Veterinary Microbiology 175 (2015) 114–122

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Characterization of *Mannheimia haemolytica* biofilm formation *in vitro*

Ismail Boukahil, Charles J. Czuprynski *

Department of Pathobiological Sciences, University of Wisconsin-Madison, School of Veterinary Medicine, 2015 Linden Drive, Madison, WI 53706, USA

ARTICLE INFO

Article history:
Received 17 February 2014
Received in revised form 4 November 2014
Accepted 12 November 2014

Keywords:
Biofilm
Mannheimia haemolytica
Adhesin
OmpA

ABSTRACT

Mannheimia haemolytica is the primary bacterial agent in the bovine respiratory disease complex. It is thought that *M. haemolytica* colonizes the tonsillar crypts of cattle as a commensal and subsequently descends into the lungs to cause disease. Many bacterial species persist in the host as biofilms. There is limited information about the ability of *M. haemolytica* to form biofilms. The aim of this study was to develop an *in vitro* model for *M. haemolytica* biofilm formation. We found that *M. haemolytica* required at least 36 h to form robust biofilms on plastic *in vitro* when incubated in RPMI-1640 tissue culture medium at 37 °C, with maximal biofilm formation being evident at 48 h. Biofilm formation was inhibited by adding the monosaccharides D(+) galactose and D(+) mannose to the growth medium. Addition of antibodies to the *M. haemolytica* surface protein OmpA also reduced biofilm formation. Upon evaluating the macromolecules within the biofilm extracellular polymeric substance we found it contained 9.7 $\mu g/cm^2$ of protein, 0.81 $\mu g/cm^2$ of total carbohydrate, and 0.47 $\mu g/cm^2$ of extracellular DNA. Furthermore, proteinase K treatment significantly decreased biofilms ($P < 0.05$) while α-amylase and micrococcal nuclease decreased biofilms to a lesser extent. *M. haemolytica* biofilm cells were more resistant than planktonic cells to the antibiotics florfenicol, gentamicin, and tulathromycin. These results provide evidence that *M. haemolytica* can form biofilms, which could contribute to its ability to persist as a commensal in the bovine upper respiratory tract.

1. Introduction

The bovine respiratory disease complex (BRDC) causes significant economic losses to the beef and dairy cattle industries (Zecchinon et al., 2005). Many viral and bacterial pathogens are associated with BRDC, including bovine viral diarrhea virus, bovine herpesvirus 1, bovine respiratory syncytial virus, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* (Caswell, 2014; Klima et al., 2014). The main bacterial agent associated with BRDC is *Mannheimia haemolytica*, a member of the family *Pasteurellaceae* that colonizes the nasal cavity and tonsillar crypts in cattle and sheep (Frank and Smith, 1983; Frank et al., 1993). Following stress or viral infection *M. haemolytica* cells increase in number and descend into the lungs, leading to acute pneumonia (Caswell, 2014). Vaccines against *M. haemolytica* are of variable efficacy and antimicrobial resistance of the pathogen is increasing (Klima et al., 2014; Lubbers and Hanzlicek, 2013).

* Corresponding author at: Department of Pathobiological Sciences, University of Wisconsin-Madison, 4174 Veterinary Medicine Building, 2015 Linden Drive, Madison, WI 53706, USA. Tel.: +1 608 262 8102; fax: +1 608 263 0438.
E-mail addresses: iboukahil@wisc.edu (I. Boukahil), czuprync@svm.vetmed.wisc.edu (C.J. Czuprynski).

http://dx.doi.org/10.1016/j.vetmic.2014.11.012
0378-1135/

Many bacterial species have adapted to living in host tissue by forming biofilms (Olson et al., 2002). Biofilms are organized bacterial microcolonies encased in an extracellular polymeric substance (EPS) that is comprised of polysaccharides, extracellular DNA (eDNA), and proteins (Kiedrowski et al., 2011). Bacterial cells are maintained in a steady state as a biofilm making them resistant to host defense mechanisms and antibiotic treatment (Donlan and Costerton, 2002). The related bovine respiratory pathogen *H. somni* forms biofilms, which are thought to allow this organism to persist as a commensal on the bovine mucosa (Sandal et al., 2007). We hypothesize that *M. haemolytica* also exists as a biofilm on the upper respiratory tract of cattle.

Cattle are commonly treated with antibiotics (metaphylaxis) to prevent *M. haemolytica* associated BRDC when they enter feedlots (Klima et al., 2014). Despite its success, this strategy is limited by the growing problem of antibiotic resistance (Lubbers and Hanzlicek, 2013). Furthermore biofilm cells are often more resistant to antibiotics than planktonic cells (Olson et al., 2002).

Previous studies have shown the importance of bacterial surface proteins in initiating biofilm formation (Rohde et al., 2007; Heilmann, 2011). Such proteins, known as adhesins, facilitate bacterial adhesion to both inanimate surfaces and host cells. It is thought that the adhesins tether the bacterial cells to a surface, allowing the bacteria to then form a biofilm by secreting their EPS (Mack et al., 1996). Several adhesins of *M. haemolytica* have been described. Outer membrane protein A (OmpA) and lipoprotein 1 (Lpp1) are two bacterial adhesins that bind to bovine respiratory epithelial cells *in vitro* (Kisiela and Czuprynski, 2009). OmpA previously was implicated in *M. haemolytica* colonization of the respiratory tract (Hounsome et al., 2011).

In this study we sought to develop an *in vitro* model system to study *M. haemolytica* biofilm formation. We define conditions that allow *M. haemolytica* serotype A1 to form a robust biofilm in polystyrene wells *in vitro*. We use this model system to identify for the first time the macromolecular constituents of the *M. haemolytica* EPS. One method used to block bacterial adhesins is through the use of monosaccharides, which can competitively inhibit the binding of adhesins to host cell surfaces by mimicking the chemical structure of the binding partner of the adhesin (Jaramillo et al., 2000). This in turn would reduce subsequent biofilm formation. We noted decreased biofilm formation by the addition of certain monosaccharides, indicating a role for these sugar residues in biofilm formation. Furthermore, we identify OmpA as a bacterial adhesin that contributes at least in part to biofilm formation *in vitro*. We also observed increased resistance of biofilm cells to gentamicin, florfenicol, and tulathromycin. The information gained from our model system provides insights into biofilm formation by *M. haemolytica in vitro*, which may be relevant to its ability to colonize the upper respiratory tract in cattle.

2. Materials and methods

2.1. Primary cells and bacterial strains

Primary bovine bronchial epithelial cells (BBEC) were a gift from Dr. D.S. Allen-Gipson (Omaha, NE) and maintained as described previously (Kisiela and Czuprynski, 2009). *M. haemolytica* A1 (SH 1217, derived from a virulent strain) (Gioia et al., 2006), were grown without shaking for 18 h in 10 mL of brain heart infusion broth (Becton Dickinson, NJ) at 37 °C to achieve a concentration of 10^9 CFU/mL, as determined by dilution and plating onto tryptic soy agar (TSA) with 5% sheep red blood cells (Becton Dickinson). In a single set of experiments we used additional isolates including a ΔlktC mutant strain (SH 1562), and two recent clinical isolates of *M. haemolytica* (strains 153 and 328) that originated from different farms. These bacterial strains were grown as described above.

2.2. Biofilm assay

Approximately 10^6 CFU of *M. haemolytica* were added to 2.5 mL of the various media tested. The media tested were tryptic soy broth (TSB) (Becton Dickinson), RPMI-1640 without L-glutamine (Lonza, MD), and RPMI with 10% fetal bovine serum (FBS) (Atlanta Biologicals, GA). The bacterial suspension was dispensed into wells (800 μL per well) of a flat bottom polystyrene 24 well plate (Corning, NY) and incubated at 37 °C with 5% CO_2 for up to 72 h. Wells containing media alone were included as a negative control. Afterwards, the media were removed, the wells gently rinsed two times with 1 mL deionized water, and the wells allowed to air dry for 1 h. Filtered crystal violet (Harleco, EMD Millipore, MA) then was added to the wells and incubated at room temperature for 15 min. The crystal violet was removed, the wells washed two times with 1 mL of water, and the plate air dried for 1 h. The stained biofilms then were solubilized with 30% glacial acetic acid (Sigma–Aldrich, MO) and the absorbance at 595 nm determined using a plate reader (DTX 880 Multimode Detector, Beckman Coulter, CA). In a single set of experiments we compared the numbers of CFU in the supernatant and biofilm; the planktonic bacterial cells were enumerated by serial dilution and plating onto TSA with 5% sheep red blood cells. The biofilms in the wells were rinsed three times with 1 mL deionized water. The fluid from the third wash was diluted in saline and plated onto TSA with 5% sheep red blood cells to assess bacterial detachment. The washed biofilms were disrupted in 1 mL of phosphate-buffered saline (PBS) by scraping and vigorous pipetting, followed by serial dilution and plating onto TSA with 5% sheep red blood cells.

2.3. Coating wells with extracellular matrix (ECM) proteins

To assess the effect of ECM proteins on biofilm formation, FBS-coated polystyrene wells were prepared as follows: 500 μL of a 1:1 dilution of FBS and ultra-pure water were added to each well of a flat bottom polystyrene 24 well tissue culture plate and the plate incubated at 37 °C for 30 min. The FBS was removed, and the wells gently rinsed with 1 mL of PBS. The wells were then immediately used in a biofilm assay. Fibronectin-coated wells were prepared as described previously (Zulfakar et al., 2012), with modifications. Briefly, bovine plasma fibronectin (Sigma) was reconstituted to 50 μg/mL in ultra pure water, and 250 μL was added to each well of a polystyrene 24 well tissue culture plate. The plate was incubated at 37 °C for