:K228, A:Y264, A:L267, A:G268, A:Y269, A:R270, A:D271, A:A272, A:V273, A:I274, A:V275, A:S276, A:S278, A:W280, A:R281, A:N282, A:E288, A:N290, A:V291, A:K292, A:L293, A:N294, A:E295, A:G296, A:K297, A:K298, A:Y299, A:Y300, A:I301, A:G302, A:D303, A:I304, A:N305, A:F306, A:I307, A:G308, A:N309, A:T310, A:A311, A:Y312, A:S313, A:T314, A:E315, A:F316, A:L317, A:V320, A:L321, A:G322, A:Y323, A:K324, A:K325, A:G326, A:D327, A:I328, A:Y329, A:D330, A:F334, A:K336, A:K337, A:V338, A:G339, A:D349, A:I350, A:L353, A:Y354, A:N356, A:N357, A:G358, A:Y359, A:L360, A:F361, A:S362, A:Q363, A:V364, A:T365, A:P366, A:V367, A:E368, A:K369, A:A370, A:I371, A:K372, A:G373, A:D374, A:S375, A:I376, A:D377, A:L378, A:E379, A:I380, A:R381, A:I382, A:K383, A:E384, A:G385, A:E386, A:K387, A:A388, A:S389, A:W390, A:D391, A:R392, A:R413, A:P414, A:G415, A:N416, A:L417, A:F418, A:I445, A:K446, A:P447, A:N448, A:P449, A:Q450, A:N451, A:N452, A:T453	132	0.697

Table 3 (continued)

No	Residues	Number of residues	Score
4	A:F521, A:I550, A:K552, A:Y553, A:A554, A:D555, A:L556, A:T557, A:G558, A:G559, A:S560, A:Q561, A:L563, A:N564, A:F566, A:Y591, A:G593, A:Y594, A:N595, A:F596, A:F599, A:P600, A:F601, A:Q602, A:F603, A:G604, A:S605, A:E606, A:Q607, A:Y608, A:S609, A:N610, A:G611, A:S612, A:A613, A:N614, A:N615, A:F616, A:F646, A:T647, A:P648, A:P649, A:Y650, A:S651, A:L652, A:F653, A:S654, A:N655, A:K656, A:D657, A:Y658, A:S659, A:T660, A:M661, A:S662, A:T663, A:A664, A:A665, A:K666, A:Y667, A:N668, A:W669, A:L670, A:E671, A:E672, A:Y673, A:D701, A:G702, A:Y703, A:N704, A:R705, A:Q706, A:L707, A:G708, A:A709, A:P710, A:P711, A:E713, A:R714, A:G737, A:Y738, A:Q739, A:N740, A:A741, A:S742, A:M743, A:T744, A:G745, A:G746, A:L747, A:T748, A:E749, A:D750, A:V751, A:T752, A:P753, A:T754, A:G755, A:G756, A:G757, A:T758, A:W791, A:N792, A:S793, A:F794, A:S795, A:T796, A:Y797, A:N798, A:P799, A:F800, A:Q801, A:L802, A:K803, A:R804, A:V806, A:F824, A:A825, A:Y826, A:G827, A:F828, A:D829, A:R830, A:T831, A:I832, A:G833, A:G834, A:T835, A:Q836, A:P837, A:S838, A:G839, A:W840, A:N841, A:T842	135	0.682
5	A:G498, A:K499, A:D500, A:F501, A:K502, A:P503, A:V504	7	0.594
6	A:R493, A:N494, A:F495, A:L496	4	0.551
7	A:I771, A:S772, A:M773, A:N774, A:Q775, A:T776	6	0.522

Fig. 4: 3D structure of the discontinuous epitopes in YaeT protein. Red, no. 2; yellow, no. 3; green, no. 4; white, no. 5



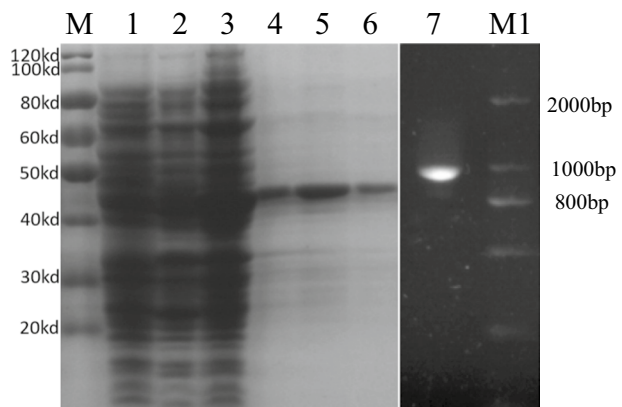
Expression and Purification of YaeT Recombinant Protein

YaeT gene was cloned from ATCC 11,845 strain and sequenced. The sequence of this gene was with 100% identity to which in CH-1 serotype. The gene was then inserted into pET-28a. The recombinant protein was expressed in BL21 cells. After purification, the purity of the protein was about 85%, which was analyzed by gel electrophoresis and shown in Fig. 5.

YaeT Protein Induces Production of High-Affinity Antibodies

The results of iELISA are shown in Fig. 6. After immunization with YaeT protein, the antibody content in duck serum has been increasing, reaching the highest level at 14 days (27 days old) after the second immunization. The antibody titers to YaeT protein and RA strains reached 1:583,200 and 1:194,400, respectively. The antibody titers in the PBS group and the PBS + adjuvant group did not exceed 1:300. It showed that the YaeT protein has stimulated the production of high levels of antibodies in ducks.

Fig. 5 Expression and purification of YaeT. M, Prestained Protein Ladder; 1, Empty pet-28 a carrier; 2, BL 21 empty strain; 3, self-induced expression of recombinant expression plasmid in *E. coli* BL 21; 4–6, purification of YaeT protein; 7, PCR products of YaeT. M1: D2000 DNA Marker



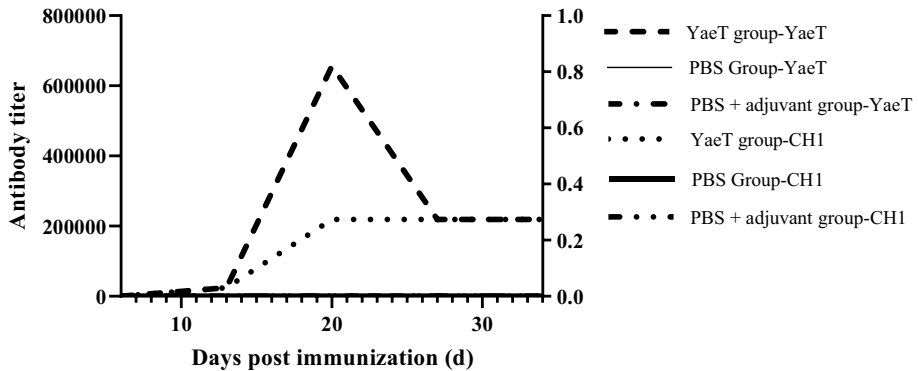


Fig. 6 Sera antibody titers of YaeT protein analyzed by iELISA. YaeT group-YaeT—the antibody titer of YaeT and adjuvant vaccination group against YaeT. PBS group-YaeT—the antibody titer of PBS vaccination group against YaeT. PBS + adjuvant group-YaeT—the antibody titer of PBS and adjuvant vaccination group against YaeT. YaeT group-CH1—the antibody titer of YaeT and adjuvant vaccination group against *RA* CH-1 strain. PBS group-CH1—the antibody titer of PBS vaccination group against *RA* CH-1 strain. PBS + adjuvant group-CH1—the antibody titer of PBS and adjuvant vaccination group against *RA* CH-1 strain

Serum Antibodies Could Promote Complement to Kill Pathogens

The *RA* strain was mixed with different concentrations of immune serum. The SBA results are shown in Fig. 7a. The number of colonies in the YaeT group decreased with increasing serum concentration. The number of colonies with 50% and 100% YaeT serum concentrations was lower than that of PBS group or PBS + adjuvant group, and the difference is extremely significant ($P < 0.01$). The number of colonies with 25% YaeT serum concentration was lower than that of PBS group or PBS + adjuvant group, and the difference was significant ($P < 0.05$). This result showed that serum antibodies could promote complement to kill pathogens *in vitro*.

Serum Antibodies Could Promote Phagocytosis of *RA* Strain

The results of phagocytosis are shown in Fig. 7b. After incubation, compared with the PBS group (100%) and the PBS + adjuvant group (58.3%), the percentage of colonies in the YaeT group was 2.08%, the difference was very significant ($P < 0.01$). This result shows that YaeT protein-immunized serum could promote phagocytosis of *RA* strain.

YaeT Protein Immunization Reduced the Mortality of *RA*-Infected Ducks

At 14 days (31 days old) after the second immunization, ducks were injected intramuscularly in the legs with 10 times of LD_{50} dose of the *RA* (2.5×10^6 CFU) each, and the survival rate was shown in Fig. 8. After challenge, the survival rate was 40% in the PBS group, 50% in the PBS + adjuvant group, and 90% in the YaeT group. This result showed that ducks vaccinated with YaeT protein could improve the survival rate of ducks after the *RA* infection.

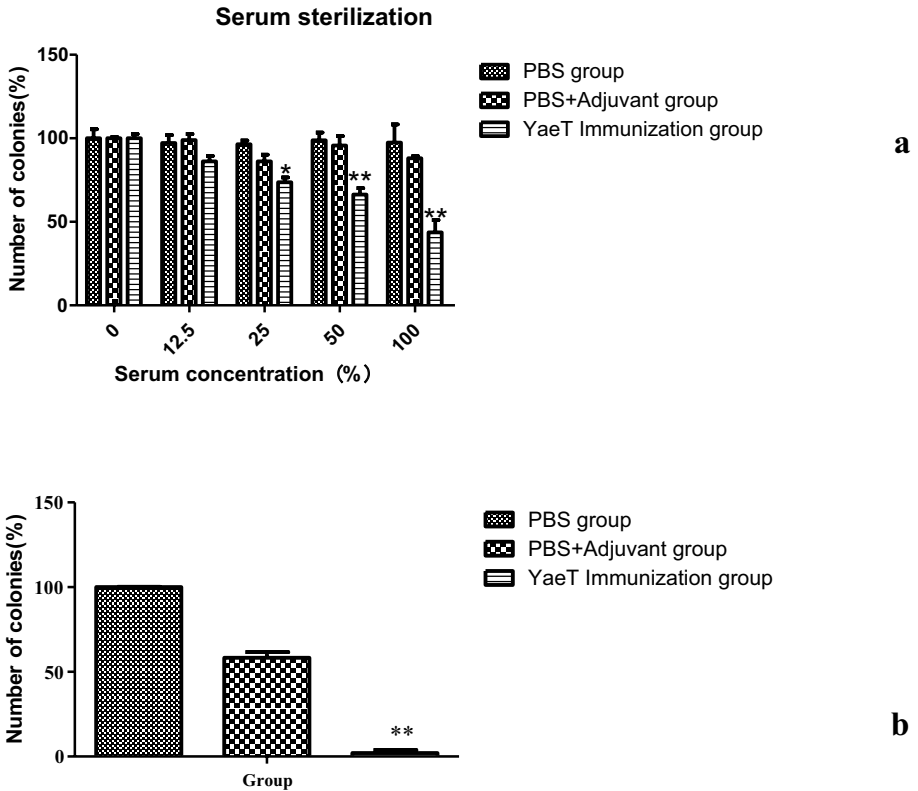
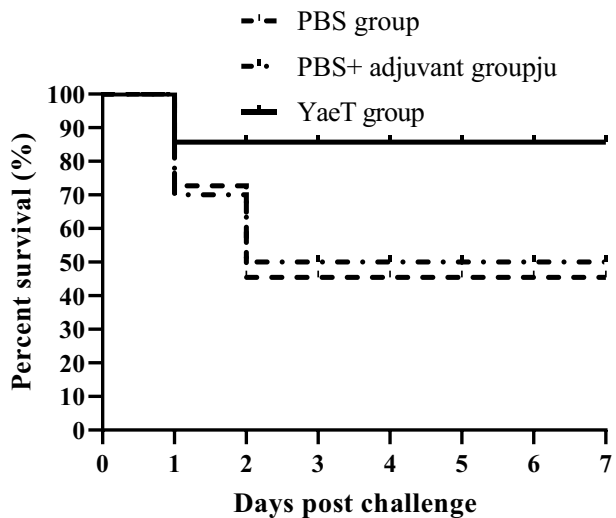


Fig. 7 Bactericidal activity and phagocytosis of the anti-YaeT sera in vitro. **A** Bactericidal activity of the anti-YaeT sera in vitro. *RA* CH-1 strain was incubated with serial dilution of YaeT group sera, PBS + adjuvant group sera and PBS group sera. **B** Phagocytosis of the anti-YaeT sera in vitro. *RA* CH-1 strain was incubated with complement, whole blood, and sera of different groups

Fig. 8 Protection efficacy of the anti-YaeT sera against *RA* CH-1 strain in vivo



Discussion

RA is harmful to waterfowl. Subunit vaccine has the advantages of good conservation and low harm to animals, so it is a good candidate for *RA* vaccine. OmpA protein is considered to have a certain immune protection [33]. However, there are few studies on *RA* outer membrane proteins, many of which were sequenced without expression and functional verification. This makes it difficult for *RA* protein to be used in a vaccine. Bioinformatics provides an analytical tool used to identify vaccine antigens in *Staphylococcus aureus* [28], *E. coli* [25], *Burkholderia pseudomallei* [22], and other pathogens [21, 23, 24]. Therefore, in this paper, bioinformatics tools were used to analyze the *RA* outer membrane protein YaeT.

An ideal vaccine should meet the following requirements. Firstly, this vaccine should confer strong immune protection. To evaluate the antigenicity of proteins, their B-cell epitopes are usually analyzed. There are many algorithms for predicting discontinuous epitopes, and the results obtained here with different programs were not identical. In this study, various models, including BepiPred-2.0 [10], Emini's surface accessibility prediction [5], and the Kolaskar and Tongaonkar antigenicity methods, were used to ensure comprehensive prediction. The iELISA assay showed that Bama protein in *RA* contains high-affinity epitopes, which can stimulate humoral immune response in ducks. This is consistent with our bioinformatics analysis. Secondly, effective epitopes must be extracellular. So, the Viterbi method, N-best method, and posterior decoding methods [2] were used to analyze the topological structure of the YaeT protein at the outer membrane. These continuous and discontinuous epitopes require further screening, in combination with the topology of this outer membrane protein. The epitopes in the extracellular domain could be selected, whereas epitopes in the transmembrane and intracellular regions must be removed. The duck serum immunized with the recombinant YaeT protein had affinity for the protein and *RA*-CH1 strain, indicating that the predicted YaeT protein is actually located in the outer membrane.

In this paper, the conservation of the YaeT protein was also analyzed based on sequence similarities and it was found that the protein shares >97% similarity among the *RA* serotypes, but <75% with those of other genera. A phylogenetic tree showing the genetic distances between the YaeT proteins within *RA* and between *RA* and other bacteria confirmed that this protein is conserved in *RA*. If an antigen is strongly expressed in the pathogen and is well conserved throughout the bacterial serotypes, it may be a candidate vaccine antigen. In previous studies, we showed that some OmpA and BamA outer membrane proteins are conserved in *E. coli*, *Salmonella*, and *Shigella*, and exert good cross-immunoprotection against these bacteria. Antibodies from ducks immunized with the rOmpA of *RA* could detect *RA* serotypes 1 and 6 in western blot analysis [20]. The ATCC 11,845 strain shares the same YaeT gene as the CH-1 serotype strain. Therefore, we speculate that the recombinant YaeT protein also has an immunoprotection against the CH-1 serotype *RA* strain.

To understand the YaeT protein in more detail, its physical and chemical properties and structures were analyzed. The 3D structure was predicted by SWISS-MODEL and AlphaFold 2. SWISS-MODEL is an automatic online software for predicting the tertiary structure of proteins by homology modeling. The more consistent the target sequence is with the template sequence, the more accurate the homology modeling method is. However, this method relies on the similar spatial structure of proteins in the database. AlphaFold 2 provides a computational method that can predict protein structures with atomic accuracy even if no similar structure is known [11], and has good application potential. The structures of the two prediction methods were evaluated by RMSD

value and TM score. RMSD value provides the average distance between alpha-carbon backbones of the two models, and higher RMSD values predict greater variant structure deviation. TM score provides the topological similarity between two proteins. If $0.5 < \text{TM score} < 1.00$, the predictions can be thought about the same fold [37]. Superposition of the two proteins showed that the protein forms a β -barrel structure, which is a typical structure for an outer membrane protein of the Gram-negative bacteria. This further confirms that the protein is an outer membrane protein. Because of the hydrophobic region, transmembrane proteins cannot fold correctly in aqueous solution. So, after the inclusion body is complex, LDAO is added as a mild decontamination agent, which is conducive to the correct folding of Bama protein.

In vivo and in vitro experiments also further confirmed that the Bama protein immunization of RA had protective effect on ducks. After the second immunization, the affinity of duck serum antibody to purified YaeT protein was 1:583,200, indicating that YaeT protein can stimulate a strong immune response in ducks. At the same time, the affinity of the serum to the RA strain was 1:194,400, indicating that the YaeT protein was distributed in the outer membrane of RA, and part of the structure was an extramembrane structure. This is consistent with previous bioinformatics analyses. SBA and opsonophagocytosis assays were used to analyze the killing of RA strains by duck serum antibodies. Within a certain range (25 ~ 100% of YaeT serum), the higher the antibody concentration, the stronger the killing effect of complement on RA. Antibodies and complement also enhanced phagocytosis of RA by phagocytes. After the second immunization, ducks in different groups were injected with the same dose of RA. On the second day after immunization, the duck survival rate in the YaeT group remained at 90%, indicating that the YaeT group had a strong immune protection effect. The survival rates of the PBS + adjuvant group and the control group remained at 50% and 40% respectively after the 3rd day, which may be due to the stimulating effect of the adjuvant, which partially enhanced the immunity of ducks. These results indicate that YaeT protein has a great potential to be developed into a RA vaccine.

Author Contribution HZ and QH conceived and designed research. QG and HZ conducted experiments. CL contributed new reagents or analytical tools. JZ and JW analyzed data. QG completed the writing and subsequent revisions of the manuscript. All authors read and approved the manuscript.

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Data Availability The data presented in the study are included in the article. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. Further inquiries can be directed to the corresponding authors.

Declarations

Ethics Approval The study was approved by the Animal Welfare and Ethical committee of the Hainan University.

Consent for Publication All the authors would like to submit the enclosed manuscript entitled “In silico analysis and immune response of YaeT protein against *Riemerella anatipestifer* in ducks,” which we wish to be considered for publication in Applied Biochemistry and Biotechnology.

Competing Interests The authors declare no competing interests.

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