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Characterization of biofilm formation by *Riemerella anatipestifer*

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ABSTRACT

Riemerella anatipestifer (RA) causes epizootics of infectious disease in poultry and results in serious economic losses, especially for the duck industry. The present study focuses on understanding the biofilm-producing ability of RA strains in attempt to explain the intriguing persistence of RA post-infection on duck farms. Four RA serotype reference strains and 39 field RA isolates were measured for the biofilm formation by crystal violet staining. Eighteen out of the 43 RA strains produced biofilms. Furthermore, RA isolate CH3 was treated with carbohydrates (sucrose; glucose), disodium EDTA (EDTA), antibiotics (ampicillin; chloramphenicol) or detergent (Triton X-100) to determine the effect of the treatments on biofilm formation. Biofilm formation by RA isolate CH3 was independent of sucrose but significantly inhibited by 5% glucose and 0.1 mmol/L EDTA. Biofilmed CH3 culture (CH3 grown with a biofilm) was 5–31 times more resistant to the treatments of ampicillin, chloramphenicol or Triton X-100 than planktonic CH3 culture on the basis of minimal inhibitory concentration and minimal bactericidal concentration. The development and architecture of the biofilm formed by CH3 were also assessed using confocal laser scanning microscopy, scanning electron microscopy and fluorescence microscopy. In addition, animal experiment was performed to determine the median lethal doses (LD_{50}) of three RA isolates with different biofilm formation abilities. Despite the result that virulence is strain-dependent as a result of various factors other than biofilm-producing ability, the fact that biofilmed isolate is more resistant to antibiotic and detergent treatments than planktonic isolate suggest that biofilm formation by RA may contribute to the persistent infections on duck farms.

1. Introduction

Riemerella anatipestifer (RA) is a Gram-negative, non-motile, nonspore-forming, rod-shaped bacterium. It belongs to the family *Flavobacteriaceae* in rRNA superfamily V based on 16s rRNA gene sequence analyses (Subramaniam et al., 1997). Currently, there are at least 21 known serotypes of RA (Pathanasophon et al., 2002). RA causes the anatipestifer syndrome in ducks, characterized by diarrhea, lethargy, and respiratory and nervous symptoms, which can lead to high mortality and consequently great economic losses (Asplin, 1955; Glunder and Hinz, 1989). RA infection has been a continued problem in many duck farms (Subramaniam et al., 2000). The precise mechanisms of RA persistence in duck farms are still unknown. The facts that a number of bacteria achieve their persistence via formation of biofilm lead us to investigate whether this is a mechanism for RA persistence as well.

Biofilm is a microbial community that consists of microorganisms surrounded by an extracellular polymeric matrix. Biofilm formation has been demonstrated for numerous pathogens such as *Escherichia coli*, *Streptococcus suis*, and *Haemophilus influenzae* (Aparna and Yadav, 2008; Hall-Stoodley and Stoodley, 2009). A large amount of data suggested that the biofilm formation was relevant to bacterial persistence due to their recaltitrance to eradica-

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tion by antibiotics (Jayaraman, 2008; Lewis, 2005, 2007). In this study, we first investigated the ability of 43 RA strains to produce biofilm, tested the effect of various chemical treatments on biofilmed versus planktonic CH3 isolates, and challenged ducklings with RA isolates to elucidate whether the ability to form biofilm plays a role in the persistence of RA infections.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Thirty-nine isolates of RA were obtained from 39 outbreaks on duck farms in China during the period of 1997–2009. The isolates were identified by phenotypic characters (Pathanasophon et al., 1994) and by amplification of 16s ribosomal RNA (Huang et al., 1999) and the *ompA* gene (Qinghai Hu et al., 2002). The serotypes of RA isolates were identified by the slide agglutination test with anti-RA rabbit antisera (Bisgaard, 1982). Four RA serotype reference strains were donated generously by Dr. Guoqiang Zhu and Dr. Zhizhong Cui. *E. coli* DH5α was purchased from Invitrogen (CA, USA). All the strains were cultured in Tryptic Soy Agar (TSA, Difco, NJ, USA) at 37 °C for 24 h in 5% CO_2 or Tryptic Soy Broth (TSB, Difco, NJ, USA) at 37 °C, 150 rpm for 16 h. The RA strains used in this study are listed in Table 1.

2.2. Biofilm quantification

A modified assay involving crystal violet (CV) staining was used to quantify the biofilm formation by RA strains and *E. coli* DH5α (Mohamed et al., 2007). Briefly, the strains were grown in TSB at 37 °C, 150 rpm for 16 h. The cultures were diluted to an optical density of 0.1 at 655 nm (OD_{655}), and 200 μl of each cell suspension was transferred to a 96-well microtitre plate (Corning, NY, USA). The plates were incubated at 37 °C in 5% CO_2 for 24 h. The wells were washed gently three times with 200 μl of 0.01 mol/L phosphate-buffered saline (PBS), dried in an inverted position, and stained with 200 μl of 0.1% CV for 30 min at room temperature. The wells were then rinsed with distilled water four times, air-dried, and 100 μl of 95% ethanol was added to dissolve the crystal violet. The optical density at 595 nm (OD_{595}) was determined using a Synergy 2 microplate reader (Biotek, VT, USA). *E. coli* DH5α was used as a negative control for biofilm formation (Mohamed et al., 2007). The RA isolates were identified as having formed biofilm if the OD_{595} reading exceeded 0.31, a value equivalent to the mean OD_{595} of *E. coli* DH5α plus two standard deviations. We classified the samples as strong biofilm producer ($OD_{595} \geq 1.00$), weak biofilm producer ($0.31 < OD_{595} < 1.00$), or non-biofilm producers ($OD_{595} \leq 0.31$) as described previously (Mohamed et al., 2007). All samples were measured in triplicate, and the mean ± one standard deviation for each isolate was calculated from three independent experiments.

2.3. The influence of carbohydrates and EDTA on biofilm formation

Sucrose, glucose and disodium EDTA (EDTA, Sigma, MO, USA) were tested for their effect on the formation of biofilm by RA isolate CH3. The bacteria were cultured and assessed for biofilm formation as described above, except for the addition of sucrose (0.5%, 1%, 2% or 5%), glucose (0.5%, 1%, 2% or 5%) or EDTA (0.1, 0.5 or 1 mmol/L) to the TSB, respectively. In addition, the influence of EDTA on the mature biofilm of CH3 was tested. Specifically, the supernatant was removed from wells containing 24 h cultures of CH3 with mature biofilm, the non-adherent cells were removed by thorough washing with PBS, 200 μl of TSB containing 0.1, 0.5, 1, 2, 5 or 10 mmol/L EDTA were added to the wells, and the bacteria were further cultured for 24 h at 37 °C with 5% CO_2. The formation of biofilm was quantified by CV staining, as described above.

2.4. The influence of antibiotics and detergents on biofilm formation

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of ampicillin, chloramphenicol, and Triton X-100 were determined for planktonic and biofilmed CH3 culture (CH3 grown with a biofilm) as described (Grenier et al., 2009) with minor modifications. Briefly, for plantonic CH3, an overnight culture was diluted to $OD_{655} = 0.1$ with TSB containing different concentrations of the three chemicals, produced by two-fold serial dilutions (1000–0.024 μg/ml, TSB-A). Two-hundred microliters of each cell suspension was

Table 1
RA strains used in this study.

Strains	Serotype	Year of collection	Reference or source
P2123	6		NADC[b]
D26220	8		DRL[c]
8785	12		CCUG[d]
D743	15		CVL[e]
DY-1; CH3; CH1; WJ4; YL4; YXb12; NJ-1; NJ-2; RA-1; CQ1; CQ2; CQ3; CQ4; CQ5; NJ-4; JY-4; YXb14	1	1997–2008	Jiangsu; Chongqing
FXb6; Yb2; Yb3; JY-1; JY-2; JY-5; NJ-3; SC1; SC2; SC3; SC4, SC5	2	2000–2008	Anhui; Jiangsu; Sichuan
HXb2; YXb11; YXb1; YXL1	10	2000–2008	Anhui; Jiangsu
YXb13; SHL1; JY-3; YXb15; YXD1; JY-6	ND[a]	2008–2009	Jiangsu; Shanghai

[a] ND: Not determined.
[b] NADC: National Animal Disease Center, Ames, IA, USA.
[c] DRL: Duck Research Laboratory, New York, USA.
[d] CCUG: Culture Collection, University of Goteborg, Sweden.
[e] CVL: Central Veterinary Laboratory, Singapore.