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A homolog of glyceraldehyde-3-phosphate dehydrogenase from *Riemerella anatipestifer* is an extracellular protein and exhibits biological activity*

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Abstract: *Riemerella anatipestifer* is the causative agent of septicemia anserum exsudativa in ducks. Its pathogenesis and virulence factors are still unclear. The glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an anchorless and multifunctional protein on the surface of several pathogenic microorganisms, is involved in virulence and adhesion. Whether homologs of GAPDH exist, and display similar characteristics in *R. anatipestifer* (RaGAPDH) has not been determined. In our research, the RaGAPDH activity from various *R. anatipestifer* isolates was confirmed. Twenty-two *gapdh* genes from genomic DNA of *R. anatipestifer* isolates were cloned and sequenced for phylogenetic analysis. The distribution of RaGAPDH in *R. anatipestifer* CZ2 strain was confirmed by antisera to recombinant RaGAPDH. The ability of purified RaGAPDH to bind host proteins was analyzed by solid-phase ligand-binding assay. Results revealed that all *R. anatipestifer* isolates showed different levels of GAPDH activity except four strains, which contained a *gapdh*-like gene. The *gapdh* of *R. anatipestifer*, which is located phylogenetically in the same branch as enterohemorrhagic *Escherichia coli* (EHEC), belonged to class I GAPDH, and encoded a 36.7-kDa protein. All RaGAPDH-encoding gene sequences from field isolates of *R. anatipestifer* displayed 100% homology. The RaGAPDH localized on the extracellular membrane of several *R. anatipestifer* strains. Further, it was released into the culture medium, and exhibited GAPDH enzyme activity. We also confirmed the binding of RaGAPDH to plasminogen and fibrinogen. These results demonstrated that GAPDH was present in *R. anatipestifer*, although not in all strains, and that RaGAPDH might contribute to the microorganism's virulence.

Key words: *Riemerella anatipestifer*, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Extracellular protein
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1 Introduction

Riemerella anatipestifer is a Gram-negative, non-motile, non-spore-forming, rod-shaped bacterium, belonging to the family Flavobacteriaceae in the ribosomal RNA (rRNA) superfamily V based on 16S rRNA gene sequence analysis (Segers *et al*., 1993; Subramaniam *et al*., 1997; Tsai *et al*., 2005). It causes an acute or chronic primary septicemic disease in domestic ducks, geese, turkeys, and other wild birds, characterized by fibrinous pericarditis, perihepatitis, air sacculitis, caseous salpingitis, meningitis, and accounts for major economic losses to the duck-rearing industry (Leavitt and Ayroud, 1997). Currently, 16 serotypes of *R. anatipestifer* have been isolated and identified in China among the 21 *R. anatipestifer* serotypes described to date (Pathanasophon *et al*., 1995; 2002; Subramaniam *et al*., 2000). Among the known serotypes of *R. anatipestifer*, there are huge variations in the virulence between serotypes and between different strains even within the same serotype (Subramaniam *et al*., 2000). Although four genome sequences of *R. anatipestifer* of strains ATCC 11845 (Mavromatis *et al*., 2011), RA-GD

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(Yuan *et al*., 2011), RA-YM (Zhou *et al*., 2010), and RA-SG (Yuan *et al*., 2013) have been reported, little is known about the molecular basis of the pathogenicity of *R. anatipestifer* infection, and so far, few virulence factors have been characterized other than virulence-associated protein D (VapD) (Chang *et al*., 1998), CAMP cohemolysin (Crasta *et al*., 2002), and outer membrane protein A (OmpA) (Hu *et al*., 2011).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a classical glycolytic enzyme converting D-glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate. Members of the GAPDH family are classified into the ubiquitous class I enzymes that utilize nicotinamide adenine dinucleotide (NAD^+) (EC 1.2.1.12), $NADP^+$ (EC 1.2.1.13 or 1.2.1.59), class II of archaeal $NAD(P)^+$-dependent GAPDHs, and class III bifunctional enzymes (erythose-4-phosphate dehydrogenase/GAPDH) that are prevalent among γ-proteobacteria (Figge *et al*., 1999). In addition to its classical glycolytic roles, GAPDH is involved in a number of fundamental cellular pathways such as maintenance of DNA integrity, intracellular membrane trafficking, histone-gene regulation, receptor-mediated cell signaling, protection of telomeric DNA, post-transcriptional gene regulation, autophagy, apoptosis, and oxidative stress response; all these depend on the ability of GAPDH to modify its subcellular localization (Sirover, 2011). Thus, GAPDH is widely used as a model protein or control in gene regulation and catalytic-mechanism-related studies, as well as a standard in Northern- and Western-blots, because of its highly conserved structure across species (Zheng *et al*., 2003). Recent studies have demonstrated that GAPDH is presented on the surface of several microbial pathogens such as *Streptococcus agalactiae* (Seifert *et al*., 2003), *S. pneumonia* (Ling *et al*., 2004), *Listeria monocytogenes* (Schaumburg *et al*., 2004), enterohemorrhagic *Escherichia coli* (EHEC), and enteropathogenic *Escherichia coli* (EPEC) (Egea *et al*., 2007), and may facilitate their colonization and invasion of host tissues by interacting directly with host-soluble proteins and surface ligands (Kenny and Finlay, 1995; Pancholi and Chhatwal, 2003). Its roles are also implicated in some pathogenic microorganisms like *Candida albicans*, *Schistosoma bovis*, and *Mycoplasma genitalium* (Alvarez *et al*., 2007). Furthermore, secreted GAPDH by *S. pyogenes* and enteropathogenic *E. coli* strains (Kenny and Finlay, 1995; Eichenbaum *et al*., 1996; Aguilera *et al*., 2012) plays a role in their adhesion and virulence (Modun and Williams, 1999; Daubenberger *et al*., 2000; Modun *et al*., 2000; Parker and Bermudez, 2000; Nagradova, 2001; Schaumburg *et al*., 2004; Jin *et al*., 2005; Alvarez *et al*., 2007; Colell *et al*., 2007; Egea *et al*., 2007; Tunio *et al*., 2010). Although one copy homolog of GAPDH-encoding gene was identified in the genomes of *R. anatipestifer* strains ATCC11845, RA-GD, RA-CH-1, and RA-SG in our previous bioinformatics analysis, it remains to be addressed whether GAPDH from these strains is in the form of an extracellular protein and displays similar characteristics as reported in other pathogens.

In the present study, the genes of GAPDH from various *R. anatipestifer* (RaGAPDH) isolates were sequenced for phylogenetic analysis. We generated antisera to recombinant RaGAPDH of *R. anatipestifer* CZ2 strain, and identified the biologically active RaGAPDH secreted in the culture of *R. anatipestifer*.

2 Materials and methods

2.1 Bacterial strains, culture conditions, and genomic DNA extraction

Twenty-two *R. anatipestifer* field isolates from Sichuan Province and Chongqing Municipality in China are described in Table 1. The strains of *R. anatipestifer* were grown on trypticase soy agar (OXOID Ltd., UK) or cultured in tryptic soybean broth (TSB; OXOID Ltd., UK) at 37 °C in an atmosphere containing 5% CO_2. *E. coli* strains DH5α and BL21 (λDE_3), used as hosts for cloning and expression of *gapdh* gene from *R. anatipestifer*, respectively, were grown routinely on Luria broth (LB) agar or in LB broth at 37 °C. Genomic DNA (gDNA) was extracted from all the field isolates using the Mini-BEST bacterial gDNA extraction kit V.2.0 (TaKaRa Biotech Co., Ltd., China). The concentration and quality of the gDNA were established by optical density measurements at 260/280 nm.

2.2 Isolation of secreted proteins in culture medium

Soluble proteins in *R. anatipestifer* culture medium were isolated as previously described (Egea *et al*.,

2007) with some modification. Briefly, exponential phase cultures in TSB were diluted 1:50 in the indicated culture media and incubated at 37 °C in a 5% CO_2 atmosphere. At different times, the bacterial cells were removed by centrifugation (5000*g*, 10 min, 4 °C) and the supernatant removed. The proteins in the filtrate were precipitated with 15% trichloroacetic acid (TCA) by incubation on ice for 3 h. The protein pellet was washed in 90% ice-cold acetone, air-dried, and suspended in loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Table 1 Twenty-two *R. anatipestifer* strains

Isolate	Year of isolation	Serotype	Extracellular GAPDH activity*	Sequencing result (bp)
AF[a]	2001	2	0.536±0.038	996
CZ1[a]	2007	1	0.678±0.033	990
CZ2[a]	2003	1	0.484±0.019	992
DX1[a]	2013		0.009±0.002	
DY1109[a]	2011		0.215±0.012	994
DY1202[a]	2012		0.636±0.033	998
DY1210[a]	2012		0.478±0.035	989
DZ1108[a]	2011		0.628±0.024	987
DZ1209[a]	2012		0.573±0.030	964
DZ1212[a]	2012		0.341±0.032	991
gHN1210[b]	2012	2	0.371±0.027	992
gXJY12[b]	2012		0.545±0.027	990
JJ1212[a]	2012		0.309±0.025	991
LS[a]	2004		0.587±0.035	994
MY[a]	2002		0.427±0.024	996
PN1209[a]	2012		0.004±0.003	
RL[a]	2009		0.578±0.034	998
SC12[a]	2012	1	0.157±0.039	998
TL[a]	2010		0.624±0.038	991
WS1210[a]	2012		0.414±0.037	1001
YC1[a]	2012		0.006±0.002	
YC2[a]	2012		0.003±0.002	

[a] Isolated from duck; [b] Isolated from goose. * Data are shown as mean±standard error (SE) (n=3) of the absorbance at 340 nm determined by the method described in Section 2.3

2.3 Enzyme activity

The RaGAPDH activity on the bacterial cell surface was measured according to Egea *et al*. (2007) with some modification. Briefly, bacterial cells were centrifuged at 4500*g* for 5 min and the pellets were washed twice in 50 mmol/L Na_2HPO_4, pH 7.5, and then resuspended in the same buffer. Bacterial suspensions of 4×10^8 CFU (colony-forming unit)/200 μl were incubated with 14 μl glyceraldehyde-3-phosphate (50 mg/ml), 200 μl NAD^+ (10 mmol/L), and assay buffer (40 mmol/L triethanolamine, 50 mmol/L Na_2HPO_4, and 5 mmol/L EDTA; pH 8.6) in a final volume of 2 ml. After 15 min at 30 °C, cells were removed by centrifugation and the supernatant was examined for the presence of nicotinamide adenine dinucleotide (NADH) by measuring the absorbance at 340 nm. Control reactions were used without bacterial cells or glyceraldehyde-3-phosphate.

The GAPDH activity of the secreted protein mixture from three strains (two showed positive GAPDH activity on the surface of bacterial cells) was measured, following the increase in absorbance at 340 nm by the formation of NADH (Egea *et al*., 2007). Briefly, supernatants from *R. anatipestifer* bacterial cultures were passed through a 0.22-μm filter and precipitated with 95% $(NH_4)_2SO_4$. The protein pellets were resuspended in a buffer solution (50 mmol/L Na_2HPO_4, pH 7.5) and used for determining the enzymatic activity of secreted GAPDH.

2.4 Identification of the *gapdh* in *R. anatipestifer*

The GAPDH amino acid sequences of 13 species obtained from GenBank, including *Xenopus laevis* (P51469), *Gallus gallus* (P00356), *E. coli* (P0A9B4), *Homo sapiens* (P0034), *Drosophila melanogaster* (Q01597), *Trypanosoma brucei* (P00097), *Plasmodium falciparum* (AF030440), *Gonyaulax polyedra* (AF028562), *Leishmania mexicana* (Q01558), *Candida albicans* (Q92211), and three *R. anatipestifer* strains (ATCC11845 (CP003388), RA-CH-1 (NC018609), and RA-GD (CP002562)), were aligned and analyzed by the clustal W method of the MegAlign II program (DNAStar, Madison, WI). Two degenerate primers RaGAPDH1 and RaGAPDH2 (Table 2) based on the conserved region of the GAPDH amino acids were designed and used for polymerase chain reaction (PCR) amplification. The PCR amplifications were performed using the above primers and the Taq™ System (TaKaRa) under the following conditions: 30 cycles of denaturation (95 °C, 1 min), annealing (52 °C, 1 min), and extension (72 °C, 1 min). The first round PCR products (diluted 200 times) were amplified by the second PCR in a 50-μl reaction mixture under the same conditions. All the second round PCR products were purified using the MiniBest Agarose Gel DNA Extraction Kit Ver. 4.0 (TaKaRa) and inserted in the pMD19^R-T (TaKaRa) cloning vector, according to the manufacturer's instructions.

Table 2 Primers used in this study

Primer	Sequence
Primers for cloning *gapdh*	
RaGAPDH1	5'-GGNTTYGGNCGNATHGGNCGN-3' (N=A/G/C/T,Y=C/T,H=A/C/T)
RaGAPDH2	5'-YTCRTTRTCRTACCANGA-3' (Y=C/T, R=A/G, N=A/G/C/T)
Primers for genomic walking	
uSP1	5'-CGCACCTACTTCATTCCATTTTAGATTG-3'
uSP2	5'-CTCCATCAAATTTTCCGTGAACAGA-3'
uSP3	5'-CTAAACTTCCGATTCTTCCGAAACCG-3'
dSP1	5'-AGGTATGTCTTTCCGTGTACCAACG-3'
dSP2	5'-GGGAGAACTTAAAGGTATCCTTGGT-3'
dSP3	5'-AGTATCTTGGTACGATAACGAAACAGG-3'
Primers for expression	
Egap1F	5'-GAGGAATTCATGTCAACAATCAAAG-3' (*Eco*RI site underlined)
Egap1R	5'-ATTCTCGAGTAAAGAAGCAGAGTGT-3' (*Xho*I site underlined)

Plasmid DNA containing *gapdh* of *R. anatipestifer* was purified and sequenced at BGI Biotech Co., Ltd., China. Comparison and alignments of deduced amino acid sequences of GAPDH from *R. anatipestifer* strains were conducted using SeqMan program (DNAStar, Madison, WI). Clustal multiple alignment algorithms were used to calculate the similarity and divergence of the sequences. The phylogenetic tree was generated using the molecular evolutionary genetics analysis (MEGA) program V5.2 with 10 000 bootstrap replicates. Sixty-six other amino acid sequences (Table 3) obtained from GenBank were also included in the analysis.

2.5 Genomic walking of flanking sequences of *gapdh* gene

A genome walking kit (TaKaRa) was used to clone the left and right flanking sequences of the *gapdh* gene in *R. anatipestifer* CZ2 strain according to the manufacturer's instructions. The *gapdh*-specific primers (Table 2), which were designed on the basis of the nucleotide sequence of the target fragments and used for the 1st, 2nd, and 3rd nested PCR, and the sense primer AP3 provided with the kit, were used to amplify the 5′- and 3′-terminus. The PCR products were cloned into the pMD19^{R}-T vector (TaKaRa). DNA sequencing was performed in the Beijing BGI-GBI Biotech Co., Ltd., China.

2.6 Preparation of mouse antiserum against recombinant RaGAPDH

Open reading frame (ORF) of RaGAPDH was amplified from gDNA of *R. anatipestifer* CZ2 strain by PCR using primers Egap1F and Egap1R containing *Eco*RI and *Xho*I sites, respectively. The products were digested with *Eco*RI and *Xho*I, purified and then cloned in frame into the bacterial expression vector pET-28a(+) (Novagen). The recombinant plasmid was sequenced to confirm that the RaGAPDH insert was in the proper reading frame and transformed into the competent *E. coli* BL21 (DE3) cells. The cells harboring the recombinant plasmid were grown at 37 °C in a rotary shaker at 160 r/min, in 100 ml LB broth supplemented with 100 μg/ml ampicillin. At the point when the absorbance at 600 nm (A_{600}) reached 0.6, 0.5 mmol/L isopropyl thiogalactoside (IPTG) was added to induce the recombinant protein expression. The cells were induced at 37 °C for 3 h and total cell lysates were prepared and analyzed by SDS-PAGE. The expressing recombinant protein was harvested and purified using Magnehis™ protein purification system (Promega, USA) according to the manufacturer's instructions. Antisera against recombinant RaGAPDH were obtained by immunization of 4-week-old female BALB/c mice subcutaneously injected four times at 2-week intervals with 30 μg of protein emulsified in Freund's complete (first immunization only) or incomplete adjuvant. Mice sera were collected at 7 d after the fourth immunization and antibody titer was determined.

2.7 Western-blot analysis

For Western-blot analysis, recombinant RaGAPDH or secreted proteins in eight *R. anatipestifer* culture media were electrophoresed on 10% gels and transferred to a polyvinylidene difluoride

Table 3 Species and identification numbers of origin in GenBank for the sequences used in this study

Species/strain	Sequence ID	Species/strain	Sequence ID
Ginkgo biloba	Q39769	*Galdieria sulphuraria*	AJ012286
Methanothermus fervidus	ADP77079	*Ralstonia solanacearum*	NP520870
Dirofilaria immitis	AFL46382	*Anabaena variabilis*	YP321014
Mycoplasma agalactiae	YP003303052	*Chlamydophila caviae*	NP828990
Mycoplasma mycoides subsp.	YP004400344	*Thermotoga maritime*	NP228497
Streptococcus pneumoniae	AAK76079	*Deinococcus geothermalis*	YP604599
Streptococcus dysgalactiae	CAA66377	*Physicomitrella patens*	P34923
Streptococcus agalactiae	AF3384161	*Dianthus caryophyllus*	P34921
Streptococcus pyogenes	AAK33348	*Atriplex nummularia*	P34783
Streptococcus suis	AAN86058	*Aspergillus niger*	Q12553
Chondrus crispus	X73033	*R. anatipestifer* RA-CH-1	NC018609
Drosophila melanogaster	NM080352	*Caenorhabditis elegans*	NM076134
Cyanophora paradoxa	AJ313316	*Karenia mikimotoi*	AB164183
Bacteroides fragilis	YP098251	*Heterosigma akashiwo*	AF319448
Synechocystis sp. PCC 6803	NP440929	*Guillardia theta*	CPU40032
Treponema denticola	NP972094	*Candida albicans*	XM714816
Thermus thermophilus	YP004524;	*Pyrenomonas salina*	PSU40033
Geobacillus kaustophilus	YP148579	*Agrobacterium fabrum*	AAK89669
Parastagonospora nodorum	AJ271155	*Candida albicans*	Q92211
Paracoccidioides brasiliensis	AF396657	*Drosophila melanogaster*	Q01597
Gallus gallus	P00356	*Ustilago maydis*	P09317
Escherichia coli O157	P06977	*Neurospora crassa*	XM951884
R. anatipestifer ATCC11845	CP003388	*Ustilago maydis*	X07879
Trypanosoma cruzi	XM814806	*Pyropia yezoensis*	AY273819
Saccharomyces cerevisiae	NM001181485	*Escherichia coli* CFT073	NP753744
Schizosaccharomyces pombe	NM001021142	*Thermosynechococcus elongatus*	NP680834
Pseudomonas aeruginosa	NP249242	*Leptospira interrogans serovar*	NP711885
Moorella thermoacetica	YP429140	*Corynebacterium diphtheriae*	NP939663
Bacillus halodurans	NP244015	*Thermobifida fusca*	YP290073
Leishmania mexicana	Q01558	*Nicotiana tabacum*	CAB39974
Gonyaulax polyedra	AF028562	*Xenopus laevis*	P51469
Plasmodium falciparum	AF030440	*Pinus sylvestris*	P34924
Trypanosoma brucei	P00097	*Homo sapiens*	P0034
Caenorhabditis elegans	NM076134	*R. anatipestifer* RA-GD	CP002562
Phaffia rhodozyma	AF006483		

(PVDF) membrane using a Bio-Rad mini Trans-Blot apparatus. The membrane was blocked and then incubated with anti-recombinant RaGAPDH sera (500× dilution in blocking solution) or with anti-OmpA sera (500× dilution in blocking solution) for 16 h at 4 °C. After several washing steps, the membrane was incubated with 1000× dilution of a rabbit anti-mouse IgG horseradish peroxidase (HP)-conjugated IgG for 2 h. Blots were developed using the enhanced chemiluminescence (ECL) plus Western-blot detection system (Chimi DOX-XRS, Bio-Rad, US). In this experiment, AF, CZ1, CZ2, gHN1210, and SC12 were selected to detect the secreted RaGAPDH because serotypes 1 and 2 are two of the most prevalent serotypes in China.

2.8 Solid-phase ligand-binding assay

The binding of RaGAPDH to host proteins (including actin, plasminogen, fibrinogen, and fibronectin) was determined according to Egea *et al*. (2007).

Briefly, actin, plasminogen, fibrinogen, or fibronectin (5 μg/ml) was bound to the wells (100 μl/well) of a 96-well high-binding microtiter plate for 9-h incubation at room temperature. Positive and negative control wells coated with RaGAPDH and 0.5% gelatin, respectively, were prepared in parallel. After 12-h incubation with Tris-buffered saline (TBS) blocking buffer (containing 1% gelatin) and then 3-h reaction with purified RaGAPDH (0.125–2.500 μg/ml, 100 μl/well), the plates were washed three times in TBS containing 0.05% Tween-20 and once in TBS buffer. The amount of RaGAPDH bound to host proteins was determined spectrophotometrically (492 nm) in an enzyme-linked immunosorbent assay (ELISA)-based assay using anti-recombinant-RaGAPDH sera (1:500) followed by peroxidase-labelled rabbit anti-mouse antibody (1:1000) and *o*-phenylenediamine as chomogen. The specificity of the absorbance values was assessed in ELISA assays in which incubation with RaGAPDH was omitted. The background values in the negative control obtained with the indicated antibody dilutions were less than 0.05 for all proteins tested, but they were nevertheless subtracted from the test values to obtain specific absorbance.

3 Results

3.1 Secreted protein fraction and bacterial surface of *R. anatipestifer* exhibiting GAPDH activity

To determine the GAPDH activity on the bacterial cell surface, suspensions of whole cells were used as the enzymatic source and resulted in an increase in absorbance at 340 nm due to the NADH formation. Eighteen of 22 *R. anatipestifer* isolate suspensions (the exceptions were DX1, PN1209, YC1, and YC2 strains) co-catalyze the oxidative phosphorylation of D-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate in the presence of NAD^+ (Fig. 1a). The presence of secreted RaGAPDH in the culture medium of *R. anatipestifer* CZ2, SC12, and YC1 strains was confirmed by assaying its catalytic activity. To this end, cell-free supernatants at different time intervals were concentrated by TCA and analyzed for GAPDH activity. Supernatants from cultures of CZ2 and SC12 showed a significant NADH formation coupled to glyceraldehyde-3-phosphate oxidation, while YC1 did not display GAPDH activity (Fig. 1b).

3.2 RaGAPDH nucleotide sequence and homology

PCR of gDNA from 18 strains of *R. anatipestifer* with RaGAPDH1 and RaGAPDH2 primers homologous to conserved regions of 13 GAPDH amino acid sequences obtained from GenBank produced a single amplicon of about 1000 bp, while the other two stains, PN1209 and DX1, produced one or two amplicons of less than 1000 bp; YC1 and YC2 strains did not produce any amplicon (Fig. 2). The products were sequenced and deduced amino acid sequences were compared with the other 69 GAPDH sequences retrieved from GenBank. A phylogenetic tree (Fig. 3) was built using the MEGA5.2 program to determine the origin and identity of the RaGAPDH. The RaGAPDH was localized on the same branch as EHEC, belonging to the class I of bacteria GAPDH (EC 1.2.1.12). Fig. 3 also shows that the origin of RaGAPDH was monophyletic.

The complete sequence of the RaGAPDH-coding gene in *R. anatipestifer* CZ2 strain was identified by genomic walking. The nucleotide sequence predicts an ORF of 334 amino acids, with a predicted molecular mass of approximately 36.7 kDa. Hydrophilic analysis of the RaGAPDH protein did not indicate the presence of any transmembrane spanning regions or a signal sequence. Despite considerable divergence from other characterized GAPDH molecules, the RaGAPDH is also composed of two basic domains common to members of the GAPDH family of enzymes: the NAD^+-binding domain (residues 4–152 amino acids) and the catalytic domain (residues 157–334 amino acids).

3.3 Antigenic nature of recombinant RaGAPDH

To detect and characterize the extracellular RaGAPDH, we were interested in the expression of RaGAPDH and the generation of specific polyclonal antibodies. After several attempts to determine the optimal conditions for RaGAPDH expression in *E. coli* BL21 (DE3), a high level of expression of recombinant RaGAPDH was obtained by adding 0.5 mmol/L of IPTG and incubating the culture at 37 °C for 3 h. The expressed protein was analyzed by SDS-PAGE and Western-blot for purity and homogeneity. As demonstrated in Fig. 4a, a protein band of a subunit of approximately 53.7 kDa was detected after protein staining with Coomassie blue. The

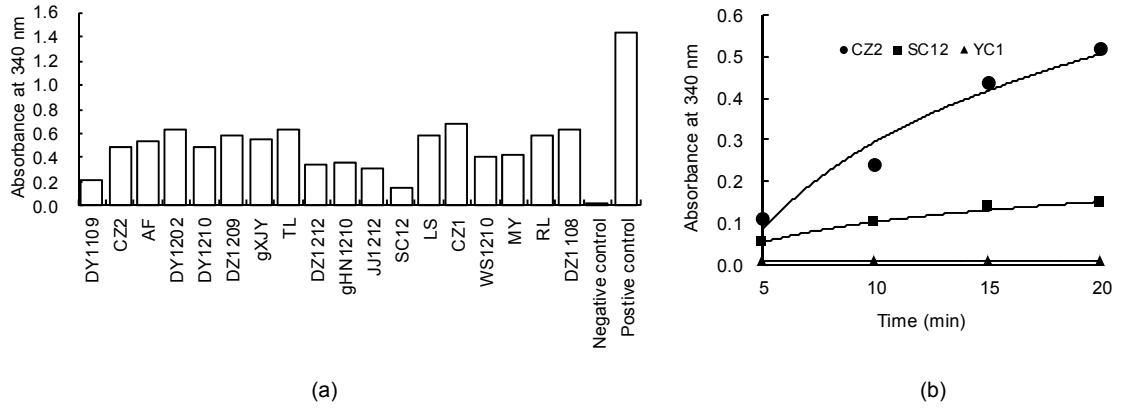

Fig. 1 Extracellular GAPDH activity of whole cells (a) and cell-free supernatants (b)

The GAPDH activity was measured by monitoring the NADH formation coupled to the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate (absorbance at 340 nm) at different time intervals or as an endpoint reading after 15 min of incubation. (a) GAPDH activity on the surface of bacterial cells. The GAPDH activity was determined at 30 °C by measuring the absorbance at 340 nm as an endpoint reading of the supernatant obtained from whole cells (4×10^8 CFU/200 μl) after 15 min of incubation in 2 ml of the assay mixture. Data are presented as the mean of three independent experiments. Negative control reactions were performed in the absence of glyceraldehyde-3-phosphate. (b) Cells of *R. anatipestifer* strains CZ2, SC12, and YC1 were collected by centrifugation, and the cell-free supernatants were processed by TCA as described in Section 2.3

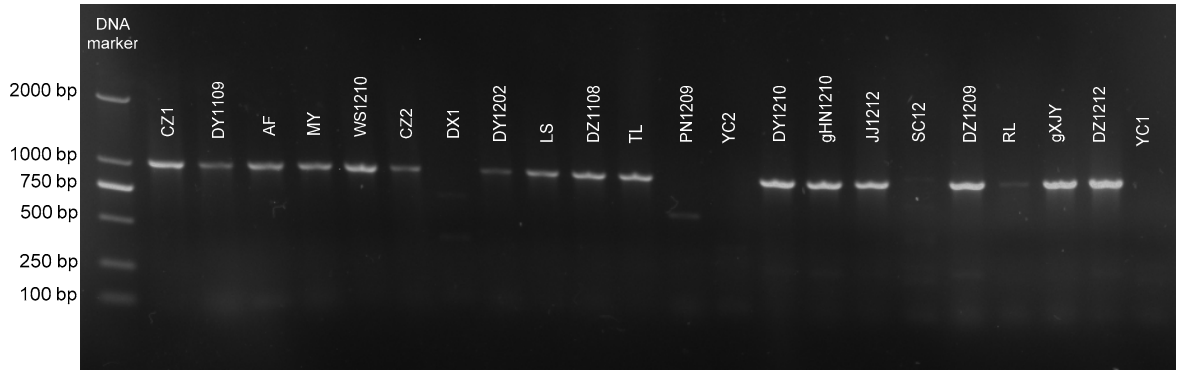

Fig. 2 Identification of a homolog of GAPDH from *R.anatipestifer* field isolates by PCR

Lane M: DNA molecular weight marker; Lanes CZ1 to YC1: twenty-two strains of *R.anatipestifer*

recombinant RaGAPDH runs slightly higher than native RaGAPDH, which could be explained by the additional 17 kDa tag-protein not present in the native sequence (Fig. 4c).

3.4 Identification of RaGAPDH as an extracellular protein in *R. anatipestifer* culture

The proteins secreted by exponential phase cultures of *R. anatipestifer* grown in TSB were extracted and used for immunoblotting analysis. The extracellular proteins were detected in supernatants of *R. anatipestifer* AF, CZ1, CZ2, gHN1210, SC12, PN1209, DX1, and YC1 strains (Fig. 4b). To exclude the possibility that the presence of RaGAPDH in the culture medium was due to the contamination from cell lysis, immunoblots were run in parallel using antibodies against the OmpA of *R. anatipestifer*. No extracellular specific band was detected for this control protein in the supernatant cultures of the three strains. The results indicated that the presence of RaGAPDH in the supernatant of exponentially growing bacteria was attributable to a secretion process rather than cell lysis.

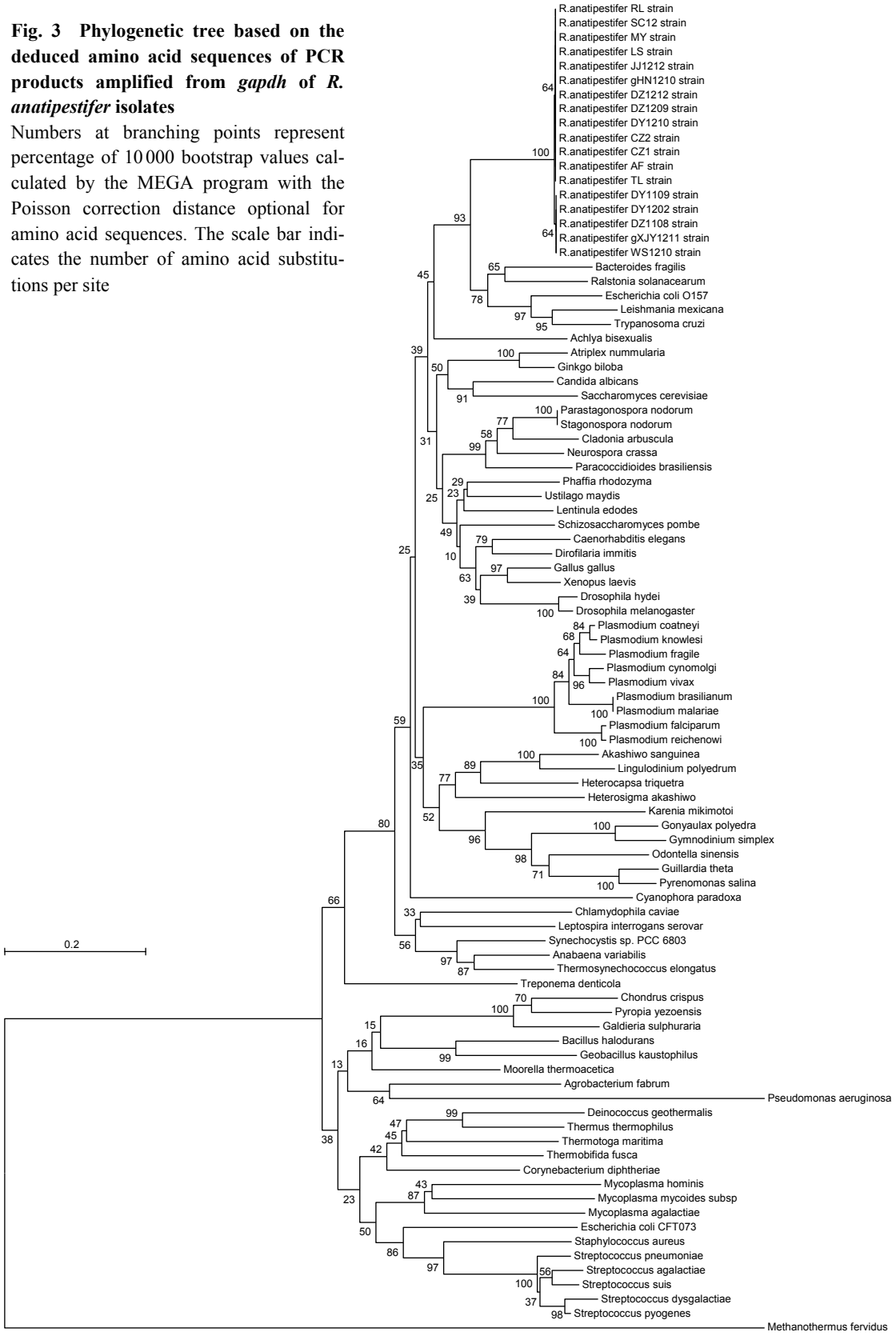

Fig. 3 Phylogenetic tree based on the deduced amino acid sequences of PCR products amplified from *gapdh* of *R. anatipestifer* isolates

Numbers at branching points represent percentage of 10 000 bootstrap values calculated by the MEGA program with the Poisson correction distance optional for amino acid sequences. The scale bar indicates the number of amino acid substitutions per site

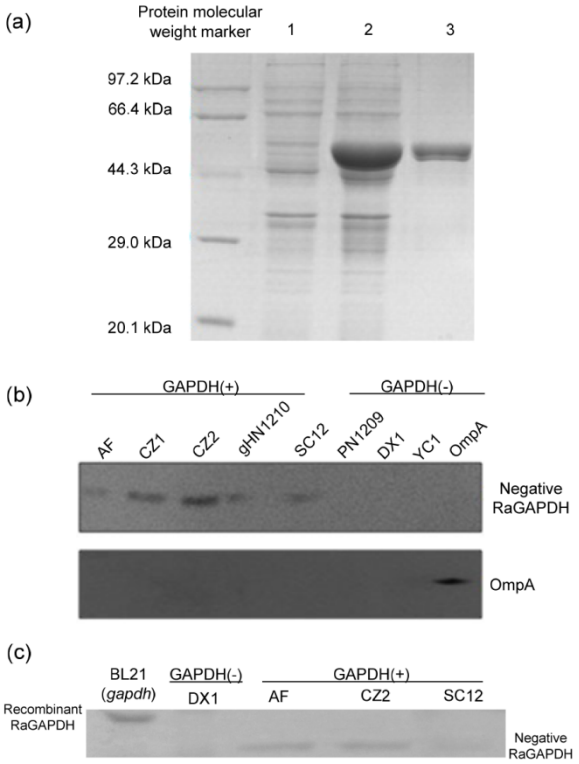

Fig. 4 SDS-PAGE analysis of recombinant RaGAPDH (a) and Western-blot analysis of RaGAPDH secretion (b) and recombinant RaGAPDH (c)

(a) SDS-PAGE (10%) of the recombinant RaGAPDH produced in *E. coli* BL21 (DE3). Lane 1: total proteins extracted from uninduced cells carrying expression vector; Lane 2: total proteins extracted from induced cells with 0.5 mmol/L IPTG at 37 °C for 3 h; Lane 3: soluble recombinant RaGAPDH fractions after Ni-NTA affinity chomatography. (b) Western-blot analysis of RaGAPDH secretion. Aliquots of 30 ml of the supernatant cultures were precipitated with TCA and analyzed by Western-blot using anti-RaGAPDH specific antibodies, or anti-OmpA as a control of cytosolic contamination. The representative positive strains (AF, CZ1, CZ2, gHN1210, and SC12) and negative strains (PN1209, DX1, and YC1) were tested for extracellular RaGAPDH. (c) Western-blot analysis of recombinant RaGAPDH in *E. coli* BL21 (*gapdh*) and native RaGAPDH in cell-free supernatants of *R. anatipestifer* DX1, AF, CZ2, and SC12 strains

3.5 Binding of purified RaGAPDH to host proteins

The binding of purified RaGAPDH to host proteins including actin, fibrinogen, plasminogen, and fibronectin was analyzed by solid-phase ligand-binding assays. These proteins were coated on 96-well ELISA plates (0.5 μg/well) and incubated with different concentrations of GAPDH (0.125, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 μg/ml). The amount of RaGAPDH bound to host proteins was determined as described in Section 2.8. The results indicated that RaGAPDH interacted with plasminogen and fibrinogen, but not with actin or fibronectin (Fig. 5).

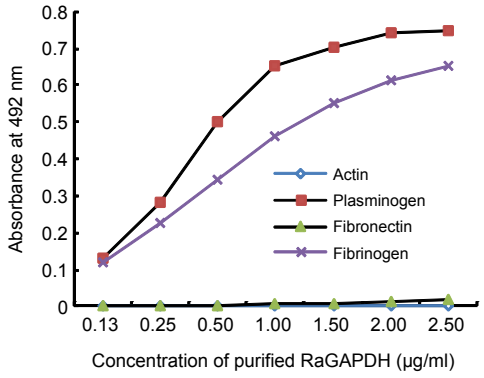

Fig. 5 Binding of purified RaGAPDH to host proteins by solid-phase ligand-binding assay

Actin, plasminogen, fibronectin, and fibrinogen were coated on 96-well microtiter plates and reacted with different concentrations of purified RaGAPDH. Data are presented as mean from three independent experiments

4 Discussion

As a cytoplasmic housekeeping enzyme with no detectable secretion or retention signal, GAPDH has been studied not only as a classical protein for its role in glycolysis but also as a model protein for enzyme kinetic analyses, crystallographic modeling, as well as for gene isolation and characterization. A large number of reports have shown that these enzymes are present on the surface of microbial pathogens, where they exert functions related to adhesion and/or virulence (Pancholi and Chhatwal, 2003). However, most studies are limited to fungi and Gram-positive pathogens, and there are few studies on Gram-negative bacteria besides *E. coli*. This is the first report documenting GAPDH activity in *R. anatipestifer*. Our results indicated that RaGAPDH is secreted by the Gram-negative *R. anatipestifer*, which is exposed and exported on the surface of the cell. The presence in the extracellular medium of RaGAPDH, but not OmpA, rules out cell lysis to explain this extracellular localization. Extracellular secretion of proteins is the major mechanism by which pathogens communicate with and intoxicate host cells. Previous reports showed that GAPDH presented on the surface of *S.*

pyogenes was able to bind several mammalian proteins including the urokinase-type plasminogen activator receptor (uPAR/CD87) membrane protein on pharyngeal cells (Lottenberg *et al*., 1992; Pancholi and Fischetti, 1992; Winram and Lottenberg, 1996; Jin *et al*., 2005), regulate intracellular host-cell signaling events (Pancholi and Fischetti, 1997), and contribute to host immune evasion (Terao *et al*., 2006). Therefore, the secretion and association with the cell membrane of RaGAPDH might have biotechnological implications that are worth further investigation.

Members of the GAPDH family are grouped into three classes, and different microbial pathogens belong to different classes. NAD^+-dependent GAPDH is the most common form and is usually located in the cytoplasm. In EHEC, *gapA* and *gapC* genes encode GAPDH proteins with highly similar sequences, and the *gapA* gene accounted for the exported GAPDH enzyme (Egea *et al*., 2007). In the *Pseudomonas syringae* genome, there are three paralogous *gapdh* genes encoding distinct GAPDHs, namely two class I enzymes having different molecular mass subunits and one class III biofunctional D-erythose-4-phosphate dehydrogenase/GAPDH enzyme (Elkhalfi *et al*., 2013). However, our results showed that there were several *R. anatipestifer* strains which did not have a homologous amplicon of GAPDH and showed no extracellular GAPDH activity. Whether lack of RaGAPDH in these strains is linked to attenuated virulence remains to be studied. Bioinformatics analysis of *R. anatipestifer* references genome, ATCC11845, RA-CH-1, RA-GD, however, showed the presence of only one copy of the *gapdh* gene. In addition, the PCR and sequence analysis results showed that there were one or two *gapdh*-like genes in *R. anatipestifer* DX1 and PN1209 strains. Therefore, we hypothesized that not all *R. anatipestifer* strains showed GAPDH activity, and there was a possibility that more than one *gapdh*-like gene existed in some *R. anatipestifer* strains, but not accounting for the extracellular GAPDH activity.

A recent proteomic study on *L. monocytogenes* showed classical cytosolic proteins like enolase that did not display a typical secretion signal, which could be exported through the SecA2-dependent secretion system (Lenz *et al*., 2003). Therefore, Gram-negative bacteria had developed one strategy consisting of vesicle-mediated export to enable such proteins to reach the extracellular space (Lenz *et al*., 2003). However, it is unclear whether GAPDH can be secreted in a similar way. Analysis of the RaGAPDH protein revealed its homology to the GAPDH family of glycolytic enzymes. There is no apparent signal sequence or transmembrane regions indicated by stretches of hydrophobic amino acid residues. However, it has a significant homology to the GAPDH proteins in EHEC, which had been shown to be membrane-associated and contain no recognized signal sequences. Our results also indicated that RaGAPDH was secreted by *R. anatipestifer* and its secretion mechanism needs to be further studied.

Attachment to host components is the critical first step in the establishment of infection by pathogens. Based on the putative role of extracellular RaGAPDH in *R. anatipestifer* infection, the ability of RaGAPDH to bind host proteins, known to interact with the extracellular GAPDH of other pathogens, was analyzed by solid-phase ligand-binding assays. We selected actin, fibrinogen, plasminogen, and fibronectin for this study. The results indicated that RaGAPDH interacted with plasminogen and fibrinogen, but not with actin or fibronectin. This is consistent with the fact that the C-terminal Lys residue of streptococcal GAPDH, important for plasminogen binding (Winram and Lottenberg, 1998), is conserved in the RaGAPDH sequence. Plasminogen is a serine protease that is abundant in extracellular fluids and associates with the surface of many cells in the body. Plasminogen-binding activity of extracellular bacterial GAPDH has been related to mechanisms of pathogenesis. Plasmin, the resulting activated form of plasminogen, can degrade extracellular matrix proteins and promote bacterial migration (Bergmann *et al*., 2004). In this way, the extracellular RaGAPDH, either exposed on the bacterial surface or secreted near the host cell, may contribute to the migration of these pathogens through digestive or respiratory tracts.

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Compliance with ethics guidelines

Ji-ye GAO, Cui-lian YE, Li-li ZHU, Zhi-ying TIAN, and Zhi-bang YANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要：

本文题目： **鸭疫里默氏杆菌的 GAPDH 同源体:一种有生物活性的胞外蛋白**

A homolog of glyceraldehyde-3-phosphate dehydrogenase from *Riemerella anatipestifer* is an extracellular protein and exhibits biological activity

研究目的： 对鸭疫里默氏杆菌的三磷酸甘油醛脱氢酶（GAPDH）进行鉴定和生物学特征分析。

创新要点： 首次证实鸭疫里默氏杆菌具有 GAPDH 的同源体酶（RaGAPDH）是一种无信号肽和跨膜区的胞外蛋白酶，具有将 3-磷酸甘油醛转化为 1,3-二磷酸甘油酸的活性，可与纤维蛋白溶酶原及纤维蛋白原发生结合，推测该酶可能是鸭疫里默氏杆菌的一个新发现的毒力因子。

研究方法： 1．对分离自重庆、四川地区的鸭疫里默氏杆菌（表 1）菌体细胞表面蛋白（图 1a）和 CZ2、SC12、YC1 三株菌胞外蛋白（图 1b）的 GAPDH 活性进行检测，对其编码基因进行 PCR 鉴定（图 2）和克隆测序分析（图 3）；2．采用染色体步移技术获得 CZ2 的 GAPDH 编码基因进行原核表达（图 4a 和 4c）；3．以获得的具有活性的重组 GAPDH 为抗原，制备鼠原多克隆抗体并采用 Western-blot 方法对鸭疫里默氏杆菌的胞外分泌蛋白进行检测分析（图 4b）；4．采用固相配体结合试验检测 RaGAPDH 与纤维蛋白溶酶原、血纤维蛋白原、肌动蛋白和纤连蛋白的结合作用（图 5）。

重要结论： 鸭疫里默氏杆菌具有三磷酸甘油醛脱氢酶同源体，具有 GAPDH 活性，能与纤维蛋白溶酶原和血纤维蛋白原结合，可能是其重要的毒力因子。

关键词组： 鸭疫里默氏杆菌；三磷酸甘油醛脱氢酶；胞外蛋白