Veterinary Microbiology 179 (2015) 277–286

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Sub-inhibitory concentrations of penicillin G induce biofilm formation by field isolates of *Actinobacillus pleuropneumoniae*

S. Hathroubi, S.-È. Fontaine-Gosselin, Y.D.N. Tremblay, J. Labrie, M. Jacques *

Groupe de recherche sur les maladies infectieuses du porc, Faculté de médecine vétérinaire, Université de Montréal, C. P. 5000, St-Hyacinthe, Québec J2S 7C6, Canada

ARTICLE INFO

Article history:
Received 3 October 2014
Received in revised form 5 June 2015
Accepted 17 June 2015

Keywords:
Actinobacillus pleuropneumoniae
Biofilm
Penicillin G
eDNA
Poly-N-acetyl-D-glucosamine (PGA)
CpxRA

ABSTRACT

Actinobacillus pleuropneumoniae is a Gram-negative bacterium and causative agent of porcine pleuropneumonia. This is a highly contagious disease that causes important economic losses to the swine industry worldwide. Penicillins are extensively used in swine production and these antibiotics are associated with high systemic clearance and low oral bioavailability. This may expose *A. pleuropneumoniae* to sub-inhibitory concentrations of penicillin G when the antibiotic is administered orally. Our goal was to evaluate the effect of sub-minimum inhibitory concentration (MIC) of penicillin G on the biofilm formation of *A. pleuropneumoniae*. Biofilm production of 13 field isolates from serotypes 1, 5a, 7 and 15 was tested in the presence of sub-MIC of penicillin G using a polystyrene microtiter plate assay. Using microscopy techniques and enzymatic digestion, biofilm architecture and composition were also characterized after exposure to sub-MIC of penicillin G. Sub-MIC of penicillin G significantly induced biofilm formation of nine isolates. The penicillin G-induced biofilms contained more poly-N-acetyl-D-glucosamine (PGA), extracellular DNA and proteins when compared to control biofilms grown without penicillin G. Additionally, penicillin G-induced biofilms were sensitive to DNase which was not observed with the untreated controls. Furthermore, sub-MIC of penicillin G up-regulated the expression of *pgaA*, which encodes a protein involved in PGA synthesis, and the genes encoding the envelope-stress sensing two-component regulatory system CpxRA. In conclusion, sub-MICs of penicillin G significantly induce biofilm formation and this is likely the result of a cell envelope stress sensed by the CpxRA system resulting in an increased production of PGA and other matrix components.

1. Introduction

Actinobacillus pleuropneumoniae is a Gram-negative bacterium belonging to the *Pasteurellaceae* family. It is the causative agent of porcine pleuropneumonia, a highly contagious disease that causes important economic losses to the swine industry worldwide (Zimmerman et al., 2012). Vaccines currently available only provide a partial protection (Ramjeet et al., 2008) and antibiotics still represent the most effective measures for controlling *A. pleuropneumoniae* outbreaks. Antibiotic therapy is used at the onset of symptoms to prevent mortality and the spread of the infection. Commonly used agents for infection control include tiamulin, chlortetracycline, ceftiofur, tilmicosin, florfenicol, enrofloaxacin and penicillin (Archambault et al., 2012). Despite an increase in the number of penicillin G resistant isolates reported in the last decade in Canada, the United States, and Europe (Archambault et al., 2012; Gutierrez-Martin et al., 2006; Salmon et al., 2003; Vanni et al., 2012), penicillin is recommended as the first-choice antibiotic by guideline of prudent use of antimicrobials in animals (Guardabassi et al., 2008; CVMA, 2008). It is well known that β-lactamases represent the main mechanism of bacterial resistance to penicillin G but a number of reports demonstrate that biofilms also contribute to β-lactam resistance (Archambault et al., 2012; Ciofu and Høiby, 2008; Hengzhuang et al., 2013; Olson et al., 2002).

Biofilms are sessile communities of microorganisms enclosed within a matrix of extracellular polymeric substances and attached to biotic or abiotic surfaces (Costerton, 1999a; Jacques et al., 2010). Biofilms are frequently associated with chronic diseases in humans (Costerton et al., 1999b) and in animals (Clutterbuck et al., 2007; Gardner et al., 2011; Jacques et al., 2010), and protect bacteria by decreasing their susceptibility to conventional biocides and the host immune system (Gardner et al., 2011). We have recently

* Corresponding author at: Faculté de médecine vétérinaire, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, Québec J2S 2M2, Canada. Tel.: +1 450 773 8521x8348; fax: +1 450 778 8108.
E-mail address: mario.jacques@umontreal.ca (M. Jacques).

http://dx.doi.org/10.1016/j.vetmic.2015.06.011
0378-1135/

shown that *A. pleuropneumoniae* is able to produce a dense biofilm on abiotic and biotic surfaces (Labrie et al., 2010; Tremblay et al., 2013a,c). The biofilm matrix of *A. pleuropneumoniae* is mainly composed of an extracellular polymer of N-acetyl-D-glucosamine (PGA) (Kaplan et al., 2004).

Numerous studies have shown that sub-minimum inhibitory concentrations (sub-MIC) of some antibiotics can affect growth, morphology, surface properties, pathogenicity and biofilm formation (Andersson and Hughes, 2014; Davies et al., 2006; Fonseca and Sousa, 2007; Gomes et al., 2013; Kaplan, 2011; Wu et al., 2014). Specifically, antibiotics at sub-MIC may induce bacterial biofilm. Among the β-lactam family, ampicillin, imipenem, nafcillin, and oxacillin increased biofilm formation by *Streptococcus intermedius* (Ahmed et al., 2009), *Pseudomomas aeruginosa* (Bagge et al., 2004), *Acinetobacter baumanni* (Nucleo et al., 2009), *Staphylococcus lugdunensis* (Frank et al., 2007) and *Staphylococcus aureus* (Mirani and Jamil, 2011), respectively. Mechanisms associated with antibiotic-induced biofilm formation are dependent on the nature of the antibiotic and the bacterial species. Recent studies have shown that sub-MIC of ampicillin induced biofilm formation by *S. intermedius* via the autoinducer-2/LuxS signalling pathway (Ahmed et al., 2009). On the other hand, exposing *P. aeruginosa* to sub-MIC of imipenem resulted in the up-regulation of genes involved in alginate biosynthesis (Bagge et al., 2004). Recently, Gomes et al. (2013) demonstrated that sub-MIC of penicillin induced bacterial filamentation, cell-surface hydrophobicity of *Corynebacterium diphtheriae* and increased biofilm formation. Thus, the purpose of this study was to evaluate the effect of sub-MIC of penicillin G on the biofilm formation by field isolates of *A. pleuropneumoniae*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of thirteen *A. pleuropneumoniae* field isolates were selected for this study based on their susceptible to penicillin G (Archambault et al., 2012) and their biofilm forming ability (weak or moderate) as previously determined in our laboratory (Labrie et al., 2010). These isolates are from clinical cases from herds located in Saskatchewan, Ontario and Québec. The serotype and the antibiotic minimum inhibitory concentration (MIC) for each isolates used in this study are listed in Table 1. All isolates were grown on Brain Heart Infusion (BHI; Oxoid Ltd, Basingstoke, Hampshire, UK) agar or in BHI broth supplemented with 15 μg/mL β-NAD (BHI-NAD) at 37 °C with 5% CO_2. The growth (OD_{600nm}) of isolates in the presence of 1/2 MIC of penicillin G was monitored and compared to a control of the same isolates without antibiotics.

Table 1
Serotypes and MICs of the *A. pleuropneumoniae* field isolates used in the present study.

Bacterial isolate	Serotype[a]	MIC (μg/mL) of Penicillin G[b]	Biofilm forming ability (OD_{600nm})[c]
7430	1	0.5	0.230 ± 0.10
7431	1	0.5	0.155 ± 0.10
2398	1	0.25	0.341 ± 0.16
111A	1	0.5	0.317 ± 0.18
2521	1	0.5	0.269 ± 0.14
508	5a	0.5	0.395 ± 0.14
996	5a	0.5	0.510 ± 0.14
37943	5a	0.5	0.488 ± 0.21
3128	5a	0.25	0.205 ± 0.08
3060	7	1	0.349 ± 0.09
06-4108	7	0.25	0.050 ± 0.02
13146	15	1	0.167 ± 0.06
05-2080	15	0.5	0.496 ± 0.54

[a] Labrie et al. (2010).
[b] Archambault et al. (2012).
[c] This study.

2.2. Biofilms assay

A microtiter plate biofilm assay was used as described previously (Labrie et al., 2010). Briefly, wells of a 96-well microtiter plate (Costar 3599, Corning, NY, USA) were filled in triplicate with a dilution (1/100) of an overnight culture. Penicillin G (Sigma–Aldrich, St. Louis, MO, USA) was added as sub-MIC (1/2, 1/4 and 1/8 MIC) to the appropriate wells. A culture without antibiotic was included as a control for each isolates. After 24 h incubation at 37 °C with 5% CO_2, the culture medium was removed by aspiration and the plate was washed once by immersing in water. The biofilms were stained with 100 μL of crystal violet (0.1%, w/v) for 2 min at room temperature, washed with distilled water then dried at 37 °C for 30 min. The stain was then released by adding 100 μL of ethanol (70%, v/v) to each well. Absorbance was measured at 590 nm using a spectrophotometer (Powerwave, BioTek Instruments, Winooski, VT, USA). For the autoaggregation assay, biofilms were done in glass tube using a dilution (1/100) of an overnight culture in 5 mL of BHI-NAD and incubated 24 h 37 °C with 5% CO_2.

2.3. Reduction of resazurin

Viability of cells in biofilms formed in the absence or presence of penicillin G was evaluated using the CellTiter-Blue® Reagent (CTB, Promega Corporation, Madison, MI, USA). Briefly, the CTB reagent contains resazurin, a molecule converted to resorufin, a fluorescent end product, by living cells. Nonviable cells do not convert resazurin to resofurin and, thus, do not generate fluorescence. Biofilms were prepared in 96-well microtiter plate as described above. After the 24 h incubation period, 20 μL of CTB reagent was added directly to the growth medium and fluorescence was measured every 30 min for up to 4 h (λ_{ex}: 560 nm and λ_{em}: 590 nm) using a Synergy™ HT multi-detection microplate reader (BioTek Instruments).

2.4. Confocal laser scanning microscopy

Biofilms of isolates S1_7430, S5a_508 and S7_3060 were prepared as described above and stained with Wheat Germ Agglutinin (WGA)–Oregon Green 488 (Invitrogen, Eugene, OR, USA), FilmTracer™ FM®1–43 (Invitrogen), BOBO™-3 Iodide (Invitrogen), FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain (Invitrogen) or the FilmTracer LIVE/DEAD Biofilm Viability Kit (Invitrogen) according to manufacturer's instructions. WGA will bind specifically to N-acetyl-D-glucosamine and N-acetylneuraminic acid residues, FM®1–43 will stain membranes of bacteria, BOBO-3 is a cell-impermeable DNA stain and will stain extracellular DNA and SYPRO Ruby labels most classes of proteins. Stained biofilms were visualized by confocal laser scanning microscopy (CLSM; FV1000 IX81; Olympus, Markham, ON, Canada) and images were acquired using the Fluoview software (Olympus).

2.5. Biomass analysis of the biofilms

Biomass analysis of the biofilms was carried using WGA stained z-stack images obtained by CLSM from randomly-selected areas. The biomass and average thickness of biofilms were determined using Image Pro (Media Cybernetics, MD, USA) as described before (Tremblay et al., 2013a).