Université de Montréal

ZINC AS AN AGENT FOR THE PREVENTION OF BIOFILM FORMATION BY PATHOGENIC BACTERIA

par

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Résumé

Les biofilms sont des communautés structurées de micro-organismes enrobées dans une matrice extracellulaire. Les biofilms sont impliqués dans la persistance de plusieurs maladies infectieuses et la matrice extracellulaire du biofilm protège les bactéries contre les cellules du système immunitaire de l'hôte, les antibiotiques et les désinfectants. Récemment notre laboratoire a démontré que le zinc inhibe la formation de biofilm chez *Actinobacillus pleuropneumoniae*, une bactérie pathogène du porc.

Le but de cette étude est d'évaluer l'effet du zinc sur la croissance et la formation du biofilm chez différentes bactéries pathogènes du porc, telles que *Bordetella bronchiseptica*, *Escherichia coli*, *Haemophilus parasuis*, *Salmonella*, *Staphylococcus aureus* et *Streptococcus suis*. Les bactéries ont été cultivées dans des plaques de 96 puits sous condition optimale de formation de biofilm et les biofilms ont été colorés au cristal violet. La présence du biofilm a été confirmée par microscopie confocale à balayage laser à l'aide du marqueur fluorescent FilmTracer™ FM ® 1-43. À des concentrations micromolaires, le zinc inhibe faiblement la croissance bactérienne et bloque d'une manière dose-dépendante la formation de biofilm d'*A. pleuropneumoniae*, *Salmonella* Typhimurium et *H. parasuis*. De plus, la formation de biofilm de *E. coli*, *S. aureus* et *S. suis* a été faiblement inhibée par le zinc. Nos résultats indiquent que le zinc a un effet inhibiteur sur la formation de biofilm de la plupart des pathogènes bactériens d'origine porcine. Cependant, le mécanisme sous-jacent de l'activité anti-biofilm du zinc reste à être caractérisé.

Mots-clés: Biofilm, Zinc, Inhibition, Bactéries pathogènes, Porcs

Abstract

Biofilms are structured communities of microorganisms enclosed in a self-produced extracellular matrix. Biofilms are responsible for the persistence of most infectious diseases, because the biofilm matrix acts as a form of protection for the bacteria against the host immune system, antibiotic and disinfectants. Recent work in our laboratory demonstrated that zinc could inhibit biofilm formation of *Actinobacillus pleuropneumoniae*, a swine pathogen.

The aim of this study was to evaluate the effect of zinc on growth and biofilm formation of other bacterial swine pathogens, such as *Bordetella bronchiseptica, Escherichia coli, Haemophilus parasuis, Salmonella, Staphylococcus aureus,* and *Streptococcus suis*. Bacteria were grown on 96-well plates under optimal biofilm forming conditions and the biofilms were stained with crystal violet. The presence of biofilms was confirmed by confocal laser scanning microscopy with FilmTracer™ FM® 1-43. At micromolar concentrations, zinc weakly inhibited bacterial growth and effectively blocked biofilm-formation by *A. pleuropneumoniae*, *Salmonella* Typhimurium, and *H. parasuis* in a dose-dependent manner. Additionally, biofilm formation of *E. coli, S. aureus* and *S. suis* was slightly inhibited by zinc. Our results indicate that zinc has an inhibitory effect on biofilm formation of most bacteria of porcine origin. However, the mechanism behind the antibiofilm activity of zinc has yet to be characterized.

Keywords: Biofilm, zinc, inhibition, bacterial pathogens, swine

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LIST OF ACRONYMS AND ABBREVIATIONS

AIPs: Autoinducer peptides

ANOVA: One-way analysis of variance

Bap: Biofilm associated protein

BBM: Basal broth

BHI: Brain Heart Infusion

CBD: Calgary biofilm device

c-di-GMP: Cyclic diguanylic acid

CFA: Colonization Factor Antigen

CLSM: Confocal Laser Scanning Microscopy

DGC: Diguanylate cyclase

DspB: Dispersin B

DTPA: Diethylenetriaminepentaacetic acid

eDNA: Extracellular DNA

EAEC: Enteroaggregative *Escherichia coli*

ETEC: Enterotoxigenic *Escherichia coli*

EPEC: Enteropathogenic *Escherichia coli*

EPS: Extracellular polymeric substances

GlcNAc: N-acetyl-D-glucosamine

HSL or AHL: Acyl homoserine lactones

LB: Luria-Bertani

MRSA: Methicillin-resistant *Staphylococcus aureus*

NAD: Nicotinamide adenine dinucleotide

PCP: Porcine contagious pleuropneumonia

PDEA: Phosphodiesterase A

PIA: Polysaccharide intercellular adhesin

PGA: Poly-N-acetyl-D-glucosamine

PQS: *Pseudomonas* quinolone signal

QS: Quorum sensing

TA: Toxin-antitoxin

TC: Tissue culture

WGA: Wheat Germ Agglutinin

DEDICATION

This thesis is dedicated to my family who never stop believing in me

My mother,

My father,

My sister.

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I. Introduction

Microorganisms exist in two forms in nature, a planktonic form and a sessile form. Free-swimming cells in planktonic form are related to proliferation and the sessile form is called biofilm, which helps a bacterial population to persist. Biofilm are not only involved in human diseases but also in animal diseases. Biofilm bacteria can grow on living or nonliving surfaces. Biofilms are difficult to eradicate once they are established. Five steps are involved in biofilm formation: initial attachment, irreversible attachment, early development of biofilm architecture, maturation of biofilm, and dispersion. Two fundamental bacterial small-molecule signalling pathways, extracellular quorum-sensing (QS) and intracellular cyclic dinucleotide signalling (c-di-GMP) are associated with biofilm formation. An interaction seems to exist between QS and c-di-GMP. QS might regulate biofilm formation through the regulation of c-di-GMP systems.

Biofilm matrix protects embedded bacteria from host immune system and harmful environmental conditions such as antimicrobial agents. Several properties of the biofilm are attributed to this resistance, including limited penetration of the compound, limited growth of the bacteria, the formation of persister cells (a subpopulation of bacteria differentiated into a dormant state), and antibiotic-induced resistance phenotypes. Biofilm matrix is composed of different types of biopolymers, called extracellular polymer substance (EPS). The main components of biofilm matrix are extracellular polysaccharides, proteins and nucleic acids. These molecules contribute to the structures and functions of biofilms.

Various veterinary infections associated with biofilm are difficult to treat with antibiotics or disinfectants. *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Escherichia coli*, *Haemophilus parasuis*, *Salmonella* spp, *Staphylococcus aureus*, and *Streptococcus suis* are important pathogenic bacteria involved in swine infectious diseases. The aim of this study was to evaluate the effect of zinc on growth and biofilm formation of these bacterial swine pathogens.

II. Literature review

1. Definition of the biofilm

In virtually all ecosystems, microorganisms can grow in an enclosed community known as biofilms. Generally, biofilm can be defined as a structured community of microorganisms enclosed in self-produced extracellular polymer substances (EPS) (Costerton et al. 1999; O'Toole et al. 2000; Donan 2002; Branda et al. 2005; Hall-Stoodley and Stoodley 2009). Microorganisms exist in two states, a planktonic form (free-swimming) and sessile form. Bacteria in the planktonic form are involved in proliferation and those in the sessile form allow a population to persist. Biofilm development is a highly complex process and involves the switch from the planktonic form to a sessile life-style. Biofilms are seen as complex differentiated communities (Stoodley et al. 2002). It is widely accepted that the biofilm life-style can protect microorganisms against the host immune system and harmful environmental conditions, and this protection enables microorganism to survive and thrive (Anderson and O'Toole 2008).

Anton van Leeuwenhoek is credited with the discovery of microbial biofilm. In 1683, he scraped the plaque from his teeth, and first observed microorganisms on tooth surfaces using his simple microscopes (Donan 2002). In the 1970s, sessile bacteria were first described as biofilms, and were considered to constitute a major component of the bacterial biomass in the environments. In the 1980s and 1990s, researchers began to appreciate that attached bacteria were organized in elaborate ways (Costerton et al. 1999). For example, in 1991, Lawrence took the first confocal laser scanning microscopy (CLSM) images of living biofilms, and these images showed the sessile bacteria grown in matrix-covered microcolonies (Lawrence et al. 1991). Although biofilms have been well known for several years, the importance of biofilm in animal diseases has been overlooked until recently (Jacques et al. 2010).

2. The importance of biofilm

A wide range of human infections are associated with bacterial biofilms, including otitis media, osteomyelitis, endocarditis and cystic fibrosis pneumonia (Costerton et al. 1999). Biofilms are also involved in various diseases of veterinary importance, such as pneumonia, liver abscesses, enteritis, wound infections and mastitis infections (Clutterbuck et al. 2007; Jacques et al. 2010).

2.1 Biofilm chronic infections

Although bacterial attachment is a feature of all infections, biofilm infections are differentiated by the aggregation of microcolonies attached on a surface. Biofilms lead to several clinical problems, including inflammation, antibiotic resistance, recurrence or persistence and metastasis or the spread of infectious emboli. It is widely recognized that biofilms cause or exacerbate a large number of chronic infections (Hall-Stoodley et al. 2004; Hall-Stoodley and Stoodley 2009). Biofilm infection is difficult to diagnose because accurate predictor have yet to be identified. Parsek and Singh (2003) made an outline of specific criteria for diagnosis of biofilm infections. For example, infecting bacteria should be adherent or attached to the substratum; bacterial clusters or microcolonies encased in an extracellular matrix should be directly visualized; infections should be localized to a particular anatomical site; bacteria should show increased resistance to antibiotics compared to their planktonic counterparts.

Biofilm infections are mainly located at epithelial sites. The infections are recurrent or long-lasting in spite of host immune response and antibiotic therapy. At the beginning of bacterial infections, antibiotics, antibodies and phagocytes can clear the free bacteria. Once attached to the surface, the sessile bacterial cells produce the biofilm and these cells become resistant to antibodies, phagocytes as well as antibiotics. Phagocytes are attracted

to the biofilms. Phagocytic enzymes are released to surrounding tissues which cause damages to the tissues. Planktonic bacteria are also released which causes dissemination of the infection to a different site (Figure 1).

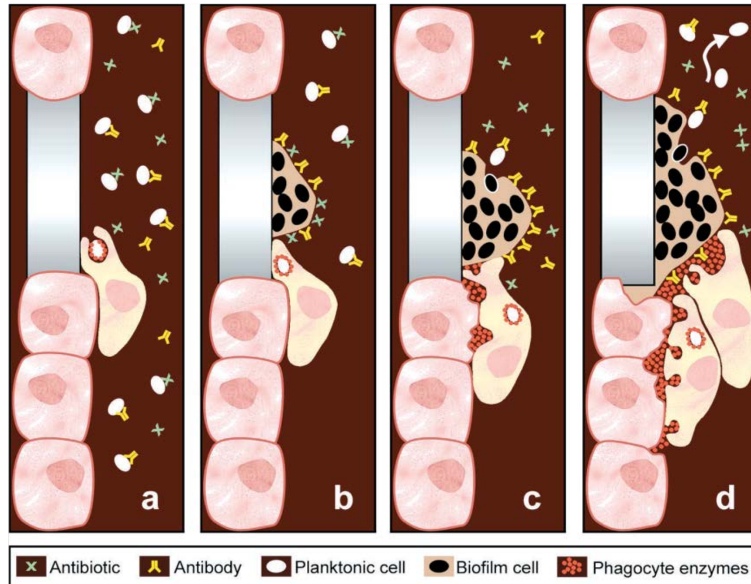


Figure 1: The biofilm formation and its resistance to immune system of host. Antigens released by sessile bacteria stimulate an antibody response which neutralizes the bacteria, but bacteria within the biofilm are not susceptible to those antibodies. **(Costerton et al. 1999)**

2.2 Biofilms in animal and human diseases

Using the Calgary Biofilm Device (CBD), biofilms were demonstrated to be associated with veterinary infectious diseases of cattle, sheep, pigs, chicken, and turkeys (Olson et al. 2002). Additionally, biofilm formation is also related to numerous human chronic infections such as periodontitis, device-related infections, CF pneumonia, chronic urinary tract infection, recurrent tonsillitis, chronic rhinosinusitis, chronic otitis media and chronic wound infections. Chronic wounds include diabetic foot ulcers, pressure ulcers as well as venous leg ulcers. (Hall-Stoodley and Stoodley 2009). In veterinary diseases,

infected wounds are a typical biofilms-associated infection and are regarded as a key problem in the hospitalized animal (Clutterbuck et al. 2007). Bacterial biofilm are more prevalent in chronic wounds than acute wounds. Biofilm-related wound diseases are typically persistent infections that develop slowly, are rarely resolved by immune defences, and respond poorly to antimicrobial therapy (James et al. 2008).

3. Biofilm formation

Microorganisms can grow a biofilm on both biotic and abiotic surfaces including those found in soil and aquatic systems, or indwelling medical devices. Once biofilms are attached, it becomes difficult to eradicate them. Biofilm formation involves the following five steps: reversible attachment, irreversible attachment, growth, maturation, and detachment and dispersion (Figure 2).

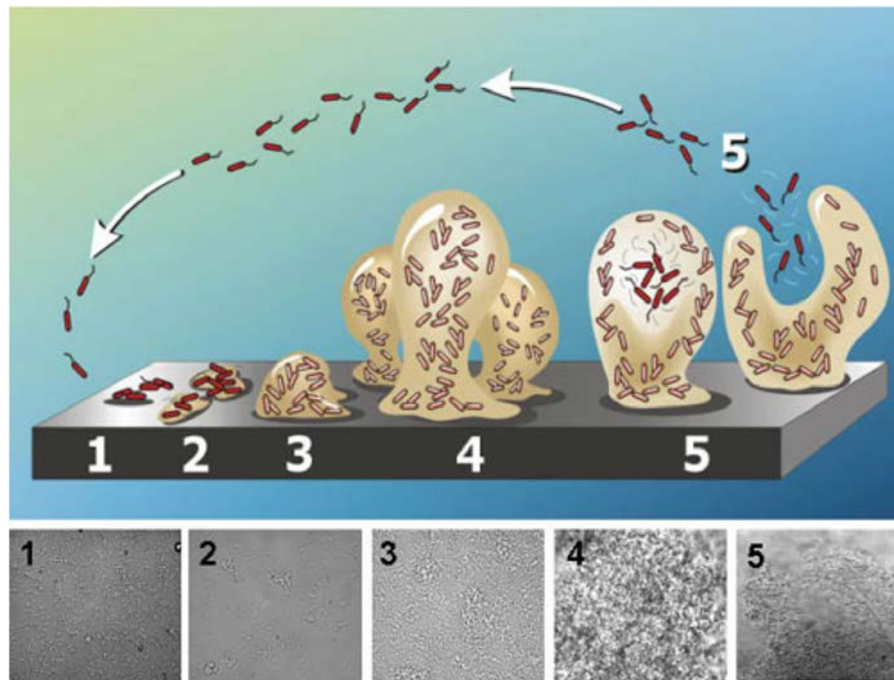


Figure 2: Diagram of the five steps involved in biofilm development: 1) initial attachment, 2) irreversible attachment, 3) early development of biofilm architecture, 4) maturation of

biofilm architecture and 5) dispersion. Images below the cycle steps show the microscopic appearance of the biofilm at each step. **(Stoodley et al. 2002)**

3.1 Reversible and irreversible attachment

Specific environmental cues are needed to stimulate biofilm development, and include temperature, pH, nutrient availability. These cues vary between species (O'Toole et al. 2000). For the first step to be initiated, bacteria need to be very close to the surface to allow for the initial attachment. This initial reversible attachment is associated with non-specific interactions such as electrostatic, van der Waals, and hydrophobic interactions. Surface charge and hydrophobic interactions are the factors responsible for the adherence of the cell to the surface. During this initial contact, bacteria can be easily removed by shear forces such as rinsing. The following step is the transition from reversible attachment to irreversible attachment. In this attachment process, bacteria lock onto the surface by the production of extracellular polysaccharides and/or specific adhesins, such as pili or fimbriae that may form a complex with the surface. Much stronger physical or chemical forces are required to remove the bacteria from the surface after this step (Palmer et al. 2007).

3.2 Biofilm growth and maturation

Once bacteria are strongly attached to the surfaces, the bacteria begin to multiply and develop the extracellular polymer substances (EPS) to help maintain the microcolony and biofilm structure (Stoodley et al. 2002). The biofilm matrix keeps the bacterial cells together and firmly attaches the bacterial mass to the surface. The mature biofilm is characterized by the presence of three-dimensional structures containing a large number of tightly organized cells. In some cases, the structure of a biofilm will look like a mushroom (Hall-Stoodley et al. 2004). The three dimensional organization of the biofilm is important for liquid flow to permit the distribution of nutrients and the disposal of wastes (McLandsborough et al. 2006). EPS help bacteria to enhance nutrient capture and to resist

environmental stress and antimicrobial agents (Mah and O'Toole 2001). Since pH, oxygen concentration, nutrient availability and cell density within biofilms vary, heterogeneity in metabolic activity and replication exists among cells located in different parts of the biofilm (Kaplan 2010).

3.3 Biofilm dispersal

Biofilm detachment and dispersal is the final stage of the biofilm development, this step is characterized by the detachment of cells from the biofilm and their dispersal into the environment. Biofim dispersal is the crucial step in the biofilm life cycle due to its contribution to the dispersal and survival of bacteria, and disease transmission. Three distinct phases are involved in biofilm dispersal: detachment of cells from the biofilm, translocation of the cells to a new surface and reattachment of the cells to a new surface (Kaplan 2010). Extracellular enzymes produced by bacteria can degrade matrix components and it is a well-known mechanism of dispersal. For example, dispersin B (DspB), a glycoside hydrolase produced by *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* and *A. pleuropneumoniae*, degrades poly-N-acetylglucosamine (PGA), an important component of some biofilm matrices (Kaplan et al. 2004; Kerrigan et al. 2008). In addition, several extracellular proteases have been reported as crucial player in biofilm detachment. For example, the increasing production of extracellular proteases accelerates the detachment of biofilm of *S. aureus* and this indicates that biofilm detachment requires extracellular protease (Boles and Horswill 2008). Degradation of biofilm matrix extracellular DNA (eDNA) is also associated with biofilm detachment. The staphylococcal thermonuclease degrades eDNA and plays a significant role in biofilm dispersal (Mann et al. 2009).

3.4 Extracellular and intracellular signalling in biofilm formation

Small molecules are used by bacteria as extracellular and intracellular signals. This kind of signalling information is integrated by bacteria and allows bacteria to respond to various changes in the environment. Two fundamental signalling pathways, the extracellular quorum-sensing signalling and the intracellular cyclic dinucleotide signalling, are involved in the regulation of biofilm formation (Camilli and Bassler 2006).

3.4.1 Quorum sensing

Specific extracellular signals regulate activation of the metabolic pathways that induce biofilm formation. These signals are produced by the bacterial community, and are called autoinducers, whose concentration is related to the density of the cell population. Autoinducers lead to multicellular responses in the bacterial population by triggering signal transduction cascades. This mechanism of cell-cell communication in bacteria is termed quorum sensing (Figure 3). QS associates with the production, release and detection of chemical signalling molecules, and then allows microbial cells to regulate gene expression according to cell-density (Camilli and Bassler 2006; Vu et al. 2009; Lopez et al. 2010;).

In a QS system, individual cells release small QS signalling molecules and cells respond to the signals from the surrounding environment in a coordinated manner. Among QS systems, several major types have been identified. Two types of small molecule autoinducers, acyl homoserine lactones (called as HSL or AHL) signals and autoinducer peptides (AIPs) are predominately used in Gram-negative and Gram-positive bacteria, respectively (Figure 3).

3.4.2 c-di-GMP

The second messenger cyclic diguanylic acid (c-di-GMP) is widely known as a central regulator for the formation and maintenance of biofilms in a large number of organisms. High c-di-GMP levels can stimulate various biofilm-associated functions (Jenal and Malone 2006; Romling and Amikam 2006; Cotter and Stibitz 2007; Hengge 2009).

The c-di-GMP is synthesized by diguanylate cyclase (DGC) and degraded by phosphodiesterase A (PDEA). DGCs have similar sequences, and are called GGDEF proteins after the conserved residues in their active site. PDEAs are members of the EAL (conserved protein domain with PDEA activity) domain family, and their name is also based on the conserved residues of their active site. Other cellular functions are also regulated by c-di-GMP (Figure 4). In the c-di-GMP regulatory system, GGDEF and EAL domain proteins control intracellular c-di-GMP levels, thus regulate the transition between biofilm and planktonic lifestyles (Cotter and Stibitz 2007).

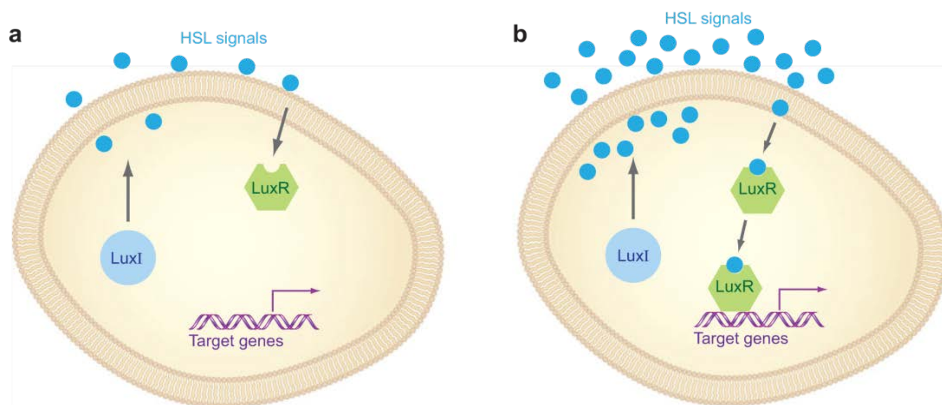
3.4.3 Relation between quorum-sensing and c-di-GMP systems

Bacteria use both the QS via AI molecules and c-di-GMP signalling to control the formation and dispersion of biofilm. c-di-GMP acts as an intracellular secondary messenger that stimulates biofilm formation. QS influences the transcription of genes involved in the production of c-di-GMP, GGDEF and/or EAL domains. Therefore, QS might have the ability to regulate biofilm formation through modulation of intracellular c-di-GMP levels. There seems to be an interaction between QS and c-di-GMP during biofilm formation of bacteria (Waters et al. 2008).

4. Biofilm resistances

Bacteria grown in biofilms are protected from antimicrobial agents and the host immune system that normally eradicate planktonic cells. This type of resistance is unique to biofilm-associated bacteria and various resistance mechanisms have been proposed (Costerton et al. 1999; Donlan and Costerton 2002; del Pozo and Patel 2007; Lewis 2010).

A



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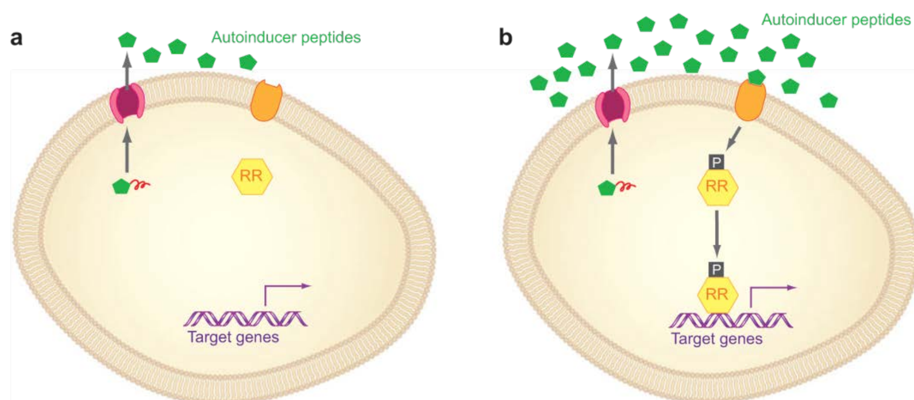

Figure 3: Different quorum sensing systems in Gram-negative bacteria and Gram-positive bacteria. Quorum sensing systems in Gram-negative bacteria (A). Acyl-homoserine lactone

(HSL or AHL) signals (*blue circles*) are produced by the LuxL enzyme homologues that bind to LuxR homologues to activate expression of target genes. (Aa) At low cell densities, concentration of the signal is low inside and outside the cell, and results in minimal activation of LuxR. (Ab) At high cell densities, acyl-HSL binds and activates LuxR which leads to the regulation of the expression of target genes. Quorum sensing in Gram-positive bacteria (B). AIPs are produced as precursor peptides and exported outsite the cell. (Ba) At low cell densities, concentration of the AIP signal is low outside the cell and there is no activation of the response regulator (RR). (Bb) At high cell densities, concentration of the AIP is high which results in the AIP binding to a histidine kinase receptor. Activation of the histidine kinases leads to phosphorylation of the RR and regulation of the expression of genes targeted by the RR. **(Jayaraman and Wood 2008).**

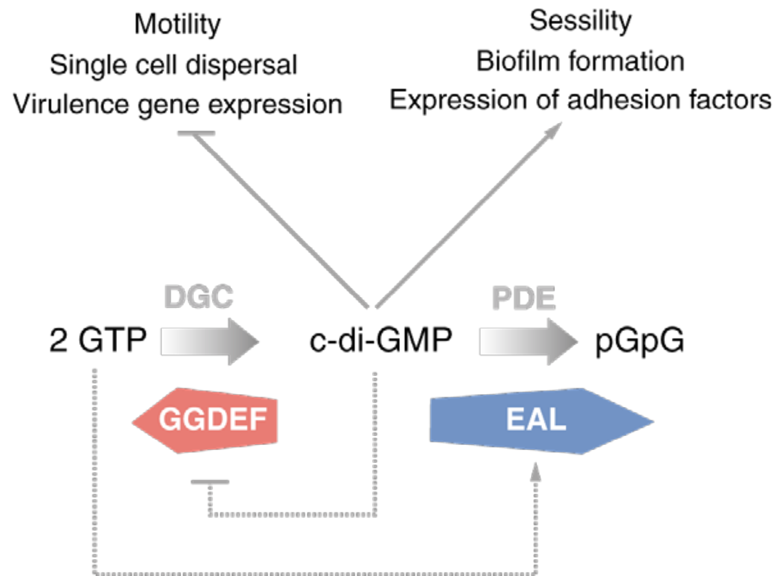

Figure 4: Schematic of the synthesis and hydrolysis of c-di-GMP. Stippled lines show the allosteric control of DGC and PDEactivities. **(Jenal and Malone 2006)**

4.1 Biofilm resistance to host immune system

Bacteria embedded in the biofilm are hard to be destroyed by the first line of host defences, phagocytic cells. Several factors account for the failure of the host's immune system to kill biofilms (Clutterbuck et al. 2007). The mechanisms of biofilm resistance to leukocyte killing and clearance may include several factors, such as limited penetration of leukocytes into the biofilm matrix, inhibitory effect of biofilm matrix on leukocyte-specific processes, decreased ability to phagocytize biofilm bacteria by leukocytes, increased resistance to leukocytes by a global response regulation in biofilm, genetic switch inducing the increase production of components associated in immune evasion (Leid et al. 2005). For example, polysaccharide intercellular adhesin (PIA) produced by *S. aureus*, is essential for immune evasion. PIA is involved in biofilm formation and protects bacterial cells against innate host defence. A PIA-mutant strain was more susceptible to major antibacterial peptides (Vuong et al. 2004a, b; Foster 2005). *Staphylococcus epidermidis* biofilm protect *S. epidermidis* from phagocytic uptake and inhibit macrophage activation (Schommer et al. 2011). Furthermore, *S. aureus* biofilms attenuate the production of inflammatory mediator and inhibit the invasion of macrophages into the biofilm. *S. aureus* biofilms do not activate the classical TLR recognition pathways, which likely limit the ability of tissue macrophages to invade biofilms (Thurlow et al. 2011).

4.2 Antimicrobial resistance of biofilm

Bacteria in biofilm are 10 to 1000 fold more resistant to various antimicrobial agents than their planktonic counterpart (Mah and O´Toole 2001). Antibiotic therapy has been commonly applied to prevent bacterial colonization and to eradicate existing infections. However, biofilm-associated infection results in antibiotic resistance which leads to ineffective antibiotic treatment of these infections (Stewart and Costerton 2001; Davies 2003; Clutterbuck et al. 2007). Growth as a biofilm results in an altered genetic regulatory patterns. These changes protect bacteria from antibiotic killing. Several factors

associated with the innate properties of biofilm affect antibiotic resistance. For example, the biofilm matrix acts as a diffusion barrier and limits antibiotic diffusion through the biofilm, and prevents antibiotic from reaching their targets. In biofilms, limited penetration result in the cells death in the outer layer of bacteria and low level of antimicrobial exposure to deeper regions of the biofilm (Szomolay et al. 2005). Some antimicrobial agents, such as tobramycin and ciprofloxacin can penetrate biofilms to kill the bacteria (Walters et al. 2003).

The reduced levels of oxygen or nutrient result in the slow growth of bacteria embedded in the biofilm. Metabolic activity is stratified in biofilms. Higher activity present at the surface of the biofilm is observed whereas low or no activity is recorded in the inner part of biofilm. The reduced growth rate results in resistance of biofilms to some antimicrobial agents (Costerton et al. 1999; Mah and O'Toole 2001; Hoiby et al. 2010). Tobramycin and ciprofloxacin are only able to kill metabolically active bacterial located in zones with high oxygen concentration.

Additionally, a small subpopulation of bacteria within biofilms is thought to differentiate into persister cells. Persister cells are not metabolically active and will not be killed by antibiotics. In chronic infections, the majority of cells are killed by antibiotics, and the immune system eliminates the regular cells and persister cells from the bloodstream. The remaining live cells persist in biofilm. These persisters seem to be the main factor responsible for the persistance of chronic infections during antimicrobial therapy (Lewis 2010)

Within biofilms, some resistance genes are specifically regulated leading to decreased growth and altered metabolism, and persister cells (Donan 2002; Anderson and O'Toole 2008). The resistant phenotype of biofilm cells might be induced by nutrient limitation, low oxygen stress response, high cell density. Some genes involved in the increased-resistance phenotype were related to biofilm phenotype (Mah et al. 2003). Many

antibiotics do not kill the cells at subinhibitory concentrations, but induce gene expression associated with stress response (Yim et al. 2007).

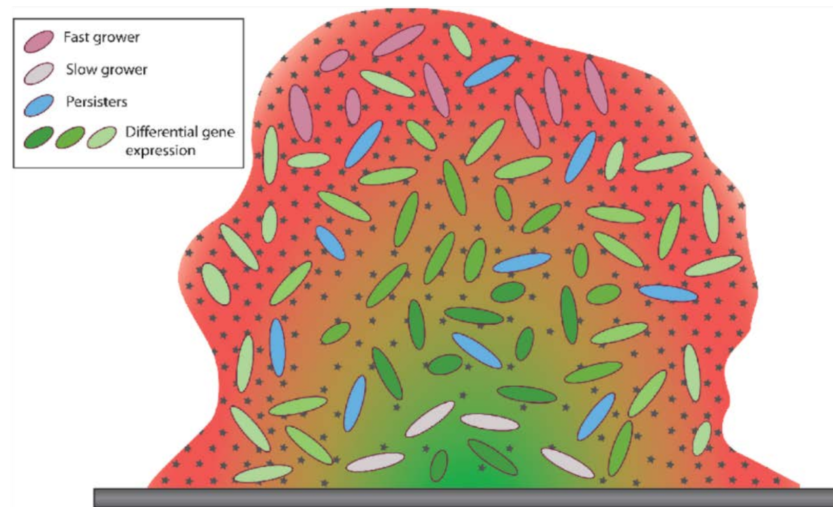

Figure 5: Mechanism of antibiotic resistance by biofilms. The single biofilm macrocolony is composed of bacteria (*ovals*) surrounded by an extracellular matrix (multicolored background). *Small dark dots* represent the antibiotic molecules to which the biofilm has been exposed. Limited antibiotic diffusion through the matrix might protect bacteria buried deep within the biofilm from antibiotic action. Oxygen and nutrient concentrations also decrease in the deeper parts of the biofilms and this is represented by a color gradient form *red* (aerobic and high nutrient concentrations) to *green* (anaerobic and low nutrient concentrations). The gradients slow the growth of bacteria in the deepest zone of the biofilm (*tan*), and thus facilitate survival of bacteria in the presence of antibiotic that typically kill only fast growing microorganisms (*magenta*). The *red* to *green* gradient also represents other possible variations within the heterogeneous biofilm, such as pH. Persister cells are considered non-growing or slow-growing, and are represented by *blue ovals*

scattered throughout the biofilm. Finally, the *green ovals* denote biofilm bacteria expressing specific biofilm activated resistance genes. Differential expression of these genes (different shade of *green*) in response to environmental gradients in the community might influence the antibiotic resistance state of individual bacteria within the biofilm. **(Anderson and O'Toole 2008)**

5. The composition of biofilms

In most biofilms, the matrix accounts for over 90% of the dry mass and the microorganisms account for less than 10%. The matrix consists of different types of biopolymers, known as extracellular polymeric substances (EPS). The EPS is like the "house of the biofilm cells", if the biofilm is "a city of microbes" (Watnick and Kolter 2000; Flemming et al. 2007). In all biofilms, the EPS is a complex and extremely important component, which provides the architectural structure and mechanical stability to the population (Allison 2003). Figure 6 shows the mushroom-like structure of biofilms. Sessile cells constitute a small part of the matrix-enclosed community. Water channels are well organized for conducting water in convective flow and delivering nutrients to other parts of the community. EPS formed an additional barrier between the bacterium and it surrounding environment (Bazaka et al. 2011).

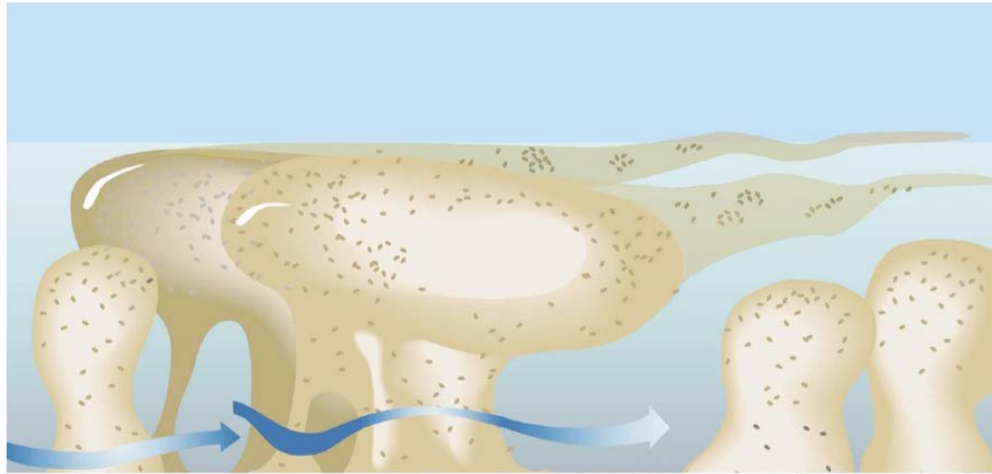

Figure 6: Diagram of biofilm structure. The arrows indicate the flow of water and nutrients within water channels at the base of the biofilm reaching most parts of the community. **(Costerton, 2003)**

Much of the biofilm matrix is water, and it accounts for up to 97% of the wet mass. Water can be bound within the capsules of microbial cells or can exist as a solvent whose physical properties are determined by the solutes dissolved in it (Sutherland 2001; Allison 2003). Water hydrates the EPS matrix which reduces the rate of drying and thus buffers the biofilm cells against fluctuation in water potential (Flemming and Wingender 2010). The EPS forms a three-dimensional network of cross-linking polymeric strands than can retain water more than 15 times its weight. EPS can protect the biofilm-embeded bacteria in unsaturated soils resulting in hydraulic decoupling during rapid wetting or drying events (Or et al. 2007).

Polysaccharides were thought to be the main component in EPS and therefore the abbreviation ''EPS'' was mostly used to describe the extracellular polysaccharides. Recent studies have shown that the biofilm matrix is composed of several components. Therefore, the term EPS refers to polysaccharides, proteins, nucleic acids and other biopolymers situated outside the cell (Eboigbodin and Biggs 2008; Vu et al. 2009). These components are secreted and organized into a structure by biofilm cells such that it encases and

immobilizes the microorganisms as aggregate and help the retention of water (Flemming et al. 2007; Flemming and Wingender 2010). The chemical structure of EPS secreted by the biofilm cells into the environment varies among species. EPS compounds may also differ within bacterial species. The variability of EPS among microorganism can be applied to identify and classify cells (Czaczyk and Myszka 2007).

5.1 Extracellular polysaccharides

Bacteria produce extracellular polysaccharides that significantly impact bacterial virulence. Extracellular polysaccharides can be classified as capsular polysaccharides or exopolysaccharides. When bacteria are grown in growth medium, and then centrifuged, extracellular polysaccharides that remain cell-associated are considered to be part of the capsule, while those remaining in the supernatant are referred to as the exopolysaccharides. This distinction is not easy to make since many extracellular polysaccharides produced in biofilms are insoluble and hard to separate from the cells (Branda et al. 2005).

Bacterial extracellular polysaccharides are key components and features of the extracellular matrix of biofilms. Extracellular polysaccharides contribute various functions in biofilm matrix such as adherence to surfaces and other cells, structural support, and resistance to host and environmental stress (Sutherland 2001). The nature of biofilm extracellular polysaccharide depends on the variety of growth conditions, medium, and substrates (Lopez et al. 2010). Some extracellular polysaccharides are homopolysaccharides such as cellulose. Other extracellular polysaccharides are heteropolysaccharides with neutral and charged sugar residues. Some extracellular polysaccharides are polyanionic due to the presence of uronic acids, such as alginate, xanthan and colonic acid. Meanwhile, other extracellular polysaccharides belong to polycationic ones, such as PIA, which is composed of a linear chain of N-acetyl-D-glucosamine (GlcNAc) residues in β (1, 6) linkage (Flemming and Wingender 2010). This polymer is referred as poly-*N*-acetylglucosamine, called PGA in *A. pleuropneumoniae* or PIA in *Staphylococcus spp* (Kaplan et al. 2004).

One of the best characterized matrix polysaccharide is PGA/PIA which functions as a biofilm extracellular polysaccharide matrix in phylogenetically diverse bacterial species. PGA is associated with intercellular adhesion and attachment of cells to the surfaces. PIA synthesis is involved in biofilm formation by various bacteria (Rohde et al. 2010). PIA is the major component for intercellular adhesion, and can be synthesized by enzymes encoded by the *icaADBC* operon. PIA is also found in *S. epidermidis*, *S. aureus*, *S. caprae*, *S. lugdunensis* and *S. haemolyticus*.

Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans* and *A. pleuropneumoniae* have the ability to produce an enzyme which can hydrolyze PGA/PIA, called dispersin B (DspB), to release their biofilms. DspB appears to be a potential antibiofilm drug (Kaplan et al. 2003; Kerrigan et al. 2008). DspB, although not produced by staphylococci, still can degrade PGA/PIA, and break staphylococcal biofilms (Kaplan et al. 2004; Otto 2008).

5.2 Extracellular DNA

Extracellular DNA (eDNA) play a key role in the composition and formation of biofilms (Whitchurch et al. 2002). In Bordetella biofilm, eDNA is a crucial structural component of the biofilm matrix formed *in vitro* and *in vivo* (Conover et al. 2011). DNA release and transformation is referred as one part of a biofilm-related life cycle and eDNA can be considered as a source of genes for horizontal gene transfer. Meanwhile, the released DNA keeps the stability of the biofilm structure (Molin and Tolker-Nielsen 2003). Initial adhesion to surfaces and aggregation of bacteria are vital steps in the process of biofilm formation. The presence of eDNA on bacterial cell surfaces enhances adhesion and surface aggregation (Das et al. 2010). eDNA plays an important role in the initial phase of biofilm development by *S. epidermidis* on polystyrene or glass surfaces under static or hydrodynamic conditions (Qin et al. 2007).

The origin of eDNA appears to be different among species (Flemming and Wingender 2010). Different bacteria within biofilm can release DNA at different levels in

the biofilm matrix (Cheng et al. 2011). eDNA is considered to be the result of cell lysis (Molin and Tolker-Nielsen 2003). On the other hand, *Helicobacter pylori* biofilm is composed of eDNA that largely did not originate from the bacteria but from another source (Grande et al. 2011).

5.3 Extracellular Proteins

The biofilm matrix includes a large amount of proteins. Some extracellular proteins in biofilm matrix are involved in biofilm formation. For example, many proteinaceous cellular appendages, such as pili, flagella and fimbriae, which are major structural components used to connect cells to each other or to different surfaces (Lopez et al. 2010).

Other extracellular proteins, such as cell surface-associated and extracellular carbohydrate-binding proteins (lectins), are related to the formation and stabilization of the polysaccharide matrix and act as a connection between the bacterial surface and extracellular EPS (Flemming and Wingender 2010).

6. Biofilm formation of selected veterinary pathogens

Various veterinary organisms have the ability to form the biofilms (Olson et al. 2002). Biofilm formation in animal pathogens has been recently reviewed (Jacques et al. 2010).

6.1 *Actinobacillus pleuropneumoniae*

A. pleuropneumoniae is a Gram-negative bacterium that belongs to the *Pasteurellaceae* family, and is the etiological agent of porcine contagious pleuropneumonia, a severe respiratory disease of swine. *A. pleuropneumoniae* binds cells of the lower respiratory tract in a process involving different adhesins and probably biofilm

formation (Izano et al. 2007; Bossé et al. 2010; Chiers et al. 2010). The *pgaBC* genes were upregulated when *A. pleuropneumoniae* adhere to SJPL cells, showing that the biofilm formation may be responsible for the colonization and persistence of *A. pleuropneumoniae in vivo* (Auger et al. 2009). Biofilm formation might be part of the response to envelop damage caused by the host immune system, resulting in the persistence of *A. pleuropneumoniae* within tonsils or sequestered lung lesions (Bossé et al. 2010).

In previous studies, *A. pleuropneumoniae* has been reported to have the ability to form biofilms under certain growth conditions (Kaplan and Mulks 2005; Labrie et al. 2010). PGA, encoded by *pgaABCD*, is a polysaccharide of the biofilm matrix. In *A. pleuropneumoniae*, PGA is responsible for the biofilm formation on polystyrene microtiter plates (Izano et al. 2007). PGA plays a role in intercellular adhesion and biofilm formation. Detachment and dispersion of *A. pleuropneumoniae* biofilms can be intiated with dispersin B, a glycosyl hydrolase produced by *A. actinomycetemcomitans*, and *A. pleuropneumoniae* (Kaplan et al. 2004). Interestingly, our laboratory recently reported that biofilm formation by *A. pleuropneumoniae* could be inhibited by low concentrations of zinc (Labrie et al. 2010).

6.2 *Bordetella bronchiseptica*

B. bronchiseptica infections lead to various respiratory syndromes and diseases in a wide range of mammals, such as dogs, pigs, cats, rabbits and rats. *B. bronchiseptica* establishes asymptomatic infection, but in pigs, it can cause atrophic rhinitis (Sloan et al. 2007). *B. pertussis* and some strains of *B. parapertussis* are responsible for whooping cough in humans. Various respiratory diseases are caused by *B. bronchiseptica*, and these infections are usually chronic. This phenomenon suggest that this organism have specific mechanisms to resist host immune responses (Irie et al. 2004).

The survival and persistence of *B. bronchiseptica* in the mammalian nasopharynx is thought to be the result of biofilm formation (Sloan et al. 2007; Conover et al. 2010). *B.*

bronchiseptica can colonize the nasal cavity of the infected host. Detaching cells from a biofilm may be responsible for the transmission from one host to another, or the persistence of chronic infections if bacteria are inhaled into the lower respiratory tract (Irie and Yulk 2007).

In *Bordetella* biofilm development, eDNA is important in maintaining biofilm stability (Conover et al. 2011). Bps is a surface polysaccharide of *B. pertussis*. The Bps polysaccharide is similar to the poly-β-1, 6-N-acetylglucosamine polysaccharides. Due to its ability to promote biofilm formation, Bps is necessary for colonization of the mouse nose and the trachea (Conover et al. 2010).

6.3 *Escherichia coli*

E. coli is an important member of the normal intestinal microflora of humans and other mammals. *E. coli* is also an important pathogen causing diarrhea, it leads to the death of pigs and occurs worldwide. Enterotoxigenic *E. coli* (ETEC), producing adhesins that mediate bacterial adherence to the intestines and enterotoxins, is the main cause of postweaning diarrhea. Enteropathogenic *E. coli* (EPEC) is another type of *E. coli*, and appears to be associated with about 6% of cases of postweaning diarrhea (Fairbrother et al. 2005).

Many isolates of *E. coli* have the ability to form biofilm *in vivo* and *in vitro.* Three extracellular polysaccharides, cellulose, PGA and colanic acid are major elements of the biofilm matrix of *E. coli*. These polymers are related to the activity of cell-to-cell contacts, contributing to the biofilm formation at liquid-solid interfaces, pellicles at air-liquid interfaces, cell aggregates and clumps in liquid cultures, and wrinkled colony morphology on agar plates (Beloin et al. 2008). PGA is produce by *E. coli* K-12 and is involved in both cell-cell adhesion and formation of permanent attachment to surfaces (Agladze et al. 2005). *In vivo*, when *E. coli* overexpresses genes required for aggregation and exopolysaccharide production, the virulence gene expression is at its highest (Beloin et al. 2008).

6.4 *Haemophilus parasuis*

H. parasuis, belong to the *Pasteurellaceae* family, the same family as *A. pleuropneumoniae*. *H. parasuis* exists commonly in the upper respiratory tract of pigs. This pathogen can cause severe systemic disease (Glässer’s disease) under appropriate conditions, the characteristic fibrinous polyserositis, polyarthritis and meningitis (Oliveira and Pijoan 2004). *H. parasuis* forms biofilms with variable ability among strains. Only the *H. parasuis* isolated form the nasal cavities of infected pigs could form biofilms. Isolates from the lung and brain were unable to form biofilms. This indicates that biofilm formation may be associated with persistent infection of *H. parasuis* in the porcine upper respiratory tract. In healthy pigs, *H. parasuis* can be easily isolated from the upper respiratory tract and most isolates are non-virulent. The upper respiratory tract is a suitable biotic surface for the biofilm formation by *H. parasuis*, leading to persistent infection. Pathogenic strains isolated from the lung/brain are not able to recover the ability to form biofilm. Non-virulent serovars show higher biofilm formation than virulent serovars. The biofilm formation phenotype is involved with the recovery site of strains and is maintained when bacteria are passaged *in vitro* and in the upper respiratory tract (Jin et al. 2006).

6.5 Salmonella

Salmonellosis is an important foodborne disease, it has a significant economical impact worldwide. This microorganism commonly infects both human and animals (Rabsch et al. 2001). The survival of *Salmonella* on a polypropylene surface is related to the ability to form a biofilm. The extracellular polymeric materials on the polypropylene surface can protect the bacterial cells from desiccation. High biofilm producer strains of *Salmonella* survive longer on polypropylene surfaces than the low biofilm producers. Since the wide use of plastic materials in food production and cooking, the contamination of *Salmonella* strains on plastic surfaces represents a high risk to food safety (Iibuchi et al. 2010).

Salmonella enterica serovar Typhimurium (*S*. Typhimurium) is the most frequently isolated serovar found in slaughter pigs in Europe. Persistently infected pigs lead to the contamination of porcine carcass by *S*. Typhimurium (Van Parys et al. 2010).

Biofilm formation is influenced by environmental conditions such as temperature and the culture medium as well as the origin of the strain. Curli, fimbriae and cellulose contribute specifically to the biofilm formation under low nutrient conditions at ambient temperatures. The difference in the composition of the biofilm matrix of *S.* Typhimurium grown under conditions mimicking the plant environment and the *in vivo* environment suggest that factors required to prevent biofilms in an industrial setting are different than those required to treat an infection (Castelijin et al. 2012).

6.6 *Staphylococcus aureus*

Methicillin resistant *Staphylococcus aureus* (MRSA) colonization in pigs has been reported in many regions, such as Europe, North America and Asia. MRSA could be transmitted between pigs and humans. Exposure to pigs is the main factor of MRSA infection in humans. ST398 MRSA is considered as livestock-associated MRSA, because it seems to have originated in pigs, and it could lead to human MRSA infections (Khanna et al. 2007; Smith and Pearson 2011; Fitzgerald 2012). In addition, *S. aureu*s is a major pathogen of bovine mastitis. Biofilm formation is considered to be an important virulence factor in *S. aureus*. Biofilms produced by *S. aureus* promote its adherence and colonization of the epithelium of the mammary gland (Melchior et al. 2006; Oliveira et al. 2006). Biofilm promotes the persistence of *Staphylococci* in the host tissue and reduces the susceptibility to antibiotics (Melchior et al. 2006).

The polysaccharide in *Staphylococci*, called PIA, is synthesized by enzymes encoded by the *ica* operon. Some strains rely more on the polysaccharide for biofilm formation, but other strains form polysaccharide-independent biofilms with matrices that contain primarily proteins and eDNA. Biofilms rely on polysaccharides for structural

integrity. Some surface proteins such as fibronectin-binding proteins, protein A, SasG, and Bap, are associated with cell-cell and cell-surface interactions (Boles and Horswill 2011).

6.7 *Streptococcus suis*

Streptococcus suis, a Gram-positive bacterium, is a major pathogen of pigs and it is also an emerging zoonotic agent of meningitis and streptococcal toxic shock-like syndrome (Fittipaldi et al. 2012). This pathogen is transmitted via respiratory route and colonizes the palatine tonsils of pigs. The infections by *S. suis* result in meningitis, septicemia, arthritis, and endocarditis. Among 35 serotypes of *S. suis* (1 to 34 and ½), serotype 2 is the most frequently associated with pathology. The capacity of biofilm formation is restricted to a few strains (Grenier et al. 2009). The addition of fibrinogen in the growth medium increases biofilm formation by *S. suis* (Bonifait et al. 2008; Bonifait et al. 2010; Grenier et al. 2009).

In *S. suis*, more biofilm is formed in non-virulent serovar strains than in virulent serovars. This indicates biofilm formation might be related to virulence but not the character of the virulent strains (Wei et al. 2009). Biofilm cells have lower virulence in an animal model, and some virulence genes are down-regulated in biofilm cells. A virulent strain may reduce its virulence by forming a biofilm thus resulting in persistent infection *in vivo* (Wang et al. 2011).

7. Antibiofilm agents

There are different strategies to prevent and inhibit biofilm formation. These strategies include the prevention of microbial attachment, prevention of microbial growth, disrupting cell-to-cell communication, inhibition of matrix synthesis, and disintegration of the biofilm matrix (Landini et al. 2010; Rendueles et Ghigo 2012; Yang et al. 2012). A basic strategy to discover inhibitors of biofilm formation is the direct screening of chemical

compounds during biofilm formation assays. Molecules can also be evaluated for their ability to disperse preformed biofilms. In recent years, the development of target-based screening for anti-biofilm agents has been focused on inhibitors of QS (e.g. halogenated furanones, azithromycin, 4-nitro-pyridine-N-oxide) and compounds interfering with the metabolism of the signal molecule c-di-GMP (e.g. sulfathiazole). Effective antibiofilm agents, used in combination or not with antimicrobial agents, could dramatically change the treatment of many infectious diseases. Examples of antibiofilm agents reported in the literature are given in Table 1.

Table 1. List of Antibiofilm agents.

Inhibition mechanism	Antibiofilm agent	References
Inhibition of the initial adhesion	Lactoferrin	Singh et al. (2002)
	Biosurfactants	Rodrigues (2011)
Modulators of quorum-sensing signals	Halogenated furanones	Hentzer et al. (2002), Muh et al. (2006)
	Azithromycine	Nalca et al. (2006), Hoffmann et al. (2007)
	4-nitro-pyridine-N-oxide	Rasmussen et al. (2005)
Inhibitors of the biosynthesis of c-di-GMP	Sulfathiazole	Antoniani et al. (2010)
	Fluorouracile	Walz et al. (2010)
Enzymatic degradation of biofilm matrix	DNase I	Whitchurch et al. (2002), Flemming and Wingender (2010)
	Dispersine B	Kaplan et al. (2003)
Modulation of the quorum-sensing to promote dispersion	Autoinducing peptide	Boles and Horswill (2008)
	cis-2-decenoic acid	Davies and Marques (2009)
	D-amino acids	Kolodkin-Gal et al. (2010)
	Nitric oxide	Hetrick and Schoenfisch (2006)

8. Zinc

Zinc ion (Zn^{2+}) is the second most abundant trace metal ion in the body. Zinc is an indispensable metal for the growth and development for all organisms (Maret 2001). Zinc plays a crucial role in several aspects of the immune system, from the barrier of the skin to gene regulation within lymphocytes and zinc is also important in the normal development and function of cells mediating nonspecific immunity (Shankar and Prasad 1998; Haase and Rink 2009).

There are a large number of genes encoding zinc binding proteins, and thus zinc is a factor that will influence various biological processes (Devirgiliis et al. 2007). Zinc is essential for the normal activity of more than 300 enzymes, including all six classes of enzymes. These zinc enzymes are oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. There are three functions in zinc enzymes, such as catalytic, coactive and structural. Zinc acts as a catalyst. For example, the enzymes will loss its activity when zinc is removed by chelation. For coactive acitivity, zinc atom functions as an activator or as an inhibitor when working with another zinc active site in the same enzyme. Structural zinc atoms are necessary elements in stabilizing the quaternary structure of oligomeric holozymes (Vallee and Falchuk 1993; McCall et al. 2000). In addition, zinc could be considered as an intracellular signal (Hasse and Rink 2009).

There are two other zinc-dependent protein groups that have been studied intensively: metallothioneins and gene regulatory proteins. Metallothioneins are small cytosolic proteins with high cysteine content (25-30%) that can bind zinc with high affinity. They belong to the intra-cellular metal-binding proteins, and they are vital factors in zinc-related cell homeostasis, including the immune response and protecting cells against oxidative stress (Maret 2000; Stefanidou et al. 2006; Devirgiliis et al. 2007). Zinc is a functional part in many nucleoproteins that directely participate in replication and transcription of DNA (Vallee and Falchuk 1993).

Zinc also protects the upper respiratory epithelium which might be related to its antioxidant activity. Several factors could contribute to the antioxidant function of zinc. For example, zinc only has one oxidation state (II), and zinc reacts poorly with oxidants when compared to other metals, such as Fe and Cu (Truong-Tran et al. 2000).

Zinc must be supplied regularly to keep a stable level of bioavailable zinc. Zinc deficiency could result in an increased risk of several infectious diseases, such as diarrhea, pneumonia, malaria as well as skin and wound infections (Walker and Black 2004).

Zinc supplementation improves the treatment and prevention of infectious diseases. ZnO has a wide range of antibacterial effects on both Gram-positive and Gram-negative bacteria (Raghupathi et al. 2011). However, Roselli et al. (2003) also demonstrated that zinc protects intestinal cells from ETEC infection by inhibiting the adhesion and internalization of the bacteria. Zinc also prevented an increase in tight junction permetability and modulated cytokine gene expression. In addition, our laboratory demonstrated that zinc could inhibit biofilm formation of *A. pleuropneumoniae* in a dose-dependent manner (Labrie et al. 2010). In some chronic infections, bacteria hide in a protective biofilm, making them both more persistent and less invasive (Parsek and Singh 2003).

Feeding higher concentration of zinc to pigs may result in the production of manure with a higher concentration of zinc which may lead to the environmental problem (Carlson et al. 2004). Zinc supplementation with MMT (montmorillonite, a controlled-release carrier for drug molecules and for gene delivery) improved pig performance, alleviated postweaning diarrhea and enhanced intestinal mucosal integrity and the active of digestive enzyme in the pancreas and small intestinal (Hu et al. 2012).

III. Approach and scientific steps

Biofilm formation by bacterial pathogens is important for the transmission of infections and persistence of bacteria in hostile environments. Furthermore, bacteria grown as a biofilm are protected against a variety of environmental stresses such as antibiotics, disinfectants and host defence. A metal cation (zinc) has been previously shown by our laboratory to inhibit biofilm formation of *A. pleuropneumoniae* (Labrie, et al. 2010). We propose in this work to evaluate the antibiofilm potential of zinc on other bacterial swine pathogens.

Thus, the aim of this project is to evaluate the effect of zinc on growth and biofilm formation of important bacterial pathogens of swine. Biofilms will be studied in the 96-well polystyrene plates under optimal conditions for biofilm formation. Biofilms will be stained with crystal violet and quantified by measuring the absorbance at 590 nm. Confocal laser scanning microscopy will be used to visualize biofilm stained with FilmTracer™ FM® 1-43, a fluorescent marker of the cell membrane. Varying concentration of zinc will be added to the biofilm-formation medium to measure the inhibitory effect of zinc on biofilm formation. Florescent molecules, which include Wheat Germ Agglutinin, FilmTracer™ SYPRO® Ruby or BOBO™ 3 iodide will be used to stain PGA, extracellular protein and eDNA in biofilm matrix, separately. If zinc can inhibit biofilm formation of most of the pathogens tested, zinc could therefore be a potential antibiofilm agent in combination with antibiotic or disinfectant treatments.

IV. Methods and Results

Article

Zinc as an agent for the prevention of biofilm formation by pathogenic bacteria

Zinc as an agent for the prevention of biofilm formation by pathogenic bacteria

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ABSTRACT

Aims: Biofilm formation is important for the persistence of bacteria in hostile environments. Bacteria in a biofilm are usually more resistant to antibiotics and disinfectants than planktonic bacteria. Our laboratory previously reported that low concentrations of zinc inhibit biofilm formation of *Actinobacillus pleuropneumoniae*. The aim of this study is to evaluate the effect of zinc on growth and biofilm formation of other bacterial swine pathogens. **Methods and Results:** To determine the effect of zinc on biofilm formation, biofilms were grown with or without zinc in 96-well plates and stained with crystal violet. At micromolar concentrations (0 – 250 μM), zinc weakly inhibited bacterial growth and it effectively blocked biofilm-formation by *A. pleuropneumoniae*, *Salmonella* Typhymurium, and *Haemophilus parasuis* in a dose-dependent manner. Additionally, biofilm formation of *Escherichia coli, Staphylococcus aureus* and *Streptococcus suis* was slightly inhibited by zinc. However, zinc did not disperse preformed biofilms. To determine if zinc inhibits biofilm formation when poly-N-acetylglucosamine (PGA) is present, PGA was detected with the lectin wheat germ agglutinin. Only *A. pleuropneumoniae* and *S. aureus* biofilms were found to contain PGA. **Conclusion**: Zinc used at non-bactericidal concentrations can inhibit biofilm formation by several Gram-negative and Gram-positive bacterial swine pathogens. **Significance and Impact of Study:** The antibiofilm activity of zinc could provide a tool to fight biofilms and the non-specific inhibitory effect may well extend to other important human and animal bacterial pathogens.

Key words: zinc, biofilm, matrix, bacterial pathogens, swine

1. Background

Biofilms are a structured community of microorganisms enclosed in a self-produced extracellular polymer matrix adhered to biological or non-biological surfaces (Costerton et al. 1999). In nature, bacteria predominantly exist in a sessile form (biofilm) rather than a free-swimming form (planktonic) (O'Toole et al. 2000, Stoodley et al. 2002). The biofilm matrix is responsible for adhesion to surfaces and for cohesion in the biofilm, and may contain polysaccharides, proteins, and extracellular DNA (eDNA). The composition of the matrix varies greatly between different microorganisms (Flemming and Wingender 2010). Biofilm formation by bacterial pathogens is important for the transmission of infections and persistence of bacteria in hostile environments (Hall-Stoodley et al. 2004, Lewis 2010). Furthermore, bacteria grown as a biofilm are protected against a variety of environmental stresses such as antibiotics, disinfectants and host defences (Hall-Stoodley and Stoodley 2009, Hoiby et al. 2010, Bridier et al. 2011).

The negative impact of biofilm formation by pathogens of medical and veterinary importance on the efficacy of antibiotics and disinfectants is a major problem in animal and human health (Parsek and Singh 2003, Clutterbuck et al. 2007, Jacques et al. 2010). Due to the general properties of biofilms, the prevention, diagnosis and treatments of diseases associated with biofilms require novel approaches. The discovery and development of agents with the ability to limit biofilm formation or eradicate established biofilms would have the potential to enhance the efficacy of biocides that are relatively ineffective against biofilm bacteria (for recent reviews see (Rendueles et al. 2013, Worthington et al. 2012). Furthermore, there is a growing interest in the discovery of nonbiocidal antibiofilm molecules because the selective pressure on bacteria to develop resistance to nonbiocidal agents should be significantly reduced.

Our laboratory recently reported that low concentrations of zinc inhibit biofilm formation by *Actinobacillus pleuropneumoniae* (Labrie et al. 2010). *A. pleuropneumoniae* is the Gram-negative bacterium responsible for porcine pleuropneumonia, a respiratory disease of swine (Chiers et al. 2010). Biofilm formation by *A. pleuropneumoniae* on

polystyrene microtiter plates is dependent on the production of PGA, a polymer of β-1, 6-N-acetyl-D-glucosamine (Kaplan et al. 2004). Biofilm formation has also been demonstrated in other swine pathogen including *Bordetella bronchiseptica*, *Escherichia coli, Haemophilus parasuis*, *Salmonella* Typhymurium, *Staphylococcus aureus*, and *Streptococcus suis* (Jacques et al. 2010). The observation we made concerning the antibiofilm activity of zinc is of interest because zinc supplementation has been associated with the reduction of diarrheal and respiratory diseases in humans and in animals (Aggarwal et al. 2007, Crane et al. 2011, Molist et al. 2011) and is frequently added to piglet feed (Molist et al. 2011, Shelton et al. 2011).

Thus, the aim of the present study was to evaluate the effect of zinc on bacterial growth and biofilm formation of other important swine pathogens.

2. Materials and Methods

2.1 Bacterial strains

All strains used in this study are listed in Table 1. *A. pleuropneumoniae* was grown on Brain Heart Infusion (BHI; Oxoid Ltd, Basingstoke, Hampshire, UK) agar supplemented with 15 µg/mL nicotinamide adenine dinucleotide (NAD). *Bordetella bronchiseptica, Haemophilus parasuis, Salmonella, Staphylococcus aureus, Streptococcus suis* were grown on BHI agar. *Escherichia coli* strains were grown on Luria-Bertani (LB; Becton, Dickinson and Company, Sparks, MD, USA) agar. Plates were incubated overnight at 37ºC with 5% CO_2.

2.2 Biofilm assay

Growth conditions to obtain mature biofilms for the assay are summarized in Table 2. Briefly, overnight cultures of *A. pleuropneumoniae, B. bronchiseptica, E. coli, Salmonella, S. aureus,* or *S. suis* were diluted 1/100 in their corresponding broth and a volume (100 µL) was aliquoted in triplicate in a flat-bottom 96-well polystyrene plate. For *H. parasuis*, colonies from overnight agar cultures were resuspended in BHI and the

suspension was aliquoted (100 µL) in triplicate in a flat-bottom 96-well polysterene plate. With the exception of *E. coli*, the microtiter plate used was a Costar® 3599 96-well plate (Corning, NY, USA). For *E. coli*, Costar® 3370 96-well plates (Corning) were used. Wells containing sterile broth were used as negative control.

Following incubation (Table 2), biofilms were treated as described by Labrie et al. (2010) with some modifications. Briefly, the liquid medium was removed using a vacuum and unattached cells were removed by immersing the plate once in MilliQ water. The water was removed with a vacuum and excess water was removed by inverting plates onto a paper towel. Biofilms were then stained with 0.1% (w/v) crystal violet for 2 min. Biofilms were washed once with distilled water and then dried at 37°C for 15 min. The stain was then released with 100 µL of 70% (v/v) ethanol and the amount of released stain was quantified by measuring the absorbance at 590 nm with a microplate reader (Powerwave, BioTek Instruments, Winooski, VT, USA). Unstained replicate plates were used to evaluate growth by measuring the absorbance at 600 nm.

2.3 Confocal laser scanning microscopy (CLSM)

Biofilms were prepared as described above. After the desired incubation time (Table 2), biofilms were stained with FilmTracer™ FM® 1-43 fluorescent marker (Molecular Probes, Eugene, OR, USA) according to manufacturer's instructions. To determine the composition of the biofilm matrix, biofilms were stained with Wheat Germ Agglutinin (WGA-Oregon Green 488, Molecular Probes), FilmTracer™ SYPRO® Ruby biofilm matrix stain (Molecular Probes) or BOBO™-3 iodide (Molecular Probes) according to manufacturer's instructions. After a 30 min incubation at room temperature, the fluorescent marker solution was removed, biofilms were washed with water and the wells were then filled with 100 µL of water or PBS for WGA-stained biofilms. Stained biofilms were visualized by CLSM (Olympus FV1000 IX81, Markham, ON, Canada).

2.4 Effect of zinc on biofilm formation

Biofilms were prepared as described above with some modifications. Prior to

inoculation, varying concentration of zinc was added to the biofilm medium by adding an identical volume of serial dilutions of a stock solution of $ZnCl_2$ or ZnO in water. With the exception of *S. suis*, 0, 100, 250, 500, 750, or 1000 μM of zinc was added to the biofilm medium. Since *S. suis* growth was more sensitive to zinc, 0, 50, 100, 150, 200, or 250 μM of zinc was added. The biofilms were incubated as described in Table 2 and were processed as described in section 2.2.

2.5 Dispersion of preformed biofilms by zinc

Biofilms were prepared as described in section 2.2. After the desired incubation time, the biofilms were washed with water and aliquots (100 μL) of growth medium containing different concentration of $ZnCl_2$ (0, 100, 250, 500, 750, or 1000 μM) were added to preformed biofilms. The biofilms were incubated for an additional 24 hours in the presence of $ZnCl_2$. Biofilms were then stained with crystal violet as described above. Dispersion was measured by comparing the amount of biofilm in the control and treated well. A biofilm was considered dispersed if the amount of the amount zinc-treated biofilm was significantly reduced.

2.6 Statistical analysis

The effect of zinc concentration on the percent biofilm formation from the untreated control were compared with one-way analysis of variance (ANOVA) taking into account the bacterial growth and considering the runs of the ELISA as random effects (package lme4 [Bates et al., 2011] of R statistical software [R Development Core Team, 2012]). Multiple comparisons to the control concentration were realized by Dunnett's test.

3. Results

3.1 Biofilm formation

3.1.1 Optimal conditions for biofilm formation

In this study, optimal conditions for biofilm formation by different bacterial swine pathogens were determined based on information available in the literature (Table 2). The

incubation period were selected to yield mature biofilms for each species. For *E. coli* and *Salmonella*, the bacteria need to be incubated at 30ºC to form biofilms. The other species were able to form mature biofilms at 37ºC. The typical time of incubation was 24h but *Salmonella* and *H. parasuis* required 48h to form a mature biofilm whereas 5h was sufficient for *A. pleuropneumoniae* to form a mature biofilm. With the exception of *E. coli*, every species formed a biofilm in a polystyrene microtiter plate that was treated for tissue culture (TC) (Costar® 3599). *E. coli* did not form biofilms on the TC treated polystyrene and required non-treated polystyrene to form biofilms (Costar® 3370). Biofilm formation for all bacteria was tested first in BHI. Most bacteria formed biofilms in BHI, however *E. coli, S. suis* and *Salmonella* required the defined minimal media M9, Basal Broth medium (BBM) and Colonization Factor Antigen medium, respectively. Finally, the *S. aureus* and *S. suis* biofilm-medium required supplementation with glucose and fibrinogen, respectively, to form robust biofilms.

3.1.2 Typical biofilm assay results

Biofilm formation was assayed using a static microtiter plate assay and by staining the biofilm with crystal violet (Table 3). In a typical assay, *A. pleuropneumoniae* and *S. suis* strains were the strongest biofilm producers with an average A_{590} of dye that ranged from 1 to 3, followed by *E. coli*, *S.* Typhimurium and *S. aureus* with A_{590} ~1.0. *B. bronchiseptica*, *H. parasuis* and *S.* Heidelberg were the weakest biofilm formers with an average A_{590} ranging from 0.34 to 0.77.

3.1.3 Confocal laser scanning microscopy

To confirm the results obtained with the crystal violet assay, biofilms were visualized by CLSM. The biofilms were stained with FilmTracer™ FM® 1-43, a molecule that becomes fluorescent once it is inserted in the cell membrane. Biofilm structure characteristics varied among the different bacterial species. Representative CLSM images of the different biofilms are shown in Figure 1. To further characterize the biofilms, 15 images of biofilm layers were recorded and stacked, and 3D-images of the biofilms were generated (Fig. 2A). Based on these reconstructions, the thickness of the biofilm produced

by each bacterial species was evaluated. The thickness of *A. pleuropneumoniae* serotype 5b strain L20 biofilm was around 60 µm (Fig. 2B). The biofilm thickness for the other bacterial species ranged from 20 µm to 40 µm (Table 2).

3.2 Effect of zinc on biofilm formation

Once growth conditions for optimal biofilm formation were determined, the effect of different zinc ($ZnCl_2$) concentration on biofilm formation was assessed (Fig. 3). To test the relationship between the effect of zinc on bacterial growth and biofilm formation, A_{600} was recorded to assess the growth of the bacteria and, with a replicate plate, crystal violet staining was measured to assess the amount of biofilm formed. For the purpose of statistical analyses, the values were transformed so that they are represented as the percentage of the no-treatment (without zinc) control. Similar results were obtained with ZnO and are, therefore, not shown.

3.2.1 *A. pleuropneumoniae*

Zinc inhibited biofilm formation by *A. pleuropneumoniae* in a dose dependent manner as previously shown by our group (Labrie et al. 2010; Fig. 3A). Zinc treatment up to 250 µM did not affect bacterial growth. However, biofilm formation by *A. pleuropneumoniae* was significantly decreased ($p<0.001$), when the same concentration of zinc (250µM) was added.

3.2.2 *B. bronchiseptica*

Zinc did not have any effect on bacterial growth and on biofilm formation of *B. bronchiseptica* strain 276 (Fig. 3B).

2.2.3 *E. coli*

When zinc was added, a significant decrease ($p< 0.001$) in biofilm formation was observed for *E. coli* (Fig. 3C). However, the amount of biofilm was ~50% of the no-treatment control. Bacterial growth was reduced to at least 80% of the control at 100, 250, and 500 µM (see Fig. 3C). Therefore, zinc was considered to have a slight effect on *E. coli*

biofilm formation.

3.2.4 *H. parasuis*

Biofilm formation by *H. parasuis* was significantly reduced ($p<0.001$) at a concentration as low as 100 μM of $ZnCl_2$ (Fig. 3D). At higher concentrations (250 μM-1000 μM), the percentage of biofilm formation was stable at ~40% of the no-treatment control. Furthermore, zinc did not have a bactericidal effect (Figure 3D).

3.2.5 *S.* Typhimurium

S. Typhimurium formed significantly ($p<0.001$) less biofilms in the presence of zinc. At lower concentrations (100 and 250μM), biofilm formation was reduced to ~60% ($p<0.001$) and bacterial growth was reduced to ~80% of the control (Fig. 3E). At higher concentrations of zinc, there was almost no biofilm formed and growth remained at ~80% of the control (Fig. 3E).

3.2.6 *S.* Heidelberg

In the case of *S.* Heidelberg, zinc decreased biofilm formation compared to the no-treatment control but a similar decrease was observed in bacterial growth (Fig. 3F). Therefore, the effect of zinc on biofilm formation was not considered to be significant.

3.2.7 *S. aureus*

Unlike most bacteria tested, biofilm formation by *S. aureus* seemed stimulated in the presence of 100 μM of zinc (Fig. 3G). Biofilm formation was reduced to 80% of the control when 500 μM $ZnCl_2$ was added ($p<0.001$). Bacterial growth was slightly affected by zinc. Therefore, high concentrations of zinc slightly decreased biofilm formation by *S. aureus*.

3.2.8 *S. suis*

Growth of *S. suis* was more sensitive to zinc when compared to the other bacterial swine pathogens tested. A significant ($p<0.001$) reduction in biofilm formation was

observed at 150µM $ZnCl_2$ (Fig. 3H). At 200µM $ZnCl_2$, *S. suis* biofilm formation was completely inhibited but growth was also markedly affected. Despite the effect of zinc on growth, we concluded that zinc had a significant effect on biofilm formation of *S. suis* at higher concentrations of zinc.

3.2.9 Inhibitory effect of zinc confirmed by CLSM

To confirm the inhibitory effect of zinc on biofilm formation, we used CLSM and fluorescent staining to visualize the zinc-treated and control biofilms. Biofilm formation by *S*. Typhimurium in the presence of $ZnCl_2$ is shown as an example (Fig. 4). As observed with the microtiter plate assay and crystal violet staining, *S*. Typhimurium formed markedly less biofilm than the control when grown in the presence of 250 and 500 µM of $ZnCl_2$ (Fig. 4).

3.3 Effect of zinc on dispersion of preformed biofilms

The ability of zinc to disperse preformed biofilms was also evaluated. The addition of zinc ($ZnCl_2$) followed by an additional incubation for 24h did not result in a reduction in the amount of biofilm when compared to the control biofilm. Therefore, it was concluded that zinc did not disperse preformed biofilms (data not shown).

3.4 Composition of biofilm matrix

The matrix of the different biofilms was stained with fluorescent probes specific for poly-N-acetyl-D-glucosamine (PGA) (Wheat Germ Agglutinin), eDNA (BOBO™-3 iodide) and proteins (FilmTracer™ SYPRO® Ruby). The composition of the matrix for the different bacterial pathogens is summarized in Table 4. The biofilm matrices of *A. pleuropneumoniae* and *S. aureus* were positive for all three components (PGA, eDNA and proteins) whereas *B. bronchiseptica* was negative for all three (Fig. 5; Table 4). *E. coli* was also negative for the three components and *H. parasuis* and *S.* Heidelberg were only positive for eDNA. Both *S. suis* and *S.* Typhimurium were positive for eDNA and proteins.

4. Discussion

Given that biofilm-associated infections are often chronic and difficult to eradicate,

the identification of anti-biofilm molecules is of high importance (Hall-Stoodley and Stoodley 2009, Jacques et al. 2010). The use of metal ions to eradicate biofilms has received some attention (Harrison et al. 2005, Workentine et al. 2008). The potential of zinc as anti-biofilm molecule has not been fully explored but recent study have demonstrated that biofilms of enteroaggregative *E. coli* (EAEC; Pereira et al. 2010), uropathogenic *E. coli* (UPEC; Hancock et al. 2010) and dental plaque bacteria (Gu et al., 2012) were sensitive to zinc. Furthermore, our laboratory previously demonstrated that sub-bactericidal concentration of zinc could inhibit biofilm formation of the swine pathogen, *A. pleuropneumoniae* (Labrie et al. 2010). The objective of our study was to evaluate the effect of zinc on biofilm formation of other important bacterial swine pathogens including *B. bronchiseptica, E. coli, H. parasuis, Salmonella, S. aureus* and *S. suis*. Under optimal conditions for biofilm formation, the addition of sub-bactericidal concentration of zinc ($ZnCl_2$ or ZnO) effectively blocked biofilm-formation of *A. pleuropneumoniae*, *S.* Typhymurium, and *H. parasuis* in a dose-dependent manner. Additionally, biofilm formation of *E. coli, S. aureus* and *S. suis* was slightly inhibited by the presence of zinc.

In our study, zinc was able to inhibit the biofilm formation of both intestinal and respiratory pathogens. Furthermore, the use of zinc to reduce diarrheal and respiratory diseases in humans and animals has already been demonstrated (Aggarwal et al. 2007, Crane et al. 2011, Molist et al.. 2011). Thus, the reduction of intestinal and respiratory diseases by zinc can probably be attributed to both the antimicrobial and antibiofilm activity of zinc. In addition to preventing infectious diseases, zinc supplementation has been used in the diet of pigs to improve feed intake. For example, ZnO supplementation altered the development of the small intestine mucosa of weaned pigs (Slade et al. 2011) and improved feed intake and growth of piglets (Molist et al. 2011). Additionally, the combination of an antibacterial agent and ZnO supplementation lead to an improvement of performance markers (Hill et al. 2001). In combination with our data, these indicate the clinical value of zinc as an additive in diet of pigs. Furthermore, it highlights the possibility that zinc may act synergistically with biocides.

Synergic effects of metal ions and biocides on biofilms have also been investigated. For example, when copper was combined with quaternary ammonium cations, synergistic bactericidal and antibiofilm activity was observed against *Pseudomonas aeruginosa* (Harrison et al. 2008). This suggested that zinc could increase the effectiveness of disinfectants. If such phenomenon is observed, zinc could be used with other disinfectant to control environmental biofilms in the farm and food-processing plants. Environmental biofilms are important in the persistence of bacterial pathogens, such as *E. coli* and *Vibrio cholera* (Shikuma and Hadfield 2010).

Despite our positive results, the use of zinc may face some limitation given that bacterial evolution is a fairly rapid process. For example, the use of zinc could apply selective pressure for strain that are able to form biofilm in the presence of antibiofilm concentration of zinc. Furthermore, a selection pressure could also be applied on pathogens to increase the subpopulations that do not form biofilm. In our study, strains from a species respond similarly to presence of zinc; however, a larger set of isolates representing different genotype should be included in future studies to ensure that antibiofilm effect of zinc is not genotype specific within a species.

The mechanism behind the antibiofilm activity of zinc has yet to be characterized, but zinc could interact with components of the matrix. It has been recently reported that PgaB activity, involved in de-N-acetylation of *E. coli* PGA, is decreased by zinc (Little et al. 2012). However, in our study, only *A. pleuropneumoniae* and *S. aureus* biofilms were found to contain PGA. Under our experimental conditions, species that did not produce PGA were also inhibited, indicating that the inhibitory effect of zinc does not appear to be solely dependent on the presence of PGA in the biofilm matrix. eDNA was one component that was present in most of the biofilm matrices. eDNA can act as a zinc chelator and this interaction has an impact on biofilm stability (Mulcahy et al. 2008). Zinc may also interfere with other cellular mechanisms such as signalling and gene-regulation. Zinc can bind to the ferric uptake regulator and may affect iron homeostasis (Klemm et al. 2010). Zinc can also interfere with a toxin-antitoxin (TA) system, MqsR/MqsA, which is associated with biofilm

formation and the development of persister cells (Papadopoulos et al. 2012). Homologues to this TA system are found in many pathogenic bacteria (Gerdes et al. 2005). Finally, zinc can inhibit the EAL domains of cyclic diguanylate phosphodiesterases and this inhibition blocks the degradation of c-di-GMP (Tamayo et al. 2005, Jenal and Malone 2006). c-di-GMP is an important player in the regulation of biofilm formation and interference in the c-di-GMP pathway will likely have consequences on the biofilm formation process (Jonas et al., 2009).

In conclusion, micromolar concentrations of zinc can inhibit biofilm formation by several Gram-negative and Gram-positive bacteria of porcine origin. The mechanism behind the antibiofilm activity of zinc has yet to be characterized. It does not, however, appear to be solely dependent on the presence of PGA in the biofilm matrix as initially thought. Given that zinc is a simple and inexpensive molecule, it would be worth to test if synergic effects are observed with antibiotic and disinfectant treatments, and thus reduce the virulence, persistence and transmission of pathogenic bacteria. In addition, the non-specific inhibitory effect of zinc on biofilm formation may well extend to other important human and animal bacterial pathogens.

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References

Aggarwal, R., Sentz, J., and Miller, M.A. (2007) Role of zinc administration in prevention of childhood diarrhea and respiratory illnesses: a meta-analysis. *Pediatrics* **119**:1120-1130.

Bates, D., Maechler, M., and Bolker, B. (2011) lme4: Linear mixed-effects models using S4 classes. [http://cran.r-project.org/web/packages=lme4]

Bonifait, L., Grignon, L., and Grenier, D. (2008) Fibrinogen induces biofilm formation by *Streptococcus suis* and enhances its antibiotic resistance. *Appl Environ Microbiol* **74**:4969-4972.

Bridier, A., Briandet, R., Thomas,V., and Dubois-Brissonnet, F. (2011) Resistance of bacterial biofilms to disinfectants: a review. *Biofouling* **27**:1017-1032.

Charbonneau, M.E., Berthiaume, F., and Mourez, M. (2006) Proteolytic processing is not essential for multiple functions of the *Escherichia coli* autotransporter adhesin involved in diffuse adherence (AIDA-I). *J Bacteriol* **188**:8504-8512.

Clutterbuck, A.L., Woods, E.J., Knottenbelt, D.C., Clegg, P.D., Cochrane, C.A., and Percival, S.L. (2007) Biofilms and their relevance to veterinary medicine. *Vet Microbiol* **121**:1-17.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284**:1318-1322.

Chiers, K., De Waele, T., Pasmans, F., Ducatelle, R., and Haesebrouck, F. (2010) Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. *Vet Res* **41**:65.

Crane, J.K., Byrd, I.W., and Boedeker, E.C. (2011) Virulence inhibition by zinc in shiga-toxigenic *Escherichia coli*. *Infect Immun* **79**:1696-1705.

Flemming, H.C., and Wingender, J. (2010) The biofilm matrix. *Nat Rev Microbiol* **8**:623-633.

Gerdes, K., Christensen, S.K., and Lobner-Olesen, A. (2005) Prokaryotic toxin-antitoxin stress response loci. *Nat Rev Microbiol* **3**:371-382.

Gu, H., Fan, D., Gao, J., Zou, W., Peng, Z., Zhao, Z., Ling, J., and LeGeros, R.Z. (2012) Effect of ZnCl2 on plaque growth and biofilm vitality. *Arch Oral Biol* **57**:369-375.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**:95-108.

Hall-Stoodley, L., and Stoodley, P. (2009) Evolving concepts in biofilm infections. *Cell Microbiol* **11**:1034-1043.

Hancock, V., Dahl, M., and Klemm, P. (2010) Abolition of biofilm formation in urinary tract *Escherichia coli* and *Klebsiella* isolates by metal interference through competition for Fur. *Appl Environ Microbiol* **76**:3836-3841.

Harrison, J.J., Turner, R.J., and Ceri, H. (2005) High-throughput metal susceptibility testing of microbial biofilms. *BMC Microbiol* **5**:53.

Harrison, J.J., Turner, R.J., Joo, D.A., Stan, M.A., Chan, C.S., Allan, N.D., Vrionis, H.A., Olson, M.E., and Ceri, H. (2008) Copper and quaternary ammonium cations exert synergistic bactericidal and antibiofilm activity against *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **52**:2870-2881.

Hill, G.M., Mahan, D.C., Carter, S.D., Cromwell, G.L., Ewan, R.C., Harrold, R.L., Lewis, A.J., Miller, P.S., Shurson, G.C., and Veum, T.L. (2001) Nutrition N-CS: Effect of pharmacological concentrations of zinc oxide with or without the inclusion of an antibacterial agent on nursery pig performance. *J Anim Sci* 2001, **79**:934-941.

Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010) Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* **35**:322-332.

Jacques, M., Aragon, V., and Tremblay, Y.D.N. (2010) Biofilm formation in bacterial pathogens of veterinary importance. *Anim Health Res Rev* **11**:97-121.

Jenal, U., and Malone, J. (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* **40**:385-407.

Jonas, K., Melefors, O., and Römling U. (2009) Regulation of c-di-GMP metabolism in biofilms. *Future Microbiol* **4**:341-358

Kaplan, J.B., Velliyagounder, K., Ragunath, C., Rohde, H., Mack, D., Knobloch, J.K.M., and Ramasubbu, N. (2004) Genes involved in the synthesis and degradation of matrix polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* biofilms. *J Bacteriol* **186**:8213-8220.

Klemm, P., Vejborg, R.M., and Hancock, V. (2010) Prevention of bacterial adhesion. *Appl Microbiol Biotechnol* **88**:451-459.

Labrie, J., Pelletier-Jacques, G., Deslandes, V., Ramjeet, M., Auger, E., Nash, J.H., and Jacques, M. (2010) Effects of growth conditions on biofilm formation by *Actinobacillus pleuropneumoniae*. *Vet Res* **41**:3.

Lewis, K. (2010) Persister cells. *Annu Rev Microbiol* **64**:357-372.

Little, D.J., Poloczek, J., Whitney, J.C., Robinson, H., Nitz, M., and Howell, P.L. (2012) The structure and metal dependent activity of *Escherichia coli* PgaB provides insight into the partial de-N-acetylation of poly-beta-1,6-N-acetyl-D-glucosamine. *J Biol Chem* **287**:31126-31137.

Molist, F., Hermes, R.G., de Segura, A.G., Martin-Orue, S.M., Gasa, J., Manzanilla, E.G., and Perez, J.F. (2011) Effect and interaction between wheat bran and zinc oxide on productive performance and intestinal health in post-weaning piglets. *Br J Nutr* **105**:1592-1600.

Mulcahy, H., Charron-Mazenod, L., and Lewenza, S. (2008) Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog* **4**:e1000213.

O'Toole, G., Kaplan, H.B., and Kolter, R. (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* **54**:49-79.

Papadopoulos, E., Collet, J.F., Vukojevic, V., Billeter, M., Holmgren, A., Graslund, A., and Vlamis-Gardikas, A. (2012) Solution structure and biophysical properties of MqsA, a Zn-containing antitoxin from *Escherichia coli*. *Biochim Biophys Acta* **1824**:1401-1408.

Parsek, M.R., and Singh, P.K. (2003) Bacterial biofilms: An emerging link to disease pathogenesis. *Annu Rev Microbiol* **57**:677-701.

Pereira, A.L., Silva, T.N., Gomes, A.C., Araujo, A.C., and Giugliano, L.G. (2010) Diarrhea-associated biofilm formed by enteroaggregative *Escherichia coli* and aggregative *Citrobacter freundii*: a consortium mediated by putative F pili. *BMC Microbiol* **10**:57.

R Development Core Team (2012) R: a language and environment for statistical computing. [www.R-project.org]

Rendueles, O., Kaplan, J.B., and Ghigo, J.M. (2013) Antibiofilm polysaccharides. *Environ Microbiol* **15**:334-346

Shelton, N.W., Tokach, M.D., Nelssen, J.L., Goodband, R.D., Dritz, S.S., DeRouchey, J.M., and Hill, G.M. (2011) Effects of copper sulfate, tri-basic copper chloride, and zinc oxide on weanling pig performance. *J Anim Sci* **89**:2440-2451.

Shikuma, N.J., and Hadfield, M.G. (2010) Marine biofilms on submerged surfaces are a reservoir for *Escherichia coli* and *Vibrio cholerae*. *Biofouling* **26**:39–46

Slade, R.D., Kyriazakis, I., Carroll, S.M., Reynolds, F.H., Wellock, I.J., Broom, L.J., and Miller, H.M. (2011) Effect of rearing environment and dietary zinc oxide on the response of group-housed weaned pigs to enterotoxigenic *Escherichia coli* O149 challenge. *Animal* **5**:1170-1178.

Stoodley, P., Sauer, K., Davies, D.G., and Costerton J.W. (2002) Biofilms as complex differentiated communities. *Annu Rev Microbiol* **56**:187-209.

Suzuki, K., Wang, X., Weilbacher, T., Pernestig, A.K., Melefors, O., Georgellis, D., Babitzke, P., and Romeo, T. (2002) Regulatory circuitry of the CsrA/CsrB and BarA/UvrY systems of *Escherichia coli*. *J Bacteriol* **184**:5130-5140.

Tamayo, R., Tischler, A.D., and Camilli, A. (2005) The EAL domain protein VieA is a cyclic diguanylate phosphodiesterase. *J Biol Chem* **280**:33324-33330.

Workentine, M.L., Harrison, J.J., Stenroos, P.U., Ceri, H., and Turner, R.J. (2008) *Pseudomonas fluorescens*' view of the periodic table. *Environ Microbiol* **10**:238-250.

Worthington, R.J., Richards, J.J., and Melander, C. (2012) Small molecule control of bacterial biofilms. Org Biomol Chem 10: 7457-7474.

Table 1. Bacterial strains used in this study.

Bacterial species	Strains	Relevant traits	Inhibitory concentration		Source
			$ZnCl_2$	ZnO	
Actinobacillus pleuropneumoniae	S4074	serotype 1; reference strain	1000 µM	1000 µM	K.R.Mittal[1]
	719	serotype 1	750 µM	7500 µM	D. Slavic[2]
	L20	serotype 5b; reference strain	1000 µM	1000 µM	K.R. Mittal
Bordetella bronchiseptica	276		>1000 µM	>1000 µM	J.M. Rutter[3]
Escherichia coli	ECL 17608	STb: AIDA: EAST1	>1000 µM	>1000 µM	J. M. Fairbrother[1]
	ECL 17659	F18: AIDA	>1000 µM	>1000 µM	J. M. Fairbrother
	ECL 17635	Eae: Paa	>1000 µM	>1000 µM	J. M. Fairbrother
Haemophilus parasuis	Nagasaki	serotype 5; reference strain	>1000 µM	>1000 µM	M. Gottschalk[1]
Salmonella Typhimurium	ATCC 14028		>1000 µM	>1000 µM	A. Letellier[1]
	STF07-8567-3		>1000 µM	>1000 µM	A. Letellier
Salmonella Heidelberg	STF08-453		>1000 µM	>1000 µM	A. Letellier
Staphylococcus aureus	154N	methicillin resistant; nasal isolate	>1000 µM	>1000 µM	M. Archambault[1]
	294	methicillin resistant; skin isolate	>1000 µM	>1000 µM	M. Archambault
	327N	methicillin resistant; nasal isolate	>1000 µM	>1000 µM	M. Archambault
Streptococcus suis	735	serotype 2; reference strain	250 µM	250 µM	M. Gottschalk

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[2] Ontario Veterinary College, University of Guelph, Guelph, ON, Canada.
[3] Institute for Research on Animal Disease, Compton, UK.

Table 2. Growth conditions used for each bacterial species to obtain mature biofilms.

Bacterial Species	Growth Conditions	Corning Plate ID	Incubation temperature and atmosphere	Incubation time	Reference
A. pleuropneumoniae	O/N in 5mL of BHI with NAD (5 µg/mL) at 37ºC with shaking (200 rpm); dilution 1/100 in BHI with NAD (5 µg/mL)	3599	37ºC, 5% CO_2	5h	Labrie et al. 2010
B. bronchiseptica	O/N in 5mL of BHI at 37ºC with shaking (200 rpm); dilution 1/100 in BHI	3599	37ºC, 5% CO_2	24h	This study
E. coli	O/N in 5mL of M9 minimal medium at 30ºC with shaking (200 rpm); dilution 1/100 in M9 minimal medium	3370	30ºC	24h	Charbonneau et al. 2006
H. parasuis	Resuspend colonies from a BHI agar plate in 3mL of BHI	3599	37ºC, 5% CO_2	48h	This study
Salmonella	O/N in 5mL of Colonization Factor Antigen (CFA) medium at 37ºC with shaking (200 rpm); dilution 1/100 in CFA	3599	30ºC	48h	Suzuki et al. 2002
S. aureus	O/N in 5mL of BHI with glucose (0.25% [w/v]) at 37ºC with shaking (200 rpm); dilution 1/100 in BHI with glucose (0.25% [w/v])	3599	37ºC, 5% CO_2	24h	This study
S. suis	O/N in 5mL of Basal Broth Medium (BBM) with fibrinogen (5 mg/mL) at 37ºC with shaking (200 rpm); dilution 1/100 in BBM with fibrinogen (5 mg/mL)	3599	37ºC, 5% CO_2	24h	Bonifait et al. 2008

Table 3. Biofilm formation in a microtiter plate.

Bacterial strains	Range of $OD_{590\ nm}$ after staining with crystal violet	Biofilm thickness (in µm) as determined by CLSM
A. pleuropneumoniae S4074	1.85±0.24	35
B. bronchiseptica 276	0.60±0.27	25
E. coli ECL 17608	1.04±0.07	23
H. parasuis Nagasaki	0.77±0.47	20
S. Typhimurium ATCC 14028	0.99±0.17	21
S. Heidelberg STF08-453	0.34±0.10	20
S. aureus 154N	1.09±0.63	40
S. suis 735	2.63±0.26	35

Table 4. Composition of the biofilm matrix as determined by staining and CLSM.

Bacterial strains	Component		
	PGA (WGA)	Extracellular DNA (BOBO-3)	Protein (SYPRO Ruby)
A. pleuropneumoniae S4074	+	+	+
B. bronchiseptica 276	-	-	-
E. coli ECL 17608	-	-	-
H. parasuis Nagasaki	-	+	-
S. Typhimurium ATCC 14028	-	+	+
S. Heidelberg STF08-453	-	+	-
S. aureus 154N	+	+	+
S. suis 735	-	+	+

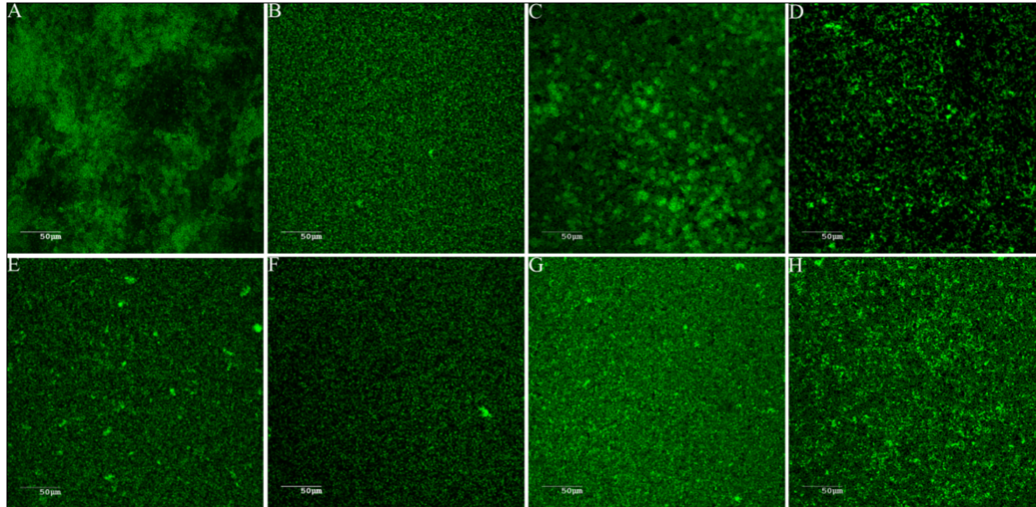


Figure 1. CLSM of FilmTracer™ FM® 1-43 stained biofilms of *A. pleuropneumoniae* S4074 (A), *B. bronchiseptica* 276 (B), *E. coli* ECL17608 (C), *H. parasuis* Nagasaki (D), *S.* Typhimurium ATCC14028 (E), *S.* Heidelberg STF08-453 (F), *S. aureus* 154N (G), *S. suis* 735 (H).

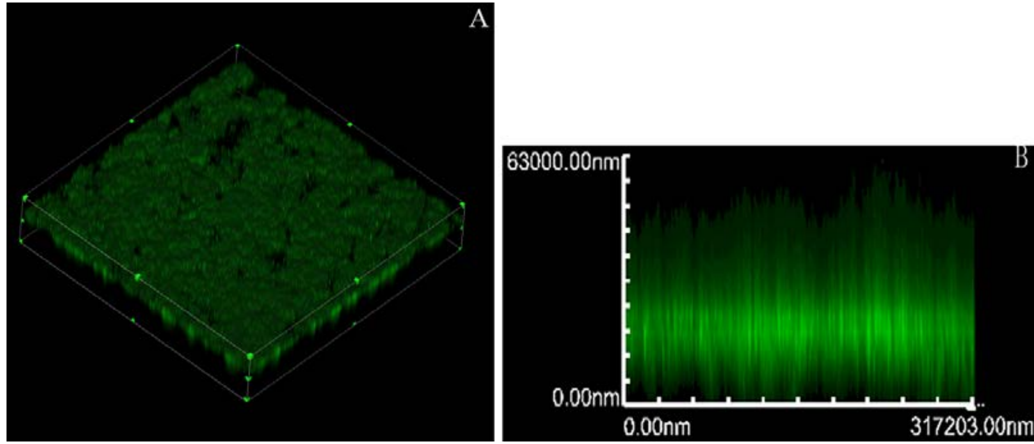
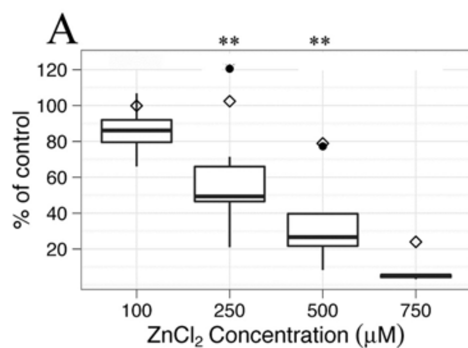
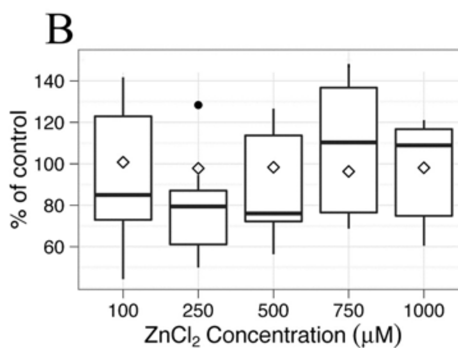
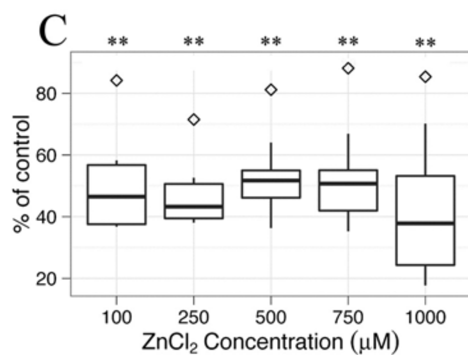
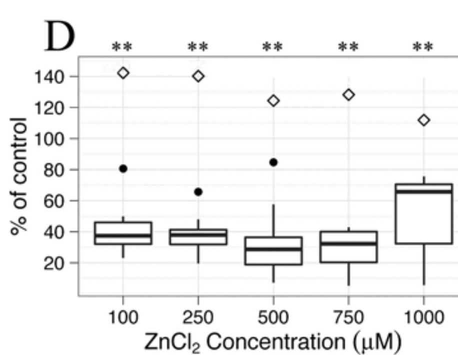


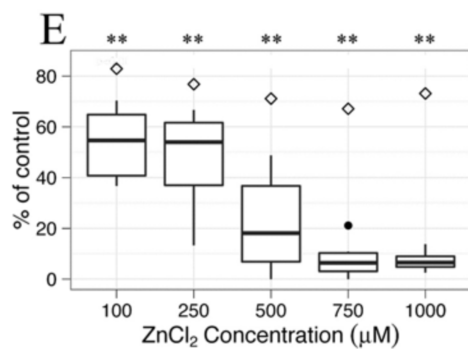
Figure 2. CLSM three-dimensional images of biofilm formation by *A. pleuropneumoniae* strain L20 stained with FilmTracer^{TM} FM$^{®}$ 1-43 (A) and stack of sections of the X-Z plane of the biofilm (B).

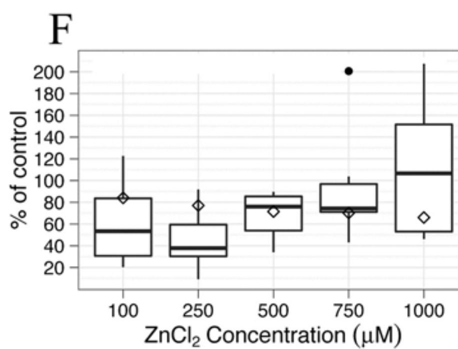

A
** **
% of control
120
100
80
60
40
20
100 250 500 750
$ZnCl_2$ Concentration (μM)

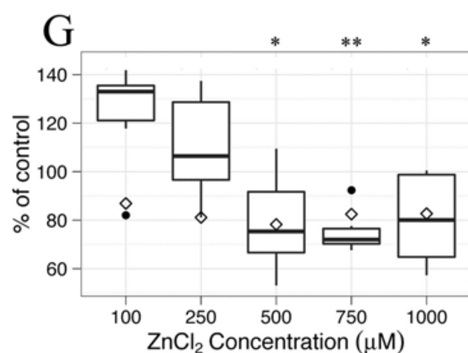

B
% of control
140
120
100
80
60
100 250 500 750 1000
$ZnCl_2$ Concentration (μM)


C
** ** ** ** **
% of control
80
60
40
20
100 250 500 750 1000
$ZnCl_2$ Concentration (μM)


D
** ** ** ** **
% of control
140
120
100
80
60
40
20
100 250 500 750 1000
$ZnCl_2$ Concentration (μM)


E
** ** ** ** **
% of control
80
60
40
20
0
100 250 500 750 1000
$ZnCl_2$ Concentration (μM)


F
% of control
200
180
160
140
120
100
80
60
40
20
100 250 500 750 1000
$ZnCl_2$ Concentration (μM)


G
* ** *
% of control
140
120
100
80
60
100 250 500 750 1000
$ZnCl_2$ Concentration (μM)

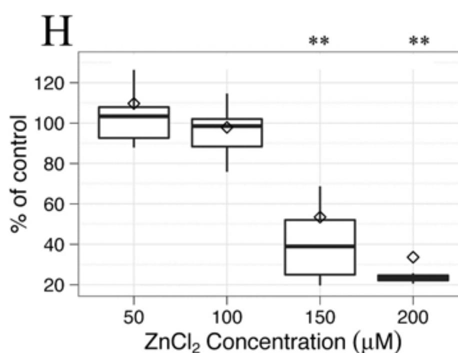

H
** **
% of control
120
100
80
60
40
20
50 100 150 200
$ZnCl_2$ Concentration (μM)

Figure 3. Effect of $ZnCl_2$ on the formation of biofilm and growth of *A. pleuropneumoniae* S4074 (A), *B. bronchiseptica* 276 (B), *E. coli* ECL17608 (C), *H. parasuis* Nagasaki (D), *S.* Typhimurium ATCC14028 (E), *S.* Heidelberg STF08-453 (F), *S. aureus* 154N (G), *S. suis* 735 (H). Values are represented as percentage of the no-treatement control. Box and whisker plots represent biofilm formation and the diamonds represent bacterial growth. Black dots outside the box and whiskers are considered outliers. Statistical significance was established by analysis of variance (ANOVA). Multiple comparisons to the control concentration were realized by the Dunnett's test. * $P<0.01$; ** $P<0.001$.

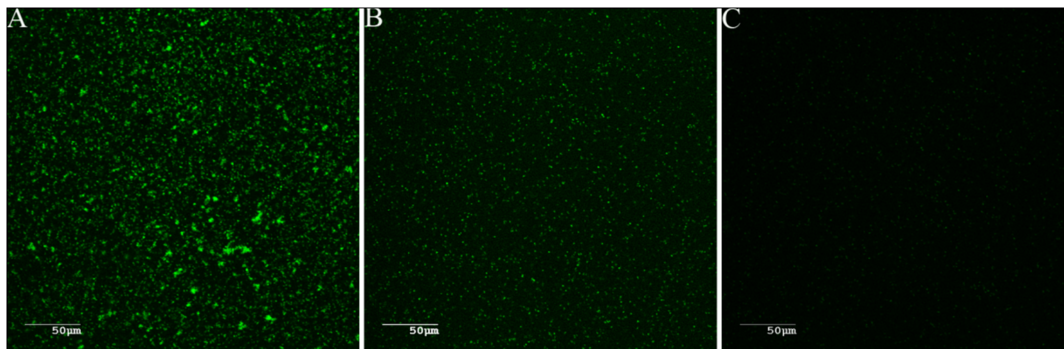


Figure 4. CLSM images of *S. Typhimurium* ATCC 14028 biofilms grown in the presence of different $ZnCl_2$ concentrations (0:A, 250:B or 500 μM:C). Biofilms were stained with FilmTracer^{TM} FM$^{®}$ 1-43.

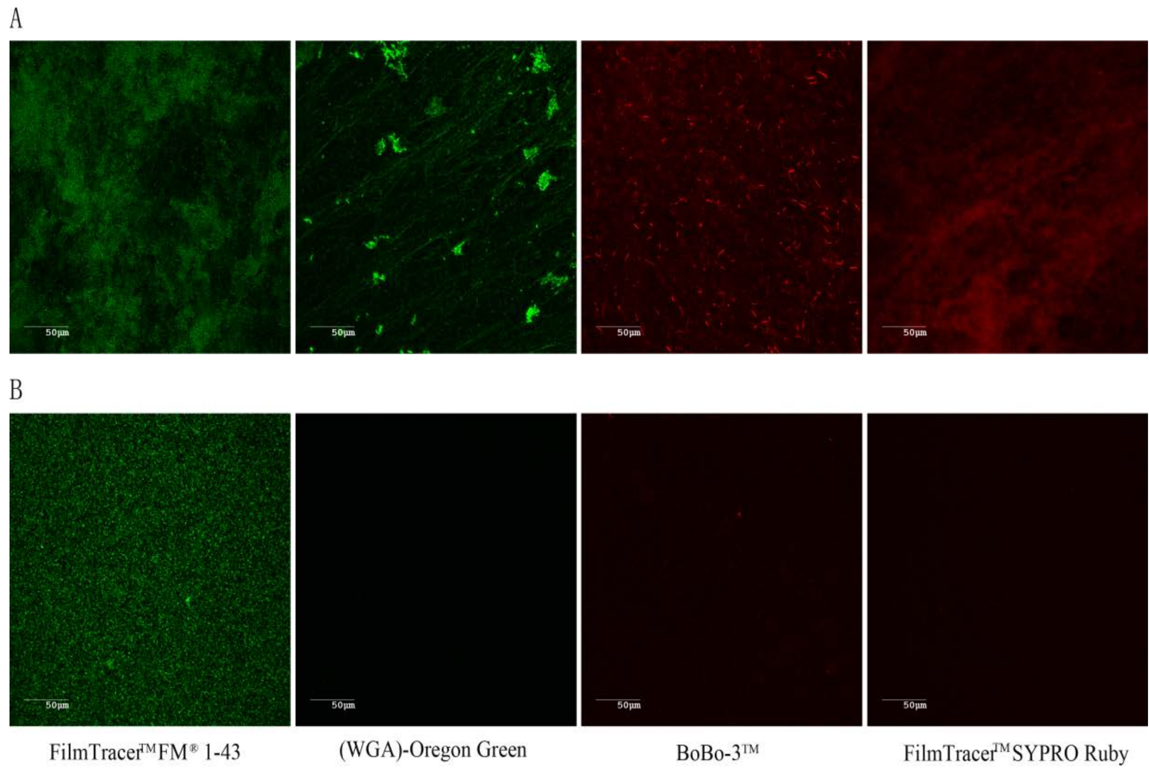


Figure 5. CLSM images of the biofilm matrix of *A. pleuropneumoniae* S4074 (A) and *B. bronchiseptica* 276 (B) stained with FilmTracer™ FM® 1-43, Wheat Germ Agglutinin (WGA) conjugated to Oregon Green 488, BOBO-3™ and FilmTracer™ SYPRO Ruby.

V. Discussion

Bacteria that attach to biotic and non-biotic biological surfaces can form biofilms. Biofilm infections are considered to be a major problem in clinical settings because biofilms are hard to eradicate (Costerton et al. 1999). Biofilms often result in chronic infections, and are resistant to the host immune response and antibiotic treatment. All these factors enable pathogens to persist (Hall-Stoodey and Stoodley 2009).

A large number of studies have focused on biofilm infections of humans. For example, the organism is found in the lesions of the disease and can be isolated in pure culture on artificial media; a similar symptom would be seen by inoculating the culture in experimental animals; from the lesions of infected animals, the organism can be recovered (Donlan and Costerton 2002). Unfortunately, few studies on biofilm in animal diseases have been published (Clutterbuck et al. 2007; Jacques et al. 2010). Persistent microbial contaminations resulting from biofilm formation have lead to food spoilage or disease transmission in food-processing environments (Van Houdt and Michiels 2010). Biofilm infections induce morbidity and mortality associated with various diseases due to their reduced susceptibility to antibiotics and this represents a serious threat to society. The prominence of biofilm in infectious disease requires intense research on the development of anti-biofilm molecules that have different mode of action than antibiotics or microbicides. Such molecules should be able to modulate bacterial biofilm formation resulting in the inhibition of biofilm-associated infections (Worthington et al. 2012).

The administration of zinc is a useful tool for the treatment and prevention of several diseases of humans and animals, especially those that occur at epithelial sites. Zinc supplementation effectively reduced the frequency and severity and duration of diarrhea and respiratory illnesses (Aggarwal et al. 2007). The inclusion of ZnO in the diet of post-weaning piglets improved their feed intake and growth, and reduced the incidence of diarrhea (Molist et al. 2011). In addition, our laboratory demonstrated that zinc could inhibit biofilm formation of *A. pleuropneumoniae* in a dose-dependent manner (Labrie et al. 2010). Considering that zinc has inhibitory effect on biofilm formation by *A. pleuropneumoniae* and that zinc is used to reduce disease burden on farms, we were

interested in evaluating the effect of zinc on biofilm formation of other important bacterial pathogens of swine including *B. bronchiseptica, E. coli, H. parasuis, Salmonella, S. aureus* and *S. suis*.

After determining the optimal conditions for biofilm formation, biofilm formation was evaluated using a 96-well plate and biofilms were stained with crystal violet. The presence of reproducible biofilms was confirmed by CLSM. CLSM was used to visualize the biofilms and get a better understanding of biofilm structure and thickness. CLSM is a great tool for biofilm analysis because it allows the study of living, and fully hydrated biofilms (Denkhaus et al. 2007). Three dimensional (3D) structure of biofilm revealed the typical mushroom shape and a network of water channels for distribution of water and nutrients in the community (Stoodley et al. 2002). In addition, the mean thickness of biofilm is an important parameter for describing the structure of biofilms (Beyenal et al. 2004).

The effect of zinc on biofilm formation was evaluated. Zinc was added in the growth culture at the beginning of the biofilm formation. Zinc ($ZnCl_2$ or ZnO) weakly inhibited bacterial growth at micromolar concentrations (0 – 250 μM) indicating that it possesses antimicrobial activity. At micromolar concentrations, zinc was able to effectively block biofilm formation of *A. pleuropneumoniae*, *S.* Typhymurium, and *H. parasuis* in a dose-dependent manner. Additionally, biofilm formation of *E. coli, S. aureus* and *S. suis* was slightly inhibited by the presence of zinc. At low concentration (100 μM to 150 μM), the addition of zinc resulted in reduction in biofilm formation and bacterial growth, suggesting that the *S. suis* strain was more sensitive to zinc than the other pathogens tested.

Zinc supplementation has been reported as beneficial in previous studies, and zinc has an inhibitory effect on biofilm formation of some pathogenic bacteria. Therefore, the reduction in intestinal and respiratory diseases burden associated with zinc could be attributed to both its antimicrobial and antibiofilm activity. The addition of zinc results in the reduction in Shiga-toxigenic enteropathogenic *E. coli* (STEC) infections (Crane et al.

2011). Zinc has also a strong inhibitory effect on Stx expression which is responsible for the extraintestinal symptoms associated with STEC infection. Furthermore, Zn (II) could significantly reduce attachment and biofilm formation of urinary tract *E. coli*. The addition of Zn (II) (500μM) impaired biofilm formation in microtiter plate. Biofilms formed in flow cell chamber system, which mimic conditions encountered in urinary tract, were affected more by Zn (II) than the biofilms formed in a microtiter plate (Hancock et al. 2010). In addition, zinc has a significant antibacterial effect on the outer and middle layers of dental plaque, a good example of an *in vivo* biofilm. Zinc could be a potential and effective supplement in dentifrices and mouth rinses to combat dental plaque (Gu et al. 2012).

The mechanism behind the antibiofilm activity of zinc has yet to be characterized, but zinc could interact with components of the biofilm matrix. For example, zinc could decrease the activity of PgaB, which is associated with the de-*N*-acetylation of *E. coli* PGA (Little et al. 2012). However, only *A. pleuropneumoniae* and *S. aureus* biofilms were stained by WGA and were considered positive for PGA. Some species that did not produce PGA under our growth conditions were also inhibited by zinc. Those results indicate that the antibiofilm effect of zinc might not be dependent on the presence of PGA but other components in the biofilm matrix could influence the antibiofilm activity of zinc. eDNA play a key role in the composition and formation of biofilms (Whitchurch et al. 2002). For most species tested in this study, eDNA was one of the components of their matrix. eDNA was reported to function as a zinc chelator and this chelating property can influence the stability of biofilms (Mulcahy et al. 2008).

For *S. aureus* biofilm, we observed that biofilm formation was stimulated by zinc, at low concentration. In our test, this phenomenon was not observed for other bacteria. In previous studies on *S. aureus* biofilm, diethylenetriaminepentaacetic acid (DTPA), a metal ion chelator, could inhibit biofilm formation, and this inhibitory effect was attributed to the chelation of zinc, and not with other metal ions (Conrady, 2008). This might explain the positive effect of zinc on *S. aureus* biofilm formation.

In our studies, zinc failed to disperse established biofilms. Our results suggest that the antibiofilm activity of zinc is mostly associated with the early steps of biofilm formation and not with the dispersion event. One effective method of biofilm control is to target community signalling and targeting this type of signalling with antibiofilm molecules will prevent biofilm formation (Costerton, 1999).

Zinc may interfere with cellular mechanisms during biofilm development, such as signalling and regulation. For examples, Zn (II) has a high affinity for Fur (ferric uptake regulator) and excess of Zn (II) might result in the down-regulation of Fur-regulated genes (Klemm, 2010). Zinc can interfere with toxin-antitoxin systems, which are involved in biofilm formation (Papadopoulos, 2012). The MqsR/MqsA, a toxin-antitoxin system, regulates biofilm formation and development of persister cells of *E. coli*. MqsR, as a toxin, contains a well-defined N-terminal domain with a zinc finger motif, which binds zinc. The toxin-antitoxin system can be found in many pathogenic bacteria (Gerdes, 2005). c-di-GMP plays an important role in the regulation of biofilm formation, and the interference in the c-di-GMP pathway will have an impact on the biofilm formation. Zn (II) could inhibit the EAL domains of cyclic diguanylate phosphodiesterases and this inhibitory effect will block the degradation of c-di-GMP (Tamayo, 2005; Jenal and Malone, 2006).

Zinc has already been used in the diet of pigs to treat diseases and to increase feed intake. Previous studies using zinc as additive in pigs diet indicate its clinical value. For example, ZnO supplement reduced ETEC excretion (Slade, 2011). ZnO as an additive in diets benefits weaned pigs by suppressing infection and mediating the development of the small intestine mucosa (Slade, 2011). In addition, the inclusion of ZnO (3000 mg/kg) in the diet of post-weaning piglets improved their feed intake growth and reduced the incidence of diarrhea (Molist, 2011). Zinc supplementation at 500 or 750 mg/kg with MMT (montmorillonite hybrid) was effective to reach pharmacological levels of zinc (2000 mg/kg of zinc) (Hu et al. 2012).

In our studies, zinc inhibited biofilm formation of most bacterial species tested. This antibiofilm activity of zinc could be beneficial during antibiotics or disinfectants treatment. ZnO has been previously combined with an antibacterial agent, which resulted in an improvement of performance marker (Hill, 2001). For the 28-d postweaning period, gains and feed intakes of pigs increased when carbadox and zinc was added as dietary supplement in the feed. The performance responses to ZnO and the antibacterial agent carbadox were additive (NB. Health Canada issued an order to stop the sale of carbadox in 2001). However, it is not known whether this effect would occur with other antibacterial agents (Hill, 2001). Therefore, zinc might have a high potential of synergistic effect with biocides.

Other metals have been studied for the effect of biofilm formation, such as copper, which could prevent the formation of *S. aureus* biofilms by repressing the positive biofilm regulator Agr and Sae. However, the addition of manganese, magnesium, and calcium has no significant effect on biofilm formation (Baker et al. 2010). Gallium shows inhibitory effect on biofilm infection by disrupting bacterial iron metabolism (Kaneko et al. 2007).

The development of antibiofilm agents as novel therapeutic molecules is given promising results; however, the main problems with the development of such molecules are largely economic costs (Romero and Kolter, 2011). Zinc is a potential agent for the prevention of biofilm-associated infection. Considering zinc is a simple and inexpensive molecule with a wide spectrum of antibiofilm effect, it would be worth to further research the synergic effects of zinc with antibiotic or disinfectant treatment. In addition, the present study only measured the effect of zinc on a single–species biofilm, and the effects of zinc on multiple species biofilm should be investigated in future studies.

VI. Conclusion

Biofilm formation is considered as a major virulence factor in many bacterial infections. Microorganisms that form biofilms are of major significance for animal health and public health due to the reduced susceptibility of biofilms to antimicrobial agents. An effective agent should also take into consideration the impact of drug resistance resulting from biofilm formation, and the environmental toxicity.

We have shown that at micromolar concentrations (0 – 250 μM), zinc ($ZnCl_2$ or ZnO) weakly inhibited bacterial growth and effectively blocked biofilm-formation of several Gram-positive and Gram-negative pathogens of swine. Zinc has a wide spectrum of antibiofilm activity that possibly affects the biofilm matrix components and the biofilm formation process.The components of biofilm matrix function as the structure and adhesion agents. Small molecules that interact with components of the biofilms may break the biofilm. In addition, cell-to-cell communication systems play an important role in biofilm maturation and development. Small molecules that interfer with cell-to-cell communication may prevent biofilm formation and keep the cells in a planktonic state. The antibiofilm property of zinc could perhaps enhance the susceptibility of biofilms to antimicrobial agents. The inhibitory effect of zinc on biofilm formation may not be limited to specific bacteria, but may extend to other important human and animal bacterial pathogens.

Zinc has already been used as an additive in feed of production animals, especially pigs. The antibiofilm property of zinc has the potential to have synergic effect with antibiotics or disinfectants in the fight against bacterial infections. This should be the focus of future studies.

VII. References

Aggarwal R, Sentz J, Miller MA. 2007. Role of zinc administration in prevention of childhood diarrhea and respiratory illnesses: a meta-analysis. *Pediatrics* 119: 1120-1130

Antoniani D, Bocci P, Maciag A, Raffaelli N, Landini P. 2010. Monitoring of diguanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. *Appl Microbiol Biotechnol* 85: 1095-1104

Agladze K, Wang X, Romeo T. 2005. Spatial periodicity of *Escherichia coli* K-12 biofilm microstructure initiates during a reversible, polar attachment phase of development and requires the polysaccharide adhesin PGA. *J Bacteriol* 187: 8237-8246

Allison DG. 2003. The biofilm matrix. *Biofouling* 19: 139-150

Anderson GG, O'Toole GA. 2008. Innate and induced resistance mechanisms of bacterial biofilms. *Curr Top Microbiol Immunol* 322: 85-105

Auger E, Deslandes V, Ramjeet M, Contreras I, Nash JH, Harel J, Gottschalk M, Olivier M, Jacques M. 2009. Host-pathogen interactions of *Actinobacillus pleuropneumoniae* with porcine lung and tracheal epithelial cells. *Infect Immun* 77: 1426-1441

Bazaka K, Crawford RJ, Nazarenko EL, Ivanova EP. 2011. Bacterial extracellular polysaccharides. *Adv Exp Med Biol* 715: 213-226

Baker J, Sitthisak S, Sengupta M, Johnson M, Jayaswal RK, Morrissey, JA. 2010. Copper stress induces a global stress response in *Staphylococcus aureus* and represses sae and agr expression and biofilm formation. *Appl Environ Microbiol* 76: 150-160

Beloin C, Roux A, Ghigo JM. 2008. *Escherichia coli* biofilms. *Curr Top Microbiol Immunol* 322: 249-289

Beyenal H, Donovan C, Lewandowski Z, Harkin G. 2004. Three-dimensional biofilm structure quantification. *J Microbiol Methods* 59: 395-413

Boles BR, Horswill AR. 2008. Agr-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog* 4: e1000052

Boles BR, Horswill AR. 2011. Staphylococcal biofilm disassembly. *Trends Microbiol* 19: 449-455

Bonifait L, Gottschalk M, Grenier D. 2010. Cell surface characteristics of nontypeable isolates of *Streptococcus suis*. *FEMS Microbiol Lett* 311: 160-166

Bonifait L, Grignon L, Grenier D. 2008. Fibrinogen induces biofilm formation by *Streptococcus suis* and enhances its antibiotic resistance. *Appl Environ Microbiol* 74: 4969-4972

Bossé JT, Sinha S, Li MS, O'Dwyer CA, Nash JH, Rycroft, AN, Kroll, JS, Langford, PR. 2010. Regulation of pga operon expression and biofilm formation in *Actinobacillus pleuropneumoniae* by sigmaE and H-NS. *J Bacteriol* 192: 2414-2423

Branda SS, Vik S, Friedman L, Kolter R. 2005. Biofilms: the matrix revisited. *Trends Microbiol* 13: 20-26

Camilli A, Bassler BL. 2006. Bacterial small-molecule signaling pathways. *Science* 311: 1113-1116

Carlson MS, Boren CA, Wu C, Huntington CE, Bollinger DW, Veum TL. 2004. Evaluation of various inclusion rates of organic zinc either as polysaccharide or proteinate complex on the growth performance, plasma, and excretion of nursery pigs. *J Anim Sci* 82: 1359-1366

Castelijn GA, van der Veen S, Zwietering MH, Moezelaar R, Abee T. 2012. Diversity in biofilm formation and production of curli fimbriae and cellulose of *Salmonella* Typhimurium strains of different origin in high and low nutrient medium. *Biofouling* 28: 51-63

Cheng M, Cook AE, Fukushima T, Bond PL. 2011. Evidence of compositional differences between the extracellular and intracellular DNA of a granular sludge biofilm. *Lett Appl Microbiol* 53: 1-7

Chiers K, De Waele T, Pasmans F, Ducatelle R, Haesebrouck F. 2010. Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. *Vet Res* 41: 65

Clutterbuck AL, Woods EJ, Knottenbelt DC, Clegg PD, Cochrane CA, Percival SL. 2007. Biofilms and their relevance to veterinary medicine. *Vet Microbiol* 121: 1-17

Conover MS, Mishra M, Deora R. 2011. Extracellular DNA is essential for maintaining *Bordetella* biofilm integrity on abiotic surfaces and in the upper respiratory tract of mice. *Plos One* 6: e16861

Conover MS, Sloan GP, Love CF, Sukumar N, Deora R. 2010. The Bps polysaccharide of *Bordetella pertussis* promotes colonization and biofilm formation in the nose by functioning as an adhesin. *Mol Microbiol* 77: 1439-1455

Conrady DG, Brescia CC, Horii K, Weiss AA, Hassett DJ, Herr AB. 2008. A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. *Proc Natl Acad Sci U S A* 105: 19456-19461

Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284: 1318-1322

Costerton W, Veeh R, Shirtliff M, Pasmore M, Post C, Ehrlich G. 2003. The application of biofilm science to the study and control of chronic bacterial infections. *J Clin Invest* 112: 1466-1477

Cotter PA, Stibitz S. 2007. c-di-GMP-mediated regulation of virulence and biofilm formation. *Curr Opin Microbiol* 10: 17-23

Crane JK, Byrd IW, Boedeker EC. 2011. Virulence inhibition by zinc in shiga-toxigenic *Escherichia coli*. *Infect Immun* 79: 1696-1705

Czaczyk K, Myszka K. 2007. Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. *Pol J Environ Stud* 16: 799-806

Das T, Sharma PK, Busscher HJ, van der Mei HC, Krom BP. 2010. Role of extracellular DNA in initial bacterial adhesion and surface aggregation. *Appl Environ Microbiol* 76: 3405-3408

Davies D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov* 2: 114-122

Davies DG, Marques CNH. 2009. A Fatty Acid messenger is responsible for inducing dispersion in microbial biofilms. *J Bacteriol* 191: 1393-1403

del Pozo JL, Patel R. 2007. The challenge of treating biofilm-associated bacterial infections. *Clin Pharmacol Ther* 82: 204-209

Denkhaus E, Meisen S, Telgheder U, Wingender J. 2007. Chemical and physical methods for characterisation of biofilms. *Microchim Acta* 158: 1-27

Devirgiliis C, Zalewski PD, Perozzi G, Murgia C. 2007. Zinc fluxes and zinc transporter genes in chronic diseases. *Mutat Res* 622: 84-93

Donlan RM. 2002. Biofilms: microbial life on surfaces. *Emerg Infect Dis* 8: 881-890

Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15: 167-193

Eboigbodin KE, Biggs CA. 2008. Characterization of the extracellular polymeric substances produced by *Escherichia coli* using infrared spectroscopic, proteomic, and aggregation studies. *Biomacromolecules* 9: 686-695

Fairbrother JM, Nadeau E, Gyles CL. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Anim Health Res Rev* 6: 17-39

Fischer Walker C, Black RE. 2004. Zinc and the risk for infectious disease. *Annu Rev Nutr* 24: 255-275

Fittipaldi N, Segura M, Grenier D, Gottschalk M. 2012. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent *Streptococcus suis*. *Future Microbiol* 7: 259-279

Fitzgerald JR. 2012. Livestock-associated *Staphylococcus aureus*: origin, evolution and public health threat. *Trends Microbiol* 20: 192-198

Flemming HC, Neu TR, Wozniak DJ. 2007. The EPS matrix: the "house of biofilm cells". *J Bacteriol* 189: 7945-7947

Flemming HC, Wingender J. 2010. The biofilm matrix. *Nat Rev Microbiol* 8: 623-633

Foster TJ. 2005. Immune evasion by Staphylococci. *Nat Rev Microbiol* 3: 948-958

Gerdes K, Christensen SK, Lobner-Olesen A. 2005. Prokaryotic toxin-antitoxin stress response loci. *Nat Rev Microbiol* 3: 371-382

Grande R, Di Giulio M, Bessa LJ, Di Campli E, Baffoni M, Guarnieri S, Cellini L. 2011. Extracellular DNA in *Helicobacter pylori* biofilm: a backstairs rumour. *J Appl Microbiol* 110: 490-498

Grenier D, Grignon L, Gottschalk M. 2009. Characterisation of biofilm formation by a *Streptococcus suis* meningitis isolate. *Vet J* 179: 292-295

Gu H, Fan D, Gao J, Zou W, Peng Z, Zhao Z, Ling J, LeGeros, RZ. 2012. Effect of $ZnCl_2$ on plaque growth and biofilm vitality. *Arch Oral Biol* 57: 369-375

Hall-Stoodley L, Costerton JW, Stoodley P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2: 95-108

Hall-Stoodley L, Stoodley P. 2009. Evolving concepts in biofilm infections. *Cell Microbiol* 11: 1034-1043

Hancock V, Dahl M, Klemm P. 2010. Abolition of biofilm formation in urinary tract *Escherichia coli* and *Klebsiella* isolates by metal interference through competition for fur. *Appl Environ Microbiol* 76: 3836-3841

Haase H, Rink L. 2009. Functional Significance of Zinc-Related Signaling Pathways in Immune Cells. *Annu Rev Nutr* 29: 133-152

Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 7: 263-273

Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR, Rice SA, Eberl L, Molin S, Hoiby N, Kjelleberg S, Givskov M. 2002. Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* 148: 87-102

Hetrick EM, Schoenfisch MH. 2006. Reducing implant-related infections: active release strategies. *Chem Soc Rev* 35: 780-789

Hill GM, Mahan DC, Carter SD, Cromwell GL, Ewan RC, Harrold RL, Lewis AJ, Miller, PS, Shurson GC, Veum TL, Nutrition NCS. 2001. Effect of pharmacological concentrations of zinc oxide with or without the inclusion of an antibacterial agent on nursery pig performance. *J Anim Sci* 79: 934-941

Hoffmann N, Lee B, Hentzer M, Rasmussen TB, Song ZJ, Johansen HK, Givskov M, Hoiby N. 2007. Azithromycin blocks quorum sensing and alginate polymer formation and increases the sensitivity to serum and stationary-growth-phase killing of *Pseudomonas aeruginosa* and attenuates chronic *P-aeruginosa* lung infection in Cftr(-/-) mice. *Antimicrob Agents Chemother* 51: 3677-3687

Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. 2010. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 35: 322-332

Hu C, Song J, You Z, Luan Z, Li W. 2012. Zinc oxide-montmorillonite hybrid influences diarrhea, intestinal mucosal integrity, and digestive enzyme activity in weaned pigs. *Biol Trace Elem Res* 149: 190-196

Iibuchi R, Hara-Kudo Y, Hasegawa A, Kumagai S. 2010. Survival of *Salmonella* on a polypropylene surface under dry conditions in relation to biofilm-formation capability. *J Food Prot* 73: 1506-1510

Irie Y, Mattoo S, Yuk MH. 2004. The Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica*. *J Bacteriol* 186: 5692-5698

Irie Y, Yuk MH. 2007. *In vivo* colonization profile study of *Bordetella bronchiseptica* in the nasal cavity. *FEMS Microbiol Lett* 275: 191-198

Izano EA, Sadovskaya I, Vinogradov E, Mulks MH, Velliyagounder K, Ragunath C, Kher WB, Ramasubbu N, Jabbouri S, Perry MB, Kaplan JB. 2007. Poly-N-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*. *Microb Pathog* 43: 1-9

Jacques M, Aragon V, Tremblay YD. 2010. Biofilm formation in bacterial pathogens of veterinary importance. *Anim Health Res Rev* 11: 97-121

James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart PS. 2008. Biofilms in chronic wounds. *Wound repair regen* 16: 37-44

Jayaraman A, Wood TK. 2008. Bacterial quorum sensing: signals, circuits, and implications for biofilms and disease. *Annu Rev Biomed Eng* 10: 145-167

Jenal U, Malone J. 2006. Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* 40: 385-407

Jin H, Zhou R, Kang MS, Luo R, Cai XW, Chen HC. 2006. Biofilm formation by field isolates and reference strains of *Haemophilus parasuis*. *Vet Microbiol* 118: 117-123

Kaneko Y, Thoendel M, Olakanmi O, Britigan BE, Singh PK. 2007. The transition metal gallium disrupts *Pseudomonas aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. *J Clin Invest* 117: 877-888

Kaplan JB, Ragunath C, Ramasubbu N, Fine DH. 2003. Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous beta-hexosaminidase activity. *J Bacteriol* 185: 4693-4698

Kaplan JB, Ragunath C, Velliyagounder K, Fine DH, Ramasubbu N. 2004. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother* 48: 2633-2636

Kaplan JB, Mulks MH. 2005. Biofilm formation is prevalent among field isolates of *Actinobacillus pleuropneumoniae*. *Vet Microbiol* 108: 89-94

Kaplan JB. 2010. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *J Dent Res* 89: 205-218

Kerrigan JE, Ragunath C, Kandra L, Gyemant G, Liptak A, Janossy L, Kaplan JB, Ramasubbu N. 2008. Modeling and biochemical analysis of the activity of antibiofilm agent Dispersin B. *Acta Biol Hung* 59: 439-451

Khanna T, Friendship R, Dewey C, Weese JS. 2008. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. *Vet Microbiol* 128: 298-303

Klemm P, Vejborg RM, Hancock V. 2010. Prevention of bacterial adhesion. *Appl Microbiol Biotechnol* 88: 451-459

Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R 2010. D-amino acids trigger biofilm disassembly. *Science* 328: 627-629

Labrie J, Pelletier-Jacques G, Deslandes V, Ramjeet M, Auger E, Nash JH, Jacques M. 2010. Effects of growth conditions on biofilm formation by *Actinobacillus pleuropneumoniae*. *Vet Res* 41: 3

Landini P, Antoniani D, Burgess JG, Nijland R. 2010. Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Appl Microbiol Biotechnol* 86: 813-823

Lawrence JR, Korber DR, Hoyle BD, Costerton JW, Caldwell DE. 1991. Optical Sectioning of Microbial Biofilms. *J Bacteriol* 173: 6558-6567

Leid JG, Willson CJ, Shirtliff ME, Hassett DJ, Parsek MR, Jeffers AK. 2005. The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-gamma-mediated macrophage killing. *J Immunol* 175: 7512-7518

Lewis K. 2010. Persister cells. *Annu Rev Microbiol* 64: 357-372

Little DJ, Poloczek J, Whitney JC, Robinson H, Nitz M, Howell PL. 2012. The structure and metal dependent activity of *Escherichia coli* PgaB provides insight into the partial de-N-acetylation of poly-beta-1,6-N-acetyl-D-glucosamine. *J Biol Chem* 287: 31126-31137

Lopez D, Vlamakis H, Kolter R. 2010. Biofilms. *Cold Spring Harb Perspect Biol* 2: a000398

Mah TF, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 9: 34-39

Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 426: 306-310

Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, Chandramohan L, Tsang LH, Smeltzer MS, Horswill AR, Bayles KW. 2009. Modulation of eDNA Release and Degradation Affects *Staphylococcus aureus* Biofilm Maturation. *PLoS One* 4: e5822

Maret W. 2000. The function of zinc metallothionein: A link between cellular zinc and redox state. *J Nutr* 130: 1455-1458

Maret W. 2001. Zinc biochemistry, physiology, and homeostasis - recent insights and current trends. *Biometals* 14: 187-190

McCall KA, Huang C, Fierke CA. 2000. Function and mechanism of zinc metalloenzymes. *J Nutr* 130: 1437-1446

McLandsborough L, Rodriguez A, Perez-Conesa D, Weiss J. 2006. Biofilms: At the interface between biophysics and microbiology. *Food Biophys* 1: 94-114

Melchior MB, Fink-Gremmels J, Gaastra W. 2006. Comparative assessment of the antimicrobial susceptibility of *Staphylococcus aureus* isolates from bovine mastitis in biofilm versus planktonic culture. *J Vet Med B* 53: 326-332

Melchior MB, Vaarkamp H, Fink-Gremmels J. 2006. Biofilms: a role in recurrent mastitis infections? *Vet J* 171: 398-407

Molin S, Tolker-Nielsen T. 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr Opin Biotechnol* 14: 255-261

Molist F, Hermes RG, de Segura AG, Martin-Orue SM, Gasa J, Manzanilla EG, Perez JF. 2011. Effect and interaction between wheat bran and zinc oxide on productive performance and intestinal health in post-weaning piglets. *Br J Nutr* 105: 1592-1600

Muh U, Schuster M, Heim R, Singh A, Olson ER, Greenberg EP. 2006. Novel *Pseudomonas aeruginosa* quorum-sensing inhibitors identified in an ultra-high-throughput screen. *Antimicrob Agents Chemother* 50: 3674-3679

Mulcahy H, Charron-Mazenod L, Lewenza S. 2008. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog* 4: e1000213

Nalca Y, Jansch L, Bredenbruch F, Geffers R, Buer J, Haussler S. 2006. Quorum-sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: a global approach. *Antimicrob Agents Chemother* 50: 1680-1688

Oliveira M, Bexiga R, Nunes SE, Carneiro C, Cavaco LM, Bernardo F, Vilela CL. 2006. Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *Vet Microbiol* 118: 133-140

Oliveira S, Pijoan C. 2004. *Haemophilus parasuis*: new trends on diagnosis, epidemiology and control. *Vet Microbiol* 99: 1-12

Olson ME, Ceri H, Morck DW, Buret AG, Read RR. 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can J Vet Res* 66: 86-92

Or D, Phutane S, Dechesne A. 2007. Extracellular polymeric substances affecting pore-scale hydrologic conditions for bacterial activity in unsaturated soils. *Vadose Zone J* 6: 298-305

O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. *Annu Rev Microbiol* 54: 49-79

Otto M. 2008. Staphylococcal biofilms. *Curr Top Microbiol* 322: 207-228

Palmer J, Flint S, Brooks J. 2007. Bacterial cell attachment, the beginning of a biofilm. *J Ind Microbiol Biotechnol* 34: 577-588

Papadopoulos E, Collet JF, Vukojevic V, Billeter M, Holmgren A, Graslund A, Vlamis-Gardikas A. 2012. Solution structure and biophysical properties of MqsA, a Zn-containing antitoxin from *Escherichia coli*. *Biochim Biophys Acta* 1824: 1401-1408

Parsek MR, Singh PK. 2003. Bacterial biofilms: An emerging link to disease pathogenesis. *Annu Rev Microbiol* 57: 677-701

Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S, Qu D. 2007. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. *Microbiology* 153: 2083-2092

Rabsch W, Tschape H, Baumler AJ. 2001. Non-typhoidal salmonellosis: emerging problems. *Microbes Infect* 3: 237-247

Raghupathi KR, Koodali RT, Manna AC. 2011. Size-dependent bacterial growth inhibition and mechanism of antibacterial activity of zinc oxide nanoparticles. *Langmuir* 27: 4020-4028

Rasmussen TB, Skindersoe ME, Bjarnsholt T, Phipps RK, Christensen KB, Jensen PO, Andersen JB, Koch B, Larsen TO, Hentzer M, Eberl L, Hoiby N, Givskov M. 2005. Identity and effects of quorum-sensing inhibitors produced by Penicillium species. *Microbiology* 151: 1325-1340

Rendueles O, Ghigo JM. 2012. Multi-species biofilms: how to avoid unfriendly neighbors. *FEMS Microbiol Rev* 36:972-989

Rodrigues LR. 2011. Inhibition of bacterial adhesion on medical devices. *Adv Exp Med Biol* 715: 351-367

Rohde H, Frankenberger S, Zahringer U, Mack D. 2010. Structure, function and contribution of polysaccharide intercellular adhesin (PIA) to *Staphylococcus epidermidis* biofilm formation and pathogenesis of biomaterial-associated infections. *Eur J Cell Biol* 89: 103-111

Romero D, Aguilar C, Losick R, Kolter R. 2010. Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. *Proc Natl Acad Sci U S A* 107: 2230-2234

Romero D, Kolter R. 2011. Will biofilm disassembly agents make it to market? *Trends Microbiol* 19: 304-306

Romling U, Amikam D. 2006. Cyclic di-GMP as a second messenger. *Curr Opin Microbiol* 9: 218-228

Roselli M, Finamore A, Garaguso I, Britti MS, Mengheri E. 2003. Zinc oxide protects cultured enterocytes from the damage induced by *Escherichia coli*. *J Nutr* 133: 4077-4082

Schommer NN, Christner M, Hentschke M, Ruckdeschel K, Aepfelbacher M, Rohde H. 2011. *Staphylococcus epidermidis* uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1. *Infect Immun* 79: 2267-2276

Shankar AH, Prasad AS. 1998. Zinc and immune function: the biological basis of altered resistance to infection. *Am J Clin Nutr* 68: 447-463

Singh PK, Parsek MR, Greenberg EP, Welsh MJ. 2002. A component of innate immunity prevents bacterial biofilm development. *Nature* 417: 552-555

Slade RD, Kyriazakis I, Carroll SM, Reynolds FH, Wellock IJ, Broom LJ, Miller HM. 2011. Effect of rearing environment and dietary zinc oxide on the response of group-housed weaned pigs to enterotoxigenic *Escherichia coli* O149 challenge. *Animal* 5: 1170-1178

Sloan GP, Love CF, Sukurnar N, Mishra M, Deora R. 2007. The Bordetella Bps polysaccharide is critical for biofilm development in the mouse respiratory tract. *J Bacteriol* 189: 8270-8276

Smith TC, Pearson N. 2011. The emergence of *Staphylococcus aureus* ST398. *Vector Borne Zoonotic Dis* 11: 327-339.

Stefanidou M, Maravelias C, Dona A, Spiliopoulou C. 2006. Zinc: a multipurpose trace element. *Arch Toxicol* 80: 1-9

Stewart PS, Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. *Lancet* 358: 135-138

Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56: 187-209

Sutherland IW. 2001. The biofilm matrix--an immobilized but dynamic microbial environment. *Trends Microbiol* 9: 222-227

Szomolay B, Klapper I, Dockery J, Stewart PS. 2005. Adaptive responses to antimicrobial agents in biofilms. *Environ Microbiol* 7: 1186-1191

Tamayo R, Tischler AD, Camilli A. 2005. The EAL domain protein VieA is a cyclic diguanylate phosphodiesterase. *J Biol Chem* 280: 33324-33330

Thurlow LR, Hanke ML, Fritz T, Angle A, Aldrich A, Williams SH, Engebretsen IL, Bayles KW, Horswill AR, Kielian T. 2011. *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation *in vivo*. *J Immunol* 186: 6585-6596

Truong-Tran AQ, Ho LH, Chai F, Zalewski PD. 2000. Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. *J Nutr* 130: 1459-1466

Vallee BL, Falchuk KH. 1993. The biochemical basis of zinc physiology. *Physiol Rev* 73: 79-118

Van Houdt R, Michiels CW. 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. *J Appl Microbiol* 109: 1117-1131

Van Parys A, Boyen F, Volf J, Verbrugghe E, Leyman B, Rychlik I, Haesebrouck F, Pasmans F. 2010. *Salmonella* Typhimurium resides largely as an extracellular pathogen in porcine tonsils, independently of biofilm-associated genes csgA, csgD and adrA. *Vet Microbiol* 144: 93-99

Vu B, Chen M, Crawford RJ, Ivanova EP. 2009. Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules* 14: 2535-2554

Vuong C, Kocianova S, Voyich JM, Yao Y, Fischer ER, DeLeo FR, Otto M. 2004. A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem* 279: 54881-54886

Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, Otto M. 2004. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell Microbiol* 6: 269-275

Walker CF, Black RE. 2004. Zinc and the risk for infectious disease. *Annu Rev Nutr* 24: 255-275

Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* 47: 317-323

Walz JM, Avelar RL, Longtine KJ, Carter KL, Mermel LA, Heard SO. 2010. Anti-infective external coating of central venous catheters: A randomized, noninferiority trial comparing 5-fluorouracil with chlorhexidine/silver sulfadiazine in preventing catheter colonization. *Crit Care Med* 38: 2095-2102

Wang Y, Zhang W, Wu ZF, Lu CP. 2011. Reduced virulence is an important characteristic of biofilm infection of *Streptococcus suis*. *Fems Microbiol Lett* 316: 36-43

Waters CM, Lu W, Rabinowitz JD, Bassler BL. 2008. Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of vpsT. *J Bacteriol* 190: 2527-2536

Watnick P, Kolter R. 2000. Biofilm, city of microbes. *J Bacteriol* 182: 2675-2679

Wei ZG, Li R, Zhang AD, He HK, Hua YF, Xia J, Cai XH, Chen HC, Jin ML. 2009. Characterization of *Streptococcus suis* isolates from the diseased pigs in China between 2003 and 2007. *Vet Microbiol* 137: 196-201

Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. 2002. Extracellular DNA required for bacterial biofilm formation. *Science* 295: 1487

Worthington RJ, Richards JJ, Melander C. 2012. Small molecule control of bacterial biofilm. *Org Biomol Chem* 10: 7457-7474

Yang L, Liu Y, Wu H, Song Z, Hoiby N, Molin S, Givskov M. 2012 Combating biofilms. *FEMS Immunol Med Microbiol* 65:146-157

Yim G, Wang HH, Davies J. 2007. Antibiotics as signalling molecules. *Philos Trans R Soc Lond B Biol Sci* 362: 1195-1200