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Biofilm formation in bacterial pathogens of veterinary importance

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Abstract

Bacterial biofilms are structured communities of bacterial cells enclosed in a self-produced polymer matrix that is attached to a surface. Biofilms protect and allow bacteria to survive and thrive in hostile environments. Bacteria within biofilms can withstand host immune responses, and are much less susceptible to antibiotics and disinfectants when compared with their planktonic counterparts. The ability to form biofilms is now considered a universal attribute of micro-organisms. Diseases associated with biofilms require novel methods for their prevention, diagnosis and treatment; this is largely due to the properties of biofilms. Surprisingly, biofilm formation by bacterial pathogens of veterinary importance has received relatively little attention. Here, we review the current knowledge of bacterial biofilms as well as studies performed on animal pathogens.

Keywords: biofilm, bacteria, animal pathogens, resistance to antibiotics, resistance to disinfectants

Introduction

The generally accepted definition of a biofilm is a structured community of bacterial cells enclosed in a self-produced polymer matrix that is attached to an abiotic or biotic surface (Costerton *et al*., 1999; Hall-Stoodley and Stoodley, 2009). Virtually all bacteria can grow as a biofilm and biofilms can be found in every ecosystem including natural, engineered and pathogenic settings. Growth as a biofilm is considered to be a protective mode that allows for survival in hostile environments. Additionally, bacteria grown in biofilms can form intricate and complex structures such as channels that allow nutrients to circulate. The structure of a biofilm has an impact on gene expression. Cells located in different areas of the biofilm will exhibit different patterns of gene expression (Costerton *et al*., 1999). Although the biofilm is composed of sessile individuals, biofilm communities can give rise to non-sessile (or planktonic) individuals that can rapidly multiply, disperse and start a new biofilm.

The clinical significance of biofilm etiology was first established in device-related infections (Donlan and Costerton, 2002; Hall-Stoodley *et al*., 2004). Electron microscopy of medical device surfaces, which had been foci of device-related infections in humans, showed the presence of large numbers of slime (matrix)-enclosed bacteria (Costerton *et al*., 1999). Furthermore, biofilms have also been isolated in the absence of medical devices. For example, tissues isolated from chronic infections also showed the presence of bacteria surrounded by an exopolysaccharide (EPS) matrix (Donlan and Costerton, 2002). The visual characteristics of biofilms growing in diverse environments are strikingly similar, indicating that there are important convergent survival strategies that are conferred in part by structural specialization (Hall-Stoodley *et al*., 2004). Bacterial biofilms share several common features: (i) cells are held together by an extracellular matrix composed mainly of EPS, proteins and nucleic acids; (ii) biofilm development occurs in

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response to extracellular signals, both environmental and self-produced; (iii) biofilms protect bacteria from a diverse and wide array of environmental stresses such as antibiotics, predators and the immune system (Lemon *et al.*, 2008).

Interestingly, biofilm aggregates have also been observed within epithelial cells. Uropathogenic *Escherichia coli* (UPEC) undergo cycles of invasion of the bladder epithelium, intracellular proliferation in polysaccharide-containing biofilm-like masses called intracellular bacterial communities (IBC), and then dispersal into the bladder lumen to initiate further rounds of epithelial colonization and invasion (Anderson *et al.*, 2003).

The ability to form biofilms is now seen as a universal attribute of micro-organisms (Lemon *et al.*, 2008). Approximately 80% of the world's microbial biomass resides as biofilm populations. The National Institutes of Health estimates that up to 75% of human infections are caused by the formation and persistence of biofilms (Richards and Melander, 2009). Although a PubMed search (15 July 2010) gave more than 13,500 hits for 'biofilm', relatively few studies concerning veterinary bacterial pathogens have been perfomed. Considering the extensive involvment of biofilms in infections and diseases in human, biofilms are likely responsible for a wide variety of infections in veterinary medicine (Clutterbuck *et al.*, 2007). Here, we review the current knowledge on bacterial biofilms, which was mostly gained from environmental, industrial and human clinical samples. This review is intended to cover biofilm formation in animal pathogens and to increase awareness about the potential impact of biofilms on the treatment options for animals and disinfection protocols used in both farming and food industries.

Model for biofilm formation

The currently accepted model for biofilm formation is based on observations obtained using various bacterial species (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004; Hall-Stoodley and Stoodley, 2009). Multiple steps are involved in the formation of biofilm by bacteria (Fig. 1A). The bacteria must first attach to a surface. Specific components found at the bacterial surface are required for adhesion. Once the bacteria have attached to a surface, they will autoaggregate into microcolonies. In addition to adhesion factors, other bacterial surface components are required for autoaggregation to occur. Following microcolony formation, the biofilm starts to take form by the maturation of the attached bacteria. During maturation, the bacterial community produces EPS, which surrounds and binds the bacterial community. Bacteria in a biofilm coordinate their behavior resulting in the formation of complex three-dimensional structures. Despite the coordinate effort in the biofilm, the bacterial biofilm community displays functional heterogeneity. The overall three-dimensional structure of the biofilm can be flat or mushroom shaped; biofilm shape is influenced by nutrient sources and the presence or absence of shear force. It is well established that bacteria can adapt to environmental changes by sensing cell density (quorum sensing (QS)) or other environmental cues. These environmental signals and conditions influence several biological functions, including biofilm formation. Some environmental signals have been identified; however, other signals are yet to be studied (Karatan and Watnick, 2009). Environmental signals can be divided into three categories: mechanical (e.g. surface sensing), nutritional and metabolic (e.g. concentration of glucose, iron and phosphate) and secondary messenger and signalling networks (e.g. c-di-GMP, two-component systems). The final step in biofilm formation is detachment and dispersal of bacteria from the biofilm. The released bacteria can then colonize new surfaces.

As with the previous steps, dispersion is greatly influenced by environmental cues (e.g. nutrients, oxygen depletion, c-di-GMP and QS) (Karatan and Watnick, 2009). Detachment can be initiated by several factors including mechanical perturbations (e.g. changes in shear forces or abrasion), enzymatic degradation of the biofilm matrix (e.g. dispersin B and alginate lyase), enzymatic degradation of the biofilm substrate (e.g. hyaluronidase), induction of motility, production of surfactants (e.g. rhamnolipids), release of EPS and surface-binding proteins or cell death and cell lysis (Hall-Stoodley *et al.*, 2004; Karatan and Watnick, 2009; Kaplan, 2010). Recently, new factors controlling biofilm dispersion have been identified. Kolodkin-Gal *et al.* (2010) discovered that D-amino acids (D-leucine, D-methionine, D-tyrosine and D-tryptophan) prevented biofilm formation by *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. D-amino acids did not have any effect when D-alanine was present. This suggests that D-amino acids prevent biofilm formation by replacing D-alanine in the peptide side chain of peptidoglycan. D-amino acids are produced by many bacteria and, thus, D-amino acids may act as biofilm dispersal signals in several bacterial species. The detachment of bacteria from an existing biofilm plays an important role in the transmission of bacteria from environmental reservoirs to human or animal hosts, in horizontal and vertical transmission between hosts, and in the exacerbation and spread of infection within a host (Kaplan, 2010).

The biofilm matrix

The biofilm matrix is believed to be highly hydrated (up to 97%) (Karatan and Watnick, 2009). Several components such as polysaccharides, proteins, DNA, surfactants, lipids, glycolipids, membrane vesicles and ions (Ca^{2+}) have been identified in the matrices of biofilm. The composition of the biofilm matrices varies according to

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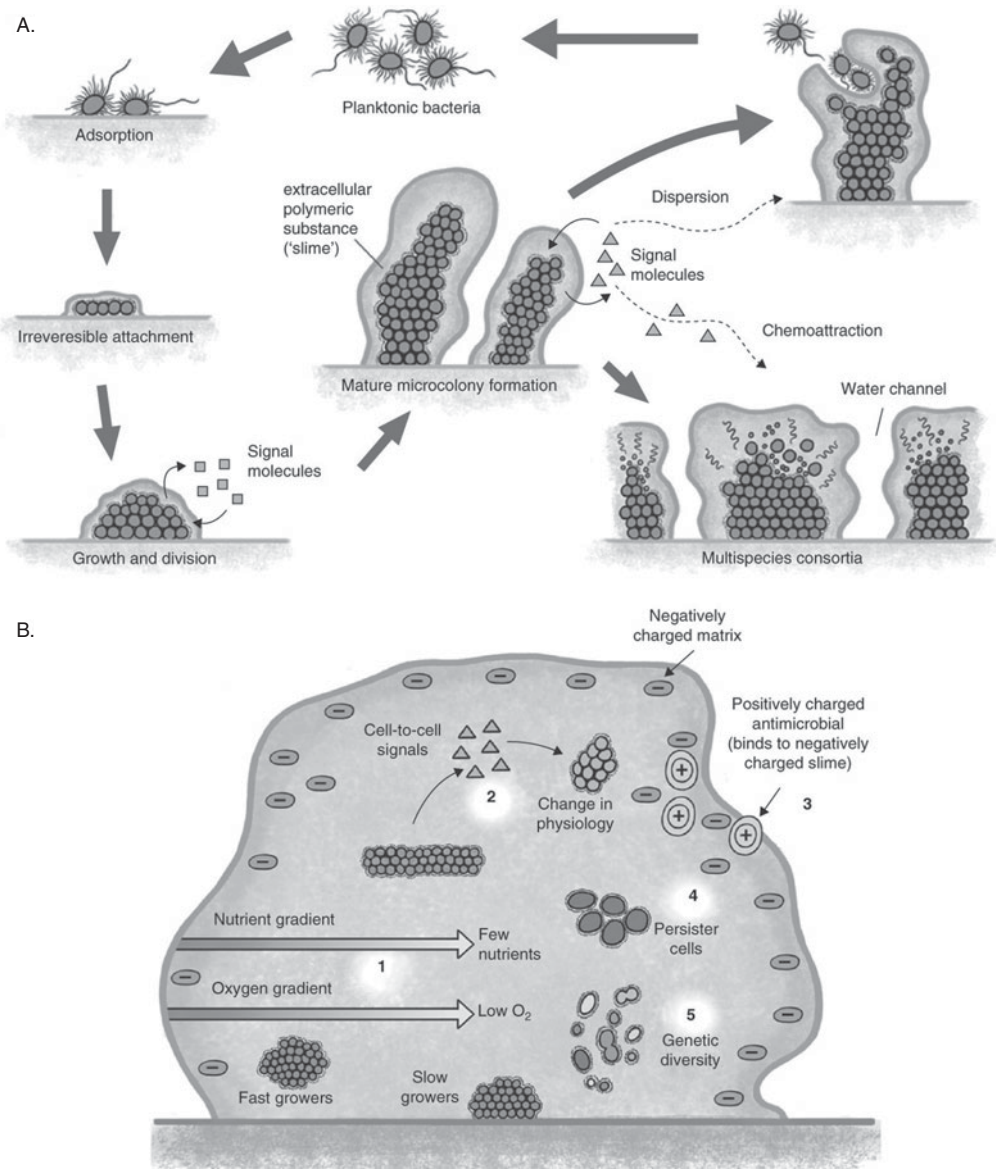

Fig. 1. (A) Growth and development of biofilms. In the center is seen the microcolony formation observed in biofilms. The lower right demonstrates polymicrobial biofilms formed through specific cell–cell signaling and attraction. The upper right demonstrates the mechanism of biofilm spread where cells become motile, swim away as a planktonic population and following to the left go through a cycle of reversible adherence, tight adherence and microcolony formation again under regulation of specific cell–cell communication. (B) Multifactorial mechanisms that contribute to antibiotic tolerance developed within a biofilm (from Ceri *et al.*, 2010; reproduced with permission). Ceri *et al.*, Expert Opinion in Pharmacotherapy, 11/8, copyright ©2010, Informa Healthcare. Reproduced with permission of Informa Healthcare.

bacterial species and growth conditions (Karatan and Watnick, 2009). One of the most common and most extensively studied matrix EPS is a polymer of β-1,6-N-acetyl-D-glucosamine called poly-glucosamine (PGA), poly-N-acetyl-glucosamine (PNAG) or polysaccharide intercellular adhesin (PIA). Several bacterial species such as *E. coli*, *S. aureus*, *Staphylococcus epidermidis*, *Yersinia pestis*, *Actinobacillus* spp., *Aggregatibacter actinomycetemcomitans* and *Bordetella* spp. produce PGA in their biofilm matrices (Fig. 2). Another EPS that is commonly found in biofilm matrices is cellulose, a linear polymer of β-1-4-linked glucose. Cellulose is found in some strains of *E. coli*, *Salmonella*, *Citrobacter*, *Enterobacter* and *Pseudomonas*. Alginate, a polymer of β-1-4-linked mannuronic acid and guluronic acid, is found in *P. aeruginosa*.

Biofilms are resistant to various agents

Biofilms found in natural and industrial environments are resistant to bacteriophages, amoebae and biocides used in industrial processes (Costerton *et al.*, 1999). Furthermore, biofilms formed by medically important bacteria can withstand host immune responses and there

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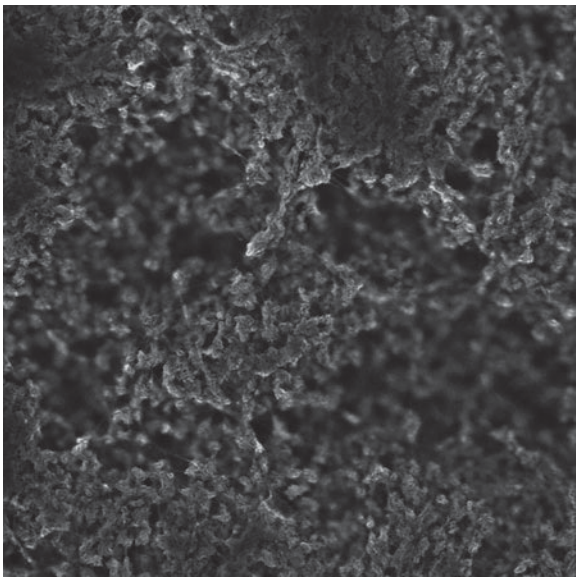

Fig. 2. Confocal scanning laser microscopic image of *Actinobacillus pleuropneumoniae* serotype 5b strain L20 biofilm stained with wheat germ agglutinin (WGA)-Oregon Green 488. WGA binds to PGA of the biofilm matrix.

is good evidence that bacteria in a biofilm are less susceptible to antibiotics and biocides than their planktonic counterparts (Costerton *et al.*, 1999; Russell, 2002; Hall-Stoodley *et al.*, 2004; Anderson and O'Toole, 2008; Hall-Stoodley and Stoodley, 2009). In fact, infections associated with biofilms are 10 to 1000 times more resistant to the effects of antimicrobial agents (Olson *et al.*, 2002; Ceri *et al.*, 2010).

Several mechanisms and factors are likely employed by biofilms to resist antimicrobial agents (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004; Anderson and O'Toole, 2008; Ceri *et al.*, 2010) (Fig. 1B). The barrier created by the biofilm matrix is considered one of the major factors that increase the resistance of bacteria to antimicrobial agents. This is particularly relevant for reactive (bleach or superoxides), charged (metals) or large (immunoglobulins) antimicrobial agents that are neutralized or bound by the matrix. Another proposed mechanism of antimicrobial resistance is related to the different metabolic states of bacteria found within the biofilm. In a biofilm, nutrients are limited and as a result, bacteria can exist in low metabolic states or in a starved/dormant state. This might result in the formation of persister cells which represent a small subpopulation of bacteria (spore-like cells) that spontaneously enter a dormant, non-dividing state. Persister cells have a greatly reduced susceptibility to antibiotics, and therefore, can also contribute to increased resistance of the biofilm to antimicrobial agents. Additionally, multispecies biofilms exhibit a decrease in antimicrobial susceptibility when compared to their single species counterparts. For example, a bacterial species can be protected against the action of an antibiotic by another species or strain that produces a β-lactamase (Hall-Stoodley and Stoodley, 2009).

Antibiotics can degrade fairly quickly in the environment; however, antibiotic resistance genes have now been identified in water and sediment surrounding animal feedlots (Zhang *et al.*, 2009). A recent study has demonstrated the accumulation of tetracycline (*tet*) resistance genes in aquatic biofilms due to periodic loadings from swine lagoons (Zhang *et al.*, 2009). Similar trends and patterns were seen with extended-spectrum β-lactamase (*bla*) and macrolide (*erm*) resistance genes (Knapp *et al.*, 2010). Antibiotic resistance genes can spread rapidly to biofilms and these genes will persist longer in the biofilm when compared to adjacent waters. This suggests that biofilms likely act as reservoirs for antibiotic resistance genes.

The minimal inhibitory concentration (MIC) has been used as a gold standard for the determination of antimicrobial sensitivity of planktonic populations of animal and human pathogens (Olson *et al.*, 2002). The inherent problem with the MIC of planktonic bacteria is that it does not correlate with the concentration required to eradicate biofilms. Therefore, the misuse of antibiotics to treat biofilm-associated bacterial infections likely contributed to the development and spread of antibiotic resistance in bacteria (Ceri *et al.*, 2010). It is now possible to determine the minimal antibiotic concentration to eradicate a biofilm and this is referred to as the minimal biofilm eradication concentration (MBEC) (Ceri *et al.*, 1999). Determination of the MBEC might permit the selection of the appropriate antibiotic for the treatment of a specific bacterial infection (Olson *et al.*, 2002). Optimal antibiotic treatment could decrease the spread of antibiotic resistance and reduce complications related to chronic infections. The need for updated standard guidelines for testing the susceptibility of biofilms is not limited to antibiotics and also applies to biocides (Toté *et al.*, 2010).

In vitro systems to study biofilms

Several systems are widely used to study biofilm formation (Lemon *et al.*, 2008). Flow cells are small chambers that allow a submerged biofilm to form in the presence of shear force and with a continuous supply of fresh nutrients; this system is amenable to observation through confocal scanning laser microscopy (CSLM). One of the advantages of the flow cell system is that it mimics conditions that are naturally encountered by bacterial biofilm. Submerged biofilm can also be studied as batch cultures in microtiter plates (Fig. 3A); the main advantage of this commonly used system is its effectiveness for high-throughput screens. Until recently, the absence of shear force was one of the major limiting factors of the batch culture. Benoit *et al.* (2010) developed a flow-cell system that utilizes a 96-well plate, and is compatible with CSLM and plate readers. Overall, this new method combines the advantages of the batch format (i.e. high-throughput screen) with those of the flow cell (i.e. natural biofilm). The floating pellicles that form at the liquid–air interface

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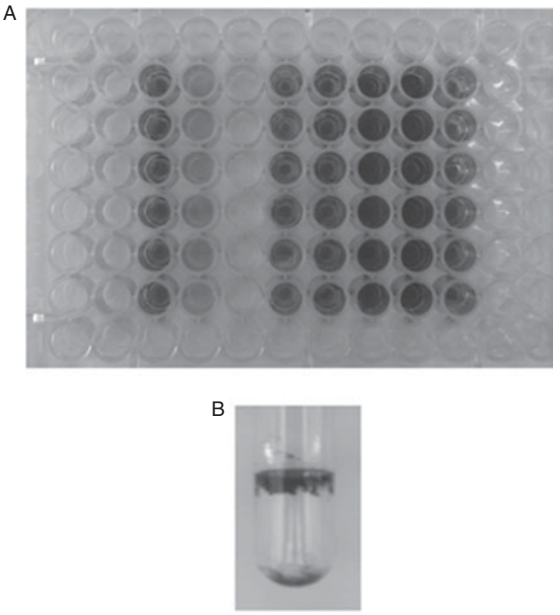

Fig. 3. Biofilm assays. (A) Biofilms of *Actinobacillus pleuropneumoniae* isolates formed in a microtiter plate and stained with crystal violet. (B) Biofilm of *A. pleuropneumoniae* serotype 5b strain L20 formed in a glass tube and stained with crystal violet.

of standing cultures represent another form of biofilm (Fig. 3B). In addition, colonies growing on the surface of agar that demonstrate macroscopically complex architecture (e.g. *Bacillus*) are now recognized as a type of biofilm (Lemon *et al.*, 2008). A method to grow biofilms under low shear conditions at the air–liquid interface was recently described (Goeres *et al.*, 2009). This method is called the drip flow reactor and this reactor allows fresh culture medium to drop onto diverse surfaces such as a glass microscope slide or a catheter. This system can model environments such as the lungs and oral cavity.

Ceri *et al.* (1999) described a technology, originally called the Calgary Biofilm Device, for the rapid and reproducible screening of the antibiotic or biocide susceptibility of a biofilm. The device is now called the MBEC Assay System. The device consists of a two-part reactive vessel. The top component forms a lid with 96 pegs that are designed to sit in the channels at the bottom component and fit into the wells of a standard 96-well plate. The bottom of the vessel serves to channel the flow of medium across the pegs to create consistent shear force across all pegs. This results in the formation of 96 equivalent biofilms. The MBEC Assay System is used to determine the MBEC of bacterial pathogens of medical and veterinary importance.

Biofilm formation by animal pathogens

In their seminal work, Olson *et al.* (2002) used the Calgary Biofilm Device to test the biofilm-forming ability of several Gram-negative and Gram-positive pathogens of veterinary importance, isolated from cattle, sheep, pigs, chicken and turkeys. In addition, they determined the MIC and MBEC of various antibiotics. This study clearly demonstrated the diversity of organisms that can form biofilms. It also showed that biofilms formed by veterinary pathogens were resistant to commonly used antibiotics in veterinary medicine. In the next sections, we review the literature regarding biofilm formation of pathogenic bacteria of veterinary importance (Table 1).

Actinobacillus pleuropneumoniae

Actinobacillus pleuropneumoniae is an important swine pathogen and member of the *Pasteurellaceae* family. *A. pleuropneumoniae* has the ability to form biofilms under specific growth conditions, when cultured under static (Kaplan and Mulks, 2005; Labrie *et al.*, 2010) and low shear conditions at the air–liquid interface (Y.D.N. Tremblay and M. Jacques, unpublished data). In *A. pleuropneumoniae*, the formation of biofilm on polystyrene microtiter plates is dependent on the production of PGA (Kaplan *et al.*, 2004; Izano *et al.*, 2007). PGA biosynthesis is dependent on the proteins encoded within the *pgaABCD* operon (Kaplan *et al.*, 2004). PGA is the substrate for dispersin B (DspB) which is a glycosyl hydrolase produced by *Aggregatibacter (Actinobacillus) actinomycetemcomitans* and *A. pleuropneumoniae* (Kaplan *et al.*, 2004; Kerrigan *et al.*, 2008). Dispersin B releases biofilms formed by *A. pleuropneumoniae*, *A. actinomycetemcomitans* and other PGA-producing bacterial pathogens. In *A. pleuropneumoniae*, mutants lacking *pgaC* cannot form biofilm on polystyrene surfaces (Izano *et al.*, 2007; Liu *et al.*, 2008). Furthermore, overexpression of the *pga* operon was associated with enhanced biofilm formation in an *rseA* mutant, which is deficient in the anti-sigma factor for σ^E, and an H-NS mutant (Dalai *et al.*, 2009; Bossé *et al.*, 2010). It was recently demonstrated that σ^E and H-NS independently regulate the expression of the *pga* operon (Bossé *et al.*, 2010). Positive regulation by σ^E indicates that biofilm formation in *A. pleuropneumoniae* is part of the extracytoplasmic stress response. The association between the extracytoplasmic stress response and biofilm formation has also been observed in other Gram-negative bacteria such as *E. coli* (Dorel *et al.*, 2006; Yang *et al.*, 2008) and *Burkholderia pseudomallei* (Korbsrisate *et al.*, 2005). Interestingly, both *pgaB* and *pgaC* were upregulated in *A. pleuropneumoniae* attached to St. Jude Porcine Lung (SJPL) cells (Auger *et al.*, 2009a). This upregulation suggests that PGA produced by *A. pleuropneumoniae* might play a role during infection and may participate in biofilm formation *in vivo*.

PGA appears to play a significant role in the properties of the *A. pleurepneumoniae* biofilm. The presence of PGA in the matrix impedes fluid convection and transport of

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Table 1. Studies on biofilm formation of bacterial pathogens of veterinary importance

Bacterial species	References
Actinobacillus pleuropneumoniae	Auger *et al.* (2009a), Bossé *et al.* (2010), Buettner *et al.* (2008), Dalai *et al.* (2009), Ganeshnaryan *et al.* (2009), Izano *et al.* (2007), Kaplan *et al.* (2004), Kaplan and Mulks (2005), Kerrigan *et al.* (2008), Labrie *et al.* (2010), Li *et al.* (2008), Liu *et al.* (2008), Tegetmeyer *et al.* (2009)
Aeromonas hydrophila	Asha *et al.* (2004), Gavìn *et al.* (2002), Kozlova *et al.* (2008), Lynch *et al.* (2002), Truchado *et al.* (2009)
Arcanobacterium pyogenes	Jost and Billington (2005), Olson *et al.* (2002)
Bacillus cereus group	Auger *et al.* (2006), Auger *et al.* (2009b), Houry *et al.* (2010), Lee *et al.* (2007), Schuch and Fischetti (2009), Shaheen *et al.* (2010), Shi *et al.* (2004), Wijman *et al.* (2007)
Bartonella henselae	Kyme *et al.* (2003)
Bordetella bronchiseptica *Bordetella parapertussis*	Irie *et al.* (2004), Irie *et al.* (2005), Irie *et al.* (2006), Mishra *et al.* (2005), Parise *et al.* (2007), Sloan *et al.* (2007)
Brucella melitensis	Uzureau *et al.* (2007)
Burkholderia pseudomallei	Boddey *et al.* (2006), Korbsrisate *et al.* (2005), Lee *et al.* (2010), Sawasdidoln *et al.* (2010), Taweechaisupapong *et al.* (2005), Tunpiboonsak *et al.* (2010)
Campylobacter coli *Campylobacter jejuni*	Fields and Thompson (2008), Gunther and Chen (2009), Hanning *et al.* (2008), Hanning *et al.* (2009), McLennan *et al.* (2008), Moe *et al.*, (2010), Murphy *et al.* (2006), Naito *et al.* (2010), Peyrat *et al.* (2008), Reeser *et al.* (2007), Reuter *et al.* (2010), Sulaeman *et al.* (2010), Svensson *et al.* (2009), Trachoo and Frank (2002), Trachoo *et al.* (2002)
Clostridium perfringens	Varga *et al.* (2008)
Corynebacterium pseudotuberculosis *Corynebacterium renale*	Olson *et al.* (2002)
Enterococcus faecalis *Enterococcus faecium*	Ballering *et al.* (2009), Ciftci *et al.* (2009), Guiton *et al.* (2009), Macovei *et al.* (2009), Mohamed and Huang (2007), Oliveira *et al.* (2010), Teng *et al.* (2009)
Erysipelothrix rhusiopathiae	Shimoji *et al.* (2003)
Escherichia coli	Agladze *et al.* (2005), Beloin *et al.* (2008), Hancock *et al.* (2010), Olson *et al.* (2002), Prigent-Combaret *et al.* (2000), Puttamreddy *et al.* (2010), Uhlich *et al.* (2010), Wood (2009), Zogaj *et al.* (2001)
Francisella novicida *Francisella tularensis*	Amer *et al.* (2010), Durham-Colleran *et al.* (2010), Margolis *et al.* (2010)
Haemophilus parasuis	Jin *et al.* (2006, 2008)
Histophilus somni	Olson *et al.* (2002), Sandal *et al.* (2007, 2009)
Leptospira	Ristow *et al.* (2008)
Listeria monocytogenes	Amalaradjou *et al.* (2009), Gandhi and Chikindas (2007), Habimana *et al.* (2009), Harmsen *et al.* (2010a, 2010b), Latorre *et al.* (2010), Riedel *et al.* (2009), Takahashi *et al.* (2010), Todhanakasem and Young (2008)
Mannheimia haemolytica	Olson *et al.* (2002)
Mycobacterium	Carter *et al.* (2004), Cook *et al.* (2010), Johansen *et al.* (2009), Ojha *et al.* (2008), Wu *et al.* (2009), Yamazaki *et al.* (2006a, 2006b)
Mycoplasma	Daubenspeck *et al.* (2009), Justice-Allen *et al.* (2010), McAuliffe *et al.* (2006, 2008), Simmons and Dybvig (2007, 2009)
Pasteurella multocida	Olson *et al.* (2002)
Pseudomonas aeruginosa	Bazire *et al.* (2010), Davies and Marques (2009), Deligianni *et al.* (2010), Fuxman Bass *et al.* (2010), Harmsen *et al.* (2010b), Lenz *et al.* (2008), Ma *et al.* (2009), Olson *et al.* (2002), Pérez-Osorio *et al.* (2010), Ryder *et al.* (2007)
Riemerella anatipestifer	Hu *et al.* (2010)
Salmonella	Jain and Chen (2007), Kim and Wei (2009), Marin *et al.* (2009), Olson *et al.* (2002), Römling (2005), Van Parys *et al.* (2010), Wong *et al.* (2010)
Staphylococcus	Boles *et al.* (2010), Dhanawade *et al.* (2010), Fox *et al.* (2005), Futagawa-Saito *et al.* (2006), Melchior *et al.* (2006a, 2006b, 2009), Nemati *et al.* (2009), Oliveira *et al.* (2006, 2007), Olson *et al.* (2002), Pérez *et al.* (2009), Tormo *et al.* (2005), Vancraeynest *et al.* (2004)
Streptococcus	Bonifait *et al.* (2008), Grenier *et al.* (2009), Konto-Ghiorghi *et al.* (2009), Moscoso *et al.* (2009), Olson *et al.* (2002), Rinaudo *et al.* (2010), Tanabe *et al.* (2010), Wei *et al.* (2009)
Yersinia	Coquet *et al.* (2002), Darby (2008), Hinnebusch and Erickson (2008), Kim *et al.* (2008), Sun *et al.* (2009), Truchado *et al.* (2009), Wortham *et al.* (2010)

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cetylpyridinium chloride (CPC) through *A. pleuropneumoniae* biofilms (Ganeshnarayan *et al.*, 2009). CPC binds reversibly to the biofilm matrix suggesting that PGA sequesters CPC. Therefore, PGA prevents contact between CPC and the bacteria located within the biofilm. Additionally, treatment of a biofilm from field isolates (serotype 5) with dispersin B increased the sensitivity of *A. pleuropneumoniae* to ampicillin. This indicates that *A. pleuropneumoniae* cultured as a biofilm exhibits a higher resistance to antibiotics than by its planktonic counterpart (Izano *et al.*, 2007). Our group observed that zinc could completely inhibit biofilm formation by *A. pleuropneumoniae* and *A. actinomycetemcomitans* (Labrie *et al.*, 2010). It is well established that PGA functions as a matrix polysaccharide in phylogenetically diverse bacteria. Therefore, it would be worth investigating if zinc interferes with PGA biosynthesis in other bacteria.

In addition to the *pga* operon, σ^E and H-NS, other genes have been associated with biofilm formation in *A. pleuropneumoniae*. Enhanced biofilm formation was observed in a QS (*luxS*) mutant (Li *et al.*, 2008), whereas deficient biofilm formation was observed for a mutant (*arcA*) in the ArcAB two-component system facilitating metabolic adaptation to anaerobicity (Buettner *et al.*, 2008) and an autotransporter serine protease (*aasP*) mutant (Tegetmeyer *et al.*, 2009).

Aeromonas hydrophila

Aeromonads are ubiquitous water-borne bacteria that are significant pathogens of amphibians, fish and reptiles. Aeromonad infections are also associated with gastroenteritis cases in humans (Gavìn *et al.*, 2002). *Aeromonas hydrophila* readily attaches to surfaces to produce a thin biofilm with a complex 3D structure. Lynch *et al.* (2002) were the first to demonstrate a role for N-acylhomoserine lactone (AHL)- or autoinducer-1 (AI-1)-dependent QS in the development of biofilm by *A. hydrophila*. Kozlova *et al.* (2008) have shown that mutation in the *luxS* gene, involved in autoinducer-2 (AI-2) QS, also affects biofilm formation by *A. hydrophila*. Furthermore, lateral flagella are essential for adherence and biofilm formation on epithelial cells by *A. hydrophila* (Gavìn *et al.*, 2002).

Arcanobacterium pyogenes

Arcanobacterium (*Actinomyces*) *pyogenes* is a commensal bacterium and also an opportunistic pathogen of economically important livestock such as dairy and beef cattle, and swine. *A. pyogenes* causes a wide array of diseases including mastitis, liver abscesses and pneumonia (Jost and Billington, 2005). *A. pyogenes* can readily form biofilms (Olson *et al.*, 2002); this appears to be controlled by the two-component regulatory system PloSR (Jost and Billington, 2005). This two-component regulatory system is considered a global regulator of *A. pyogenes* virulence. The implication of the PloSR system in biofilm formation suggests that biofilm formation may also be a virulence factor in *A. pyogenes*. Furthermore, when *A. pyogenes* was grown as a biofilm it was highly resistant to antibiotics (Olson *et al.*, 2002).

Bacillus cereus group

The *Bacillus cereus* group includes three genetically related species: *B. cereus* sensu stricto, *Bacillus anthracis* and *Bacillus thuringensis* (Auger *et al.*, 2009b). *B. cereus* biofilm formation is highly dependent on the strain, the assay and environmental conditions (Wijman *et al.*, 2007). Furthermore, the ability of *B. cereus* to form biofilms in polyvinylchloride (PVC) microtiter plates at 30°C is strongly dependent on the origin of the strain; strains isolated from soil or from digestive tract infections were efficient biofilm-formers, whereas strains isolated from other locations were poor biofilm-formers (Auger *et al.*, 2009b). This organism appears to form biofilms preferentially at the air–liquid interface (Wijman *et al.*, 2007) and motility promotes biofilm formation in this condition (Houry *et al.*, 2010). The addition of exogenous AI-2 inhibits biofilm formation by *B. cereus*; furthermore, the addition of exogenous AI-2 also promotes the dispersion of cells from preformed biofilms (Auger *et al.*, 2006). *B. cereus* biofilms can be a site for spore formation and release. Biofilm formation represents a problem for the food industry, because *B. cereus* biofilms are more resistant to cleaning procedures than their planktonic counterparts (Wijman *et al.*, 2007). For example, hot-alkali resistant spores from *B. cereus* isolated from dairy silo tanks were able to germinate and form biofilms in whole milk (Shaheen *et al.*, 2010).

B. anthracis is an endospore-forming bacterium and is the etiological agent of pulmonary, gastrointestinal and cutaneous anthrax. Anthrax infections are part of the natural lifecycle of many ruminants in North America, including cattle and bison, and in other parts of the world (Lee *et al.*, 2007). *B. anthracis* readily forms biofilms under static and shear conditions and these biofilms are inherently resistant to commonly used antibiotics (Lee *et al.*, 2007). In *B. anthracis*, sporulation is regulated during biofilm growth which is likely the result of nutrient limitation and other stresses. Inactivation of the genes encoding enzymes involved in the biosynthesis of polyphosphate in *B. cereus* resulted in motility and biofilm-formation defects (Shi *et al.*, 2004). Homologous enzymes have been identified in *B. anthracis* and they may represent attractive targets for the treatment of anthrax. Recently, Schuch and Fischetti (2009) demonstrated that lysogeny can block or promote sporulation. The effects of lysogeny are dependent on the type of bacteriophage present. Lysogeny can also induce EPS expression and

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biofilm formation in *B. anthracis*. The complete role of biofilm in the ecology of this pathogen is yet to be understood.

Bartonella henselae

Bartonella henselae is a fastidious bacterial pathogen of cats and humans. Pilin expression is associated with auto-agglutination in liquid cultures. Pilins are also required for the agar-pitting phenotype and for biofilm formation; however, components other than pilins are also required for the mentioned phenotype (Kyme *et al.*, 2003).

Bordetella bronchiseptica and Bordetella parapertussis

Bordetellae colonize the respiratory tracts of humans and animals and cause a wide array of respiratory diseases. These bacteria are capable of living as sessile communities on a number of abiotic surfaces (Parise *et al.*, 2007). *B. bronchiseptica* is associated with a variety of respiratory diseases in animals, whereas *B. parapertussis* is associated with non-progressive pneumonia in sheep. Various *Bordetella* species, including *B. bronchiseptica* and *B. parapertussis*, produce PGA (Parise *et al.*, 2007). Biofilm formation under static hydrodynamic conditions does not require PGA in the initial stages, but PGA contributes to the stability and the maintenance of the complex architecture of *Bordetella* biofilms. In addition to PGA, a xylose polymer was also detected in the biofilm matrix of *B. bronchiseptica* (Irie *et al.*, 2006). The BvgAS signal transduction system regulates biofilm development in *Bordetella* (Irie *et al.*, 2004; Mishra *et al.*, 2005). Biofilm also appears to develop during *Bordetella* infection. For example, when nasal tissues of mice infected with *B. bronchiseptica* were examined by immunofluorescence and scanning electron microscopy (Sloan *et al.*, 2007), *B. bronchiseptica* was able to form robust biofilms attached to nasal epithelium. The biofilm formed *in vivo* displayed the same architectural characteristics observed in biofilms formed *in vitro* on inert surfaces.

Bordetella biofilms are highly tolerant to a number of antimicrobial agents, which include antibiotics recommended for the treatment of veterinary and human infections caused by these bacteria (Mishra *et al.*, 2005). Interestingly, it was shown that rhamnolipids, a biosurfactant secreted by *P. aeruginosa*, dispersed *B. bronchiseptica* biofilms (Irie *et al.*, 2005).

Brucella melitensis

Brucella spp. are intracellular pathogens and the etiological agents of brucellosis, a worldwide zoonosis affecting a broad range of mammals (Uzureau *et al.*, 2007). Mutations of the QS-dependent regulator VjbR lead to drastic surface modifications in *B. melitensis* including overproduction of a matrix-forming EPS (Uzureau *et al.*, 2007). This study was the first to suggest that *B. melitensis* can form a biofilm.

Burkholderia pseudomallei

B. pseudomallei is the etiological agent of melioidosis. The ability of *B. pseudomallei* to produce biofilm varies in each isolate and the source of the isolate is not indicative of its ability to form biofilm (Taweechaisupapong *et al.*, 2005). Inactivation of the *rpoE* operon found in *B. pseudomallei* resulted in a reduced ability to form biofilms (Korbsrisate *et al.*, 2005). Biofilm formation on PVC is independent of *pilA*, which encodes the type IVA pilin (Boddey *et al.*, 2006). Mutations in the flagellin genes resulted in markedly reduced biofilm formation, whereas mutations in genes encoding the capsule and O-side chains of lipopolysaccharide (LPS) had no effect on biofilm formation (Sawasdidoln *et al.*, 2010). A polyphosphate kinase mutant, which is deficient in swimming and swarming motility, forms a biofilm that is less dense than that of the wild-type strain (Tunpiboonsak *et al.*, 2010). Finally, a c-di-GMP phosphodiesterase (*cdpA*) mutant had higher intracellular levels of c-di-GMP, produced more EPS, autoaggregated more, lacked flagella and swimming motility and had enhanced biofilm formation (Lee *et al.*, 2010).

When isolates of *B. pseudomallei* were cultured as biofilms, their resistance to all antimicrobial agents increased despite uneven amounts of biofilm production among isolates (Sawasdidoln *et al.*, 2010). In contrast, virtually all isolates cultured as planktonic cells were susceptible to the antimicrobial agents studied, which included doxycycline, ceftazidime, imipenem and trimethoprim/sulfamethoxazole.

Campylobacter coli and Campylobacter jejuni

Several members of the *Campylobacter* genus are pathogenic and are responsible for causing a range of diseases in humans and domesticated animals. Thermophilic campylobacters, especially *C. jejuni* and *C. coli*, are the major etiological agents of foodborne gastrointestinal infections in the developed world (Murphy *et al.*, 2006; Peyrat *et al.*, 2008; Gunther and Chen, 2009; Sulaeman *et al.*, 2010). Post-infection complications include reactive arthritis and Guillain–Barré syndrome, an immune-mediated disorder affecting the peripheral nervous system (Sulaeman *et al.*, 2010).

The most important source of *Campylobacter* is poultry, pig and bovine meat. *Campylobacter* has a high occurrence throughout the meat production and processing chain (Sulaeman *et al.*, 2010). A recent study by Peyrat

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et al. (2008) indicates that *C. jejuni* is able to survive on the surface of meat processing equipment despite robust cleaning and disinfection. The surviving *C. jejuni* may subsequently contaminate carcasses during the slaughter process.

In general, *Campylobacter* are fastidious and very sensitive to atmospheric oxygen levels (Gunther and Chen, 2009). They encounter many stresses in the host intestinal tract, on processed meat and in the environment. However, they have developed survival mechanisms to overcome these stresses (reviewed by Murphy *et al*., 2006). Biofilm formation has been suggested as a possible means of persistence for *Campylobacter* (Murphy *et al*., 2006; Gunther and Chen, 2009). For example, *C. jejuni* starts to develop into a biofilm in response to aerobic stress or other stressful conditions. The formation of biofilms allows for survival during detrimental conditions, and the biofilm can act as a reservoir of planktonic cells (Reuter *et al*., 2010). The ability to form biofilm on different surfaces (glass, stainless steel and polystyrene plastic) by representative strains of 14 *Campylobacter* species was investigated (Gunther and Chen, 2009). The results obtained suggest that the anaerobic *Campylobacter* species are able to form biofilm more readily than the microaerophilic species; however, further investigation is required to determine if those trends apply to a wider range of isolates.

Campylobacter can form monospecies biofilms as wells as join pre-established multispecies biofilms (Sulaeman *et al*., 2010). For example, it was shown that biofilms isolated from chicken houses enhance the survival of *C. jejuni* (Trachoo *et al*., 2002). The number of viable *C. jejuni* determined by direct viable count was greater than by standard enumeration method. This suggests that *C. jejuni* can adopt a viable but non-culturable state within biofilms. Recently, it was determined that *C. jejuni* attachment is facilitated by pre-established biofilms found in poultry environments (Hanning *et al*., 2008). Additionally the survival of culturable *C. jejuni* was extended in certain pre-established biofilms (Hanning *et al*., 2008). Further investigation showed no evidence of interspecies signaling indicating that the attachment of *C. jejuni* to pre-established biofilm was mediated by bacterial surface components. The above-mentioned examples clearly indicate that the control of any type of biofilms is critical because pre-established biofilm communities can trap *C. jejuni* and may therefore be important in the transmission and prevalence of *C. jejuni*. The presence of biofilm microbiota also decreased the effectiveness of many sanitizers used to control *C. jejuni*; however, chlorine completely inactivated *C. jejuni* enclosed in a biofilm (Trachoo and Frank, 2002).

C. jejuni has the ability to form biofilms in the water supplies and plumbing systems of animal husbandry facilities and animal-processing plants. These biofilms may provide a continual inoculum for domesticated animals (Reeser *et al*., 2007). *C. jejuni* can form biofilms on a variety of abiotic surfaces commonly used in water systems, such as acrylonitrile butadiene styrene and PVC plastics. Biofilm formation by *C. jejuni* is inhibited in the presence of nutrient-rich media or high osmolarity, and is enhanced under thermophilic and microaerophilic conditions. Therefore, nutritional and environmental conditions affect biofilm formation in *C. jejuni*. Recently, it was determined that biofilm formation in *C. jejuni* increased under aerobic conditions when compared to microaerobic conditions, but the final biofilm levels were comparable after 3 days (Reuter *et al*., 2010). The aerobic conditions utilized were similar to those found during meat production and processing.

Adhesion to an inert surface (the first step of biofilm formation) by *C. jejuni* and *C. coli* strains was compared using the BioFilm Ring Test® (Sulaeman *et al*., 2010). The adherence ability of the strains tested ranged from no adhesion to strong adhesion; no strains of *C. coli* were strongly adherent to the surface. Interestingly, strains isolated from animals or carcasses were less adherent than those isolated from food-processing equipment and clinical cases suggesting that certain environments such as food-processing equipment and the human body can select for strains with greater adhesion. A recent study postulated that agar-grown *C. jejuni* do not colonize young chickens as well as planktonic cells, suggesting that 'biofilm cells' may have poor colonizing abilities (Hanning *et al*., 2009).

Both flagella (*flaAB*) and QS (*luxS*) appear to be required for optimum biofilm formation in *C. jejuni* (Reeser *et al*., 2007). Furthermore, a flagellated but non-motile (*motA*) mutant did not form biofilm to the level exhibited by the wild-type strain. This suggests that motility as well as flagella is required for biofilm formation (Moe *et al*., 2010). At an early stage, flagella are likely to play an important role in biofilm formation, because motility is necessary for cells to gather and form microcolonies. Thereafter, the flagella appear to act as bridges for the formation of net-like connections between the organisms. Although the flagella appear to be important for biofilm formation, aflagellated strains were able to form more biofilm under aerobic conditions. This suggests that biofilm formation can occur in a flagella-dependent and flagella-independent manner in *C. jejuni* (Reuter *et al*., 2010). Interestingly, mutants (*waaF* and *lgtF*) lacking the lipooligosaccharide (LOS) outer core exhibited enhanced biofilm formation, implicating the cell envelope in biofilm formation by *C. jejuni* and highlighting the dynamic nature of the cell envelope (Naito *et al*., 2010). A number of regulatory proteins have been shown to have a role in biofilm formation by *C. jejuni*. Deletion of a gene encoding a histidine kinase sensor (*cprS*) enhanced biofilm formation (Svensson *et al*., 2009), whereas the absence of the global regulator CsrA reduced biofilm formation (Fields and Thompson, 2008). Finally, a *spoT* mutant, defective for the stringent response, reacted more to calcofluor white and formed more biofilm than the

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wild-type strain (McLennan *et al.*, 2008). Calcofluor binds to β1-3 and β1-4 carbohydrate linkages.

Clostridium perfringens

Clostridium perfringens is an anaerobic pathogen that causes a variety of infections in animals and humans, including gas gangrene and intestinal diseases. Varga *et al.* (2008) were the first to study biofilm formation by *C. perfringens*. Biofilms were formed under static conditions with an anaerobic atmosphere for a period of up to 5 days. Under these conditions *C. perfringens* formed a flat biofilm that was 30–40 μm thick. Type IV pilus-dependent gliding motility and the catabolite control protein (CcpA), a key regulator of the response to carbohydrate limitation, were needed for maximal biofilm formation. Finally, biofilm cells had a 5- to 15-fold-increase in resistance to penicilin G when compared to planktonic cells.

Corynebacterium pseudotuberculosis and Corynebacterium renale

To form biofilms, a lymphadenitis isolate of *Corynebacterium pseudotuberculosis* and a pyelonephritis isolate of *Corynebacterium renale* required the addition of fetal bovine serum and incubation under 10% CO_2 for 24 h (Olson *et al.*, 2002). When cultured as biofilms, isolates of *C. renale* and *C. pseudotuberculosis* were highly resistant to the antimicrobial agents tested, but were sensitive to the same antimicrobial agents when cultured as planktonic cells. Infections caused by these bacteria require prolonged antimicrobial therapy and are often unresponsive to treatment (Olson *et al.*, 2002). This suggests that *C. renale* and *C. pseudotuberculosis* form biofilms during infection.

Enterococcus

Enterococci are recognized as opportunistic pathogens. They are natural inhabitants of the oral cavity, the intestinal tract and the female genital tract of humans and animals (Mohamed and Huang, 2007). Enterococci are common human nosocomial agents and vancomycin-resistant strains are of particular interest. The two most common enterococci species are *Enterococcus faecalis* and *Enterococcus faecium*, and both are capable of forming biofilms (Mohamed and Huang, 2007). Many environmental and genetic factors are or have been proposed to be associated with the production of biofilm (reviewed by Mohamed and Huang, 2007). A gene cluster involved in polysaccharide biosynthesis (*epa*, enterococcal polysaccharide antigen) was shown to be uniformly present in *E. faecalis* strains (Teng *et al.*, 2009). Disruption of the genes in this cluster resulted in mutants with deficient biofilm formation. The *E. faecalis* biofilm matrix contains DNA and it was shown by Guiton *et al.* (2009) that sortase A (SrtA) and autolysin (Atn) have a role in the release of DNA during biofilm development. Recently, a comprehensive analysis of genetic determinants of biofilm formation in the core genome of *E. faecalis* was carried out (Ballering *et al.*, 2009). Sixty-eight genetic loci predicted to be involved in biofilm formation were identified by RIVET (recombinase *in vivo* expression technology). Most of these genes had not been studied previously and many are highly conserved in Gram-positive pathogens and may thus constitute a pool of uncharacterized genes that may be targeted for drug discovery.

Biofilm formation was observed in 50–60% of commensal enterococci isolated from the intestine of broilers (Oliveira *et al.*, 2010) and of *E. faecalis* strains isolated from chicken arthritis (Ciftci *et al.*, 2009). Fsr-regulated gelatinase-positive *E. faecalis* that formed biofilm were relatively common in the agricultural environment such as swine and cattle feces. The agricultural environment may therefore represent a source/reservoir of clinically relevant strains (Macovei *et al.*, 2009). As observed with other bacteria, enterococci cultured as biofilms are more resistant to antibiotics than their planktonic counterparts (Mohamed and Huang, 2007). Biofilm production in enterococci isolated from broilers also correlated with an increase in antibiotic resistance (Oliveira *et al.*, 2010). Additionally, slime-producing *E. faecalis* strains isolated from chickens suffering from arthritis were found to be more resistant to antibiotics (Ciftci *et al.*, 2009).

Erysipelothrix rhusiopathiae

Erysipelothrix rhusiopathiae causes erysipelas in animals and erysipeloid in humans. Two surface proteins (RspA and RspB) of *E. rhusiopathiae* bind to fibronectin, and type I and type IV collagens. These two surface proteins also participate in the initiation of biofilm formation by binding to abiotic and biotic surfaces (Shimoji *et al.*, 2003).

Escherichia coli

With thousands of serotypes, *E. coli* is a very diverse species that is a part of the normal intestinal microbiota, and can be an important intestinal pathogen or invade the blood stream and cause meningitis (Beloin *et al.*, 2008). Biofilm formation by several *E. coli* strains has been studied extensively. Furthermore, *E. coli* strains will compete among themselves to establish colonies within a host or a niche. For example, it was established that the probiotic *E. coli* strain Nissle 1917 (serotype O6 : K5 : H1) was a good biofilm former and can out-compete pathogenic strains of *E. coli* (Hancock *et al.*, 2010). The

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biofilm matrix of *E. coli* is composed of several EPS, but LPS and capsular polysaccharides do not usually accumulate in the matrix; however, LPS and capsule play an important role during biofilm formation. Cellulose (Zogaj *et al.*, 2001), PGA (Agladze *et al.*, 2005) and colanic acid (Prigent-Combaret *et al.* 2000) have been detected in the biofilm matrix of *E. coli*. All three EPS are important for biofilm formation (Beloin *et al.*, 2008). Several key factors (e.g. various fimbriae) are implicated in *E. coli* surface colonization. To ensure proper biofilm formation, the expression and activity of these factors are finely regulated at specific times and at various locations in the biofilms (Beloin *et al.*, 2008; Wood, 2009). Microarray analysis has elucidated several aspects of biofilm formation in *E. coli*. The analysis resulted in the discovery of the role of stresses, intra- and inter-species cell signaling (e.g. AHLs and AI-2), toxin/antitoxin genes (e.g. Hha/TomB and MqsR/B3021) and small RNAs (e.g. CsrB) on biofilm formation and dispersal (reviewed by Wood, 2009). One common trend among different biofilm transcriptome studies was that stress genes were induced during biofilm formation.

Olson *et al.* (2002) found that *E. coli* strains isolated from cases of bovine or turkey enteritis were able to form biofilms. Olson *et al.* (2002) also noted that enrofloxacin and gentamicin were effective antibiotics against the biofilms of these enteritis *E. coli* isolates. The data collected by Olson *et al.* (2002) suggest that established *E. coli* biofilms may be difficult to treat with some antibiotics and this is supported by observations made in clinical cases involving pig, cattle and poultry.

Some strains of enterohemorrhagic *E. coli* O157 : H7, a worldwide foodborne pathogen, are able to form biofilms. Recently, a genome-wide transposon mutagenesis of *E. coli* O157 : H7 strain EDL933 revealed that virulence plasmid pO157 plays an essential role during biofilm formation. Specifically *espP,* an autotransporter serine protease, and *ehxD,* the enterohemolysin translocator, were identified as mediators of biofilm formation (Puttamreddy *et al.*, 2010). Strain EDL933 was cured of plasmid pO157 and the resulting strain failed to establish a biofilm. This confirmed the essential role of pO157 during biofilm formation. Other studies indicate that the biofilm negative strains of *E. coli* O157 : H7 can associate with pre-established biofilms generated by commensal *E. coli* strains (Uhlich *et al.*, 2010). Furthermore, *E. coli* O157 : H7 strain 0475s enclosed in a multi-species biofilm survived a H_2O_2 challenge better than its planktonic counterpart.

Francisella tularensis

Francisella tularensis is associated with water and waterways and infects many species of animals, insects and protists (Durham-Colleran *et al.*, 2010). Tularemia is commonly spread by arthropod vectors such as mosquitoes, biting flies and ticks. The mechanisms utilized by *Francisella* to survive in its natural environment are yet to be elucidated; however, biofilm formation plays a critical role in the survival of another arthropod-borne bacterium, *Y. pestis* (see below). Durham-Colleran *et al.* (2010) recently demonstrated for the first time that *F. tularensis* subsp. *novicida* (or *F. novicida*) forms a biofilm *in vitro*. Biofilm formation is regulated by the orphan response regulator, QseB. *F. novicida* is less virulent than *F. tularensis*, and is widely used as a model organism for the *F. tularensis* species. The data collected by Durham-Colleran *et al.* (2010) suggest that biofilm formation may be important for the lifecycle of *F. tularensis*. It was recently shown that biofilm formation by *F. tularensis* promotes persistence on chitin surfaces which can act as a carbon source (Margolis *et al.*, 2010).

Haemophilus parasuis

Haemophilus parasuis is the etiological agent of Glässer's disease in pigs. The ability to form biofilms by field isolates and reference strains was tested in glass tubes and polystyrene microtiter plates (Jin *et al.*, 2006). In general, non-virulent serovars formed more biofilms than virulent serovars. In pigs experimentally infected with biofilm-positive strains, bacteria recovered from the nasal cavities remained biofilm positive, whereas bacteria recovered from the lung and brain became biofilm negative. These observations indicate that most serovars of *H. parasuis* can form biofilms *in vitro* and the ability to form biofilms is associated with the recovery site of the strains. The ability to form biofilms is maintained during *in vitro* passages and after infection in the upper respiratory tract. Recently, selective capture of transcribed sequences (SCOTS) was used to identify *H. parasuis* genes upregulated in necrotic lungs 7 days after the challenge (Jin *et al.*, 2008). The data collected by Jin *et al.* (2008) support the role of biofilm formation by *H. parasuis* during colonization and/or persistence. Our group has recently made a similar observation. In an *in vitro* assay, non-virulent strains isolated from the nasal cavities of pigs tend to form more biofilms than did virulent strains isolated from systemic lesions (V. Deslandes, M. Jacques and V. Aragon, unpublished results).

Histophilus somni

Histophilus somni (*Haemophilus somnus*) is an obligate inhabitant of mucosal surfaces in cattle and sheep. *H. somni* is an opportunistic pathogen associated with a variety of conditions such as respiratory disease, meningoencephalitis, myocarditis, arthritis and other systemic infections (Sandal *et al.*, 2007; Corbeil, 2008). *H. somni* is also one of the primary agents responsible for bovine respiratory disease complex (Corbeil, 2008). All *H. somni*

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strains examined were able to form biofilms on PVC wells. The majority of isolates from systemic sites produced more biofilm than commensal strains isolated from prepuces (Sandal *et al.*, 2007). *H. somni* also formed biofilms in flow cells and the biofilm development went through the stages of attachment, growth, maturation and detachment. Furthermore, *H. somni* formed biofilms in cardiopulmonary tissue following experimental respiratory infection in the bovine host (Sandal *et al.*, 2009). Biofilms were evident and most prominent in the myocardium, and were associated with a large amount of amorphous extracellular material. After transposon mutagenesis of *H. somni* strain 2336, mutants that formed enhanced biofilm or did not form biofilm were selected and identified (Sandal *et al.*, 2009). Several mutants deficient in biofilm formation had an insertion in the gene encoding for a homologue of filamentous hemagglutinin (FHA), which is predicted to be involved in attachment. The formation of biofilm may therefore be crucial to *H. somni* persistence *in vivo* and the formation of a robust biofilm may provide a selective advantage for pathogenic strains that cause systemic disease. Surprisingly, both planktonic and biofilm cells of *H. somni* (a bovine pneumonia isolate) were equally sensitive to antimicrobial agents (Olson *et al.*, 2002).

Leptospira spp.

Leptospira spp. belong to the bacterial phylum *Spirochaetes*. Leptospires exist as aquatic saprophytic organisms or as pathogens. Leptospirosis is a widespread zoonosis and humans are usually infected through cut or abraded skin that comes into contact with water contaminated with the urine of mammals. Both saprophytic (e.g. *Leptospira biflexa*) and pathogenic (e.g. *Leptospira interrogans*, *Leptospira borgpetersenii* and *Leptospira kirschneri*) species formed biofilms on glass and polystyrene surfaces (Ristow *et al.*, 2008). Electron microscopy images showed cells embedded in an extracellular matrix. The formation of such a biofilm is consistent with the aquatic life style of saprophytic strains and may help pathogenic strains to survive in their environmental habitats and to colonize their host.

Listeria monocytogenes

The food-borne pathogen *Listeria monocytogenes* is the causative agent of listeriosis, a severe disease with high hospitalization and fatality rates in humans (Gandhi and Chikindas, 2007). *L. monocytogenes* is also an important pathogen of several animal species, including ruminants. *L. monocytogenes* can survive and grow in a wide range of environmental conditions such as refrigeration temperatures, low pH and high salt concentration. It can be isolated from a variety of raw and processed foods (e.g. milk and dairy products, various meats and meat products, seafood and fish products). *L. monocytogenes* is frequently isolated in food-processing plants, especially those involved in the meat and dairy industries. Biofilms on milking equipment on a dairy farm has been implicated as a potential source of bulk tank milk contamination with *L. monocytogenes* (Latorre *et al.*, 2010). Biofilms of *L. monocytogenes* are of particular concern, because biofilm cells are more resistant to disinfectants and sanitizing agents than are planktonic cells. Many disinfectants, including quaternary ammonium compounds and hypochloride, do not effectively kill *L. monocytogenes* in the presence of soil or organic matter, or at low temperatures (Amalaradjou *et al.*, 2009). However, octenidine hydrochloride, a positively charged bispyridinamine, has been shown to effectively kill planktonic cells and biofilm cells of *L. monocytogenes* at various temperatures (from 4 to 37°C) and in the presence of organic matter (Amalaradjou *et al.*, 2009).

Several studies have shown that strains of *L. monocytogenes* differ in their ability to adhere to surfaces and form biofilms (reviewed by Gandhi and Chikindas, 2007). Cellular hydrophobicity, however, appears to be an important factor during the initial adherence to PVC (Takahashi *et al.*, 2010). *L. monocytogenes* can form monospecies or multispecies biofilms. To study multispecies biofilms, different types of *Lactococcus lactis* biofilms that exhibited different architectures, porosities, types of matrices and individual cell surface properties were used to investigate factors governing the initial attachment of *L. monocytogenes* to biofilms (Habimana *et al.*, 2009). It was observed that the adhesion of planktonic *L. monocytogenes* to *L. lactis* biofilm was lower than to an abiotic surface. Adhesion of *L. monocytogenes* was almost prevented by the presence of the EPS produced by *L. lactis*. The presence of biofilms that can prevent the contamination of surfaces by *L. monocytogenes* can perhaps constitute a novel approach for controlling *L. monocytogenes*. However, a porous *L. lactis* biofilm can also enhance *L. monocytogenes* attachment.

Biofilm development in *L. monocytogenes* involves flagellar motility; when motility is blocked, the initial attachment is decreased but it later results in the formation of a high-density biofilm (Todhanakasem and Young, 2008). The *L. monocytogenes* Agr peptide-sensing system, which encodes for a putative QS peptide (Riedel *et al.*, 2009) has also been studied for its role in biofilm formation by creating a deletion mutant in *agrD*. The *agrD* mutant had reduced ability to form biofilm and to invade Caco-2 intestinal epithelial cells. The virulence of the *argD* mutant was also attenuated in a mouse model. It has been recently demonstrated that extracellular DNA may be the central component of the matrix of the *L. monocytogenes* biofilm because DNase I treatment dispersed the biofilm and exogenous DNA could not restore biofilm formation (Harmsen *et al.*, 2010a).

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Mannheimia haemolytica

A bovine pneumonia isolate of *Mannheimia haemolytica* formed a biofilm when cultured in the presence of fetal bovine serum and incubated under 10% CO_2 for 24 h (Olson *et al*., 2002). Surprisingly, planktonic and biofilm *M. haemolytica* had similar levels of sensitivity to antimicrobial agents with the exception of trimethroprim/sulfadoxine.

Mycobacterium

Mycobacterium avium subsp. *avium* causes tuberculosis in domestic and wild birds, while *M. avium* subsp. *hominissuis* is an opportunistic pathogen of humans and swine, and occasionally of other mammals (Johansen *et al*., 2009). An optimized method to screen human, swine and avian isolates of *M. avium* was established recently (Johansen *et al*., 2009). After an incubation of 2–3 weeks, nine swine isolates produced biofilm, but none of the human or avian isolates formed biofilms. However, some human *M. avium* strains were previously shown to form biofilm and the use of a green-fluorescent protein promoter library and transposon mutagenesis allowed the identification of genes associated with biofilm formation (Yamazaki *et al*., 2006b). Most of the genes identified by Yamazaki *et al*. (2006b) were involved in glycopeptidolipids (GPL) biosynthesis. Interestingly, biofilm-negative mutants were also impaired in the invasion of the bronchial epithelial cell line BEAS-2B (Yamazaki *et al*., 2006a).

M. avium subsp. *paratuberculosis* (Map) is the causative agent of Johne's disease, a chronic enteric infection affecting ruminants (Cook *et al*., 2010). Map is transmitted through the fecal–oral route including indirect transmission via ingestion of contaminated materials in the farm environment. The ability of Map to persist in a multispecies biofilm on materials commonly used to construct livestock watering troughs (concrete, plastic, galvanized or stainless steel) was evaluated (Cook *et al*., 2010). It was found that Map encased in mixed biofilms survived for an extended period of time on livestock watering trough materials. To avoid the exposure of susceptible farm animals to Map, management practices for Johne's disease should be aimed at maintaining trough surfaces free of biofilm. It is known that biofilm formation is linked to GPLs, a major component of the mycobacterial cell wall (Wu *et al*., 2009). Recently, a new cell wall lipopeptide was shown to be important for biofilm formation and the pathogenicity of Map (Wu *et al*., 2009).

Biofilm formation has also been observed in *Mycobacterium bovis* strain BCG and *Mycobacterium tuberculosis* (Ojha *et al*., 2008). In *M. tuberculosis*, the extracellular matrix is rich in free mycolic acids and harbors an important drug-tolerant sub-population. This sub-population persisted despite exposure to high levels of isoniazid or rifampicin. Finally, it was also noted that subinhibitory concentrations of clarithromycin, but not moxifloxacin, inhibits biofilm formation by human isolates of *M. avium*; however, clarithromycin has no activity against pre-established biofilms (Carter *et al*., 2004).

Mycoplasma

Despite their small genome size, mycoplasmas cause a wide range of disease in both humans and animals (McAuliffe *et al*., 2006). For example, *Mycoplasma* spp., typically *Mycoplasma bovis*, are important bovine pathogens that cause mastitis, metritis, pneumonia and arthritis (Justice-Allen *et al*., 2010). McAuliffe *et al*. (2006) examined a variety of mycoplasma species and found considerable variation in their ability to form biofilms in an air–liquid interface model. For example, *Mycoplasma agalactiae* and *M. bovis* produced thick and dense biofilms. On the other hand, the highly virulent causative agent of contagious bovine pleuropneumonia, *Mycoplasma mycoides* subsp. *mycoides* SC, was unable to form biofilm. Compared to their planktonic counterpart, biofilm cells were more resistant to stresses, including heat and desiccation. Furthermore, a correlation between the ability to form biofilms and specific molecular genotypes was established for *M. bovis* (McAuliffe *et al*., 2006). However, when a simple model (growth on a membrane placed on an agar plate) lacking an air–liquid interface is used, *M. mycoides* subsp. *mycoides* SC can form biofilms (McAuliffe *et al*., 2008). As with the air–liquid interface model, the biofilms formed on a membrane were more resistant to many stresses, including heat, osmotic shock and oxidative stress. Additionally, mycoplasmas cultured on a membrane showed remarkable persistence and were viable for up to 20 weeks.

The murine respiratory pathogen *Mycoplasma pulmonis* can form biofilms on the epithelium of explanted trachea (Simmons and Dybvig, 2009). *M. pulmonis* was also able to form biofilms in experimentally infected mice and these biofilms exhibited similar structure and biological characteristics when compared to their *in vitro* counterparts. Transposon mutants of *M. pulmonis* that failed to produce the glucose–galactose containing EPS-I had enhanced biofilm-formation ability on glass surfaces. The increased biofilm-formation ability likely resulted from the overproduction of a second EPS (EPS-II) containing N-acetylglucosamine (Daubenspeck *et al*., 2009). When encased within a biofilm, cells of *M. pulmonis* producing a short form of the Vsa (variable surface antigen) were more resistant to complement killing and gramicidin than were dispersed mycoplamas (Simmons and Dybvig, 2007). The resistance appeared to be localized within cells found within the tower structures of biofilms.

The association between the occurrence of *Mycoplasma* spp. in recycled bedding sand and mycoplasma

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mastitis in cows has also been investigated (Justice-Allen *et al.*, 2010). It was found that concentrations of *Mycoplasma* spp. in a sand pile positively correlated with the growth of Gram-negative micro-organisms. This suggests that biofilm formation by the bacterial community could account for the survival and replication of mycoplasmas.

Pasteurella multocida

Pasteurella multocida can infect several wild and domesticated animals. Diseases caused by *P. multocida* include fowl cholera in birds, atrophic rhinitis in pigs, hemorrhagic septicemia in ungulates, enzootic pneumonia in cattle, sheep and goats and snuffles in rabbits. *P. multocida* has also been recognized as a contributor to debilitating and fatal porcine pneumonia. Recent data suggest that *P. multocida* can form biofilms in the porcine lung (Ross, 2007). Olson *et al.* (2002) used the Calgary Biofilm Device to study biofilm formation by bovine pneumonia isolates and chicken cholera isolates of *P. multocida* in the presence of fetal bovine serum and 10% CO_2. The MIC and the MBEC values of planktonic cultures and biofilm cultures were similar for the antibiotics tested with the exception of trimethroprim/sulfadoxine. These observations correlate with clinical cases because animals with pasteurellosis respond well to most antimicrobial agents if no secondary infection is present (Olson *et al.*, 2002).

Pseudomonas aeruginosa

P. aeruginosa is found in various environments, especially in soil. *P. aeruginosa* is an opportunistic pathogen responsible for chronic lung infections in cystic fibrosis patients and infections in immunocompromised individuals (Bazire *et al.*, 2010; Ma *et al.*, 2009). The biofilm lifestyle of *P. aeruginosa* is well characterized and is often used as a model organism for biofilm formation (for a recent review see Harmsen *et al.*, 2010b). *P. aeruginosa* will form biofilm both in the environment and in its host during an infection. At least three EPS are synthesized during biofilm formation: Psl (polysaccharide synthesis locus), Pel (pellicle formation) and alginate (reviewed by Ryder *et al.*, 2007). Recent data indicate that Psl is a key component required for proper scaffolding of the matrix (Ma *et al.*, 2009). Alginate is a main component of the biofilm matrix of mucoid strains. The extracellular function sigma factor AlgU (also known as AlgT, RpoE, σ^E and σ^{22}) is responsible for transcription of the alginate biosynthetic operon. An *algU* mutant showed a dramatic impairment in biofilm formation under dynamic conditions (Bazire *et al.*, 2010). Extracellular DNA is also considered an important component of the biofilm matrix of *P. aeruginosa* because extracellular DNA acts as a component for cell-to-cell connection in the biofilm (Ma *et al.*, 2009). Furthermore, the extracellular DNA found in *P. aeruginosa* biofilm matrix was shown to induce a proinflammatory response by neutrophils (Fuxman Bass *et al.*, 2010). Degradation of extracellular DNA found in the biofilm matrix by DNase I resulted in a marked reduction in the ability of *P. aeruginosa* biofilms to induce the release of the proinflammatory cytokines IL-8 and IL-1β and the expression of the activation markers CD18, CD11b and CD66b. The absence of exogenous DNA in the biofilm matrix also led to a reduction in the number of bacteria phagocytosed by neutrophils. The number of neutrophil extracellular traps also decreased in the absence of extracellular DNA in the biofilm matrix (Fuxman Bass *et al.*, 2010).

Chemical gradients established in biofilms lead to heterogeneity in bacterial gene expression. Laser capture microdissection was used in combination with quantitative real-time reverse transcriptase PCR (RT-PCR) (Lenz *et al.*, 2008) or 16S rRNA/rDNA ratios (Pérez-Osorio *et al.*, 2010) to target defined biofilm subpopulations in order to measure gene expression. In thick *P. aeruginosa* biofilms, cells in the 30-μm zone next to the air–biofilm interface actively expressed genes associated with stationary phase. Cells in the interior portions did not express stationary-phase-associated genes, and therefore, cells located in the center of a biofilm are likely in a late stationary phase-like state or are possibly dormant (Pérez-Osorio *et al.*, 2010).

Diversity in biofilm production, biofilm architecture and control of biofilm formation was observed among human cystic fibrosis isolates (Deligianni *et al.*, 2010). Interestingly, it was observed that biofilm-positive strains of *P. aeruginosa* were capable of entrapping biofilm-negative strains. The biofilm-negative strains could be observed in the mature biofilm.

The resistance to antimicrobial agents by *P. aeruginosa* increased when cultured as a biofilm (Olson *et al.*, 2002). Planktonic cultures of an animal wound isolate of *P. aeruginosa* were sensitive to enrofloxacin, gentamicin, ampicillin, oxytetracycline and trimethoprim/sufladoxine, but when cultured as a biofilm the same isolate was sensitive to enrofloxacin only (Olson *et al.*, 2002). *P. aeruginosa* infections in animals are difficult to treat and this clearly suggests that biofilm formation is an important factor during infections.

Riemerella anatipestifer

Riemerella anatipestifer causes the anatipestifer syndrome in ducks. Anatipestifer is characterized by diarrhea, lethargy and respiratory and nervous symptoms. More than 40% of the 43 isolates and reference strains tested were positive for biofilm formation (Hu *et al.*, 2010). Bacteria cultured as biofilms were more resistant to antibiotics and detergents than their planktonic counterparts. This suggests that biofilm formation in *R. anatipestifer*

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may contribute to the persistence of this infection on duck farms.

Salmonella

Animal-derived products, particularly poultry meat, are the main source of *Salmonella* infections in human. During meat and poultry processing, pathogens such as *Salmonella* may attach and subsequently form biofilms on a variety of surfaces (Jain and Chen, 2007). The red, dry and rough (rdar) colony morphotype is a multicellular behavior displayed by *Salmonella enterica* and *E. coli*. Such colonies are characterized by the production and secretion of cellulose and the expression of curli fimbriae (Römling, 2005). These rdar colonies can easily form thick biofilms on abiotic surfaces. The rdar colony phenotype has been associated with the response regulator CsgD and the c-di-GMP producing enzyme AdrA. CsgD stimulates biofilm production indirectly by activating the transcription of the curli biosynthesis operon. The production of c-di-GMP by AdrA is a crucial signal to initiate the biosynthesis of cellulose (Römling, 2005).

S. enterica subsp. *enterica* serovar Typhimurium (or *Salmonella* Typhimurium) is the primary serovar isolated from slaughtered pigs in Europe (Van Parys *et al.*, 2010). Persistent infections in pigs are a major concern for food safety and human health. The role of biofilm formation in the persistence of *Salmonella* Typhimurium in porcine tonsils was studied by examining the contribution of biofilm-associated genes *csgA*, *csgD* and *adrA*. It was concluded that *Salmonella* Typhimurium colonized porcine tonsils in a biofilm-independent manner (Van Parys *et al.*, 2010).

Salmonella Typhimurium phage type DT104 is of particular importance, because this type of *Salmonella* Typhimurium is resistant to a core group of antibiotics such as ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Kim and Wei, 2009). Transposon mutagenesis of *Salmonella* Typhimurium DT104 indicated that several factors, including the production and export of exopolymeric substances, expression of flagella and regulation of exoribonucleases and RNA-binding proteins, were involved in biofilm formation and attachment to surfaces (Kim and Wei, 2009). The Calgary Biofilm Device was used to compare the susceptibility of 3-day-old biofilms and planktonic cells of *Salmonella* Typhimurium to various disinfectants (Wong *et al.*, 2010). Wong *et al.* (2010) observed that *Salmonella* biofilms were less susceptible to disinfectants than were planktonic *Salmonella*. However, the disinfectants evaluated were able to reduce the number of viable cells enclosed in biofilms at concentrations and contact times sufficient to eliminate planktonic cells. Despite the reduction in the amount of viable cells in the biofilm, there were enough viable cells remaining to cause further contamination and potentially result in infection. In another study, the Calgary Biofilm Device was used to evaluate the susceptibily of *Salmonella* isolates from bovine, swine and turkey enteritis to various antimicrobial agents. As previously observed with other bacteria, *Salmonella* biofilms were resistant to most antibiotics tested (Olson *et al.*, 2002).

A study investigating the source of *Salmonella* contamination in broiler and layer farms in Spain found that dust, surfaces and feces were the main sources of contamination (Marin *et al.*, 2009). Approximately 50% of the isolates were able to produce biofilms, and *S.* Enteritidis was the primary serovar isolated. In addition, Marin *et al.* (2009) showed that the use of glutaraldehyde, formaldehyde and peroxygen at a concentration of 1% in field conditions was inadequate for the elimination of *Salmonella* in the farm.

Staphylococcus

Mastitis is an economically important and frequent disease of dairy cows. *S. aureus* is the primary pathogen isolated from mastitis cases (Melchior *et al.*, 2006a; 2006b; Oliveira *et al.*, 2007). Coagulase-negative staphylococci (CNS), such as *S. epidermidis*, can also infect the mammary glands of dairy cows, goats and sheep. CNS infections are usually milder than those associated with *S. aureus*. In general, susceptibility to antimicrobial agents is high, but the recovery rates are poor. This suggests that during infection pathogens associated with mastitis form biofilms which facilitate their persistence in the udder (Melchior *et al.*, 2006b).

Approximately one-third (37.5%) of *S. aureus* and *S. epidermidis* isolates from subclinical mastitis produced biofilms (Oliveira *et al.*, 2006). It was later observed that the majority of biofilm-positive *S. aureus* isolates from mastitis cases produced a detectable biofilm within 48 h. On the other hand, biofilm-positive *S. epidermidis* isolates from mastitis cases required 72 h to produce a detectable biofilm (Oliveira *et al.*, 2007). Biofilm production by *S. aureus* isolated from milk, teat skin and from milking machine unit liners has also been compared (Fox *et al.*, 2005). It was found that *S. aureus* associated with milk are more likely to produce biofilms when compared to *S. aureus* extramammary sources (teat skin and milking unit liners). This suggests that the biofilm producing strains of *S. aureus* are able to attach to mammary mucosal surfaces and cause infections at a greater rate than are biofilm-negative strains. A fluorescent *in situ* hybridization (FISH) protocol for the detection of biofilm formation by staphylococci in contaminated milk has been developed (Oliveira *et al.*, 2006). This new assay would allow for the rapid detection of biofilm-positive staphylococci in contaminated milk. Early detection is a key factor for the application of corrective measures in mastitis cases.

Several genes have been associated with biofilm formation by staphylococci. These genes encode the

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accessory gene regulator Agr, *icaADBC* which encodes the PIA/PNAG producing enzyme and transporter, and the biofilm-associated protein Bap (Tormo *et al.*, 2005; Melchior *et al.*, 2009). The *IS257* genes have also been implicated in biofilm formation (Tormo *et al.*, 2005). There is a good correlation between the ability of *S. aureus* isolates from bovine subclinical mastitis to form a biofilm and the presence of the intercellular adhesion genes, *icaA* and *icaD* (Dhanawade *et al.*, 2010). Additionally, there is a strong correlation between the ability of CNS to form biofilms and the presence of *bap,* despite the absence of the *icaADBC* operon. Bap is a surface protein encoded by a gene carried in a putative transposon that is inserted in the mobile staphylococcal pathogenicity island SaPIbov 2 (Tormo *et al.*, 2005). The presence of a gene responsible for biofilm formation on a mobile element suggests that the ability to form biofilm can be acquired horizontally. The relation between biofilm formation and Arg type carried by *S. aureus* isolates from mastitis cases was also determined (Melchior *et al.*, 2009). Strains carrying Agr II preferred extracellular niches suggesting that biofilm formation is important for the survival of these strains. On the other hand, strains possessing Agr I were adapted for an intracellular lifestyle suggesting that biofilm formation is probably not as important for their survival. Recently, transposon analysis of a *S. aureus* isolate producing a proteinaceous biofilm matrix rich in extracellular DNA identified genes important for biofilm formation in a polysaccharide-independent manner. The analysis highlighted the importance of extracellular protease activity and autolysis in biofilm development (Boles *et al.*, 2010).

In poultry, *S. aureus* is normally a part of the skin microbiota; however, it can also be associated with osteomyelitis, bumble foot and arthritis. *S. aureus* is commonly isolated from the joints, tendon sheaths and the bones of infected animals (Nemati *et al.*, 2009). *S. aureus* isolates recovered from poultry between 1970 and 1972 and in 2006 were screened for the presence of biofilm-associated genes (*bap*, *icaA* and *icaD)*, and for the presence of genes encoding microbial surface components recognizing adhesive matrix molecules (MSCRAMMs; *bbp*, *cna*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *clfA* and *clfB)* (Nemati *et al.*, 2009). No correlation was observed between the presence of MSCRAMM genes and biofilm-associated genes in old or recent *S. aureus* isolates from healthy or infected chickens. Furthermore, there is no indication that the presence of these genes has changed over time. Some of the recent isolates belonged to the animal-associated methicillin-resistant *S. aureus* (MRSA) ST398. All MRSA-related isolates were positive for *icaD*, *cna*, *ebpS*, *eno*, *fnbA*, *fnbB*, *clfA* and *clfB*. This suggests that MRSA ST398 isolates have the genetic potential for strong adherence which may facilitate the colonization of not only chickens but also humans.

In rabbits, *S. aureus* can invade subcutaneous tissues through small dermal lesions (Vancraeynest *et al.*, 2004). In rabbit flocks, two types of *S. aureus* infections can be observed: infections limited to a small sub-population caused by weakly virulent strains and a chronic infection affecting the rabbitry caused by highly virulent strains. Highly and weakly virulent isolates of *S. aureus* were screened for biofilm-associated or MSCRAMMs genes (Vancraeynest *et al.*, 2004). The distribution of the *icaA*, *icaD* and *bap* genes among highly and weakly virulent isolates was similar. This suggests that the clinical symptoms associated with *S. aureus* infections in rabbits are not related to their biofilm formation potential.

Staphylococcus intermedius is part of the normal microbiota of dogs and pigeons. *S. intermedius* is an aetiological agent of skin infections in dogs, especially pyoderma (Futagawa-Saito *et al.*, 2006). A study using a large number of *S. intermedius* isolates indicated that biofilm formation was significantly higher in canine isolates than in pigeon isolates (Futagawa-Saito *et al.*, 2006). Following the description of a new species, *Staphylococcus pseudintermedius* (Devriese *et al.*, 2005), it appeared that several strains formerly identified as *S. intermedius* should be reclassified. All canine and some equine isolates previously identified as *S. intermedius* are now considered to be *S. pseudintermedius*. *S. intermedius* strains isolated from domestic pigeons and, in some cases, horses appear to belong to the species *Staphylococcus delphini*. *S. intermedius* seems to be confined to strains isolated from feral pigeons.

The MBEC of biofilm cultures of *S. aureus* isolated from bovine mastitis or *Staphylococcus hyicus* isolated from porcine dermatitis was higher than the MIC of planktonic cultures for all antimicrobial agents tested (Olson *et al.*, 2002); similar observations were made by Melchior *et al.* (2006a). Bacteria growing in biofilms appear to be highly resistant to antimicrobial agents at the maximum concentrations used *in vivo*. Hence, it can be assumed that biofilm formation by mastitis-associated *S. aureus* can offer an explanation for the poor correlation between bacteriological cure and susceptibility to antimicrobial agents as determined by CLSI (Clinical and Laboratory Standards Institute) testing methods.

Streptococcus

Streptococcus agalactiae or Group B *Streptococcus* (GBS) can colonize the mammary glands of ruminants. *S. agalactiae* is able to survive for long periods in udders, resulting in clinical and sub-clinical mastitis (Rinaudo *et al.*, 2010). GBS can form biofilms and pili, especially type 2a that play an important role during biofilm formation (Konto-Ghiorghi *et al.*, 2009; Rinaudo *et al.*, 2010). Pili have a domain called the von Willebrand adhesion domain that is involved in adhesion to epithelial cells. However, the von Willebrand adhesion domain of pili is not required for biofilm formation. Biofilm cultures of a bovine mastitis isolate of *S. agalactiae* were not killed by

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the antimicrobial agents tested, whereas planktonic cultures of the same isolate were (Olson *et al.*, 2002). On the other hand, the MBEC and the MIC of a bovine mastitis isolate of *Streptococcus dysgalactiae* were comparable for β-lactams and oxytetracycline (Olson *et al.*, 2002).

Streptococcus suis is a major pathogen of pigs; infection by this pathogen can result in pneumonia, meningitis, and septicemia. *S. suis* is also a zoonotic agent and workers in swine farms and pork-processing plants are at a high risk of infection. Bonifait *et al.* (2008) have shown that fibrinogen-induced biofilm formation by *S. suis* in a dose-dependent manner. Futhermore, an unencapsulated mutant of *S. suis* formed more biofilm than the parental strain suggesting that the capsule masks important adhesins and hydrophobic molecules responsible for biofilm formation (Tanabe *et al.*, 2010). Additionally, avirulent strains seemed to produce more biofilms than virulent strains (Wei *et al.*, 2009). Biofilm-grown cells were more resistant to penicillin G and ampicillin than were planktonic cells (Bonifait *et al.*, 2008; Grenier *et al.*, 2009). However, there was no increased resistance to β-lactams by biofilm cultures of a porcine pericarditis isolate of *S. suis* (Olson *et al.*, 2002). Biofilm formation has also been observed with a bovine rumen isolate of *Streptococcus bovis* (Olson *et al.*, 2002).

Streptococcus pneumoniae biofilms have been detected in a chinchilla model of otitis. It has been proposed that biofilm formation *in vivo* is intertwined with the formation of extracellular neutrophil traps (reviewed by Moscoso *et al.*, 2009).

Yersinia

Yersinia enterocolitica is a food-borne pathogen inhabiting the tonsils of pigs. It tolerates a wide range of temperature and growth conditions, suggesting that it can survive in soil, water and other environments. Therefore, growth as a biofilm may allow *Y. enterocolitica* to persist in several environmental niches (Kim *et al.*, 2008). Flagella are important for the initiation of biofilm formation but biofilm development occurs differently under static conditions or in the presence of shear force (Kim *et al.*, 2008).

Yersinia ruckeri is the etiological agent of enteric redmouth disease, also called yersiniosis. Yersiniosis is an economically important infectious disease affecting the rainbow trout industry (Coquet *et al.*, 2002). The presence of *Y. ruckeri* biofilms on the surface of fish tanks used in fish farms was a potential source of contamination for recurrent infection (Coquet *et al.*, 2002). Yersiniosis is routinely treated with oxolinic acid and biofilm cells were more resistant to this agent than were their planktonic counterparts.

Y. pestis is the causative agent of bubonic plague. It has been suggested that *Y. pestis* biofilms induce starvation of fleas by blocking their digestive tract. This starvation stimulates the flea into a feeding frenzy involving repeated biting. This feeding frenzy would thereby facilitate the transmission of *Y. pestis* to new hosts (Darby, 2008). *Y. pestis* biofilms appear to play an important role during the infection of fleas but biofilms play no role in mammalian pathogenesis (Darby 2008; Kim *et al.*, 2008; Sun *et al.*, 2009). The extracellular matrix of the biofilm contains a homopolymer of PGA (Darby, 2008). The extracellular matrix is synthesized by proteins encoded by the *hmsHFRS* operon and these proteins are orthologues of proteins encoded by the *pgaABCD* operon of *E. coli* and the *icaABCD operon* of *S. epidermidis* (Darby, 2008; Hinnebusch and Erickson, 2008). Despite the presence of PGA in its matrix, the *Y. pestis* biofilm cannot be digested by the PGA-degrading enzyme dispersin B. The expression of the *hmsHFRS* operon is regulated by polyamines (Wortham *et al.*, 2010); furthermore, c-di-GMP signaling regulates biofilm formation in *Y. pestis*. The mechanism involved in c-di-GMP signaling is widespread in bacteria. However, *Y. pestis* is the only bacterium able to form biofilms in fleas (Darby 2008).

Yersinia pseudotuberculosis is a food- and water-borne enteric pathogen, from which *Y. pestis* is thought to have evolved (Haesebrouck *et al.*, 1995). Similar to *Y. pestis*, *Y. pseudotuberculosis* possesses the *hmsHFRS* operon. *Y. pseudotuberculosis* can form biofilms *in vitro*, but it is unable to do so in fleas (Hinnebusch and Erickson, 2008). The response regulator PhoP negatively regulates the formation of biofilm in *Y. pseudotuberculosis* and *Y. pestis* (Sun *et al.*, 2009). PhoP appears to downregulate the diguanylate cyclase HmsT. Interestingly, a *Caenorhabditis elegans* model has been developed to study biofilm formation by *Y. pestis* (Darby, 2008).

Biofilms and chronic wounds

It has been known for decades that microbial infections have a deleterious effect on wound healing, and the control of bioburden is considered to be an important aspect of wound management (James *et al.*, 2008). Biofilm-associated diseases are typically persistent infections that develop slowly, are rarely resolved by immune defenses, and respond poorly to antimicrobial therapy. Chronic wound infections share the above-mentioned characteristics. Molecular techniques have highlighted the polymicrobial nature of wound infections. In animal diseases, wound infections are particularly problematic (Clutterbuck *et al.*, 2007), for example lower-limb wounds in horses. These wounds pose similar problems to pressure sores and diabetic foot ulcers in humans. Recently, Cochrane *et al.* (2009) were the first to demonstrate the presence of biofilms in chronic wounds of horses. They also demonstrated the importance of combining denaturing gradient gel electrophoresis and PCR with culture techniques to analyze the microbes

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associated with chronic wounds. An *in vitro* model to study biofilms associated with chronic wounds was recently developed, this new technique uses a constant depth film fermenter to assess the efficacy of antimicrobial treatments (Hill *et al.*, 2010).

Biofilms and the food industry

The food industry is considered to be part of the realm of public health area rather than the realm of veterinary medicine. However, it is important to keep in mind the impact of biofilm formation when a farm-to-fork (or gate-to-plate) approach to food safety is used. The significance of biofilms in the food industry is well documented. Biofilm formation by bacteria on food products or food processing equipment contributes to spoilage, and contamination of food and the spread of food-borne pathogens (Wong, 1998; Uhlich *et al.*, 2010; Van Houdt and Michiels, 2010). Bacteria in biofilms are able to persist in the food industry environment, because biofilms protect bacteria from heat, desiccation, other environmental stresses and antibacterial agents. This intrinsic resistance creates obstacles for the complete cleaning and disinfection of surfaces that come into contact with food. Milk and milk products can harbor a variety of microorganisms and have the potential to be important sources of food-borne pathogens (Oliver *et al.*, 2005). The presence of food-borne pathogens in milk is due to direct contact with contaminated sources in the dairy farm and their secretion from the udder of an infected animal. Overall, biofilms represent potential reservoirs of food-borne pathogens that are difficult to eradicate.

Compounds affecting bacterial biofilm formation and dispersal

There are several strategies to prevent and inhibit biofilm formation. These strategies include the prevention of microbial attachment, prevention of microbial growth, disruption of cell-to-cell communication, inhibition of matrix synthesis and disintegration of the biofilm matrix (Stewart, 2003; Anderson and O'Toole, 2008; Cegelski *et al.*, 2008). Effective biofilm inhibitors could dramatically change the treatment of many infectious diseases (Cegelski *et al.*, 2008). A basic strategy to discover inhibitors of biofilm formation is the direct screening of chemical compounds during biofilm formation assays. In recent years, the development of target-based screening for anti-biofilm agents has been focused on inhibitors of QS (e.g. halogenated furanones, azitrhomycin and 4-nitro-pyridine-N-oxide), compounds interfering with the metabolism of the signal molecule c-di-GMP (e.g. sulfathiazole) or inhibitors of DNA and nucleotide biosynthesis (Landini *et al.*, 2010). Additionally, signal molecules inhibiting biofilm formation can also stimulate biofilm dispersal (Landini *et al.*, 2010). Since QS has a potential role during bacterial infections of animals, the use of QS inhibitors as an antibacterial agent could provide new tools for veterinary medicine. However, the current research is limited to use of QS inhibitors in aquaculture (Boyen *et al.*, 2009).

Several examples of molecules capable of interfering with biofilm formation have recently been reported. Davies and Marques (2009) described a small messenger fatty acid molecule produced by *P. aeruginosa*, *cis*-2-decenoic acid, capable of inducing the dispersion of pre-established biofilms and inhibiting biofilm development. This molecule was also capable of inducing dispersal of biofilms formed by Gram-negative and Gram-positive bacteria and yeasts. Therefore, *cis*-2-decenoic acid may function in different kingdoms. A derivative of the marine natural product bromoageliferin called TAGE (*trans*-bromoageliferin analogue) also had anti-biofilm activity against *P. aeruginosa* (Huigens III *et al.*, 2008). Truchado *et al.*, (2009) have observed that chestnut honey and its aqueous extract can act as QS inhibitors. These two compounds also significantly reduce the formation of biofilm by *Y. enterocolitica* and *A. hydrophila*. The cathelicidin LL-37 was shown to inhibit *F. novicida* biofilm formation at sub-inhibitory concentrations (Amer *et al.*, 2010). The cathelicidin family is a large and diverse collection of cationic antimicrobial peptides found in a variety of vertebrate hosts. They represent an ancient mechanism of the innate immune response used to combat infection. Interestingly, resistance to these antimicrobial peptides is uncommon.

A cross-disciplinary approach combining microbiology and chemoinformatics was recently used to identify new and effective drugs with anti-biofilm activity (Dürig *et al.*, 2010). Natural compounds from plants inhibiting the formation of biofilms by *S. aureus* and *S. dysgalactiae* were identified using this approach. The compounds did not have an effect on planktonic cells at the concentrations needed to inhibit biofilm formation. These plant compounds can be considered drugs that prevent the switch to a biofilm lifestyle. According to the authors, such compounds might be less prone to the development of bacterial resistance than are classic antibiotics.

Bacteriophages can also be used to disperse biofilms, because bacteriophages produce depolymerases and other surface enzymes that degrade bacterial polysaccharides (Anderson and O'Toole, 2008; Richards and Melander, 2009). Unfortunately phage specificity is high and a phage cocktail composed of different types of phages would be necessary to treat a multi-species biofilm.

Vaccination

Vaccination against biofilm-positive pathogens has not been thoroughly evaluated but promising data have been generated. As previously mentioned, PGA is a matrix

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polysaccharide produced by a variety of bacterial pathogens. Protective antibodies to PGA are elicited when a deacetylated glycoform is used in conjugate vaccines, whereas protective antibodies are not generated when highly acetylated PGA is used (Gening *et al.*, 2010). Chemical synthesis of a series of oligoglucosamine ligands with specific differences in their N-acetylation allowed for the identification of a formulation for a conjugate vaccine that generated protective immune responses against *S. aureus* and *E. coli* (Gening *et al.*, 2010). This potential vaccine could be used to generate protective immunity against PGA-producing pathogens. However, the production cost associated with this potential vaccine might be prohibitive for its use in veterinary medicine.

A vaccine for sheep mastitis composed of *S. aureus* bacterins, crude bacterial extracts or purified PGA from biofilm-cultured bacteria was evaluated (Pérez *et al.*, 2009). Bacterins from strong biofilm-producing bacteria triggered the highest production of antibodies to PGA and were more protective than the vaccine containing the bacterins from weak biofilm-producing bacteria in an intra-mammary challenge. The production of protective immunity was independent of the adjuvant or the *S. aureus* capsular type used in the challenge. It is suggested that PGA-specific IgG antibodies opsonized bacteria and favored phagocytosis. Thus, bacterins from bacteria producing strong biofilms could be a cost-effective vaccine against ruminant mastitis caused by *S. aureus*.

Finally, analysis of antigens expressed by a biofilm-culture of *A. hydrophila* revealed that 15 proteins were repressed and three new proteins were present when compared to a planktonic culture (Asha *et al.*, 2004). Interestingly, biofilm cells did not express the S-layer proteins and produced an additional high molecular weight LPS. The above-mentioned changes are probably key factors in the efficacy of an oral vaccine based on a biofilm-culture of *A. hydrophila* in eliciting a protective immune response in fish.

Conclusion and future challenges

Biofilm formation may not always be a virulence factor, given that many non-pathogenic bacteria produce biofilms (Hall-Stoodley and Stoodley, 2005; Hancock *et al.*, 2010). However, biofilm formation by certain pathogens appears to facilitate their survival in the environment and the host. Biofilm research is therefore an area of intense interest (Haussler and Parsek, 2010) and biofilm research has recently gained recognition in areas such as animal health and public health. Reseachers now have access to a wide arsenal of techniques including three-dimensional imaging, specific fluorescent stains and molecular-reporter technology to study biofilms (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004). Furthermore, new techniques to study biofilm are being developed to enhance research on specific bacterial sub-populations found in the biofilm. For example, it is now possible to use laser capture microdissection to isolate subsets of bacterial cells from defined locations in a biofilm and study gene expression in the selected sub-population (Lenz *et al.*, 2008).

New tools are required for the prevention, treatment and diagnosis of pathogens that form biofilms during the infection process, because biofilms have unique properties that are absent in planktonic cells (Murphy *et al.*, 2009). As we develop the next generation of antimicrobial compounds, consideration should be given to the synergies between antibiotics, and other molecules (e.g. biocides, metals, enzymes, surfactants or QS inhibitors) for the treatment of biofilms (Ceri *et al.*, 2010). However, before synergies can be measured, new standardized assays have to be developed (Ceri *et al.*, 2010). Additionally, the efficacy of biocides against bacteria has to be evaluated for biofilm cultures and cannot be restricted to planktonic cells.

The impact of bacterial biofilm formation in veterinary medicine is obvious. There is a potential for increased resistance to antibiotics, to disinfectants and to the host immune response. The development of such resistance interferes with the treatment of animals and/or effective disinfection of the farm environment. The persistence of antibiotic resistance genes within biofilms is another aspect that should not be neglected, because it has a potential impact on both animal and public health. Furthermore, bacteria rarely form monospecies biofilms and bacteria are also known to attach to pre-existing biofilms. Examples have been documented with *L. monocytogenes* (Habimana *et al.*, 2009), *E. coli* O157 : H7 (Uhlich *et al.*, 2010) and *P. aeruginosa* (Deligianni *et al.*, 2010). Therefore, the natural existence of multi-species biofilms adds complexity to the analysis of biofilms. Although studies investigating *in vitro* biofilm formation have been performed on bacterial pathogens of veterinary importance, very few studies involving the formation of biofilms *in vivo* have been performed. Further research is required to develop effective strategies to treat and prevent biofilm-associated diseases in animals (Clutterbuck *et al.*, 2007). Further research is also required to develop effective disinfection protocols to eliminate biofilms from the farm and food-processing environments, because biofilms can act as a reservoir for infectious agents.

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