Attachment and Biofilm Formation by *Salmonella* in Food Processing Environments

Efstathios Giaouris¹, Nikos Chorianopoulos², Panagiotis Skandamis³ and George-John Nychas³

¹Department of Food Science and Nutrition, University of the Aegean
²Veterinary Research Institute, National Agricultural Research Foundation (NAGREF)
³Department of Food Science and Technology, Agricultural University of Athens (AUA)
Greece

1. Introduction

During the last decades, it has become increasingly clear that bacteria, including foodborne pathogens such as *Salmonella enterica*, grow predominantly as biofilms in most of their natural habitats, rather than in planktonic mode. A biofilm can be broadly defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan & Costerton, 2002; Kuchma & O’Toole, 2000; Lazazzera, 2005; Shemesh et al., 2007). Interestingly, it has been observed that the resistance of biofilm cells to antimicrobials is significantly increased compared with what is normally seen with the same cells being planktonic (Gilbert et al., 2002; Mah & O’Toole, 2001). Thus, it is believed that biofilm formation enhances the capacity of pathogenic *Salmonella* bacteria to survive stresses that are commonly encountered both within food processing, as well as during host infection.

In food industry, biofilms may create a persistent source of product contamination, leading to serious hygienic problems and also economic losses due to food spoilage (Brooks & Flint, 2008; Carpentier & Cerf, 1993; Ganesh Kumar & Anand, 1998; Lindsay & von Holy, 2006; Zottola & Sasahara, 1994). Improperly cleaned surfaces promote soil build-up, and, in the presence of water, contribute to the development of bacterial biofilms which may contain pathogenic microorganisms, such as *Salmonella*. Cross contamination occurs when cells detach from biofilm structure once food passes over contaminated surfaces or through aerosols originating from contaminated equipment. Till now, there is only limited information on the presence of *Salmonella* in biofilms in real food processing environments. However, numerous studies have shown that *Salmonella* can easily attach to various food-contact surfaces (such as stainless steel, plastic and cement) and form biofilms under laboratory conditions (Chia et al., 2009; Giaouris et al., 2005; Giaouris & Nychas, 2006; Hood & Zottola, 1997a,b; Marin et al., 2009; Oliveira et al., 2006; Rodrigues et al., 2011; Vestby et al., 2009a,b).

The natural environments that most bacteria inhabit are typically complex and dynamic. Unfortunately, this complexity is not fully appreciated when growing microorganisms in monocultures under laboratory conditions. Thus, in real environments, biofilm communities
are usually inhabited by numerous different species in close proximity (Wimpenny et al., 2000). Spatial and metabolic interactions between species contribute to the organization of multispecies biofilms, and the production of a dynamic local environment (Goller & Romeo, 2008; Tolker-Nielsen & Molin, 2000). Indeed, cell-to-cell signalling and interspecies interactions have been demonstrated to play a key role in cell attachment and detachment from biofilms, as well as in the resistance of biofilm community members against antimicrobial treatments (Annous et al., 2009; Burmølle et al., 2006; Irie & Parsek, 2008; Nadell et al., 2008; Remis et al., 2010). Mixed-species biofilms are usually more stable than mono-species biofilms, while biofilm formation by Salmonella has also been shown to be influenced by either the natural in situ presence of other species, or just their metabolic by-products (Chorianopoulos et al., 2010; Girennavar et al., 2008; Habimana et al., 2010b; Jones & Bradshaw, 1997; Prouty et al., 2002; Soni et al., 2008).

In this chapter, we review up-to-date available voluminous literature on the attachment and biofilm formation by Salmonella strains on abiotic surfaces, simulating those encountered in food processing areas (section 4). Before this, the advantages of biofilm lifestyle for microorganisms are briefly discussed (section 2), together with the serious negative implications of biofilm formation for the food industry (section 3). Major molecular components building up Salmonella biofilm matrix are then reported (section 5). Finally, we review available knowledge on the influence of cell-to-cell communication (quorum sensing) on the establishment of Salmonella biofilms (section 6).

2. Bacterial attachment to surfaces and advantages of the biofilm lifestyle

For most of the history of microbiology, microorganisms have primarily been characterised as planktonic, freely suspended cells and described on the basis of their growth characteristics in nutritionally rich culture media. Although this traditional way of culturing bacteria in liquid media has been instrumental in the study of microbial pathogenesis and enlightening as to some of the amazing facets of microbial physiology, pure culture planktonic growth is rarely how bacteria exist in nature. On the contrary, direct observation of wide of variety of natural habitats has shown that the majority of microbes persist attached to surfaces within a structured biofilm ecosystem and not as free-floating organisms (Costerton et al., 1987, 1995; Kolter & Greenberg, 2006; Verstraeten et al., 2008). The data on which this theory is predicated came mostly from natural aquatic ecosystems, in which direct microscopic observations together with direct quantitative recovery techniques showed unequivocally that more than 99.9% of the bacteria grow as biofilms on a wide variety of surfaces. The diversity and distribution of salmonellae in fresh water biofilms has also been recently shown (Sha et al., 2011). Moreover, it is becoming clear that these natural assemblages of bacteria within the biofilm matrix function as a cooperative consortium, in a relatively complex and coordinated manner (James et al., 1995; Moons et al., 2009; Wuertz et al., 2004). Nowadays, besides natural aquatic systems, it is well established that biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices and also industrial systems, such as pharmaceutical industries, oil drilling, paper production, waste water treatment and food processing (Hall-Stoodley et al., 2004). Thus, examples of this bacterial lifestyle are abundant in daily life: the slimy material that covers flower vases, pipelines, submerged rocks, and even the surface of teeth (Marsh, 2005; Wimpenny, 2009).

Biofilm formation occurs through sequential steps in which the initial attachment of planktonic bacteria to a solid surface is followed by their subsequent proliferation and
accumulation in multilayer cell clusters, and the final formation of the bacterial community enclosed in a self-produced polymeric matrix (Goller & Romeo, 2008; Lasa, 2006; O’Toole et al., 2000; Palmer et al., 2007; Rickard et al., 2003). The initial interaction between solid surface and bacterial cell envelope appears to be mediated by a complex array of chemical and physical interactions, with each affected by the chemical and physical environment to which the bacterial cell and the surface are currently or recently exposed (Palmer et al., 2007). Mature biofilms are highly organized ecosystems in which water channels are dispersed and can provide passages for the exchange of nutrients, metabolites and waste products (Stoodley et al., 2002). Once the biofilm structure has developed, some bacteria are released into the liquid medium, in order to colonize new surfaces, probably when surrounding conditions become less favourable (Gilbert et al., 1993; Hall-Stoodley & Stoodley, 2002, 2005; Klausen et al., 2006).

According to Darwin’s theory of evolution, the only true driving force behind the course of action of any organism is reproductive fitness. Outside of the laboratory bacteria rarely, if ever, find themselves in an environment as nutrient rich as culture media, and in these conditions, there are a number of fitness advantages imparted by the biofilm mode of growth (Jefferson, 2004). The process of biofilm formation is believed to begin when bacteria sense certain environmental parameters (extracellular signals) that trigger the transition from planktonic growth to life on a surface (Lopez et al., 2010). Currently, four potential incentives behind the formation of biofilms by bacteria are considered: (i) protection from the harmful environment (as a stress response mechanism), (ii) sequestration to a nutrient rich area, (iii) utilization of cooperative benefits (through metabolic cooperativity), and (iv) acquisition of new genetic traits (Davey & O’Toole, 2000; Molin & Tolker-Nielsen, 2003).

Bacteria experience a certain degree of shelter and homeostasis when residing within a biofilm and one of the key components of this microniche is the surrounding extrapolymeric substance (EPS) matrix (Flemming & Wingender, 2010). This matrix is composed of a mixture of components, such as exopolysaccharides, proteins, nucleic acids, and other substances (Branda et al., 2005). The nature of biofilm matrix and the physiological attributes of biofilm microorganisms confer an inherent resistance to antimicrobial agents, whether these antimicrobial agents are antibiotics, disinfectants or germicides. Thus, established biofilms can tolerate antimicrobial agents at concentrations of 10-1000 times that need to kill genetically equivalent planktonic bacteria, and are also extraordinary resistant to phagocytosis, making rather difficult to eradicate biofilms from living hosts (Cos et al., 2010). Mechanisms responsible for resistance may be one or more of the following: (i) delayed penetration of the antimicrobial agent through the biofilm matrix, (ii) altered growth rate of biofilm microorganisms, and (iii) other physiological changes due to the biofilm mode of growth, e.g. existence of subpopulations of resistant phenotypes in the biofilm, which have been referred to as “persisters” (Donlan & Costerton, 2002; Gilbert et al., 2002; Lewis, 2001; Mah & O’Toole, 2001).

Scientific interest in the process of bacterial biofilm formation has erupted in recent years and studies on the molecular genetics of biofilm formation have begun to shed light on the driving forces behind the transition to the biofilm mode of existence. Evidence is mounting that up- and down-regulation of a number of genes occurs in the attaching cells upon initial interaction with the substratum (Donlan, 2002; Sauer, 2003). Thus, high-throughput DNA microarray studies have been conducted to study biofilm formation in many model microorganisms and have identified a large number of genes showing differential expression under biofilm conditions (Beloin et al., 2004; Hamilton et al., 2009; Lazazzera,
In S. Typhimurium, 10% of its genome (i.e. 433 genes) showed a 2-fold or more change in the biofilm, using a silicone rubber tubing as a substratum for growth, compared with planktonic cells (Hamilton et al., 2009). The genes that were significantly up-regulated implicated certain cellular processes in biofilm development, including amino acid metabolism, cell motility, global regulation and tolerance to stress. Obviously, the more we learn about the genetic regulation of biofilm formation, the more we understand about the relative roles of benefits and forces that drive the switch to the biofilm mode of growth.

3. Biofilm formation in food processing environments and implications

The ability of bacteria to attach to abiotic surfaces and form biofilms is a cause of concern for many industries, including the food ones (Chmielewski & Frank, 2003). Poor sanitation of food-contact surfaces is believed to be an essential contributing factor in foodborne disease outbreaks, especially those involving Listeria monocytogenes and Salmonella. This is because the attachment of bacterial cells to such surfaces is the first step of a process which can ultimately lead to the contamination of food products. Thus, biofilms formed in food processing environments are of special importance since they may act as a persistent source of microbial contamination which may lead to food spoilage or/and transmission of diseases (Brooks & Flint, 2008; Zottola & Sasahara, 1994). While food spoilage and deterioration may result in huge economic losses, food safety is a major priority in today’s globalizing market with worldwide transportation and consumption of raw, fresh and minimally processed foods (Shi & Zhu, 2009).

Besides food spoilage and safety issues, in the dairy industry, bacterial attachment in heat exchangers (a process commonly known as “biofouling”) greatly reduces the heat transfer and operating efficiency of the processing equipment, while it can also causes corrosion problems (Austin & Bergeron, 1995). Additionally, in the various filtration systems, biofilm formation reduces significantly the permeability of the membranes (Tang et al., 2009). However, it should be noted that in the industry of fermented food products (sausages, cheeses etc), biofilm formation by some useful and technological bacteria (e.g. staphylococci, lactococci, lactobacilli) can be desirable, as a mean of the enhancement of food fermentation process, and more importantly as a mean of protection against the establishment of pathogenic biofilms (Chorianopoulos et al., 2008; Zhao et al., 2006).

Adhesion of Salmonella to food surfaces was the first published report on foodborne bacterial biofilm (Duguid et al., 1966). Since that time, many documents have described the ability of foodborne pathogens to attach to various surfaces and form biofilms, including L. monocytogenes (Blackman & Frank, 1996; Chorianopoulos et al., 2011; di Bonaventura et al., 2008; Poimenidou et al., 2009), Salmonella enterica (Chia et al., 2009; Giouris et al., 2005; Giouris & Nychas, 2006; Habimana et al., 2010b; Joseph et al., 2001; Kim & Wei, 2007, 2009; Oliveira et al., 2006; Marin et al., 2009; Rodrigues et al., 2011; Solomon et al., 2005; Stepanović et al., 2003, 2004), Yersinia enterocolitica (Kim et al., 2008), Campylobacter jejuni (Joshua et al., 2006) and Escherichia coli O157:H7 (Habimana et al., 2010a; Skandamis et al., 2009).

Modern food processing supports and selects for biofilm forming bacteria on food-contact surfaces due to mass production of products, lengthy production cycles and vast surface areas for biofilm development (Lindsay & von Holy, 2006). In situ biofilms have been recognised in various food processing industries, such as processors of cheese and other milk products, raw and cooked/fermented meats, raw and smoked fish etc (Austin & Bergeron, 1995; Bagge-Ravn et al., 2005; Shemesh et al., 2007; Whiteley et al., 2001).
et al., 2003; Gounadaki et al., 2008; Gunduz & Tuncel, 2006; Sharma & Anand, 2002). Several studies were also focused on the attachment of bacterial pathogens to food surfaces such as *Escherichia coli* to beef muscle and adipose tissue (Rivas et al., 2006) and *S. Typhimurium*, *Yersinia enterocolitica* and *L. monocytogenes* to pork skin (Morild et al., 2011).

Biofilm formation depends on an interaction between three main components: the bacterial cells, the attachment surface and the surrounding medium (Van Houdt & Michiels, 2010). Adhesion of bacterial cells, the first phase of biofilm formation, is influenced by the physicochemical properties of the cells' surface, which in turn are influenced by factors such as microbial growth phase, culture conditions and strain's variability (Briandet et al., 1999; Giaouris et al., 2009). The surfaces of most bacterial cells are negatively charged, and this net negative charge of the cell surface is adverse to bacterial adhesion, due to electrostatic repulsive force. However, the bacterial cell-surface possesses hydrophobicity due to fimbriae, flagella and lipopolysaccharide (LPS) (Ukuku & Fett, 2006). Hydrophobic interactions between the cell surface and the substratum may enable the cell to overcome repulsive forces and attach irreversibly (Donlan, 2002). The properties of the attachment surface (e.g. roughness, cleanability, disinfectability, wettability, vulnerability to wear) are important factors that also affect the biofilm formation potential and thus determine the hygienic status of the material. Stainless steel type 304, commonly used in the food processing industry, is an ideal material for fabricating equipment due to its physico-chemical stability and high resistance to corrosion. Teflon and other plastics are often used for gaskets and accessories of instruments. These surfaces become rough or crevice with continuous reuse and form a harbourage to protect bacteria from shear forces in the food fluid.

Environmental factors such as pH, temperature, osmolarity, O$_2$ levels, nutrient composition and the presence of other bacteria play important roles in the process of biofilm formation (Giaouris et al., 2005; Hood & Zottola, 1997a; Stepanovic et al., 2003). The integration of these influences ultimately determines the pattern of behavior of a given bacterium with respect to biofilm development (Goller & Romeo, 2008). In food processing environments, bacterial attachment is additionally affected by food matrix constituents, which can be adsorbed onto a substratum and create conditioning films (Bernbom et al., 2009). For example, skim milk was found to reduce adhesion of *Staphylococcus aureus*, *L. monocytogenes*, and *Serratia marcescens* to stainless steel coupons (Barnes et al., 1999). Additionally, in real environments, the presence of mixed bacterial communities adds additional complexity to attachment and biofilm formation procedure. For instance, the presence of *Staphylococcus xylosus* and *Pseudomonas fragi* affected the numbers of *L. monocytogenes* biofilm cells on stainless steel (Norwood & Gilmour, 2001), while compounds present in *Hafnia alvei* cell-free culture supernatant inhibited the early stage of *S. Enteritidis* biofilm formation on the same material (Chorianopoulos et al., 2010).

Once biofilms have formed in the factory environment, they are difficult to be removed often resulting in persistent and endemic populations (Vestby et al., 2009b). Interestingly, persistent *L. monocytogenes* strains had the added ability of enhanced adhesion within shorter times to stainless steel surfaces compared to non-persistent strains (Lundén et al., 2000). It has been suggested that such persistence is likely due to physical adaptation of cells in biofilms, particularly resistance to cleaning and sanitizing regimes, since it is generally accepted and well documented that cells within a biofilm are more resistant to biocides than their planktonic counterparts (Carpentier & Cerf, 1993). For example, nine disinfectants commonly used in the feed industry and efficient against planktonic *Salmonella* cells, showed a bactericidal effect that varied considerably for biofilm-grown cells with products containing
70% ethanol being most effective (Møretrø et al., 2009). Other studies similarly indicated that compared to planktonic cells, biofilm cells of *Salmonella* were more resistant to trisodium phosphate (Scher et al., 2005) and to chlorine and iodine (Joseph et al., 2001). In a comparative study of different *S. Enteritidis* phage type 4 isolates it was found that those isolates that survived better on surfaces also survived better in acidic conditions and in the presence of hydrogen peroxide and showed enhanced tolerance towards heat (Humphrey et al., 1995).

The cellular mechanisms underlying microbial biofilm formation and behaviour are beginning to be understood and are targets for novel specific intervention strategies to control problems caused by biofilm formation in fields ranging from industrial processes like food processing, to health-related fields, like medicine and dentistry. In food industry, various preventive and control strategies, like hygienic plant lay-out and design of equipment, choice of materials, correct selection and use of detergents and disinfectants coupled with physical methods can be suitably applied for controlling biofilm formation. Right now, bacterial biofilms have not been specifically addressed in the HACCP system that has been employed in the food processing facilities. However, surveying of biofilms in food environments and developing an effective sanitation plan should be considered in the HACCP system (Sharma & Anand, 2002). An upgraded HACCP with biofilm assessment in food plants will provide clearer information of contamination, and assist the development of biofilm-free processing systems in the food industry.

4. Attachment to food-contact surfaces and biofilm forming ability of *Salmonella*

*Salmonellae* represent a group of Gram-negative bacteria that are recognized worldwide as major zoonotic pathogens for both humans and animals. In the EU, salmonellosis was the second most commonly reported zoonotic infection in 2009, with 108,614 human cases confirmed and a case fatality rate of 0.08%, which approximately corresponds to 90 human deaths (EFSA-ECDC, 2011). That year, *Salmonella* was most often found in fresh broiler, turkey and pig meat where proportions of positive samples, on average 5.4%, 8.7% and 0.7%, were detected respectively. The two most common *Salmonella* serotypes, implicated in the majority of outbreaks, are Typhimurium and Enteritidis (52.3% and 23.3% respectively of all known serovars in human cases). The native habitat of salmonellae is considered to be the intestinal tract of taxonomically diverse group of vertebrates, from which salmonellae can spread to other environments through released faeces (Littrup et al., 2010).

Interestingly, salmonellae have been shown to survive for extended periods of time in non-enteric habitats, including biofilms on abiotic surfaces (White et al., 2006). Thus, several reports have demonstrated the ability of *Salmonella* to form biofilms on abiotic surfaces outside the host, such as stainless steel (Austin et al., 1998; Chorianopoulos et al., 2010; Giaouris et al., 2005; Giaouris & Nychas, 2006; Hood & Zottola, 1997a,b; Joseph et al., 2001; Kim & Wei, 2007, 2009; Møretrø et al., 2009), plastic (Asséré et al., 2008; Iibuchi et al., 2010; Jain & Chen, 2007; Joseph et al., 2001; Ngwai et al., 2006; Stepanović et al., 2003, 2004; Vestby et al., 2009a,b), rubber (Arnold & Yates, 2009), glass (Kim & Wei, 2009; Korber et al., 1997; Prouty & Gunn, 2003; Solano et al., 1998), cement (Joseph et al., 2001), marble and granite (Rodrigues et al., 2011). Taken into account, that all these surfaces are commonly encountered in farms, slaughter houses, food industries and kitchens, it is obvious that the risk for public health is quite serious.

It is strongly believed that the ability of *Salmonella* to form biofilms on inanimate surfaces contributes to its survival and persistence in non-host environments and its transmission to
new hosts. To this direction, Vestby et al. (2009b) found a correlation between the biofilm formation capacities of 111 Salmonella strains isolated from feed and fish meal factories and their persistence in the factory environment. Another study on colonization and persistence of Salmonella on egg conveyor belts indicated that the type of egg belt (i.e. vinyl, nylon, hemp or plastic) was the most important factor in colonization and persistence, while rdar morphotype, a physiological adaptation associated with aggregation and long-term survival which is conserved in Salmonella (White & Surette, 2006), surprisingly, was not essential for persistence (Stocki et al., 2007). Interestingly, inoculation onto fresh-cut produce surfaces, as well as onto inert surfaces, such as polyethersufone membranes, was found to significantly increase the survival of salmonellae during otherwise lethal acid challenge (pH 3.0 for 2 hours) (Gawande & Bhagwat, 2002). Similarly, Salmonella strains with high biofilm productivity survived longer on polypropylene surfaces under dry conditions than strains with low productivity (Iibuchi et al., 2010).

In the food processing environments, food-contact surfaces come in contact with fluids containing various levels of food components. Under such conditions, one of the first events to occur is the adsorption of food molecules to the surface (conditioning). Both growth media and surface conditioning were found to influence the adherence of S. Typhimurium cells to stainless steel (Hood & Zottola, 1997b). A study of 122 Salmonella strains indicated that all had the ability to adhere to plastic microwell plates and that, generally, more biofilm was produced in low nutrient conditions, as those found in specific food processing environments, compared to high nutrient conditions (Stepanovic et al., 2004). A study conducted in order to identify the risk factors for Salmonella contamination in poultry farms, showed that the most important factors were dust, surfaces and faeces, and nearly 50% of the strains isolated from poultry risk factors were able to produce biofilm, irrespective of the origin of different serotypes (Marin et al., 2009).

There are some studies which have investigated the influence of physicochemical and surface properties (e.g. charge, hydrophobicity, surface free energy, roughness) of Salmonella and surface materials on the attachment process. For instance, Sinde & Carballo (2000) found that surface free energies and hydrophobicity do not affect attachment of Salmonella to stainless steel, rubber and polytetrafluorethylene, while Ukuku & Fett (2002) found that there was a linear correlation between bacterial cell surface hydrophobicity and charge and the strength of attachment of Salmonella, E. coli and L. monocytogenes strains to cantaloupe surfaces. Korber et al. (1997) found that surface roughness influences susceptibility of S. Enteritidis biofilms, grown in glass flow cells (with or without artificial crevices) to trisodium phosphate. Chia et al. (2009) studied the attachment of 25 Salmonella strains to four different materials (Teflon®, stainless steel, rubber and polyurethane) commonly found in poultry industry and found out that materials more positive in interfacial free energies had the highest number of adhering bacteria. However, in that study, authors concluded that Salmonella adhesion is strain-dependent, and probably influenced by surface structures, such as cell wall and membrane proteins, fimbriae, flagella and polysaccharides. This was also the conclusion of another similar study which compared the adhesion ability of four S. Enteritidis isolates to three different materials (polyethylene, polypropylene and granite) used in kitchens (Oliveira et al., 2006). Ngwai et al. (2006) characterized the biofilm forming ability of eleven antibiotic-resistant S. Typhimurium DT104 clinical isolates from human and animal sources and concluded that there was a general lack of correlation between this ability and bacterial physicochemical surface characteristics.
The persistence of *Salmonella* within the food chain has become a major health concern, as biofilms of this pathogen formed in food processing environments can serve as a reservoir for the contamination of food products. The development of materials to be used for food-contact surfaces with improved food safety profiles continues to be a challenge. One approach which has been developed to control microbial attachment is the manufacture of food-contact materials incorporating antimicrobial compounds. Triclosan-impregnated kitchen bench stones (silestones), although prone to bacterial colonization, were found to reduce *S. Enteritidis* biofilm development on them and also the viability of cells within the biofilm (Rodrigues et al., 2011).

### 5. Molecular components of *Salmonella* biofilms formed on abiotic surfaces

Curli fimbriae (formerly designated as thin aggregative fimbriae or Tafi) and cellulose are the two main matrix components (exopolymeric substances, EPS) in *Salmonella* biofilms (Gerstel & Römling, 2003). When co-expressed on Congo Red (CR) agar plates, curli fimbriae and the exopolysaccharide cellulose form the characteristic rdar (red, dry and rough) morphotype (also called rugose or wrinkled) (Römling, 2005). Their syntheses are co-regulated by a complex regulatory system. The LuxR type regulator CsgD protein stimulates the production of curli through transcriptional activation of the csgBAC (formerly agfBAC) operon, while the activation of cellulose production is indirect through the regulator AdrA which is a member of the GGDEF protein family regulated by csgD (Römling et al., 2000). García et al. (2004) demonstrated that most GGDEF proteins of *S. Typhimurium* are functionally related, probably by controlling the levels of the same final product, cyclic di-GMP, a secondary messenger that seems to regulate a variety of cellular functions including cellulose production and biofilm formation. The co-expression of curli fimbriae and cellulose leads to the formation of a highly hydrophobic network with tightly packed cells aligned in parallel in a rigid matrix and enhances biofilm formation on abiotic surfaces (Jain & Chen, 2007). Solomon et al. (2005) showed that 72% of 71 *S. enterica* strains, originating from produce, meat or clinical sources and belonging to 28 different serovars, expressed the rdar morphotype, with curli- and cellulose-deficient isolates being least effective in biofilm formation on polystyrene microtiter plates. White et al. (2006) showed that rdar morphotype significantly enhanced the resistance of *Salmonella* to dessication and sodium hypochlorite, suggesting that this phenotype could play a role in the transmission of *Salmonella* between hosts. However, aggregation via the rdar morphotype does not seem to be a virulence adaptation in *S. Typhimurium*, since competitive infection experiments in mice showed that nonaggregative cells outcompeted rdar-positive wild-type cells in all tissues analyzed (White et al., 2008).

A variety of environmental cues such as nutrients, oxygen tension, temperature, pH, ethanol and osmolarity can influence the expression of the transcriptional regulator CsgD, which regulates the production of both cellulose and curli (Gerstel & Römling, 2003). Transcription of csgD is dependent upon the stationary phase-inducible sigma factor RpoS, and is maximal in the late exponential or early stationary phase of growth (Gerstel & Römling, 2001). For an extensive overview on the current understanding of the complex genetic network regulating *Salmonella* biofilm formation, reader is advised to refer to the recently published review of Steenackers et al. (2011). When csgD is not expressed the morphotype is a conventional smooth and white (saw) colony, which does not produce any extracellular matrix (Römling et al., 1998b). In wild type *Salmonella* strains, rdar morphotype is restricted to low temperature (below 30°C) and low osmolarity conditions, but biogenesis of curli
fimbriae occurs upon iron starvation at 37°C. Römling et al. (2003) showed that the majority (more than 90% of 800 strains) of human disease-associated S. Typhimurium and S. Enteritidis (isolated from patients, foods and animals) displayed the rdar morphotype at 28°C, but just rarely at 37°C. Interestingly, mutants in the csgD promoter have also been found expressing rdar morphotype independently of temperature (Römling et al., 1998b). Curli fimbriae are amyloid cell-surface proteins, and are involved in adhesion to surfaces, cell aggregation, environmental persistence and biofilm development (Austin et al., 1998; Collinson et al., 1991; White et al., 2006). The csg (curli subunit genes) genes (previously called agf genes) involved in curli biosynthesis are organized into two adjacent divergently-transcribed operons, csgBAC and csgDEFG (Collinson et al., 1996; Römling et al., 1998a). Knocking out the gene encoding for the subunit of thin aggregative fimbriae, AgfA, results in pink colony formation, the pdar (pink, dry and rough) morphotype, which is characterised by production of cellulose without curli (Jain & Chen, 2007). Solano et al. (2002) stressed the importance of the applied biofilm system since they noticed that curli were not essential for biofilm mediated glass adherence under adherence test medium (ATM) conditions, while they were indispensable to form a tight pellicle under LB conditions. In addition to curli, the second component of the extracellular matrix of the Salmonella biofilms is cellulose, a β-1→4-D-glucose polymer, which is biosynthesized by the bcsABZC-bcsEFG genes (bacterial cellulose synthesis) (Zogaj et al., 2001). Both operons are responsible for cellulose biosynthesis in both S. Enteritidis and S. Typhimurium (Jain & Chen, 2007; Solano et al., 2002). Cellulose production impairment generates a bdar (brown, dry and rough) morphotype on Congo red (CR) agar plates, characteristic of the expression of curli. Solano et al. (2002) showed that cellulose is a crucial biofilm determinant for Salmonella, under both LB and ATM conditions, without however affecting the virulence of the bacterium. Additionally, cellulose-deficient mutants were more sensitive to chlorine treatments, suggesting that cellulose production and biofilm formation may be an important factor for the survival of Salmonella in hostile environments. Prouty & Gunn (2003) identified its crucial importance for biofilm formation on glass coverslips. However, cellulose was not a major constituent of the biofilm matrix of S. Agona and S. Typhimurium strains isolated from the feed industry, but it contributed to the highly organized matrix structurization (Vestby et al., 2009a). Malcova et al. (2008) found that cellulose was not crucial for S. Enteritidis adherence and biofilm formation on polystyrene. Latasa et al. (2005) also reported another matrix component, BapA, a large cell-surface protein required for biofilm formation of S. Enteritidis. This protein was found to be loosely associated with the cell surface, while it is secreted through the BapBCD type I protein secretion system, encoded by the bapABCD operon. The expression of bapA was demonstrated to be coordinated with the expression of curli and cellulose through the action of csgD (Latasa et al., 2005). Also, these authors demonstrated that a bapA mutant strain showed a significant lower colonization rate at the intestinal cell barrier and consequently a decreased efficiency for organ invasion compared with the wild-type strain. Motility was found to be important for Salmonella biofilm development on glass (Prouty & Gunn, 2003) and polyvinyl chloride (PVC) (Mireles et al., 2001). On the contrary, Teplitski et al. (2006) noticed that the presence of the flagellum on the surface of the cell, functional or not, is inhibitory to biofilm formation on polystyrene, as mutants lacking intact flagella, showed increased biofilm formation compared to the wild-type. Flagella were not found to be important for S. Typhimurium rdar expression on Congo Red (CR) agar plates (Römling & Rohde, 1999). Solano et al. (2002) noticed that flagella affect S. Enteritidis biofilm development.
only under LB but not under ATM conditions. Stafford & Hughes (2007) showed that the conserved flagellar regulon gene flhE, while it is not required for flagella production or swimming, appeared to play a role in flagella-dependent swarming and biofilm formation on PVC. Kim & Wei (2009) noticed that flagellar assembly was important during biofilm formation on PVC in different (meat, poultry and produce) broths and on stainless steel and glass in LB broth.

Colanic acid, a capsular extracellular polysaccharide, essential for *S. Typhimurium* biofilm development on epithelial cells was found not to be required for *Salmonella* biofilm formation on abiotic surfaces (Ledeboer & Jones, 2005; Prouty & Gunn, 2003). Solano et al. (2002) showed that colonic acid was important to form a tight pellicle under LB conditions, while it was dispensable under ATM conditions. De Rezende et al. (2005) purified another capsular polysaccharide (CP) from extracellular matrix of multiresistant *S. Typhimurium* DT104 which was found to be important for biofilm formation on polystyrene centrifuge tubes and was detected at both 25°C and 37°C. This was comprised principally of glucose and mannose, with galactose as a minor constituent. Malcova et al. (2008) confirmed the importance of this capsular polysaccharide in the biofilm formation capacity of strains unable to produce either curli fimbriae or cellulose. Due to mucoid and brown appearance on Congo Red agar plates, their morphotype was designated as sbam (smooth, brown and mucoid).

However, other capsular polysaccharides can be present in the extracellular biofilm matrix of *Salmonella* strains (de Rezende et al., 2005; Gibson et al., 2006; White et al., 2003), and the exact composition depends upon the environmental conditions in which the biofilms are formed (Prouty & Gunn, 2003). Another component of the EPS matrix of *Salmonella* bile-induced biofilms, the O-antigen (O-ag) capsule, while it was found to be crucial for *S. Typhimurium* and *S. Typhi* biofilm development on gallstones, this was not necessary for adhesion and biofilm formation on glass and plastic (Crawford et al., 2008). The formation of this O-ag capsule was also found to be important for survival during desiccation stress (Gibson et al., 2006). Anriany et al. (2006) highlighted the importance of an integral lipopolysaccharide (LPS), at both the O-antigen and core polysaccharide levels, in the modulation of curli protein and cellulose production, as well as in biofilm formation, thereby adding another potential component to the complex regulatory system which governs multicellular behavior in *S. Typhimurium*. Mireles et al. (2001) observed that for *S. Typhimurium* LT2, all of the LPS mutants examined were able to form a biofilm on polyvinyl chloride (PVC) but none were able to attach to a hydrophilic surface such as glass. Kim & Wei (2009) noticed that a rfbA mutant of *S. Typhimurium* DT104, showing an aberrant LPS profile, was impaired in rdar expression, pellicle formation, biofilm forming capability on PVC in meat, poultry and produce broths and biofilm formation on stainless steel and glass.

6. Cell-to-cell communication in *Salmonella* biofilms (quorum sensing)

It has been thoroughly suggested that bacterial cells communicate by releasing and sensing small diffusible signal molecules, in a process commonly known as quorum sensing (QS) (Miller & Bassler, 2001; Smith et al., 2004; Whitehead et al., 2001). Through cell-to-cell signaling mechanisms, bacteria modulate their own behaviour and also respond to signal produced by other species (Ryan & Dow, 2008). QS involves a density-dependent recognition of signaling molecules (autoinducers, AIs), resulting in modulation of gene expression (Bassler, 1999). Gram-negative bacteria primarily use a variety of *N*-acylhomoserine lactones (AHLS) as AI (autoinducer-1, AI-1), while Gram-positive bacteria
use a variety of autoinducing polypeptides (AIPs). AHLs are synthesized and recognized by QS circuits composed of LuxI and LuxR homologues, respectively (Whitehead et al., 2001). Both AHLs and AIPs are highly specific to the species that produce them. A third QS system is proposed to be universal, allowing interspecies communication, and is based on the enzyme LuxS which is in part responsible for the production of a furanone-like compound, called autoinducer-2 (AI-2) (Schauder et al., 2001). Bacteria use QS communication circuits to regulate a diverse array of physiological activities, such as genetic competence, pathogenicity (virulence), motility, sporulation, bioluminescence and production of antimicrobial substances (Miller & Bassler, 2001). Yet, a growing body of evidence demonstrates that QS also contributes to biofilm formation by many different species (Annous et al., 2009; Davies et al., 1998; Irie & Parsek, 2008; Lazar, 2011). As biofilms typically contain high concentration of cells, autoinducer (AI) activity and QS regulation of gene expression have been proposed as essential components of biofilm physiology (Kjelleberg & Molin, 2002; Parsek & Greenberg, 2005).

To date, three QS systems have been identified in *S. enterica* and are thought to be mainly implicated in the regulation of virulence (SdiA, luxS/AI-2 and AI-3/epinephrine/norepinephrine signaling system) (Boyen et al., 2009; Walters & Sperandio, 2006). Firstly, the LuxR homologue SdiA has been characterized in *Salmonella*, but there does not appear to be a corresponding signal-generating enzyme similar to LuxI in this species (Ahmer et al., 1998). Since *Salmonella* does not possess a luxI homologue, it cannot produce its own AHLs (Ahmer, 2004). However, *Salmonella* SdiA can detect AHLs produced by a variety of bacterial species, leading to the suggestion that SdiA can be used in interspecies communication within a mixed-species community (Michael et al., 2001; Smith & Ahmer 2003). Till now, SdiA is known to activate the expression of the *rck* operon and the *srgE* gene (Ahmer et al., 1998; Smith & Ahmer, 2003). In contrast to the function of SdiA in *E. coli* adherence to HEp-2 epithelial cells and also biofilm formation on polystyrene (Lee et al., 2009; Sharma et al., 2010), no direct link between SdiA and *Salmonella* biofilms has been reported. Interestingly, Chorianopoulos et al. (2010) demonstrated that cell-free culture supernatant (CFS) of the psychrotrophic spoilage bacterium *Hafnia alvei*, containing AHLs among other unknown metabolites, negatively influenced the early stage of biofilm formation by *S. Enteritidis* on stainless steel. Similarly, Dheilly et al. (2010) reported the inhibitory activity of CFS from the marine bacterium *Pseudoalteromonas* sp. strain 3J6 against biofilm formation on glass flow cells by *S. enterica* and other Gram-negative bacteria. Taking into account that *Salmonella* possess SdiA, a receptor of AHLs which may be produced by resident flora on food-contact surfaces (Michael et al., 2001; Smith & Ahmer, 2003; Soares & Ahmer, 2011), the effect of AHLs on biofilm formation by this pathogen in multispecies real food processing environments needs to be further studied.

The second QS system of *Salmonella* uses the LuxS enzyme for the synthesis of AI-2 (Schauder et al., 2001; Soni et al., 2008). The Lsr ABC transporter is known to be involved in the detection and transport of AI-2 into the cell (Taga et al., 2001), while the *rbs* transporter has recently been suggested as an alternative AI-2 uptake system (Jesudhasan et al., 2010). A *S. Typhimurium luxS* deletion mutant was impaired in biofilm formation on polystyrene (De Keersmaecker et al., 2005; Jesudhasan et al., 2010). However, this phenotype could not be complemented by extracellular addition of QS signal molecules, suggesting that AI-2 is not the actual signal involved in *Salmonella* biofilm formation (De Keersmaecker et al., 2005). To this direction, Kint et al. (2010) analyzed additional *luxS* mutants for their biofilm phenotype. Interestingly, a *luxS* kanamycin insertion mutant and a partial deletion mutant,
that only lacked the 3′ part of the luxS coding sequence, were found to be able to form mature wild-type biofilms on polystyrene, despite the fact that these strains were unable to produce AI-2. These authors concluded that a small regulatory RNA molecule, MicA, encoded in the luxS adjacent genomic region, rather than LuxS itself, influences S. Typhimurium biofilm formation phenotype. On the other hand, Prouty et al. (2002) showed that a S. Typhimurium luxS insertion mutant formed scattered biofilm on gallstones with little apparent EPS even after 14 days of incubation. Yoon & Sofos (2008) showed that biofilm formation by S. Thompson on stainless steel, under monoculture conditions (72 h at 25°C), was similar between AI-2 positive and negative strains. Altogether, these results demonstrate that the relationship between biofilm formation and the presence of an active LuxS system and AI-2 in S. enterica is not clear and further research is needed.

The third QS system of Salmonella uses the two component system PreA/B (Bearson & Bearson 2008; Merighi et al., 2006). PreA/B is similar to the luxS-dependent two component QseB/QseC of enterohemorrhagic E. coli, which has been shown to sense the QS signal AI-3, as well the eukaryotic hormones epinephrine and norepinephrine (Sperandio et al., 2002; Walters & Sperandio, 2006). In S. Typhimurium, the histidine sensor kinase QseC, which is able to detect norepinephrine, has been implicated in the regulation of virulence traits, such as motility and in vivo competitive fitness in pigs (Bearson & Bearson, 2008). Even though the role of AI-3/epinephrine/norepinephrine signaling system in the formation of biofilm by Salmonella is still unknown, given that motility is usually an important biofilm determinant in many bacterial species, it is quite possible that this third QS system may also affect Salmonella biofilm formation.

7. Conclusions

Biofilms are commonly defined as communities of microorganisms attached to a surface and producing an extracellular matrix, in which these microorganisms are embedded. Biofilms are very diverse and unique, not just to the microorganism, but to the particular environment in which they are being formed. This makes in vitro characterization of biofilms difficult and requires the establishment of laboratory conditions that mimic the natural setting being studied. Pathogenic biofilms have been of considerable interest in the context of food safety and have provoked interest of many research groups. In particular, biofilm formation by Salmonella is a serious concern in food industry, since the persistence of this bacterium in biofilms formed on food-contact surfaces may become a constant source of product contamination.

The discovery of bacterial biofilms in medical and industrial ecosystems has created an urgency to identify and characterize factors that are necessary for biofilm development, which may serve as targets for biofilm prevention and treatment. Thus, researchers in the fields of clinical, food, water, and environmental microbiology have begun to investigate microbiological processes from a biofilm perspective. As the pharmaceutical, health-care and food industries embrace this approach, novel strategies for biofilm formation and control will undoubtedly emerge. Particularly challenging is the attempt to understand the complexity of the interactions within a biofilm community, since these interactions between the different species influence the final outcome of this community. Communication between species may include extracellular compounds whose sole role is to influence gene expression, metabolic cooperativity and competition, physical contact, and the production of antimicrobial exoproducts. One or all of these interactions may be
occurring simultaneously. The challenge becomes more intriguing given that microflora on inadequately cleaned and disinfected food processing surfaces is a complex community, contrary to the laboratory studied pure-species biofilms. Undoubtedly, a clearer understanding of the factors which influence microbial attachment to abiotic surfaces could provide the information necessary to modify processes in food processing environments in order to reduce microbial persistence and therefore reduce the contamination of food products. For instance, the understanding of bacterial attachment to solid surfaces, such as stainless steel, may help in the future development of surfaces with no or reduced attachment, or in developing an effective sanitation programme and thus reducing the potential contamination of processed products by spoilage or/and pathogenic bacteria. Undoubtedly, the ability to recognize how Salmonella attach to food-contact surfaces and form biofilms on them is an important area of focus, since a better understanding of this ability may provide valuable ways towards the elimination of this pathogenic bacterium from food processing environments and eventually lead to reduced Salmonella-associated human illness.

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9. References


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More than 2,500 serotypes of Salmonella exist. However, only some of these serotypes have been frequently associated with food-borne illnesses. Salmonella is the second most dominant bacterial cause of food-borne gastroenteritis worldwide. Often, most people who suffer from Salmonella infections have temporary gastroenteritis, which usually does not require treatment. However, when infection becomes invasive, antimicrobial treatment is mandatory. Symptoms generally occur 8 to 72 hours after ingestion of the pathogen and can last 3 to 5 days. Children, the elderly, and immunocompromised individuals are the most susceptible to salmonellosis infections. The annual economic cost due to food-borne Salmonella infections in the United States alone is estimated at $2.4 billion, with an estimated 1.4 million cases of salmonellosis and more than 500 deaths annually. This book contains nineteen chapters which cover a range of different topics, such as the role of foods in Salmonella infections, food-borne outbreaks caused by Salmonella, biofilm formation, antimicrobial drug resistance of Salmonella isolates, methods for controlling Salmonella in food, and Salmonella isolation and identification methods.

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