Abstract
Salmonella enterica (S. enterica) can form multicellular structures, commonly called biofilms, on various occasions. Biofilms can be formed on diverse surfaces/interfaces such as at the epithelial cell layer of a vertebrate host, on plant surfaces, or on abiotic surfaces, at the air–liquid interface and on gallstones. In this way, S. enterica is thought to achieve environmental persistence, transmission, and colonization of host organisms. Characterization of biofilm formation at the molecular level revealed different types of biofilms that have common and distinct regulatory and structural components. The so-called “rdar morphotype,” a multicellular behavior of S. enterica on agar plates is characterized by the expression of the master regulator CsgD, which controls expression of the extracellular matrix components curli fimbriae, the large surface-associated protein BapA, the exopolysaccharide cellulose and a capsular polysaccharide. Colonization of abiotic surfaces, plant surfaces and epithelial cell layers partly require the same factors as rdar morphotype formation, while the biofilm formed by Salmonella on gallstones seems to be substantially distinct from rdar morphotype formation.

Introduction
Salmonella enterica, in particular serovar Typhimurium (S. Typhimurium) and Enteritidis (S. Enteritidis) have mainly been studied as pathogens in the process of causing disease (Hensel, 2004; Hurley and McCormick, 2003). However, often S. enterica is a regular colonizer of warm- and cold-blooded animals without causing disease. Although animals and humans act as host organisms for salmonellae, the life cycle of S. enterica is complex and includes stages outside of the host (Winfield and Grosso-man, 2003). For example, S. enterica has to be effectively transmitted between hosts either by close contact, or through animate or inanimate vehicles such as water, food and produce. Effective survival outside a host is consequently an essential component of the life cycle of S. enterica. Since life outside and inside a host is fundamentally different, S. enterica has to tightly regulate gene expression in order to survive in both environments (Mouslim et al., 2002).

A multicellular behavior, commonly called biofilm-formation, is expressed by almost all bacteria and can play roles in virulence and host colonization as well as for the survival outside a host. In this review, current knowledge about biofilm formation in S. enterica is summarized. S. enterica
forms biofilms on different surfaces in the host such as at the epithelial cell layer or on gall stones; is able to colonize plant surfaces for transmission of the pathogen and colonizes abiotic surfaces such as stainless steel and glass. Elucidation of the molecular basis of biofilm formation has revealed that similar and distinct components are required for biofilm formation on different surfaces.

**Definition of biofilm formation**
Perhaps the most general definition of biofilm formation comes from Bill Costerton, who in 1995 defined biofilm-forming bacteria as “bacterial communities enclosed in a self-produced matrix adherent to each other and/or to surfaces or interfaces” (Costerton et al., 1995). If this definition is interpreted in the broadest sense, it implies that any bacterial aggregation does not occur randomly, but is a highly controlled process. Consequently, biofilm formation is assumed to be an intrinsic developmental process in the life cycle of almost any bacterium.

**How and when does Salmonella form biofilms? An educated perspective**
*S. enterica* has many opportunities to form multicellular communities commonly defined as biofilms (Figs 7.1 and 7.2).

In warm- and cold-blooded animals *S. enterica* inhabits the gastrointestinal tract as an asymptomatic transient or persistent colonizer, and occasionally causes acute, even fatal, disease (Fierer and Guiney, 2001). Transmission from animals to humans can occur upon close contact. For example, children who keep reptiles such as turtles as pet animals are a risk group for getting *Salmonella* infections (Dessi et al., 1992). More importantly, however, there is a pandemic problem of human food-borne salmonellosis. *Salmonella* is a most important agent causing acute food-borne disease through, for example, meat and eggs (Todd, 1997), which makes *Salmonella* a particular threat to human health. A significant fraction of food-borne salmonellosis is caused by *S. Enteritidis* and *S. Typhimurium* that colonize the intestinal tract of cattle and chicken flocks world-wide (Humphrey, 2004; Rabsch et al., 2002), and whereupon the bacteria presumably grow in biofilms in the intestinal tract. In chickens, transmission of *Salmonella* to the offspring is further enabled by the ability of certain serovars to invade the oviducts and to colonize the reproductive tissue, which causes the contamination of intact eggs (Okamura et al., 2001). Effective egg colonization is aided by a biofilm component (Cogan et al., 2004).

Somewhat surprisingly, other food-borne infections caused by *Salmonella* have occurred through seeds, sprouts, green leaves, fruits and vegetables as vehicles of transmission (Brandl and Mandrell, 2002). Indeed, fresh food outbreaks caused by *Salmonella* have been increasing in the last decade (Sivapalasingam et al., 2004). *Salmonella* has been shown to adhere and to colonize plant leaves and sprouts (Barak et al., 2002; Brandl and Mandrell, 2002). Up to 4% of vegetable samples can be contaminated with *Salmonella* spp. or *Shigella* spp. (Lindow and Brandl, 2003).

Also contamination of industrial facilities has been associated with food-born outbreaks of *Salmonella* (Werber et al., 2005). These outbreaks usually involve contamination of industrial facilities with multispecies biofilms, whereby *Salmonella* is the causative agent of pathogenicity (Wolfgang Rabsch, personal communication).
Salmonellosis and especially typhoid fever can also occur as water-borne infections. Persistence of *Salmonella* in water, and transmission of *Salmonella* by water, might involve biofilm formation (Rooney et al., 2004). In a riverine system most *Salmonella* spp. isolates have been found on the sediment, but not in the water (Murray, 1991). In relation to human salmonellosis, *S. Enteritidis* survives in household toilets as a biofilm for more than four weeks (Barker and Bloomfield, 2000).

Last but not least, the carrier state of *Salmonella enterica* serovar Typhi (*S. Typhi*) with a reservoir in the gall bladder has been associated with the organisms grown in biofilms on gall stones (Forbes and Cotton, 1984; Lai et al., 1992). Consequently the formation of biofilms is an integral part of the *S. enterica* life cycle occurring in host colonization, environmental persistence, transmission and the carrier state.
**Figure 7.2** Different models of biofilm formation to abiotic surfaces. (A) Steady state model of biofilm formation. The Crystal violet-stained *S. Typhimurium* MAE52 cells are attached to the polystyrene surface of the well of a 96-well plate. Note that most of the biofilm formed by growing the cells in Luria broth (LB) without salt medium forms at the air–medium interface, but not at the bottom of the well. (B) Continuous flow model of biofilm formation. The entire design of the flow system is shown on the top. A flow chamber with four parallel flow channels is shown in detail below. Reprinted from *Methods Enzymol.*, 310, Christensen BB, Sternberg C, Andersen JB, Palmer RJ Jr., Nielsen AT, Givskov M, Molin S, Molecular tools for study of biofilm physiology.: 20–42, 1999 with permission from Elsevier. (C) Pellicle formation at the air–liquid interface. Arrow points to the extensive bacterial mat at the air–liquid interface. LB without salt medium was used to grow *S. Typhimurium* MAE52.

**Expression of the biofilm phenotype by *Salmonella enterica* isolates**

The impact of biofilm formation of *S. enterica* has been demonstrated by epidemiological studies. When 800 isolates of *S. Typhimurium* and *S. Enteritidis* from acute infections were examined, almost all expressed a biofilm phenotype under certain growth conditions in the laboratory (Römling *et al.*, 2003b). The majority of *Salmonella* isolates from animals and vegetable also display biofilm formation and expression of extracellular matrix components (Solano *et al.*, 2002; Solomon *et al.*, 2005).

The formation of biofilms by isolates from disease, asymptomatic animal carriers and contaminated vegetable is usually highly regulated by environmental conditions such as temperature, oxygen tension and osmolarity (Gerstel and Römling, 2001; Gerstel *et al.*, 2003; Römling *et al.*, 1998b).

That is, production of biofilm components does usually not occur in normal rich laboratory medium at 37°C. Indeed, for unknown reasons, very few isolates display a so-called deregulated semi-constitutive biofilm phenotype (Collinson *et al.*, 1991; Römling, 2003b). On the other hand, host-restricted serovars *S. Typhimurium* var. Copenhagen phage type DT2 and DT99, *S. Typhi* and *S. Choleraesuis* do not express the aforementioned biofilm-associated rdar morphotype under laboratory conditions (Römling, 2003b). These latter observations suggest that the loss of certain biofilm traits is associated with disease where bacteria disrupt the epithelial cell lining.

**Resistance phenotype of biofilm-forming *Salmonella***

It is well documented that biofilm-associated microorganisms exhibit decreased
susceptibility to antimicrobial agents and treatment with disinfectants. Indeed, biofilm-associated Salmonella cells can be more resistant against antibiotics, sanitizers like sodium hypochlorite, iodine and triclosan, and disinfectants like ethanol, compared to their planktonic (suspended) counterparts (Gerstel and Römling, 2001; Joseph et al., 2001; Olson et al., 2002; Scher et al., 2005; Solano et al., 2002; Tabak et al., 2006). For instance, more than 10% of pellicle-associated Salmonella survived treatment with 50 p.p.m. of chlorine for 15 minutes, whereas planktonic cells at either the stationary or logarithmic phases of growth were completely killed by the same treatment (Scher et al., 2005).

The efficiency of sanitizer treatment can also vary depending on the particular surfaces studied. Biofilm-associated bacterial cells on stainless steel, for instance, are more sensitive to hypochloride and iodine compared to cells of the same strain adherent on plastic surfaces (Joseph et al., 2001). One might argue that the structural organization of the cells in a biofilm diminishes the access of the active chemical. However, a molecular study revealed that resistance of the cells in a pellicle to the broad-spectrum biocide triclosan was probably the result of stress adaptation (Tabak et al., 2006). Pellicle-associated cells overexpressed genes required for the efflux of antimicrobial agents such as those encoding the transcriptional activator MarA and its respective target, the efflux pump AcrAB, when compared to cells in the logarithmic and stationary phases of growth.

However, there is no general resistance of biofilm-forming Salmonella to different stress conditions. In contrast to the treatment with antimicrobials and disinfectants, heat and acidification did not differentially affect biofilm-associated bacteria as compared to their planktonic counterparts (Scher et al., 2005). Somewhat surprisingly, cells in a biofilm were more susceptible to ionizing radiation (Niemira and Solomon, 2005).

Biofilm-forming Salmonella also show enhanced resistance against stress conditions such as long-term desiccation (Gibson et al., 2006; White et al., 2006).

**Model systems of biofilm formation of Salmonella**

Many models to study biofilm formation of S. enterica in various situations have been developed. On the molecular level, biofilm formation by Salmonella has been characterized as a multicellular behavior termed rdar morphotype, as the adherence phenotype to abiotic surfaces, as a pellicle at the air–liquid interface, as adherence on gallstones, and as the colonization phenotype on epithelial cells and plant tissue (Fig. 7.1; Boddicker et al., 2002). Furthermore, biofilm-formation by S. Typhimurium on urethral catheter surfaces has also been investigated (Mireles et al., 2001), although this pathogen is not known to cause catheter-associated urinary tract infections. Such model systems have enabled in-depth molecular studies, which in turn have revealed that Salmonella forms biofilms with different characteristics on various surfaces in different media (Prouty and Gunn, 2003) showing a multifactorial potential of S. enterica for multicellular behavior.

**Molecular characterization of the rdar morphotype**

The rdar (red, dry and rough) morphotype is the model of multicellular behavior in S. Typhimurium and S. Enteritidis that has been best studied genetically (Collinson et al., 1997; Römling, 2005). The rdar morphotype is defined by a characteristic colony morphology type on Congo Red
agar plates, whereby the bacteria form a multicellular network characterized by the expression of extracellular matrix components (Fig. 7.1A; Römling, 2001). Bacteria expressing the rdar morphotype also show other types of multicellular behavior such as adherence to abiotic surfaces (glass and polystyrene) in steady state (Fig. 7.2A) and continuous (Fig. 7.2B) culture, cell clumping and pellicle formation, and colonization of the air–liquid interface (Fig. 7.2C).

The extracellular matrix of the rdar morphotype is composed of proteinaceous components and exopolysaccharides. Among the proteinaceous components that have been characterized are the adhesive curli fimbriae (alternatively called Taf or thin aggregative fimbriae (agf)) (Collinson et al., 1991; Römling et al., 1998a) and the large secreted protein BapA (Latasa et al., 2005; Römling et al., 1998b). Curli fimbriae are highly aggregative surface structures that bind various bacterial and host components (Rochon and Römling, 2006; Römling et al., 2003a). The major curli protein, CsgA, contains self-associating peptide repeats, which are assembled into fibril-like formations resembling amyloid characteristic of major disorders in humans such as Alzheimer’s and prion diseases (Chapman et al., 2002; Cherny et al., 2005; Lundmark et al., 2005). In biofilm formation, curli fimbriae mediate the adherence of Salmonella to abiotic surfaces (Austin et al., 1998; Römling et al., 1998b) and establish rigid, yet fragile, cell–cell interactions (Zogaj et al., 2001). Overexpression of curli fimbriae can compensate for the absence of BapA, which is loosely associated with the cell surface (Latasa et al., 2005).

The biofilm-associated exopolysaccharides (EPSs) include cellulose and a novel O-antigenic capsular polysaccharide (Gibson et al., 2006; Zogaj et al., 2001). In biofilm formation using the standing culture model, cellulose expression mainly mediates cell–cell interactions and the formation of a biofilm at the air–liquid interface (Zogaj et al., 2001). The capsule, which is distinct from the known EPS colanic acid, resembles the lipopolysaccharide O-antigen with some modifications (Gibson et al., 2006). Although the capsule apparently does not contribute to multicellular behavior, it plays a fundamental role in the protection of the cells against desiccation stress.

The proteinaceous and exopolysaccharide components interact with each other. Curli fimbriae interact with the cellulose to build up a rigid three-dimensional network structure between the bacterial cells. By analogy to the structural function of cellulose in trees, this network has been called “bacterial wood” (Zogaj et al., 2001). Salmonella also forms distinct biofilms in a model system using continuous culture conditions. Surprisingly, the role of curli fimbriae and cellulose is different in biofilm formation in a flow cell system compared to the steady state model (Figs 7.2C and 7.3). Curli fimbriae seem to contribute significantly neither to primary attachment of cells nor to the three dimensional structure of the mature biofilm. In contrast, lack of cellulose expression lead to an almost entire loss of primary attachment with only the formation of separated microcolonies after 24 hours of incubation.

Another recently described novel capsular polysaccharide of S. Typhimurium (de Rezende et al., 2005) has a unique sugar composition and is therefore distinct from the capsule described above (Gibson et al., 2006). This capsule is expressed both at 25°C and 37°C indicating that it is not associated with rdar morphotype expression (that is limited to temperatures below 28°C), and that its expression is not regulated by the master regulator CsgD (see be-
Figure 7.3 Biofilm formation of *S. Typhimurium* UMR1 and its cellulose (Δ*bcsA*) and curli (Δ*csgA*) deficient mutants in the continuous flow model of biofilm formation using M9 minimal medium. (A) Primary attachment of the cells visualized after 2 hours of incubation. The cellulose deficient mutant *S. Typhimurium* UMR1 Δ*bcsA* did not visually attach. Images were acquired by phase contrast microscopy. Representative figures are shown for two independent experiments. Magnification, x630. (B) The biofilm-forming capacity and architecture after 24 hours post-inoculation. Images were acquired via confocal scanning laser microscopy (CSLM). The large panels represent top-down views of the biofilms, and the side panels represent orthogonal cross-sections (XZ and YZ) taken along the green and blue lines, respectively. Representative figures are shown for two independent experiments. Magnification, x630.

Nevertheless, although not involved in primary attachment to the surface, the capsule may contribute to the extracellular matrix of the biofilm in a flow chamber (de Rezende *et al.*, 2005). The authors also speculate that the capsule may have a role in other features of *Salmonella*, such as virulence, as has been demonstrated previously for the capsules of other bacteria.

**Regulation of rdar morphotype expression by the master regulator CsgD**

Expression of the biofilm-associated matrix components is regulated by the master regulator CsgD, a transcriptional regulator of the LuxR superfamily (Fig. 7.4; Gerstel and Römling, 2003). The expression of CsgD itself is highly regulated by environmental conditions, and hence the rdar morphotype is not constantly expressed. Consequently, under laboratory conditions, rdar morphotype expression is observed when bacteria are grown on/in rich medium at low osmolarity under microaerophilic conditions or in a defined medium under aerobic conditions at ambient temperature. Besides temperature, osmolarity and oxygen tension, the pH, iron availability, starvation by phosphate, nitrogen, ethanol and other uncharacterized conditions.
Figure 7.4 Regulatory network of rdar morphotype expression in S. Typhimurium. The transcriptional regulator CsgD is a central regulator of the rdar morphotype. Expression of CsgD is positively (gray arrows) and negatively (black arrows) regulated by, among other proteins, two-component systems (EnvZ/OmpR, CpxA/CpxR and RcsCD/RcsBA), GGDEF-EAL domain proteins (STM2123, STM3388) and nucleoid-binding proteins (IHF, H-NS). The GGDEF-EAL domain proteins STM2123 and STM3388 regulate CsgD expression presumably also on a post-transcriptional level. In turn, CsgD activates the transcription of at least three target operons: csgBAC, adrA, bapABCD and represses transcription of the emcIJ operon in S. Typhimurium. Thereby, the csgBAC operon encodes the structural components CsgA and CsgB of curli fimbriae (Römling et al., 1998a). The bapABCD operon (previously stm2689/stm2690/91/92) encodes the large surface protein BapA and its type I protein secretion apparatus (Latasa et al., 2005). Expression of AdrA leads to the production of the second messenger c-di-GMP required for post-transcriptional activation of cellulose biosynthesis (Simm et al., 2004; Zogaj et al., 2001). Products of emcIJ (previously yihVW) repress the divergently regulated emcA-H operon (yihU-O yshA) required for the expression of an O-antigen capsule (Gibson et al., 2006).

Various global regulatory proteins, such as the nucleoid-binding protein H-NS, integration host factor (IHF), the two-component systems EnvZ/OmpR, CpxA/CpxR and RcsCD/RcsBA, the MerR-like regulator MlrA, the stress sigma factor RpoS and the GGDEF-EAL domain proteins STM2123 and STM3388 have been demonstrated to regulate CsgD expression (Fig. 7.4; Brown et al., 2001; Gerstel et al., 2003; Huang et al., 2006; Jubelin et al., 2005; Kader et al., 2006; Römling et al., 1998a).

How environmental conditions are translated into molecular events to control expression of CsgD is largely unknown. However, several of the above-mentioned
regulatory proteins can bind upstream of the csgD promoter in the 521 bp long intergenic region localized between the divergently transcribed curli biosynthesis operons csgBA and csgDEFG, and downstream of the csgD promoter in the open reading frame of csgD (Fig. 7.5; Gerstel and Römling, 2003, Gerstel et al., 2003, Dorel et al., 2006; Gerstel et al., 2006). Thus, one could envision a three-dimensional nucleoprotein complex being formed to tightly regulate csgD expression, but molecular details of the regulation of csgD expression are not resolved yet.

The best studied link between environmental cues and CsgD expression concerns microaerophilic conditions and activation of csgD transcription by IHF (Fig. 7.5; Gerstel et al., 2003). In rich medium transcription of csgD is activated under microaerophilic conditions in contrast to aerobic conditions. The enhanced transcription of csgD is partially dependent on the presence of IHF and the IHF1 binding site in the intergenic region (Gerstel et al., 2003). The IHF1 binding site overlaps with binding sites D3-D6 for the transcriptional regulator OmpR. Under aerobic conditions, OmpR binds to its binding sites D3-D6 and thus transcription of the csgDEFG operon remains repressed (Fig. 7.5). Under microaerophilic conditions IHF effectively competes with OmpR for binding to the operator sequences, and thus by binding to IHF1, IHF activates transcription (Fig. 7.5; Gerstel et al., 2003). In support of this model, two point mutations in highly conserved nucleotides in the IHF1 binding site, which is located approximately 200 bp upstream of the transcriptional start site, significantly decrease expression of the csgD promoter (Gerstel et al., 2006).

Figure 7.5 The nucleo-protein complex in the csgD promoter region. (A) Binding sites of proteins in the csgD promoter region. RNAP=RNA-polymerase. (B) Model of the activating effect of IHF at the csgD promoter under microaerophilic conditions. Under aerobic conditions, csgD transcription is repressed by the binding of OmpR to its binding sites D2 and D3-D6. Under those conditions, IHF does not play a role in transcriptional activation (Gerstel et al., 2003). Under microaerophilic conditions, csgD transcription is activated. IHF binding to IHF1 enhances the transcriptional activity. Still OmpR is able to bind to the D1, D2 and D7 binding sites.
Regulation of rdar morphotype expression by c-di-GMP

Recently, a novel bacterial secondary messenger, diguanylic acid (c-di-GMP), has been discovered which regulates the transition between sessility and motility; and sessility and virulence (Paul et al., 2004; Ross et al., 1987; Simm et al., 2004; Tischler and Camilli, 2004, 2005). The turnover of c-di-GMP is mediated by diguanylate cyclases (GGDEF domain proteins) (Paul et al., 2004; Simm et al., 2004) and phosphodiesterases (EAL and HD-GYP domain proteins) (Ryan et al., 2006; Schmidt et al., 2005; Simm et al., 2004). The rdar morphotype is regulated by c-di-GMP on various levels. First, the expression of CsgD is activated by the complex GGDEF-EAL domain proteins STM2123 and STM3388 (Fig. 7.4). Both proteins, when overexpressed, enhance the intracellular c-di-GMP concentration, thus acting as diguanylate cyclases (Kader et al., 2006).

The positive regulation of CsgD expression exerted by c-di-GMP takes place on a transcriptional and, presumably, on a post-transcriptional level. However, c-di-GMP signaling is also involved in the regulation of processes downstream of CsgD expression. Expression of AdrA, a GGDEF domain protein with di-guanylate cyclase activity, is transcriptionally activated by CsgD (Römling et al., 2000). Subsequently, AdrA activates cellulose biosynthesis post-transcriptionally through the production of c-di-GMP (Zogaj et al., 2001). AdrA is also partially responsible for the biosynthesis of the proteinaceous curli fimbriae downstream of CsgD expression (Kader et al., 2006). However, there is no positive feedback loop for c-di-GMP signaling, shown by the fact that mutational inactivation of adrA only downregulates expression of CsgA, but not expression of CsgD. Thus cyclic di-GMP signaling activates rdar morphotype expression on various levels in the regulatory network.

Biofilm formation of S. enterica on abiotic surfaces

Adhesion of Salmonella to abiotic surfaces is a model of biofilm formation and mimics the situation in industrial facilities. Adherence to Teflon (polytetrafluoroethylene) and stainless steel has not been investigated in detail, however, it has been demonstrated that curli fimbriae and possibly type 1 fimbriae are involved in adherence of S. Enteritidis to both types of surfaces (Austin et al., 1998). Investigation of the adherence process to glass and polystyrene surfaces has shown that several components required for rdar morphotype expression are also involved in adherence to such abiotic surfaces. In particular, adherence is regulated by the transcriptional regulator CsgD, which subsequently activates the expression of the extracellular matrix components cellulose, curli fimbriae and the surface protein BapA, all of which are required for adherence (Latasa et al., 2005; Römling et al., 1998b, 2000; Zogaj et al., 2001).

Not all adherence depends on CsgD. In the so-called ATM medium, the formation of biofilm and synthesis of cellulose is controlled by the expression of the GGDEF domain protein STM1987, whereas expression of STM1987 is not dependent on CsgD (Garcia et al., 2004; Solano et al., 2002).

The response regulator FimZ is another molecular link between sticking and swimming (Clegg and Hughes, 2002). Overexpression of FimZ leads to hyper-expression of type 1 fimbriae and nonmotile cells independent of fimbrial expression.
Biofilm formation at the air–liquid interface

Formation of a bacterial mat at the air–liquid interface, a pellicle, is another mode of multicellular behavior. Examination of the two faces of the pellicle biofilm revealed that the upper air–cell interface was smooth with the cells tightly arranged in a network. The cellular network on the liquid side was rougher and less tight, however, individual cells were wrapped by an extracellular matrix (Römling and Rohde, 1999). A recent study that used Atomic Force Microscopy (AFM) to characterize the morphology of the two sides of the Salmonella biofilm at the air–liquid interface confirmed the results of the surface structure of the pellicle (Kesselman et al., 2006).

A set of genes, similar to the set required for rdar morphotype expression, is required for the formation of a firm pellicle (Römling et al., 2000; Solano et al., 2002). Although not required for the local cell–cell interactions at the air–liquid interface, flagella play a crucial role in the formation of a complete mat structure (Römling and Rohde, 1999). Cells not expressing the biofilm components cellulose and curli fimbriae can still be incorporated into a pellicle (Scher et al., 2005). However, the presence of BapA is required for bacterial aggregation at the air–liquid interface and the production of microcolonies that are built up in the pellicle (Latasa et al., 2005).

Pellicle-derived cells have been used as a model system to investigate the effect of sanitizers and stress conditions on survival of biofilm cells (Scher et al., 2005; Tabak et al., 2006). Cells in a pellicle showed a significant increase in the transcription of bcsA, encoding the cellulose synthase, and bcsE, coding for another structural gene involved in cellulose biosynthesis upon exposure to triclosan (Tabak et al., 2006). This suggests that expression of cellulose is induced by triclosan or triclosan-induced stress. On the other hand, the expression of agfB, coding for a structural gene of curli fimbriae was not enhanced upon exposure to triclosan.

Biofilm formation by Salmonella on gallstones

The chronic carrier state of Salmonella is frequently associated with gallbladder abnormalities such as gallstones (Lai et al., 1992), to which Salmonella can adhere. Investigation of the adherence behavior has shown that S. Typhimurium formed a unique biofilm on gallstones consisting of loosely assembled matrices of cells, which were formed after 14 days (Prouty et al., 2002). Biofilm formation was dependent on the medium and the surface. Biofilm formation on gallstones was only observed when bile was added to the medium, suggesting that bile components signal the formation of biofilm. The bile-dependent biofilm was only formed on the gallstone, but not on a granite pebble, indicating specificity in attachment to certain gallstone surface structures (Prouty et al., 2002). Molecular analysis demonstrated that, although different genes were required for biofilm formation on gallstones and abiotic glass surfaces, some overlap exists (Prouty and Gunn, 2003). In particular, the exopolysaccharide cellulose and curli fimbriae, which are major determinants of the rdar morphotype and adherence to abiotic surfaces, are not required for attachment to gallstones. On the other hand, the PhoP/PhoQ two-component system represses biofilm formation on gallstones. In particular, the PhoP/PhoQ repressed gene prgH involved in the formation of the type III secretion system apparatus is required for biofilm formation. Based on additional experimentation, the authors hypothesize...
that secretion of exopolysaccharide or some effects of exopolysaccharide expression could be a novel function for the type III secretion system encoded by the *Salmonella* pathogenicity island 1 (Prouty and Gunn, 2003).

**Biofilm formation by *Salmonella* on epithelial cells**

Not all natural growth conditions of microorganisms can be mimicked adequately by steady state culture conditions. In fact, many natural growth habitats are better characterized by continuous transport of fluid. The most popular experimental setup developed for studying biofilm-formation under flow is the flow-cell device (Fig. 7.2B). In this device, the bacteria adhere to a glass cover slip, while a continuous flow of fresh medium is applied (Christensen et al., 1999). The major advantage of the flow-cell system is the continuous monitoring of biofilm development in real time without disturbance of the biofilm formation process. Recently, a flow-through continuous culture system has been developed whereby bacterial communities of *S. Typhimurium* were formed on a monolayer of Hep-2 cells (Fig. 7.1D). It has been suggested that this experimental setup mimics early events in the establishment of infection (Boddicker et al., 2002). As a first determinative component of biofilm formation, two amino acid changes in the FimH adhesin of type 1 fimbriae were identified that acted synergistically in the formation of biofilms on the epithelial cell monolayer (Boddicker et al., 2002). *S. Typhimurium* strains which express this particular adhesin on the tip of the type 1 fimbriae formed extensive biofilms on the surface of epithelial cells, while strains which express type 1 fimbriae with another FimH allele, yielded virtually no bacterial accumulation at all.

Two known exopolysaccharides produced by *S. Typhimurium*, colanic acid and cellulose, also contributed to biofilm formation on epithelial cells (Ledeboer and Jones, 2005). Without the expression of colanic acid the biofilm remained as a thin layer across the surface of Hep-2 cells. On the other hand, lack of cellulose biosynthesis resulted in the appearance of small isolated microcolonies on the epithelial cell surface. A requirement of the same factors, particular FimH variant, colanic acid and cellulose biosynthesis has been observed on biofilms formed on intestinal tissue of chickens (Ledeboer and Jones, 2005), indicating that Hep-2 cells grown in continuous flow cells might be a congruent model for biofilm formation in the intestine.

**Role of biofilm formation in bacterial–host interaction**

Several components of the *Salmonella* biofilm components play important roles in pathogen–host interactions. A *bapA* mutant was described as showing decreased adherence and invasion of the epithelial tissue in the intestine, whereas the ability of the mutant to colonize internal organs was not significantly changed (Latasa et al., 2005). Curli fimbriae are also required for the adherence to mouse small intestinal epithelial cells (Sukupolvi et al., 1997), and to promote efficient egg contamination (Cogan et al., 2004). Indirect evidence for expression of curli fimbriae during colonization of the gastrointestinal tract of resistant mice originated from a positive immune response against curli fimbriae after *Salmonella* infection (Humphries et al., 2005). Interestingly, CsgA aids fluid accumulation in the calf model of human gastroenteritis (Tukel et al., 2005). Further experimentation showed that the immune response initiated by curli fimbriae...
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Biofilm-related components also play a role in the systemic phase of the infection. The EAL domain-like protein STM1344 affects expression of the rdar morphotype (R. Simm and U. Römling, unpublished data), and can protect *S. Typhimurium* from the deleterious effect of phagocyte oxidase in infected mice, a source of reactive oxygen intermediates in macrophages (Hisert *et al.*, 2005). Further experimentation showed that STM1344 increased bacterial resistance to hydrogen peroxide *in vitro* and slowed down bacterial killing of macrophages.

While clearly selected components of biofilms seem to have distinct roles in virulence, biofilm formation itself has been identified as an antivirulence factor, since a strain with semi-constitutive expression of the rdar morphotype, due to a single point mutation in the *csgD* promoter, has shown an approx. 10-fold reduced virulence in mice (Römling *et al.*, 2000).
Screening of a pool of random transposon mutants deficient in plant colonization identified 20 unique insertions, with 65% being located in uncharacterized genes, indicating that exploration of a novel environment of *Salmonella*, the plant surface, can lead to detection of novel gene function (Barak *et al.*, 2005). Previously well-characterized components involved in the attachment process to plant surfaces were the stationary phase sigma factor RpoS and curli fimbriae. RpoS is also required for rdar morphotype expression and adhesion to abiotic surfaces (Römling *et al.*, 1998a; Römling *et al.*, 1998b). RpoS was required for initial attachment of *Salmonella* to alfalfa sprouts, but a rpoS mutant reached levels similar to the wild type after two days of incubation. Curli fimbriae are required for various forms of attachment: bacterial cell–cell interaction in rdar morphotype expression, adhesion of the bacteria to abiotic surfaces and adhesion of *Salmonella* to host epithelial cells (Römling *et al.*, 1998a; Römling *et al.*, 1998b; Sukupolvi *et al.*, 1997), yet defined deletions in csgA and csgB showed that only a csgB mutant was affected in initial attachment to alfalfa sprouts as well as colonization over time (Barak *et al.*, 2005).

Previous findings have shown that, while *Salmonella* adhered tightly to alfalfa sprouts, enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) could be easily washed away (Barak *et al.*, 2002). Since the EHEC strain used in this study contained a non-functional csgD gene and therefore does not express curli fimbriae, the authors suggested that bacterial strains producing curli fimbriae are more likely to attach at high density to plant tissue (Barak *et al.*, 2005).

Mutational deletion of the extracellular matrix components cellulose and curli fimbriae did not affect the ability of *Salmonella* to attach to parsley. Even so, *Salmonella* which produced the extracellular matrix components cellulose and curli fimbriae, showed improved persistence after chlorination treatment of the parsley (Lapidot *et al.*, 2006). However, the mutant deficient in the expression of the extracellular matrix components still colonized parsley in significant numbers, indicating that additional factors contribute to the survival of *Salmonella* on parsley.

**Conclusions**

*Salmonella enterica* forms biofilm at a variety of surfaces, and the diversity of biofilm formation by *S. enterica* is just being explored. The formation of biofilms plays a role in host colonization, virulence and the carrier state. Colonization of cattle and chickens by *S. enterica* leads to contamination of meat and eggs, which are subsequent vectors for transmission of the pathogen to humans. In nature, *S. enterica* persists in biofilms in natural and artificial man-made environments. Through modern methodology in food production and handling, novel vehicles for the transmission of *S. enterica* arise, causing food-borne outbreaks with plants as novel vectors. Transmission of *S. enterica* by fresh fruit and vegetables requires efficient colonization of the plant surfaces. Contamination of industrial surfaces by *S. enterica* leads to the contamination of processed food such as chocolate. To date, biofilm formation by *S. enterica* has been studied mainly in monoculture on the different surfaces. However, under natural conditions *S. enterica* mainly exists in mixed culture biofilms. Those biofilms are just beginning to be explored (Esteves *et al.*, 2005; Brandl, 2002; Jones and Bradshaw, 1997).

The rdar morphotype, a multicellular behavior expressed by the majority of
Biofilms of *Salmonella enterica* S. *enterica* isolates, is a convenient model system to study the regulation of biofilm formation. A complex regulatory network for rdar morphotype in *S. enterica* has been characterized in which environmental conditions and global regulatory proteins tightly regulate expression of the master regulator of rdar morphotype formation, the transcriptional regulator CsgD. CsgD is also required for the colonization of plant and abiotic surfaces, and the air–liquid interface, while extracellular matrix components are necessary for surface colonization and environmental persistence.

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References


Biofilms of *Salmonella enterica*


