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Original article

Riemerella anatipestifer lacks *luxS*, but can uptake exogenous autoinducer-2 to regulate biofilm formation

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Abstract

Riemerella anatipestifer (RA) causes major economic losses to the duck industry. Autoinducer-2 (AI-2) is a quorum-sensing signal that regulates bacterial physiology. The *luxS* and *pfs* genes are required for AI-2 synthesis in many bacterial species. *pfs* encodes Pfs, which functions upstream of LuxS in the biosynthesis of AI-2. In this study, we investigated the AI-2 activity of RA using an AI-2 bioassay, which showed that RA does not produce AI-2. Bioinformatic analysis indicated that the RA genome has a *pfs*, but not a *luxS*, homologue. To investigate the function of RA *pfs*, an avian pathogenic *Escherichia coli* (APEC) *pfs* mutant was constructed, which was subsequently transformed with a recombinant plasmid carrying RA *pfs*. An AI-2 bioassay demonstrated that RA *pfs* restored AI-2 production to the APEC *pfs* mutant, suggesting that RA *pfs* functions in AI-2 synthesis. Furthermore, we found that RA utilizes exogenous AI-2 to regulate biofilm formation. RA biofilm formation decreased significantly upon addition of exogenous AI-2. Real-time quantitative PCR results showed that expression of 13 genes related to RA biofilm formation decreased significantly when exogenous AI-2 was added to the RA culture media. These findings will benefit future studies on AI-2 regulation in RA.

Keywords: *Riemerella anatipestifer*; Pfs; Autoinducer-2; Biofilm formation

1. Introduction

Riemerella anatipestifer (RA) is a Gram-negative, non-motile, non-spore-forming rod-shaped bacterium. RA infections cause major economic losses to the duck industry because of high mortality rates, low feed conversion and high treatment costs. In particular, the mortality rate has been reported to be as high as 70% in ducks with severe RA infections [1]. Currently, studies of RA have focused mainly on bacterial serotypes, virulence genes, detection methods and vaccine development [2–5]. Quorum sensing (QS) is a bacterial intercommunication system that has been reported to regulate survival and virulence-related genes or processes in many pathogenic bacteria [6]. However, little is known about QS in RA.

Autoinducer-2 (AI-2) is a quorum-sensing signal that is produced in the activated methyl cycle (AMC) by the AI-2 synthase Pfs (also called 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase) and LuxS [7,8]. The AI-2 synthase LuxS was identified in 1999 [8] and LuxS homologues have now been identified in 537 of the 1402 currently sequenced bacterial genomes [9]. AI-2 is derived from S-adenosylmethionine (SAM), which acts as a methyl donor to yield S-adenosylhomocysteine (SAH). Pfs can convert SAH to S-ribosylhomocysteine (SRH) and then SRH is used by the LuxS enzyme to produce homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD undergoes further rearrangement to produce AI-2 [10]. The activity of AI-2 may be detected by its ability to induce luminescence in *Vibrio*

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harveyi strain BB170, which was constructed to serve as a reporter strain to detect AI-2 [11].

Recent studies have shown that, depending on the bacterium, AI-2 can regulate a variety of pathological behaviours, including bioluminescence, biofilm formation, bacterial virulence, conjugation, sporulation, swarming motility and antibiotic production [6,12,13]. To date, many microbes have been shown to produce AI-2-like activities [9]. Some species of bacteria lack the AI-2 synthase gene *luxS* and thus cannot produce an interspecies AI-2 signal. However, such bacteria might use AI-2 produced by other bacteria to regulate their physiological functions. For example, *Sinorhizobium meliloti*, a plant symbiont lacking the gene for AI-2 synthase, is not capable of producing AI-2, but responds to AI-2 produced by other species [14].

In this study, we found that RA lacks the *luxS* gene and failed to produce the interspecies signal AI-2; however, it does contain a functional Pfs and responds to AI-2 by down-regulating biofilm formation. These findings will benefit future studies of AI-2 regulation in RA.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Thirty-three RA strains were isolated from infected duck farms in China between 1997 and 2009 [15], including 15 strains of serotype 1 (WJ4, CH3, NJ1, NJ2, NJ4, CQ1, CQ3, CQ4, CQ5, JY4, YL4, YXB12, YXB14, NN2 and NN3), ten strains of serotype 2 (SC2, NJ3, B2, TH4, YXB1, NN5, GD3, GD4, GD5 and GD7), three strains of serotype 10 (YXL1, HXB2 and YXB11), one strain of serotype 15 (NN6) and four strains of unknown serotype (NN11, GD1, GD6 and JY6). All the RA strains were cultured in tryptic soy broth (TSB, Difco, Franklin Lakes, NJ, USA) at 37 °C with 5% CO_2.

A clinical isolate of avian pathogenic *Escherichia coli* (APEC) strain DE17 (serotype O2) was isolated from a duck with septicaemia and neurological signs in Anhui Province, China in 2008 [6]. DE17 and *E. coli* strains DH5α (Invitrogen, Carlsbad, CA, USA) were grown routinely in Lennox broth (LB) or on solid medium containing 1.5% agar at 37 °C. *V. harveyi* strains BB170 (sensor^{1-} sensor^{2+}) and BB152 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultivated in modified autoinducer bioassay (AB) medium [16]. BB170 is the AI-2 biosensor strain and BB152 is used as a positive control for AI-2 production.

All chemicals used were of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. AI-2 bioassay

AI-2 activity was expressed as relative light units (RLUs). The AI-2 bioassay was performed according to a method previously described, with modifications [6,16,17]. Cell-free culture fluid (CF) was prepared as follows: RA strains were grown in 5 ml of TSB at 37 °C for 5 h with shaking, and pelleted by centrifugation at 12,000 *g* at 4 °C for 10 min. Then, the resulting supernatant was filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA) to obtain the CF sample. The reporter strain *V. harveyi* BB170 was diluted 1:5000 in fresh AB medium. Subsequently, 180 μl of bacterial culture was mixed with 20 μl of the CF sample and incubated at 30 °C for 4 h. Then, 100-μl aliquots were added to white, flat-bottomed, 96-well plates (Thermo Labsystems, Franklin, MA, USA) to detect AI-2 activity. A positive control was obtained from overnight cultures of BB152 and the CF from *E. coli* DH5a was used as the negative control. Luminescence values were measured with a Tecan GENios Plus microplate reader in luminescence mode (Tecan Austria GmbH, Grödig, Austria). The AI-2 activity in the CF is reported as RLUs produced by *V. harveyi* BB170. Experiments were repeated three times.

2.3. Construction and identification of the APEC pfs mutant strain DE17Δpfs

The upstream and downstream sequences of the APEC *pfs* gene (APECO1_1826) are the *yadT* (APECO1_1827) and *dgt* (APECO1_1825) genes, respectively. The mutant strain DE17Δ*pfs* was generated by creating a 590 bp in-frame deletion in the 699-bp *pfs* open reading frame (ORF) using the lambda Red recombinase method as described previously, with modifications [6,18]. Briefly, the upstream and downstream fragments (1077 bp and 1002 bp, respectively) of the *pfs* gene were amplified by PCR using the primer pairs DE17-pfsUF/DE17-pfsUR and DE17-pfsDF/DE17-pfsDR (Table 1). The upstream and downstream fragments of the *pfs* gene were ligated by overlap PCR using the primer pair Overlap-F1/Overlap-R1 (Table 1) to produce a 2079 bp PCR product and then the PCR product was subcloned into the pMD18-T vector to construct the recombinant plasmid pMD18-Up-Down, which has a 590 bp deletion within the *pfs* ORF. A chloramphenicol resistance cassette (Cm) was amplified from plasmid pKD3 by PCR using the primers pKD3-F/pKD3-R (Table 1). Then, the Cm was digested with SalI and inserted subsequently into the recombinant plasmid pMD18-Up-Down to form pMD18-Up-Cm-Down. The mutagenic construct (containing an insertion of the Cm within the *pfs* gene coding region) was amplified by PCR using the primers DE17-pfs-UF/DE17-pfs-DR. Subsequently, the PCR product (3092 bp) was purified and used for electroporation. One microgram of PCR product was added to 100 μl of DE17 competent cells containing the lambda Red recombinase expression plasmid pKD46 and electroporation was performed using a Gene Pulser II transfection apparatus (Bio-Rad, Hercules, CA, USA) at 25 μF, 2.4 kV and 250 Ω. After the electric pulse, the cells were diluted immediately in 1 ml of SOC medium and incubated at 37 °C for 2 h. They were subsequently plated on LB plates containing 8 μg/ml chloramphenicol. After 24 h incubation, the resulting chloramphenicol-resistant colonies were selected for PCR amplification with the primer pairs DE17-pfs-inF/DE17-pfs-inR and DE17-pfs-outF/DE17-pfs-outR (Table 1) to confirm the deletion of the *pfs* gene from DE17. A 428 bp PCR product was amplified from the DE17 wild-type