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Salmonella biofilms: An overview on occurrence, structure, regulation and eradication

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ABSTRACT

The ability of *Salmonella* to form complex surface-associated communities, called biofilms, contributes to its resistance and persistence in both host and non-host environments and is especially important in food processing environments. In this review, the different types of abiotic (plastic, glass, cement, rubber, and stainless steel) and biotic surfaces (plant surfaces, epithelial cells, and gallstones) on which *Salmonella* biofilms have been described are discussed, as well as a number of commonly used laboratory setups to study *Salmonella* biofilm formation (rdar morphotype, pellicle formation, and biofilms on polystyrene pegs). Furthermore, the structural components important during *Salmonella* biofilm formation are described (curli and other fimbriae, BapA, flagella, cellulose, colanic acid, anionic O-antigen capsule and fatty acids), with special attention to the structural variations of biofilms grown on different surfaces and under different conditions. Indeed, biofilm formation is strongly influenced by different environmental signals, via a complex regulatory network. An extensive overview is given on the current understanding of this genetic network and the interactions between its different components (CsgD, RpoS, Crl, OmpR, IHF, H-NS, CpxR, MlrA, c-di-GMP, BarA/SirA, Csr, PhoPQ, RstA, Rcs, metabolic processes and quorum sensing). To further illustrate that biofilm formation is a mechanism of *Salmonella* to adapt to different environments, the resistance of *Salmonella* biofilms against different stress factors including desiccation stress, disinfectants (*e.g.* hypochlorite, glutaraldehyde, cationic tensides and triclosan) and antibiotics (*e.g.* ciprofloxacin) is described. Finally, a number of *Salmonella* biofilm inhibitors, identified through bottom-up- and top-down-approaches, are discussed, such as surfactin, glucose, halogenated furanones, 4(5)-aryl 2-aminoimidazoles, furocoumarins and salicylates. Also the potential of combination therapy (*e.g.* combinations of triclosan and quaternary ammonium salts or halogenated furanones and antibiotics/disinfectants) and nano- and micro-emulsions to inhibit *Salmonella* biofilm formation is discussed. Insight into the pathogen's complex biofilm process will eventually lead to further unraveling of its intricacies and more efficient strategies to combat *Salmonella* biofilms.

1. Introduction

It is now commonly accepted that biofilms are the predominant mode of bacterial growth, reflected in the observation that approximately 80% of all bacterial infections are related to biofilms (National Institutes of Health (USA)) (Davies, 2003; Hall-Stoodley & Stoodley, 2009). Biofilms are defined as structured communities of bacterial cells enclosed in a self-produced polymeric matrix adherent to inert or living surfaces (Costerton, Stewart, & Greenberg, 1999; Donlan & Costerton, 2002; Hall-Stoodley et al., 2006; Homoe, Bjarnsholt, Wessman, Sorensen, & Johansen, 2009). Bacteria in biofilms are generally well protected against environmental stresses, antibiotics (Hoiby, Bjarnsholt, Givskov, Molin, & Ciofu, 2010), disinfectants and the host immune system (Jensen, Givskov, Bjarnsholt, & Moser, 2010) and as a consequence are extremely difficult to eradicate (Burmolle et al., 2010). Biofilm formation by *Pseudomonas aeruginosa* in the lungs of patients suffering from cystic fibrosis is a classic example of biofilm involvement in chronic infections. Because the bacteria assemble in biofilms, this chronic infection is often noncurable and eventually results in the death of CF patients (Bjarnsholt et al., 2009; Hassett et al., 2010). Given the extent of problems caused by biofilms, there has been a significant effort to develop new anti-biofilm strategies (Bjarnsholt, Tolker-Nielsen, Hoiby, & Givskov, 2010; Landini, Antoniani, Burgess, & Nijland, 2010). The general features of biofilm formation do also apply to the enteric pathogen *Salmonella*. *Salmonella* follows a cyclic lifestyle in which host colonization is alternated with periods of survival outside the host (Winfield & Groisman, 2003). Here, we describe the current knowledge of how biofilm formation contributes to both host colonization by *Salmonella* as well as to its survival in non-host conditions. In the first section, we discuss the different host and non-

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host environments in which *Salmonella* biofilms have been encountered. Subsequently, a detailed overview of the current knowledge of the structural organization as well as the complex regulation of *Salmonella* biofilm formation is given. In the next section, the resistance of *Salmonella* biofilms against various stress factors such as desiccation, disinfectants and antibiotics is illustrated and some mechanisms of the acquired resistance are proposed. Finally, we describe a number of bottom-up and top-down approaches that have been followed to develop strategies to prevent and combat *Salmonella* biofilms.

2. Occurrence of *Salmonella* biofilms

Salmonella biofilms are encountered on many biotic and abiotic surfaces and becomes clear from the examples cited below. In addition, a number of commonly used laboratory set-ups to study *Salmonella* biofilm formation are described.

2.1. Abiotic surfaces

Several reports have demonstrated the ability of *Salmonella* strains to form biofilms on abiotic surfaces outside the host such as plastic (Hurrell, Kucerova, Loughlin, Caubilla-Barron, & Forsythe, 2009; Joseph, Otta, Karunasagar, & Karunasagar, 2001; Mireles, Toguchi, & Harshey, 2001; Stepanovic, Cirkovic, Ranin, & Svabic-Vlahovic, 2004; Vestby, Moretro, Langsrud, Heir, & Nesse, 2009), rubber (Arnold & Yates, 2009), cement (Joseph et al., 2001), glass (Prouty & Gunn, 2003; Solano et al., 2002) and stainless steel (Giaouris & Nychas, 2006; Joseph et al., 2001; Moretro et al., 2009; Ramesh, Joseph, Carr, Douglass, & Wheaton, 2002), which are commonly encountered in farms, slaughter houses, food processing industry, kitchens, toilets and bathrooms. Joseph et al. for example demonstrated the ability of *S.* Weltevreden to form biofilms on plastic (polyethylene), cement and stainless steel (Joseph et al., 2001). Consistently, Stepanovic et al. tested biofilm formation of 122 *Salmonella* spp., isolated from humans, animals and food and found that all strains were able to form biofilms on polystyrene microplates (Stepanovic et al., 2004), while Giaouris and Nychas demonstrated the ability of *Salmonella* Enteritidis PT4 to form biofilms on stainless steel (Giaouris & Nychas, 2006). As *Salmonella* biofilms are more resistant to several environmental stress factors such as desiccation and disinfectants, the ability of *Salmonella* to form biofilms on these surfaces likely contributes to the survival in non-host environments and the transmission to new hosts. In support of this hypothesis, Vestby et al. found a correlation between the biofilm formation capacity of 111 *Salmonella* strains isolated from feed and fish meal factories and their persistence in the factory environment (Vestby, Moretro, Langsrud, et al., 2009). Another marked example is provided by Barker and Bloomfield, who studied the survival of *Salmonella* in toilets and bathrooms in homes, where a family member had recently suffered an attack of salmonellosis (Barker & Bloomfield, 2000). They found that *Salmonella* bacteria became incorporated into biofilm material in the toilet bowl and could persist up to 4 weeks after the diarrhea had stopped, despite the use of cleaning products.

2.2. Plant surfaces

Although plants are traditionally not considered as hosts for human enteric pathogens, numerous recent *Salmonella* outbreaks (listed and reviewed in (Berger et al., 2010; Heaton & Jones, 2008; Sivapalasingam, Friedman, Cohen, & Tauxe, 2004)) in industrialized countries have been associated with contaminated sprouted seeds (*e.g.* alfalfa (Mahon et al., 1997; Taormina, Beuchat, & Slutsker, 1999; Van Beneden et al., 1999)), fresh vegetables and fruits (*e.g.* cantaloupe (Bowen, Fry, Richards, & Beuchat, 2006), and cilantro (Brandl & Mandrell, 2002; Campbell et al., 2001)). Over the years, it has become clear that *S. enterica* is able to colonize various parts of a variety of plant species ranging from seeds (Mahon et al., 1997) over sprouts (O'Mahony et al., 1990), leaves (Campbell et al., 2001) and roots (Klerks, Franz, van Gent-Pelzer, Zijlstra, & van Bruggen, 2007) to even fruits (Guo, Chen, Brackett, & Beuchat, 2001), making plants important vectors for *Salmonella* transmission between hosts. In this context, it has been demonstrated that *S. enetrica* soil contaminations (via contaminated irrigation water and raw manure) can lead to plant contamination of a variety of agricultural crops (*e.g.* tomatoes (Barak & Liang, 2008), lettuce, parsley, radish and carrot (Islam et al., 2004a,b)) up to six months and more after the contamination event, showing the highly persistent nature of *Salmonella* in plant environments (Teplitski, Barak, & Schneider, 2009). Since the survival and persistence of *Salmonella* and other enteropathogens as epiphytes on external plant surfaces is largely affected by their ability to adapt to this 'new' ecological niche (Beuchat, 2002), it is of great importance to get molecular insight into the attachment of human pathogens (*Salmonella* in particular) to various plant tissues in order to organize effective prevention and mitigation. As such, the behaviour of enteropathogens in the phyllosphere became a growing field of research and several studies (*e.g.* (Brandl & Mandrell, 2002; Brandl, Miller, Bates, & Mandrell, 2005; Fett, 2000; Heaton & Jones, 2008; Iturriaga, Tamplin, & Escartin, 2007; Kroupitski, Pinto, Brandl, Belausov, & Sela, 2009; Lapidot, Römling, & Yaron, 2006; Rayner, Veeh, & Flood, 2004)) provided evidence for biofilm formation by *Salmonella* and other human pathogens on plant surfaces. Despite the efforts, little molecular knowledge has emerged, although some interesting studies were reported (*e.g.* (Barak, Gorski, Liang, & Narm, 2009; Barak, Gorski, Naraghi-Arani, & Charkowski, 2005; Barak, Jahn, Gibson, & Charkowski, 2007; Lapidot & Yaron, 2009)). Barak and colleagues, for instance, exploited an optimized *Salmonella*/alfalfa sprout attachment assay to study the genetic basis of *Salmonella* (*S.* Newport and *S.* Enteritidis) attachment to and biofilm formation on alfalfa (Barak et al., 2005, 2007, 2009), as will be discussed below. Next to molecular insight, efficient visualization is of crucial importance as well. Recent microscopic techniques such as *e.g.* episcopic differential interference contrast microscopy coupled to epifluorescence allowed proper *in situ* visualization of bacterial biofilms, as exemplified by the detection of *S.* Thompson on lettuce leaves (Warner, Rothwell, & Keevil, 2008). Another example was provided by Kroupitsky et al. who used confocal microscopy to show that cut surfaces of leaves were the preferential attachment sites of *Salmonella* (Kroupitski, Pinto, et al., 2009), being an important possible contamination route in post harvest processing settings.

Remarkably, several reports demonstrated that enteric pathogens associated with plants are not killed by various surface sterilization methods (Beuchat, Ward, & Pettigrew, 2001; Proctor, Hamacher, Tortorello, Archer, & Davis, 2001; Van Beneden et al., 1999; Weissinger & Beuchat, 2000; Weissinger, McWatters, & Beuchat, 2001). This not only indicates the impact of epiphytic aggregation and biofilm formation, but also stresses the importance of endophytic growth (*i.e.* inside the plant tissue) (Dong, Iniguez, Ahmer, & Triplett, 2003; Gandhi, Golding, Yaron, & Matthews, 2001; Klerks, van Gent-Pelzer, Franz, Zijlstra, & van Bruggen, 2007; Lang, Harris, & Beuchat, 2004). Endophytic bacteria colonize the inner tissues of plants without entering host cells, causing disease or forming symbiotic structures. Iniguez et al., for example, calculated that human consumption of 10 g of surface-sterilized alfalfa sprouts but yet contaminated with *S.* Typhimurium ATCC14028 was sufficient to cause salmonellosis, highlighting the importance of endophytic *Salmonella* (Iniguez et al., 2005). Kutter et al. even showed the ability of *S.* Typhimurium LT2 and DT104h to spread systemically, after root colonization, in barley, using a culture-independent PCR-based detection technique in an axenic system (Kutter, Hartmann, & Schmid, 2006). Examples generated by different research groups clearly illustrate that the extent of endophytic colonization is determined by both plant and microbe genotypes (Tyler & Triplett, 2008) and that, although plants

may be able to modulate colonization by human enteric pathogens, they do not recognize them as potentially pathogenic and as such host defense mechanisms preventing colonization are not activated (Berger et al., 2010). Whether or not *Salmonella* is able to form or incorporate in biofilms inside plant tissue remains to be shown. In this context, bacterial biofilm formation inside plants has been shown for some vascular plant pathogens (*e.g. Xylella fastidiosa* (Andersen, Brodbeck, Oden, Shriner, & Leite, 2007)) and is usually short-lived, as blockage of the plant vascular system leads to a rapid plant death from wilt or similar symptoms. Recently however, apoplast biofilm formation of *Gluconacetobacter diazotrophicus*, a nitrogen-fixing endophyte of sugarcane, has been hypothesized (Velazquez-Hernandez et al., 2011). Although *Salmonella* intrinsically differs from these mentioned bacteria (in terms of taxonomy and host adaptation), evidence generated by Schikora and co-workers indicated that *S.* Typhimurium might be seen as a true plant endopathogen overcoming the innate *Arabidopsis* immune responses, entering and proliferating inside plant tissue and even causing disease symptoms (Schikora, Carreri, Charpentier, & Hirt, 2008), making it a plausible candidate for biofilm formation inside plants. Recent findings by Lapidot and Yaron (2009) supporting this *Salmonella* endophytic biofilm forming notion, showed the ability of *S.* Typhimurium to form small aggregates at a depth of 8 to 32 μm beneath the upper leaf surface.

2.3. Animal epithelial cells

Salmonella is also able to adhere to and subsequently form bacterial communities, microcolonies and even mature biofilms on epithelial cells. This likely plays a significant role in the establishment and persistence of mucosal infections in appropriate hosts and is a possible cause of intestinal carriage in domestic animals (*e.g.* (Althouse, Patterson, Fedorka-Cray, & Isaacson, 2003; Morgan et al., 2004; Ricke, 2003)). In this context, Boddicker et al. optimized a continuous *in vitro* flow system to study *S.* Typhimurium biofilm formation on a confluent monolayer of epithelial-like HEp-2 cells, which mimics the early events in establishment of infection in appropriate *Salmonella* hosts (Boddicker, Ledeboer, Jagnow, Jones, & Clegg, 2002). Using this system they noticed that *S.* Typhimurium is able to outcompete and displace *E. coli* after heterologous infection and biofilm formation, respectively (Esteves, Jones, & Clegg, 2005). In addition, genetic determinants important for *S.* Typhimurium biofilm formation on epithelial cells were identified (Boddicker et al., 2002; Ledeboer, Frye, McClelland, & Jones, 2006; Ledeboer & Jones, 2005) and will be discussed below. The same genetic determinants were shown to be important using a more realistic chicken intestinal tissue biofilm model, further stressing the relevance of the *in vitro* HEp-2 model system as an accurate approximation (Ledeboer & Jones, 2005). Subsequent real *in vivo* studies using poultry, showed the *in vivo* relevance of the used model systems (Ledeboer et al., 2006).

2.4. Gallstones

Gallstones are another well documented biotic surface on which *Salmonella* is able to form biofilms. After crossing the intestinal epithelial layer *S.* Typhi is able to invade macrophages, which can carry the pathogen to the liver from where it can be shed into the gallbladder (as recently reviewed by (Tsolis, Young, Solnick, & Baumler, 2008)). *S.* Typhi is the etiologic agent of human typhoid fever, annually affecting around 20 million people worldwide. Once in the gallbladder, this pathogen can either cause an active (cholecystitis) or a chronic infection (carrier state). Around 5% of the people infected with *S.* Typhi become asymptomatic chronic gallbladder carriers. Shedding of *Salmonella* by these asymptomatic carriers can contaminate food and water supplies, especially in underdeveloped countries, and as such be a source of recurring *Salmonella* infections. This chronic carrier state is hard to cure with antibiotics and is often associated with gallbladder abnormalities, such as gallstones to which *Salmonella* can adhere (Dutta, Garg, Kumar, & Tandon, 2000; Lai, Chan, Cheng, Sung, & Leung, 1992; Levine, Black, & Lanata, 1982). As such, surgical gallstone and often even gallbladder removal is the only effective way to cure patients from these chronic infections. Gunn and colleagues have shown that *S.* Typhimurium, Typhi and Enteritidis can form fully developed biofilms on gallstone surfaces within 14 days *in vitro* in a bile-dependent, surface-specific way (Prouty, Schwesinger, & Gunn, 2002). They even noticed that *Salmonella* biofilm formation on glass slides was enhanced if bile was added to the culture medium, suggesting a biofilm directed signalling function for this complex liver digestive secretion product. Although bile has emulsifying and antimicrobial properties, *Salmonellae* are resistant to it and are even able to use it as an environmental signal affecting their virulence properties (Gunn, 2000). Next to this gallstone model, Gunn and colleagues recently developed a highly consistent, *in vitro*, gallstone-independent, cholesterol-coated Eppendorf tube assay to study gallstone-based *Salmonella* biofilm formation (Crawford, Gibson, Kay, & Gunn, 2008). This *in vitro* system has some advantages over the human gallstone system with efficiency and high-throughput character being the most important ones. Molecular analysis using both systems demonstrated some important determinants for gallstone-associated biofilm formation, as will be discussed in the next section (Crawford, Reeve, & Gunn, 2010; Crawford et al., 2008; Prouty & Gunn, 2003; Prouty et al., 2002). Using this new model system, it also became clear that the patchy, surface specific biofilm distribution on gallstones was due to local differences in cholesterol distribution and that bile also induced the biofilm formation on cholesterol. Recently, Crawford et al. provided *in vivo* evidence that gallstones indeed play an important role in *Salmonella* gallbladder colonization and carriage through the formation of *Salmonella* biofilms on the surface of these cholesterol coated structures, using an appropriate Nramp1$^{+/+}$ (*Salmonella*-resistant) murine model and clinical evidence of asymptomatic human carriers (Crawford, Rosales-Reyes, et al., 2010). All this *in vitro* and *in vivo* evidence fulfills the Hall-Stoodley and Stoodley criteria (Hall-Stoodley & Stoodley, 2009), based on the earlier formulated Parsek and Singh criteria (Parsek & Singh, 2003), for diagnosing biofilm infections from clinical specimens, suggesting typhoid biofilms on gallstones are indeed facilitating *S.* Typhi carriage in infected humans. Briefly, these diagnostic criteria for biofilm related bacterial infections state that (1) the pathogens are surface-associated, (2) the infected tissue demonstrates aggregated cells in clusters encased in a matrix, (3) the infection is confined to a particular site in the host organism, (4) the bacteria residing in these biofilms show recalcitrance to antibiotic treatment despite susceptibility of their planktonic counterparts, (5) culture-negative results might be obtained, despite a clinically documented high suspicion of infection, (6) ineffective host clearance occurs as evidenced by the location of macrocolonies in discrete host tissue areas associated with host inflammatory cells.

2.5. Laboratory biofilm set-ups

Pellicle formation at the air–liquid interface in Luria-Bertani (LB) growth conditions (rich medium, 28 °C, up to 96 h, non-shaking incubation) is a widely used laboratory manifestation of *Salmonella* biofilm formation (Römling & Rohde, 1999; Solano et al., 2002). Differences between both sides of the pellicle were noticed with the layer facing the air and liquid being smooth and less smooth, respectively. Under adherence test medium (ATM) conditions (nutrient-deficient medium, 37 °C, 4 h, shaking incubation), *Salmonella* biofilms could be visualized as a ring of strongly attached cells to the glass wall at the air–liquid interface (Solano et al., 2002). The specific composition of the extracellular matrix under these conditions will be discussed in the next section.

The rdar (red, dry and rough) morphotype is another extensively studied laboratory appearance of *Salmonella* multicellular behaviour, resulting from patterned, aggregative colonies when grown on media containing Congo Red (CR) linked with the expression of curli fimbriae and cellulose (Gerstel & Römling, 2003; Römling, 2005; Römling, Pesen, & Yaron, 2007). Normally this morphotype is only expressed under specific environmental conditions: at ambient temperatures (below 30 °C) on agar plates containing rich medium without salt (nutrient-limiting, low osmolarity) or at 37 °C on iron-depletion media. Exceptions on these conditions, however, are possible and will be discussed throughout the text. Furthermore, cells expressing this colony morphotype on solid medium are often also characterized by cell clumping in liquid culture, pellicle formation at the air–liquid interface and biofilm formation on abiotic surfaces (Austin, Sanders, Kay, & Collinson, 1998; Römling, Rohde, Olsen, Normark, & Reinkoster, 2000; Römling, Sierralta, Eriksson, & Normark, 1998; Solano et al., 2002). Later on, it became clear that this phenotype could also be visualized on standard trypticase soy agar (TSA) at ambient temperatures by the formation of similar colony surface patterns (rugose phenotype) (Anriany, Weiner, Johnson, De Rezende, & Joseph, 2001). This rugose phenotype (and not its smooth variant) also correlated with the ability to form surface pellicles and biofilms in glass flasks, both at ambient temperature and low osmolarity conditions (tryptic soy broth (TSB) at 25 °C), and rugose cells adhered better to polystyrene surfaces as compared to their smooth variants (Anriany et al., 2001; de Rezende, Anriany, Carr, Joseph, & Weiner, 2005). In relation to the *Salmonella* lifecycle, it was shown that aggregation via the rdar phenotype was not an obvious virulence adaptation strategy for *S.* Typhimurium, but rather an environmental persistence strategy (White, Gibson, Kim, Kay, & Surette, 2006; White et al., 2008). Another obvious characteristic of this morphotype and the major reason for its persistent character is the production of an abundant extracellular matrix consisting of proteinaceous compounds and exopolysaccharides. The exact composition of this matrix and the relation between matrix compounds and different appearances of this morphotype (rdar (curli and cellulose expressed), pdar (cellulose expressed), bdar (curli expressed), saw (curli nor cellulose expressed)) will be discussed throughout the next sections.

Different studies have been conducted to determine the prevalence of the *Salmonella* rdar morphotype among natural isolates from *S. enterica* subgroup I, containing 99% of all *Salmonella* serovars and all major human pathogens. Solano et al. found differences among 204 natural *S.* Enteritidis isolates originating from food, environmental, animal and clinical samples considering their biofilm forming abilities: 97% and 71% were able to form a biofilm under ATM and LB conditions respectively, while only 66% showed the rdar morphotype (Solano et al., 2002). Moreover, they showed that calcofluor (CF) binding and subsequent fluorescence under long-wave UV light, indicative for cellulose production, could be used as an easy screening method for isolating biofilm-deficient *S.* Enteritidis strains. Römling et al. showed that the majority (more than 90% of 800 strains) of human disease-associated *S.* Typhimurium and *S.* Enteritidis (isolated from patients, food and animals) displayed the rdar morphotype at 28 °C, but just rarely at 37 °C (Römling et al., 2003). Moreover, they noticed that most strains expressing the saw morphotype (approximately 10% of the tested strains) belonged to *S.* Typhimurium var. Copenhagen, an invasive *Salmonella* variant in pigeons. Other invasive *Salmonella* strains such as *S.* Typhi (human-adapted) and *S.* Choleraesuis (pig-adapted), causing systemic disease in their respective hosts, also produced this saw morphotype, indicating that loss of curli and cellulose production might be a way to evade host defenses leading to systemic infections. Solomon et al. showed the ability of 72% of 71 strains, from 28 different serovars, of *S. enterica* originating from produce, meat or clinical sources, to express the rdar morphotype (Solomon, Niemira, Sapers, & Annous, 2005). Malcova et al. identified that 66% of 96 *S.* Typhimurium isolates expressed the rdar morphotype, but also noticed a sbam (smooth, brown and mucoid) morphotype (Malcova, Hradecka, Karpiskova, & Rychlik, 2008). Furthermore, White et al. identified 80.5% of the natural isolates, including *S.* Typhi strains, of the *Salmonella* reference collection B (SARB) (Boyd et al., 1993) forming the rdar morphotype (White et al., 2006). Vestby et al. found that 74% of 148 *Salmonella* strains, isolated from feed industry, clinical and reference collections, showed rdar expression and up to 55% of *S.* Agona displayed a bdar morphotype (Vestby, Moretro, Ballance, Langsrud, & Nesse, 2009). In another study, White and Surette analyzed the genetic and phenotypic conservation of the rdar morphotype throughout the entire *Salmonella* genus and noticed that the rdar morphotype was conserved in 79% of 96 isolates representing all 7 *Salmonella* groups (*Salmonella* reference collection C 96, SARC96) (Boyd, Wang, Whittam, & Selander, 1996) (White & Surette, 2006). This number was reduced to 31% when a reference set of 16 strains (SARC16) was used. Altogether, it can be concluded that most natural *Salmonella* isolates are able to produce the most important extracellular matrix components curli and cellulose, which, can be seen as an important characteristic for extracellular survival.

Another frequently used, more high-throughput, experimental setup can be found in biofilm formation on the walls and bottoms of microtiter plate wells. In a more practical alternative, biofilms are formed on the polystyrene pegs of the Calgary Biofilm Device. This system consists of a platform carrying 96 polystyrene pegs that fits as a microtiter plate lid with a peg hanging into each well of the microtiter plate (Ceri et al., 1999; De Keersmaecker et al., 2005). Providing a link between *in vitro* and *in planta* biofilm formation, it was noticed that strong *Salmonella* biofilm producers on polystyrene attached better to lettuce leaves (Kroupitski, Pinto, et al., 2009; Patel & Sharma, 2010), suggesting that the high-throughput polystyrene test system may provide a suitable prediction model for *Salmonella*–lettuce (and maybe even *Salmonella*–plant) interactions.

3. Structural components of *Salmonella* biofilms

The extracellular matrix components of *Salmonella* biofilms vary considerably with the used biofilm set-up and the applied environmental conditions (as can be seen from Table 1). The rdar morphotype is the best studied form of *Salmonella* multicellular behaviour with respect to regulation and exopolysaccharide (EPS) composition. However, one should be cautious when generalizing themes about biofilm regulation and/or EPS composition between different test systems as will become clear later on in this overview. For example, comparison between the rdar phenotype, which is an agar-based multicellular phenotype, and other biofilm test systems, often liquid-based, might sometimes be problematic since it has been shown that almost 30% of the *S.* Typhimurium functional genome is differentially regulated between agar and broth culturing (Wang, Frye, McClelland, & Harshey, 2004). The rdar colonies are structurally composed of proteinaceous compounds and exopolysaccharides. The proteinaceous fraction consists of adhesive curli fimbriae (alternatively called Tafi or thin aggregative fimbriae (*agf*) in *Salmonella*) (Römling, Bian, Hammar, Sierralta, & Normark, 1998) and the secreted BapA protein (Latasa et al., 2005). The EPS fraction is largely made up by cellulose (Zogaj, Nimtz, Rohde, Bokranz, & Römling, 2001), but also contains an O-antigenic capsule (O-Ag-capsule) (Gibson et al., 2006) and additional expolysaccharides such as another capsular polysaccharide (de Rezende et al., 2005) and lipopolysaccharide (LPS) (Anriany, Sahu, Wessels, McCann, & Joseph, 2006; de Rezende et al., 2005; Gibson et al., 2006; White, Gibson, Collinson, Banser, & Kay, 2003). Fimbriae (type 1 fimbriae, plasmid encoded fimbriae (Pef), curli fimbriae (Csg), long polar fimbriae (Lpf), bovine colonization factor (Bcf) and Sth), colanic acid and cellulose are indispensable for the formation of *Salmonella* biofilms on epithelial cells (Boddicker et al., 2002; Ledeboer & Jones, 2005; Ledeboer et al., 2006), while flagella (but not flagellar motility *per se*), the O-Ag-capsule and to a lesser extent fimbriae appear to be the main structural biofilm components on hydrophobic gallstone surfaces (Crawford, Reeve et al., 2010; Crawford et al., 2008; Prouty & Gunn, 2003; Prouty et al., 2002). Cellulose, an intact

Table 1
Most important and already experimentally validated structural determinants important for *Salmonella* biofilm formation on particular surfaces.

Surface	Structural determinants important for *Salmonella* biofilm formation on this particular surface	Reference
Agar plates (rdar morphotype)	curli (*csgDEFG–csgBAC*)	(Römling, Bian, et al., 1998)
	BapA (*bapABCD*)	(Latasa et al., 2005)
	Cellulose (*bcsABZC–bcsEFG*)	(Zogaj et al., 2001)
	O-Ag-capsule (*yihU–yshA* and *yihVW*)	(Gibson et al., 2006)
	Other capsular polysaccharide	(de Rezende et al., 2005)
	LPS	(Y. Anriany et al., 2006; de Rezende et al., 2005; Gibson et al., 2006; White et al., 2003)
Epithelial cells	Type 1 fimbriae (*fim*)	(Boddicker et al., 2002; Ledeboer & Jones, 2005; Ledeboer et al., 2006)
	Plasmid encoded fimbriae (*pef*)	
	Curli fimbriae (*csg*)	
	Long polar fimbriae (*lpf*)	
	Bovine colonization factor (*bcf*)	
	Sth fimbriae (*sth*)	
	Colanic acid (*wca* genes and *wza*, *wzb* and *wzc*)	
	Cellulose (*bcsABZC–bcsEFG*)	
Gallstones	Flagella	(Crawford, Reeve, et al., 2010; Crawford et al., 2008; Prouty & Gunn, 2003; Prouty et al., 2002)
	O-Ag-capsule(*yihU–yshA* and *yihVW*)	
	Type I fimbriae (*fim*)	
Glass	Cellulose (*bcsABZC–bcsEFG*)	
	LPS	
	Type-three secretion apparatus (TTSS)	(Crawford et al., 2008; Prouty & Gunn, 2003)
	Flagella	
Alfalfa seeds	Curli (*csg* genes)	(Barak et al., 2005; Barak et al., 2007)
	Cellulose (*bcsABZC–bcsEFG*)	
	O-Ag-capsule(*yihU–yshA* and *yihVW*)	

LPS, a functional type III secretion system (TTSS) apparatus and flagellar motility are of crucial importance for biofilms grown on hydrophilic glass coverslips (Crawford et al., 2008; Prouty & Gunn, 2003). Attachment to and subsequent biofilm formation on alfalfa requires curli, cellulose and O-Ag-capsule as the main extracellular matrix compounds (Barak et al., 2005, 2007). Flagella were also shown to be important for *Salmonella*–plant interaction in certain serovars under some environmental conditions (Berger et al., 2009).

3.1. Proteinaceous fraction

Curli (encoded by *csgBAC–csgDEFG*) are highly aggregative, non-branching, amyloid-like cell-surface proteins that are important in processes such as host colonization, persistence, motility and invasion (as reviewed by (Barnhart & Chapman, 2006)). *Salmonella* curli are important during biofilm formation because they promote initial cell-surface and subsequent cell–cell interactions. White and colleagues pointed out that in the native state, curli exist as a complex with cellulose and the O-Ag-capsule, physically linking the cells together (Gibson et al., 2006; White et al., 2003). Through binding the hydrophobic dye CR, mediated by the β-strand structure of the structural CsgA and CsgB subunits, they contribute to the typical rdar appearance (Collinson, Clouthier, Doran, Banser, & Kay, 1996; Römling, Bian, et al., 1998; White et al., 2001). Failure to produce intact curli (*csgA* and *csgB* mutants) resulted in a pdar (pink, dry and rough) morphotype on CR agar plates, characteristic for cellulose synthesis (Römling, Bian, et al., 1998). While both structural curli subunits are important for the expression of the rdar morphotype (Römling, Bian, et al., 1998; White et al., 2003), only CsgB appeared to be important in initial attachment and colonization of alfalfa sprouts (Barak et al., 2005). Solano et al. also stressed the importance of the applied biofilm test system since they noticed that curli are not essential for biofilm mediated glass adherence under ATM conditions, while they are indispensable to form a tight pellicle under LB conditions (Solano et al., 2002).

In addition to curli (*csg*), the *S.* Typhimurium genome contains 12 other putative fimbrial operons (McClelland et al., 2001), some of which were shown to be important in biofilm formation. Type 1 fimbriae, for example, are an absolute requirement for adherence to and biofilm formation on epithelial cell layers as shown using HEp-2 cells (Boddicker et al., 2002) and chicken intestinal tissue (Ledeboer & Jones, 2005). This epithelial biofilm formation capacity varied significantly for very closely related *S.* Typhimurium strains expressing different alleles of the *fimH* adhesin gene, with *fimH* mutants showing no biofilm formation and strains expressing the low- and high-adherence *fimH* alleles showing very little (patchy) and extensive biofilm formation, respectively. This gene encodes the FimH adhesin, positioned on top of the type 1 fimbriae, mediating adhesion to mannose residues. Further highlighting the importance of fimbriae in biofilm formation, Ledeboer et al. found by microarray analysis that biofilm formation on a HEp-2 monolayer significantly up-regulated five fimbrial gene clusters (*pef*, *csg*, *lpf*, *bcf* and *sth*), next to genes with unknown functions and genes involved in central metabolism, conjugative DNA transfer (Ghigo, 2001; Reisner, Haagensen, Schembri, Zechner, & Molin, 2003), antibiotic resistance, intracellular survival and colanic acid biosynthesis (Ledeboer et al., 2006). Consistently, plasmid-encoded fimbrial (*pef*) and curli (*csg*) mutants are defective for biofilm formation on plastic, HEp-2 cells and chicken intestinal tissue. Curli mutants are also defective in adhesion to a murine intestinal epithelial cell line (Sukupolvi et al., 1997). Long polar fimbrial (*lpf*) mutants showed an intermediate loss of biofilm formation capacity on HEp-2 cells and plastic, while a significant reduction in biofilm capacity was detected on chicken intestinal tissue. *sth* mutants, on the other hand, had no detectable biofilm defects, while *bcf* mutants showed an increased biofilm formation capacity on HEp-2 and chicken intestinal cells and a comparable one on plastic. All *in vitro* results highlighting the importance of Type I fimbriae, Pef, Curli, Lpf and Bcf were confirmed using *in vivo* chicken trials (Ledeboer et al., 2006). Type I fimbriae were also shown to be important in the *in vivo* colonization of swine for example (Althouse et al., 2003). Altogether, these results showed that fimbriae may have separate and complementary functions that are important during genesis and maturation of *Salmonella* biofilms on eukaryotic cell surfaces and that all have to act together to develop a mature biofilm. The importance of fimbriae in biofilm formation was found to be strongly dependent on the used test system. Mutations in several fimbrial operons (*fim*, *csg*, *lpf* and *pef*) seemed not to affect

gallstone biofilm formation (Prouty et al., 2002), while overexpression of type 1 fimbriae (via *fimW* insertional activation) had a negative effect on cholesterol and hence gallstone binding and subsequent biofilm maturation (Crawford, Reeve, et al., 2010). Biofilm formation on glass and plastic, on the other hand, was not influenced. Curli also seemed not to be involved in biofilm formation on glass surfaces using a continuous-flow system (Grantcharova, Peters, Monteiro, Zakikhany, & Römling, 2010).

Burmolle et al. recently found that the conjugative plasmid pOLA52, conferring multiple antibiotic resistance to *E. coli*, enhances *S.* Typhimurium biofilm formation through plasmid-bound type 3 fimbrial expression (Burmolle, Bahl, Jensen, Sorensen, & Hansen, 2008). This stresses the potential importance of proximity and conjugation in multispecies biofilms in respect to the potential transfer of biofilm important genes such as fimbriae. Furthermore, conjugative transfer mechanisms might be involved in biofilm formation as adherence factors (conjugative pili) or in biofilm maturation (Ghigo, 2001; Reisner et al., 2003).

The large (386 kDa), proline-threonine-rich secreted, multidomain protein BapA of *S.* Enteritidis, showing homology and functional relation to the *Staphylococcus aureus* surface protein Bap (biofilm associated protein) (Cucarella et al., 2001; Latasa et al., 2005), is a second important component of the proteinaceous fraction of the rdar morphotype. BapA was shown to be important for bacterial aggregation and subsequent pellicle formation at the air–liquid interface under LB conditions. It was found to be loosely associated with the cell surface and is secreted through the BapBCD type I protein secretion system, encoded by the *bapABCD* operon. Secretion was shown to be absolutely necessary to fulfill its role in biofilm development. When overexpressed, BapA led to increased pellicle and biofilm formation (Latasa et al., 2005). The highly homologous STM4261 (*siiE*), together with BapA encoding the two largest proteins in the *Salmonella* genome, was found not to be important during *S.* Enteritidis biofilm formation, maybe because of functional redundancy between SiiE and BapA (Latasa et al., 2005). BapA was unable to complement curli or cellulose dysfunctions, while overproduction of curli, but not cellulose, was able to restore pellicle formation under LB conditions in a *bapA*-deficient strain. This observation suggested that BapA could play a role in connecting individual cells, either directly through homophilic interactions or by strengthening curli mediated associations. In relation to virulence, BapA (Latasa et al., 2005), and also SiiE (Morgan et al., 2004) contributed to invasion through the regular (oral) *Salmonella* infection route, suggesting subtle links between biofilm formation, host colonization and virulence in general. Recent findings using atomic force microscopy to study *Salmonella* biofilm morphology illustrated that BapA does not have a major impact on biofilm formation and morphology, in contrast to cellulose and curli (Jonas et al., 2007). This apparent discrepancy could partly be explained by the different biofilm test systems used (pellicle formation vs. surface growth and liquid biofilm assay).

Flagella are indispensable for swarming (and swimming) but can serve different roles during *Salmonella* biofilm formation. Swarming is a multicellular process involving the generation of slimy colonies that expand rapidly via flagella mediated motility. It is similar, but to some extent inversely related to biofilm formation (as reviewed by (Verstraeten et al., 2008)). Gunn and colleagues showed that the flagellar filament, but not motility *per se* are gallstone biofilm determinants since a *fliA* mutant did not and a *motA* mutant did form mature biofilms, respectively. Conversely, on glass (Prouty & Gunn, 2003; Prouty et al., 2002) and polyvinyl chloride (PVC) (Mireles et al., 2001) motility was important as well. They further confirmed these results utilizing the previously mentioned *in vitro* cholesterol binding assay, using *flhC*, *flgC*, *fliC*, *fljB* and *motA* mutants and showed that, although bile reduced the transcription of *flhC*, *flgC* and *fliC*, normal flagella could still be produced in the presence of bile (Crawford, Reeve, et al., 2010). Further elaboration showed that *fliC*, and not the antigenically distinct *fljB*, alternatively expressed through phase variation, is critical for cholesterol binding and that the flagellar filament is only crucial for initial attachment to the cholesterol and not for biofilm maturation. Teplitski and co-workers on the other hand, noticed, somewhat contradictory to the results of Gunn and colleagues, that the presence of the flagellum on the surface of the cell, functional or not, is inhibitory to biofilm formation on polystyrene, as mutants which lack intact flagella (*flhD*, *flhC*, *fliF*, *fliA* and *fljB*/*fliC* mutants) show an increased biofilm formation as compared to the wildtype. Non-functional flagella on the surface of the cell (*motA*), or a lack of chemotaxis (*cheZ*), were found to be even more inhibitory to biofilm formation than the presence of functional (wild-type) flagella, as a *motA* and *cheZ* mutant showed a reduced biofilm formation (Teplitski, Al-Agely, & Ahmer, 2006). Römling and Rohde showed that flagella are not important for *S.* Typhimurium rdar expression on CR plates (Römling & Rohde, 1999), while Solano et al. noticed that a defect in flagellar production (via a *S.* Enteritidis *fliS* insertion mutant) affects the biofilm formation process only under LB but not ATM conditions (Solano et al., 2002). Using a *S.* Typhimurium *flgK* mutant, Kim and Wei noticed that flagellar assembly is important during biofilm formation in different (meat, poultry, produce) broths and on different contact surfaces (PVC and steel) (Kim & Wei, 2009). Stafford et al. showed that the conserved *S.* Typhimurim flagellar regulon gene *flhE*, involved in the flagellar type III secretion specificity switch (Hirano, Mizuno, Aizawa, & Hughes, 2009) is not required for flagella production or swimming, but appeared to play a role in swarming and biofilm formation (Stafford & Hughes, 2007). A *flhE* mutant showed an altered phenotype on CR plates and heavily increased biofilm formation on PVC. Since both phenotypes appeared not to be associated with curli or cellulose alterations, FlhE might function as an additional extracellular matrix component. This notion is strengthened by its N-terminal Sec-signal (for translocation through the Sec-translocation pathway), suggesting a periplasmic or extracellular location. In the context of plant attachment, the importance of flagella became clear during a survey testing a range of *S. enterica* serovars (Berger et al., 2009). This study revealed differences in attachment capacity to leafy vegetables, suggesting that different *Salmonella* serovars use strain-specific attachment mechanisms. For example, by using *fliC* mutants, flagella were shown to be important in the attachment of an outbreak strain of *S.* Senftenberg to leaves, but not in the attachment of *S.* Typhimurium SL1344. A bit contradictory, a clear effect of *S.* Typhimurium flagella mutation (*fliGHI*) on attachment and subsequent photosynthesis driven stomata internalization was seen (Kroupitski, Golberg, et al., 2009), showing that variations in utilized plant models and physiological conditions have an important impact on the obtained results.

3.2. Exopolysaccharide fraction

Cellulose, a β-1-4-D-glucose polymer, encoded by the *bcsABZC–bcsEFG* genes, is an important biofilm-associated EPS. In relation to the characteristic rdar morphotype expression pattern, cellulose supports long-range cell–cell interactions responsible for the sticky texture (Römling et al., 2000; Solano et al., 2002; Zogaj et al., 2001). Cellulose production impairment generates a bdar (brown, dry and rough) morphotype on CR agar plates, characteristic for the expression of curli. Bacteria of the pdar morphotype, expressing cellulose also bind CF (Römling et al., 2000; Solano et al., 2002). Solano et al. showed that cellulose is a crucial *Salmonella* biofilm determinant under LB as well as ATM conditions. Moreover, Ledeboer and Jones showed the crucial importance of cellulose during *S.* Typhimurium biofilm formation (using a *bcsB* mutant) (more specifically during the maturation phase (Ledeboer et al., 2006)) on epithelial cell surfaces (HEp-2 cells and chicken intestinal tissue) (Ledeboer & Jones, 2005), while Prouty and Gunn identified its crucial importance for biofilms grown on glass coverslips (Prouty & Gunn, 2003). Using a *bcsA* mutant, Barak et al. highlighted the importance of cellulose during *S. enterica* (Enteritidis

and Newport) attachment and colonization of alfalfa sprouts (Barak et al., 2007). Cellulose, together with curli, also play a role during the transfer of contaminated water to the edible parts of parsley (Lapidot & Yaron, 2009), while it was shown not to be involved in the initial attachment properties of *S.* Typhimurium to parsley (Lapidot et al., 2006). Since cellulose is also important in plant adherence of plant-symbiotic and -pathogenic bacteria (Rodriguez-Navarro, Dardanelli, & Ruiz-Sainz, 2007), it could be hypothesized that human pathogens and plant-associated bacteria share at least one extracellular molecule, cellulose, to colonize plants. On the other hand, cellulose appears not to be an important constituent of the EPS produced by *Salmonella* spp. during gallstone biofilm formation (Prouty & Gunn, 2003). Similarly, cellulose is not a major constituent of the biofilm matrix of feed industry-isolated *S.* Agona and *S.* Typhimurium (Vestby, Moretro, Ballance, et al., 2009), but it was noticed that even the smallest amounts of cellulose contributed to the highly organized matrix structuralization. Malcova et al. also identified that cellulose is not crucial for *S.* Enteritidis adherence to and biofilm formation on polystyrene (using a *bcsA* mutant) (Malcova, Karasova, & Rychlik, 2009).

Colanic acid is a capsular extracellular polysaccharide found to be important for *Salmonella* to create extensive three-dimensional structures on epithelial cells, as shown by the thin biofilm layer across the surface of HEp-2 cells and lack of mature biofilms on chicken intestinal epithelium in colanic acid mutants (*wcaM*, *wcaA* and *wza*) (Ledeboer & Jones, 2005; Ledeboer et al., 2006). Similarly, colanic acid contributes to the complex three-dimensional architecture of *E. coli* biofilms (Danese, Pratt, & Kolter, 2000). On the other hand, this exopolysaccharide sugar was found not to be required for *Salmonella* biofilm formation on abiotic surfaces (Ledeboer & Jones, 2005; Prouty & Gunn, 2003), gallstones (using a *wcaA* mutant) (Prouty & Gunn, 2003) and alfalfa seeds (using a *wcaJ* mutant) (Barak et al., 2007). This highlights the fact that the bacterial requirements for attachment to and colonization of plant tissue differ from the ones for animal tissue attachment and colonization. Furthermore, Solano et al. showed that colanic acid was important to form a tight pellicle under LB conditions, where it was dispensable under ATM conditions (using a *wcaI* insertional mutant), again stressing the environmental importance in matrix production.

Next to cellulose, the EPS fraction of *S.* Enteritidis biofilms on agar plates consists of an anionic O-antigen capsule, different from colanic acid and covalently attached to lipids (Gibson et al., 2006; White et al., 2003). This capsule consists of more than 2300 repeating tetrasaccharide units, is highly hydrated (as are all capsules) and is linked to the membrane via a lipid anchor (Snyder, Gibson, Heiss, Kay, & Azadi, 2006). Structurally the O-Ag capsule (encoded by *yihU-yshA* and *yihVW*) is similar to the previously reported LPS O-Ag of *S.* Enteritidis considering repeating oligosaccharide unit, but differs from it considering size, charge, substitution, lipid attachment and immunoreactivity (Gibson et al., 2006; Snyder et al., 2006; White et al., 2003). This capsule was proven to be involved in desiccation tolerance, but not important in multicellular behaviour and formation of the extracellular matrix on agar plates *per se*. As such, the O-Ag capsule is hypothesized to play an important role in environmental persistence (Gibson et al., 2006). The O-Ag-capsule is also crucial for *S.* Typhimurium and *S.* Typhi (and to lesser extent *S.* Enteritidis) gallstone biofilms, but not necessary for adhesion and biofilm formation on glass or plastic (Crawford et al., 2008). Further on, it appeared to be important during alfalfa attachment and biofilm formation (Barak et al., 2007), while nothing is known about its importance for attachment to epithelial cells. This further highlights the difference between plant and animal tissue biofilms, a notion that became even strengthened by the emerging importance of genes with unknown function (FUN genes) in *Salmonella*-plant interactions and biofilm formation on plants (Barak et al., 2009). *Salmonella* indeed appears to rely on a different set of genes to interact with plant and animal hosts (Barak et al., 2009; Teplitski et al., 2009). A recent high throughput study of specific response of *S. enterica* to tomato varieties and fruit ripeness further confirmed the minor overlap between the *Salmonella* set of genes to colonize animals and plants (Noel, Arrach, Alagely, McClelland, & Teplitski, 2010).

In addition, de Rezende et al. purified another *Salmonella* capsule from the extracellular matrix fraction of the multiresistent *S.* Typhimurium DT104, which has a different chemical composition as compared to the above mentioned O-Ag-capsule, since it lacked rhamnose (de Rezende et al., 2005). This capsule was also shown to be important in biofilm formation (not in the primary attachment step, but during the maturation phase) itself and was detected at both 25 °C and 37 °C (de Rezende et al., 2005). (Gibson et al., 2006). It was speculated by de Rezende and colleagues that this capsule might also be important in environmental as well as during in-host persistence (given its constitutive expression pattern), protection to external stress factors, nutrient scavenging and virulence, however, this still needs to be proven (de Rezende et al., 2005). Recently, Malcova et al. confirmed the importance of capsular polysaccharide in the biofilm formation capacity of strains not expressing curli or cellulose, but overproducing this capsule (sbam (smooth, brown and mucoid) morphotype on CR plates) (Malcova et al., 2008).

3.3. Fatty acids and lipopolysaccharides (LPS)

The presence of different fatty acids (common LPS components as well as some saturated and unsaturated fatty acids) was also noticed in the EPS fraction of rdar expressing *S.* Enteritidis strains (Gibson et al., 2006). Solano et al. showed that LPS and the enterobacterial common antigen, or at least some components of the biosynthetic pathways leading to their production, were important for biofilm formation under LB, but not ATM conditions (using insertion *wecE*, *wzxE*, and *rffG* mutants). Mireles et al. noticed that all tested *S.* Typhimurium LT2 LPS mutants showed equal or higher biofilm formation as compared to an LT2 wild-type strain on PVC, but none was able to adhere to glass (Mireles et al., 2001). Mutational analysis of *galE* and *rfaD* mutants, both involved in the LPS O-Ag synthesis, showed that an incomplete LPS did not drastically affect biofilm formation on the hydrophobic gallstone surface, but was important for *Salmonella* biofilm formation on hydrophilic glass (Prouty & Gunn, 2003). In this context, it is important to note that *galE* and similar general metabolism mutants often suffer from pleiotropic effects and caution is needed to interpret data generated using such mutants as shown by different research groups (Gibson et al., 2006; Prouty & Gunn, 2003; White et al., 2003). *galE*, encoding uridine diphosphogalactose-4-epimerase, plays a central role in sugar metabolism, being crucial in galactose and nucleoside sugar precursor biosynthesis. Since galactose is not only involved in LPS production at different stages (*i.e.* outer core and the O-antigen synthesis), but also in colanic acid (Danese et al., 2000) and O-Ag capsular biosynthesis processes (Gibson et al., 2006), one can easily imagine that a single mutation can have several effects on cell surface and biofilm formation. Further elaborating on the importance of LPS for *Salmonella* biofilm formation, Kim and Wei noticed that a *rfbA* mutant, showing an aberrant LPS profile, was impaired in rdar expression, pellicle formation, biofilm forming capacity on PVC in meat, poultry and produce broths and biofilm formation on steel and glass, while an *rfaB* mutant was just slightly affected (Kim & Wei, 2009). Moreover, Anriany et al. showed the importance of LPS for *S.* Typhimurium DT104 and LT2 multicellular behaviour, since two LPS biosynthesis mutants, identified during a random mutagenesis experiment, expressed an altered rugose phenotype (Y. Anriany et al., 2006). These LPS alterations caused changes in the cell surface: disruption of *ddhC* (*rfbH*) resulted in a lack of the complete O-Ag, while a *waaG* (*rfaG*) mutation caused an even more truncated LPS that lacked the outer LPS core polysaccharide. Considering extracellular matrix compound production, the former mutation resulted in reduced and characteristically altered extracellular matrix production, while the latter mutant produced more,

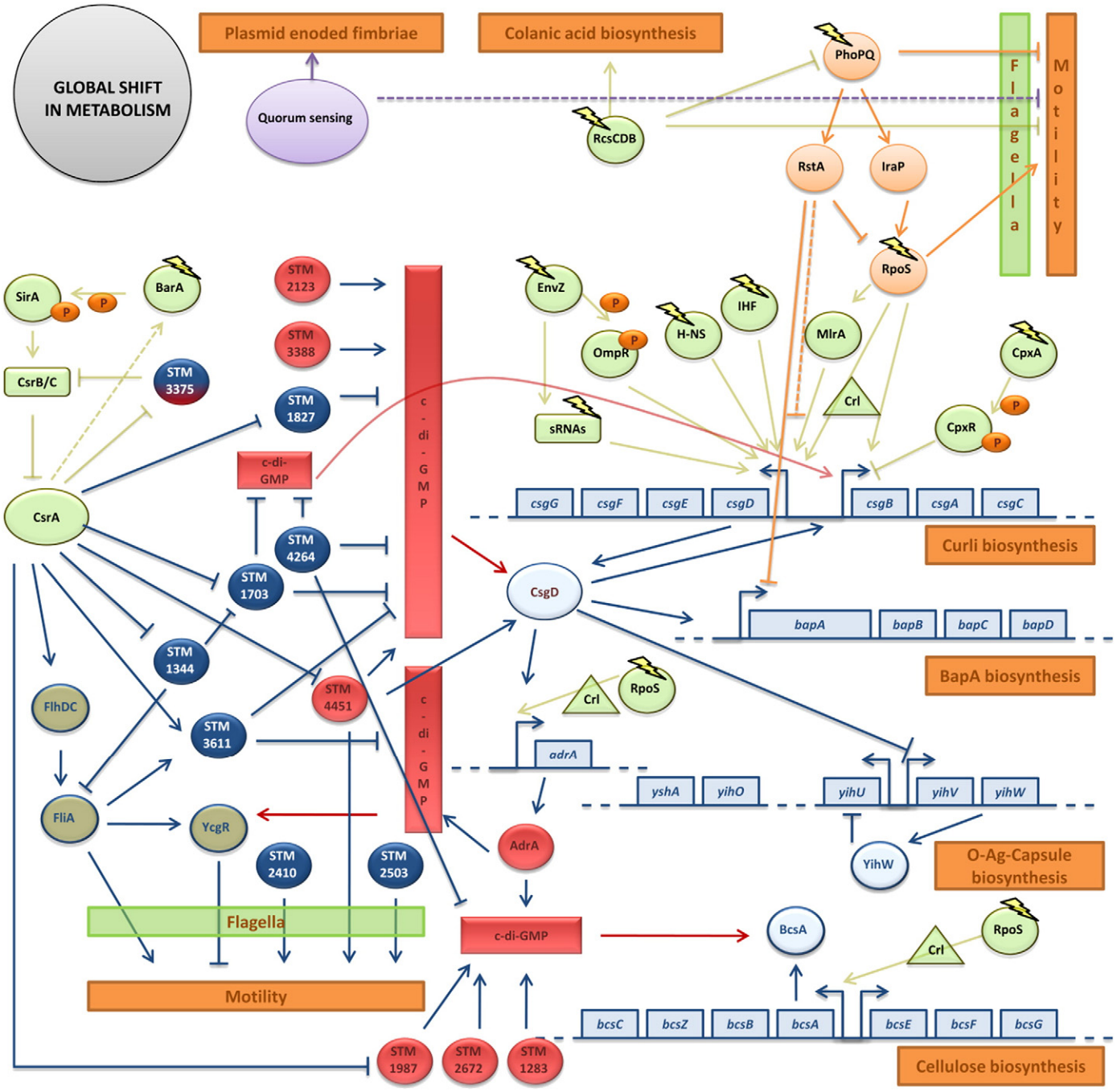


Fig. 1. Complex regulatory network governing *Salmonella* biofilm formation. Working model for the regulation of *Salmonella* biofilm formation. Arrows and flat-headed arrows represent an activating and repressing effect respectively. Broken lines indicate putative links, that need to be experimentally validated or further investigated. 'P' symbols represent transferable phosphorus groups of two component systems. Light blue rectangles represent the genomic organization of the genes encoding the major structural biofilm components, indicated by orange rectangles. The orange 'Motility' rectangle is an exception as it represents a community behaviour related to biofilm formation (regulated through flagellar genes (dark green circles) and flagella (green rectangle)). Light blue circles represent important regulators involved in the production of the major structural biofilm components. Light green circles, triangles and rectangles represent global *trans*-acting regulators, the Crl protein and sRNAs, respectively, and lightning bolt symbols represent the input and integration of different environmental signals through these general regulators into the regulatory system. Orange circles and arrows indicate the link between PhoPQ and biofilm formation. The grey and purple circles indicate the role of metabolism and quorum sensing respectively. Dark blue and red circles represent EAL and GGDEF proteins, respectively, involved in c-di-GMP turnover, of which the exact functions can be found throughout the text and in Table 2. Red rectangles represent the different, but interconnected c-di-GMP pools. CsgD is the general *Salmonella* biofilm regulator (Gerstel et al., 2003), as can be seen in the right-hand side of the figure, triggering the biosynthesis of the major extracellular matrix components consisting of a proteinaceous fraction made up by curli fimbriae (Römling, Bian, et al., 1998) and the large secreted BapA protein (Latasa et al., 2005) on the one hand, and a exopolysaccharide fraction largely made up by cellulose (Zogaj et al., 2001) and an O-antigenic capsule (O-Ag-capsule) (Gibson et al., 2006) on the other hand. Cellulose synthesis is not only regulated by CsgD, via AdrA and c-di-GMP (Simm et al., 2004; Zakikhany et al., 2010; Zogaj et al., 2001), but also via a CsgD-independent pathway in which STM1987 (and other GGDEF proteins) and c-di-GMP are involved (Da Re & Ghigo, 2006; Garcia et al., 2004; Simm et al., 2007; Solano et al., 2002). From the complex regulation of the c-di-GMP network, on the left-hand side of the figure, it becomes clear that different c-di-GMP pools exist within *Salmonella* cells. These different, but interconnected, pools serve slightly different, but intertwined, purposes (Simm et al., 2007; Solano et al., 2009), as clarified throughout the text.

profuse matrix. Further analysis revealed that curli and cellulose production was reduced and increased, respectively, with the *waaG* mutant showing the greatest changes. Both mutants also showed altered biofilm formation under various test conditions: reduced biofilm formation in rich medium under low osmolarity conditions and more biofilm formation with addition of glucose or a mixture of glucose and NaCl, both at 28 °C and 37 °C (a temperature at which curli and cellulose are not produced). Based on these observations the authors concluded that normal curli production hinders cellulose production, an inverse relation between LPS and biofilm formation exists (as already suggested by (Mireles et al., 2001)) and more profoundly that the balance in production between both curli and cellulose appears to depend to a certain extent on LPS and hence the cell surface as also shown by White and colleagues (Gibson et al., 2006; White et al., 2003). Altogether, this implies that LPS mutations and/or certain environmental conditions may be able to induce alternative pathways leading to extracellular matrix production.

4. Regulators, signal transduction and metabolism in *Salmonella* biofilms

As illustrated in Fig. 1, the synthesis of the structural components of *Salmonella* biofilms is regulated by a highly complex regulatory network. In this section, an extensive overview is given on the current understanding of this network and the interactions between its different components.

4.1. CsgD

CsgD is a major control and integration unit for *Salmonella* biofilm formation regulating the expression of specific *Salmonella* biofilm-associated matrix compounds (Gerstel & Römling, 2003), as can be seen on Fig. 1. CsgD, previously referred to as AgfD (thin aggrative fimbriae gene D), is a transcriptional response regulator containing an N-terminal receiver domain with a conserved aspartate (D59) and a C-terminal LuxR-like helix-turn-helix (HTH) DNA-binding motif belonging to the FixJ/NarL family. In a genomic context, *csgD* is an integral part of the curli biosynthesis system consisting of the divergently transcribed *csgBAC* and *csgDEFG* operons (alternatively called *agfBAC* and *agfDEFG*). A *csgD* mutant lacks any form of multicellular behaviour as visualized by a saw (smooth and white) morphotype on CR agar plates (Römling et al., 2000). In addition, individual point mutations in the highly complex *csgD* promoter region (521 bp between *csgB* and *csgD*) can even cause a switch from a highly regulated (strict environmental control) to a semiconstitutively regulated (not such a strict environmental control) rdar program (Römling, Sierralta, et al., 1998). A *csgD* insertion mutant showed no pellicle formation under LB conditions, but did show biofilm formation under ATM conditions (Solano et al., 2002). An insertion mutant in the *csgB–csgD* intergenic region, also showed reduced alfalfa sprout attachment (Barak et al., 2005). Flow cell-based biofilm experiments on glass using *csgD* mutants revealed that CsgD is required for biofilm maturation, but appeared to be dispensable for microcolony establishment (Grantcharova et al., 2010).

High degree of conservation at nucleotide and protein level between the corresponding curli operons of *S.* Typhimurium and *E. coli*, together with cross-complementation ability and similar regulation patterns, suggested these genes were already present in their common ancestor (Römling, Bian, et al., 1998). Conservation of the rdar morphotype and *csgD* promoter region in the *Salmonellae* has been described above, but an important note concerning this conservation can be found in the work of White and Surette (White & Surette, 2006). Using a comparative genetic analysis of the *csgB–csgD* intergenic region of the SARC16, they showed that, with the exception of two *S. enterica* subsp. *arizonae* isolates (belonging to *Salmonella* group IIIa), promoter functionality of the *csgD* and *csgB* genes was conserved, despite sequence differences (being the biggest for two group V *S. bongori* isolates), for six of the seven *Salmonella* subgroups. This indicates that most changes in the *csgB–csgD* intergenic region were the result of neutral mutations originating from genetic drift. Next to the two clear sequence (*cis*) mutations, generating inactive *csgB* and *csgD* promoters, reflecting a different evolutionary lifestyle (no or minimal non-host environmental passage during lifecycle), six other isolates harboured upstream regulatory (*trans*) mutations, responsible for rdar phenotype loss, probably originating from domestication. In line with this, recent evidence indeed showed that rapid domestication due to laboratory passage in rich medium was responsible for the evolutionary loss of the rdar morphotype (Davidson, White, & Surette, 2008). This loss most of the times appeared to be related to mutations in *rpoS*, within the cellulose biosynthesis pathway or in unknown upstream rdar morphotype regulators. Similar phenomena could be the reason for the appearance of spontaneous smooth variants originating from *S.* Typhimurium rugose strains after repeated passages on TSA culture medium (Anriany et al., 2001).

It is already known for a long time that CsgD regulates the transcription of the structural curli subunits encoded by *csgBAC* (Römling, Bian, et al., 1998). However, direct specific binding to an 11 bp variant of the *E. coli* predicted motif (Brombacher, Dorel, Zehnder, & Landini, 2003) and subsequent transcriptional activation of the *csgBAC* promoter region by unphosphorylated CsgD was only recently shown (Zakikhany, Harrington, Nimtz, Hinton & Römling, 2010). In the same study, it was also shown that CsgD displays reduced promoter binding after *in vitro* acetyl phosphate-driven phosphorylation and that the conserved D59 is important for CsgD functionality and stability *in vitro* and *in vivo*. Both curli operons are necessary for the production of intact, highly stable curli. *Salmonella* curli assembly, following activation by CsgD, occurs via the extracellular nucleation precipitation pathway (ENP) (Hammar, Bian, & Normark, 1996) as shown by interbacterial complementation experiments in LPS O-polysaccharide-deficient strains (*galE* mutants) (Gibson, White, Rajotte, & Kay, 2007; White et al., 2003). Using luciferase (*lux*) expression reporters, White et al. visualized that curli production probably initiates extracellular matrix production and as a consequence specific rdar surface patterns, since *csgB* expression peaks coincided with the sharp transition to these specific patterns. Similar progressive transition towards a rugose phenotype was visualized on TSA broth at ambient temperatures (Anriany et al., 2001). This transition, however, was not unequivocally confirmed to be primarily curli mediated but importance of curli was noticed in a later study (Anriany et al., 2006). Consistent with this, curli were shown to provide specific short-range cell–cell interactions yielding this adhesive structure (Römling et al., 2000; White et al., 2006).

In the context of curli regulation, it was found that in *E. coli* K-12, CsgD also altered the cell physiology to enable production of curli, a process not yet clearly identified in *Salmonella* (Chirwa & Herrington, 2003). Expression of *glyA*, encoding serine hydroxymethyltransferase (SHMT) important in glycine biosynthesis, was shown to be up-regulated by CsgD. Since the N-terminal part of CsgA contains a higher than average glycine percentage, up-regulation of SHMT activity improves CsgA and curli biosynthesis and hence biofilm formation. Further on, it was shown that CsgD also negatively regulates biofilm-inhibiting factors in *E. coli*, altering its cell physiology (Brombacher, Baratto, Dorel, & Landini, 2006; Brombacher et al., 2003).

Biosynthesis of cellulose, occurring at the inner bacterial membrane, is also positively regulated by CsgD via direct binding and subsequent transcriptional stimulation of *adrA* (AgfD regulated gene) in *S.* Typhimurium (Zakikhany et al., 2010; Zogaj et al., 2001). As for *csgBAC* activation, it was noticed that the unphosphorylated CsgD form binds specifically to the *adrA* promoter, although in a more complex manner (Zakikhany et al., 2010). This different binding pattern between the *csgB* and *adrA* promoter, together with intrinsic promoter differences, implies different transcriptional activation mechanisms for both abundant matrix components. AdrA in turn

regulates *bcsABZC*, the constitutively transcribed genes encoding the cellulose biosynthesis machinery at the post-transcriptional level (Simm, Morr, Kader, Nimtz, & Römling, 2004; Zogaj et al., 2001), by altering the cellular levels of c-di-GMP (Robbe-Saule et al., 2006; Zogaj et al., 2001). As discussed in detail in the c-di-GMP section, AdrA encodes a membrane bound GGDEF domain protein with di-guanylate cyclase activity which is involved in the production of c-di-GMP. Next to *bcsABZC*, an additional operon was found to be important for cellulose production in *S.* Typhimurium and *S.* Enteritidis: the *bcsEFG* operon (divergently transcribed according to the *bcsABZC* operon (Solano et al., 2002)). Mutants in both operons affected pellicle and biofilm formation in LB and ATM medium, respectively, and abolished their CF binding capacity (Römling et al., 2003; Solano et al., 2002; Zogaj et al., 2001).

Some lines of evidence showed that next to this CsgD-dependent pathway of cellulose production, also a CsgD-independent pathway can be involved in this process: (1) a *S.* Enteritidis disease associated strain showed a *csgD*-uncoupled cellulose production (Römling et al., 2003); (2) a clinical *S.* Enteritidis isolate showed cellulose production and subsequent biofilm formation under ATM conditions that were not affected by mutations in *adrA*, *rpoS*, *csgD* and *ompR*, but dependent on the diguanylate cyclase STM1987 (expression of which was also CsgD independent) (Garcia et al., 2004; Solano et al., 2002); (3) cellulose production was partly uncoupled from *csgD* expression in a continuous-flow model (Grantcharova et al., 2010); (4) although *adrA* is expressed *in planta* (*i.e.* alfalfa sprouts), it was shown not to be required for cellulose synthesis and attachment in this context (Barak et al., 2007) (5) cellulose overproduction in an LPS mutant suggests an alternative pathway with uncoupled cellulose and curli production (Y. Anriany et al., 2006); (6) an identified and elucidated CsgD-independent pathway in the commensal *E. coli* 1094 (Da Re & Ghigo, 2006). The latter pathway has been proven to be AdrA-independent, but dependent on RpoS and YedQ (a membrane bound GGDEF domain protein showing high similarity to STM1987), and hence c-di-GMP. Confirmation of such a CsgD-independent cellulose pathway in *Salmonella* was provided in a recent study (Simm, Lusch, Kader, Andersson, & Römling, 2007).

Expression of *bapA*, part of the *bapABCD* operon responsible for BapA synthesis and export, is also regulated by CsgD (Latasa et al., 2005). Despite the finding that the *bapA* promoter region contains a similar CsgD binding sequence as the inverted repeat of the *adrA* promoter (Latasa, Solano, Penades, & Lasa, 2006), Zakikhany et al. did not identify *bapA* as a CsgD regulated gene in *S.* Typhimurium, using a combined bioinformatics and global transcriptomic approach (Zakikhany et al., 2010). Differences in the applied experimental procedures could be a possible explanation for such a discrepancy.

The *S.* Enteritidis O-Ag-capsule, assembled and translocated by the divergently oriented operons, *yihU-yshA* and *yihVW*, is another compound of the EPS fraction that is regulated by CsgD (Gibson et al., 2006; White et al., 2003). The *yih* intergenic region and divergent operon structure was detected in all tested isolates from the SARC (Boyd et al., 1996). Differential regulation of the *yih* operons is executed by CsgD, through repression of the transcriptional repressor YihW leading to *yihU* (-*yshA*) activation, in coordination with the other extracellular matrix compounds, as could be seen using *lux*-based expression analysis (Gibson et al., 2006). Similarly as for *bapA*, the genes of the capsule operon were not retained as being CsgD regulated in *S.* Typhimurium (Zakikhany et al., 2010). Addition of bile resulted in an up-regulation of the O-Ag capsule-encoding operon in a *csgD*-independent manner, suggesting existence of an alternative pathway governing O-Ag-capsule expression (Crawford et al., 2008).

The *S.* Typhimurium DT104 capsule isolated by de Rezende et al. probably is not incorporated into the *csgD* regulon, since it is also expressed at 37 °C, a temperature at which native *csgD* is not activated (de Rezende et al., 2005).

Taken together, CsgD can be seen as the biofilm control point, regulating the expression of all major *Salmonella* biofilm constituents (under rdar conditions) and controlling the transition between planktonic and multicellular behaviour. As such and since *csgD* has a low basal transcription level (Gerstel & Römling, 2003), it is not surprising that the expression of *csgD* itself is highly regulated by different environmental stimuli (temperature, oxygen tension, nutrients and starvation, osmolarity, ethanol, iron and pH) via different transcriptional regulators (OmpR, Crl, RpoS, MlrA, CpxR, H-NS and IHF) and the secondary bacterial messenger molecule c-di-GMP, as discussed below and visualized in Fig. 1. This complex regulation enables fine-tuning of the regulatory network and the generation of quick and well-controlled responses to changing environmental conditions. Transcription of *csgD* is maximal during the late exponential and early stationary growth phase under low osmolarity conditions at ambient temperatures (below 30 °C). Microaerophilic and aerobic conditions induce this maximal expression in rich medium and minimal medium, respectively (Gerstel & Römling, 2001). However a single point mutation in the complex *csgD* promoter region renders the *S.* Typhimurium strain RpoS- and temperature-independent with respect to its curli production (Römling, Sierralta, et al., 1998), further emphasizing the importance of this regulatory pathway. In addition, specific environmental conditions, such as iron limitation, can also cause temperature-independent curli expression.

Recently, Grantcharova et al. identified the bistable nature of *csgD* expression (Grantcharova et al., 2010). Using a chromosomal *csgD-gfp* translational fusion, different biofilm model systems (rdar expression, steady-state liquid culture and continuous-flow biofilm formation), fluorescence microscopy and FACS, they identified non-uniform, bistable, cytoplasm based CsgD-GFP expression in the highly regulated rdar morphotype strain, while monomodal expression was observed at higher overall CsgD levels as encountered in the semiconstitutive rdar morphotype or by increased c-di-GMP levels. Previously however, it was noticed that the highly regulated rdar morphotype strain showed a *csgD* expression level representative for most disease-associated *S.* Typhimurium isolated strains (Römling et al., 2003). This makes the native *csgD* promoter an ideal spot for the integration of stochastic fluctuations, enabling adaptation to highly variable micro-environments, finally giving rise to a heterogeneous biofilm population (Chai, Chu, Kolter, & Losick, 2008; Stewart & Franklin, 2008). There indeed seems to exist some kind of task distribution with that part of the population showing high levels of CsgD-GFP expression being responsible for extracellular matrix expression, as could be expected from the position of CsgD in the regulatory biofilm formation cascade (Grantcharova et al., 2010). Interestingly, White et al. also noticed two *S.* Typhimurium populations, aggregated and nonaggregated, when grown under natural environment-mimicking nutrient-limiting conditions, with the aggregated subpopulation producing greater amounts of curli (White et al., 2008). This aggregated subpopulation also showed some typical physiological differences inherent to the rdar morphotype such as increased hypochlorite resistance and uninvolvement in virulence. Some possible advantages coupled to this bistable *csgD* expression and more general the existence of these kind of subpopulations include: (1) cost minimization and benefit maximization given the fact that biofilm formation is an energy costly process (Nadell, Xavier, & Foster, 2009); (2) maintenance of the developmental potential of the population; (3) maximization of the changes for survival under changing environmental conditions.

4.2. RpoS and Crl

RpoS and Crl are two other main regulators of *Salmonella* biofilm formation, influencing this highly complex process at different points (Fig. 1). The RNA polymerase of *Enterobacteriaceae* is composed of a core enzyme (E) that associates with one of the seven sigma factors (σ) to form a holoenzyme (Eσ). A σ factor directs the Eσ complex to a specific set of promoters. While σ^{70}, encoded by *rpoD*, is responsible for transcription during exponential growth, σ^{S},

encoded by *rpoS*, regulates the transcription of genes important for general stress response and stationary phase survival (as reviewed by (Hengge-Aronis, 2002)). Hamilton et al. showed via microarray analysis that more than 25% of the *S.* Typhimurium RpoS regulon is up-regulated in biofilm cells on silicone rubber (Hamilton et al., 2009). In agreement with this, White et al. found the transcription of RpoS to be almost three times higher in *S.* Typhimurium wild-type cells as compared to *csgD* mutant cells (White et al., 2010). During rdar colony growth, *rpoS* expression appeared to be maximal between 3 and 7 days, but was even still detectable after 47 days (White et al., 2006). Furthermore, *S.* Typhimurium *rpoS* mutants show altered morphotypes on CR plates (Römling et al., 2003) and are affected in proper biofilm formation on gallstones (Prouty & Gunn, 2003), glass (Prouty & Gunn, 2003) and polystyrene (Hamilton et al., 2009). Moreover, an *rpoS* insertion mutant has a defect in initial plant (alfalfa) tissue attachment (Barak et al., 2005). Altogether, these findings highlight the importance of RpoS in the survival of cells within the complex biofilm environment. As already mentioned above, a *S.* Enteritidis *rpoS* mutant, however, is not affected in its biofilm forming capacity under ATM conditions (Solano et al., 2002). In an attempt to study serovar-specific differences in multicellular behaviour, Römling et al. noticed that disease-isolated *S.* Typhimurium and *S.* Enteritidis *rpoS* mutants express a saw and bdar morphotype, respectively, pointing at a different role of RpoS in the regulatory cascade leading to biofilm formation (Römling et al., 2003). The *S.* Typhimurium ATCC14028 *rpoS* mutant laboratory strain, on the other hand, also has a bdar-like morphotype (Römling, Sierralta, et al., 1998; Römling et al., 2000).

In the highly regulated rdar morphotype, transcription of *csgD* is highly dependent upon RpoS, and was shown to be maximal at the end of the exponential and the beginning of the stationary phase at ambient temperatures, periods during which *rpoS* expression is maximal as well (Gerstel & Römling, 2001; Römling, Sierralta, et al., 1998; White et al., 2006). Iron limitation caused an increase in *csgD* expression (Römling, Sierralta, et al., 1998) and prolonged expression of *csgB*, *adrA* and *yihU* (White et al., 2008), probably due to activation of *rpoS*. The same mechanism probably holds for the lack of other nutrients such as phosphate, bicarbonate and sulfate. On the other hand, in the semiconstitutive rdar morphotype, expressed at different temperatures and with various environmental stimuli *csgD* transcription is RpoS independent, but still dependent on a functional *ompR* gene (Gerstel & Römling, 2001; Römling, Bian, et al., 1998). Further comparison between both regulatory programs showed that although they respond similarly to most environmental stimuli, the threshold of CsgD expression is often only exceeded in the unregulated strain because of its consistent and intrinsic higher *csgD* expression. The authors suggested this could be because of slight differences (only 1 bp mutations) rendering the unregulated promoter susceptible to the housekeeping sigma factor RpoD recognition and therefore abolish the need for an activator encoded by an RpoS dependent pathway.

Next to regulating *csgD* expression, it was shown that RpoS itself is also required at some steps in *csgBAC* and *adrA* expression, since an *rpoS* mutation has a more profound effect on their expression than a *csgD* mutation. Results further confirmed using *in vitro* transcription experiments (Robbe-Saule et al., 2006). Studies by the group of Römling, however, concluded that *csgB* expression does not require RpoS *in vivo* (Römling, Sierralta, et al., 1998) and showed the interchangeability between RpoS and RpoD during *in vitro* transcription of *csgB* in the presence of CsgD (Zakikhany et al., 2010). Accordingly, a more efficient recognition of the *adrA* promoter by $E\sigma^S$, as compared to $E\sigma^{70}$, in the absence of CsgD was also noticed. Besides, RpoS also positively regulates *mlrA* expression (Brown et al., 2001). Moreover, Adams et al. reported that RpoS is involved in the activation of motility gene expression, pointing at another possible link with biofilm formation (Adams et al., 2001).

Results from adaptive divergence experiments by White and Surette showed that *Salmonella* isolates lacking native *rpoS* activity could revert to rdar producing strains by acquired *cis* (promoter) or *trans* (regulator) mutations (White & Surette, 2006). Furthermore, Davidson et al. indicated the *rpoS* locus to be highly mutable, because of a yet to be identified mechanism, responsible for a lot of cases of evolutionary loss of the rdar morphotype during laboratory passage (Davidson et al., 2008). Since this phenomenon was observed independent of the presence of a functional *csgD* (and hence rdar morphotype) in a daily passage regimen (including stationary phase), it was concluded that this is because of a benefit in nutrient scavenging. Similar trade-off phenomena between nutrient scavenging and stress tolerance have been described in *E. coli*, as reviewed by Ferenci (Ferenci, 2005).

Robbe-Saule et al. showed that a functional Crl protein, a DNA-binding transcriptional regulator, is required for rdar development in *S.* Typhimurium ATCC14028 since a *crl* mutant expressed an atypical rdar morphotype (Robbe-Saule et al., 2006). Through cross-complementation experiments with plasmid-borne *crl* and *rpoS* in *crl*, *rpoS* and *rpoS crl* double mutants, it became clear that Crl exerts its function together with RpoS. Although Crl was found to be required for maximal *csgB*, *bcsA*, *csgD* and *adrA* expression, *in vitro* experiments indicated Crl only directly activates σ^S-dependent, and not σ^{70}-dependent, initiation of transcription at the latter two promoters by enhancing the rate of open complex formation and as such transcription. RpoS dependency of *bcs* gene expression, however, is not noticed in a Nal^R (nalidixic acid-resistant) strain variant (Zogaj et al., 2001), indicating minor strain differences could account for slightly different regulation pathways. Further experiments by Robbe-Saule and colleagues indicated that the magnitude of Crl activation is dependent on the promoters and on the σ^S-levels, with increasing physiological impact and Crl-mediated σ^S-dependent gene activation at decreasing σ^S levels (Robbe-Saule, Carreira, Kolb, & Norel, 2008; Robbe-Saule, Lopes, Kolb, & Norel, 2007). Indeed, upon entry into stationary phase, RpoS levels appeared to be the limiting factor for the expression of RpoS-dependent genes, with Crl increasing the efficiency of transcription. In addition, it was shown that the levels of RpoS and Crl are tightly linked and negatively correlated, with RpoS exerting a negative effect on Crl production and *vice versa*. Since the physiological effects, but not the levels, of Crl are greater at 28 °C than at 37 °C, it is not hard to imagine that Crl can function as a (or one of the) temperature sensor(s) during *Salmonella* biofilm formation.

4.3. Complex regulation of csgD expression by trans (OmpR, IHF, H-NS, CpxR and MlrA)—and cis (csgD 5′UTR)-acting regulators

OmpR (outer membrane protein R) was one of the first *trans*-acting regulators shown to be required for regulation of the *Salmonella csgD* promoter (Römling, Sierralta, et al., 1998), except under ATM conditions (Solano et al., 2002). OmpR is part of the EnvZ (membrane bound sensor kinase)/OmpR two-component regulatory system able to change the OmpR phosphorylation pattern in response to environmental changes such as osmolarity (high osmolarity leads to high levels of phosphorylated OmpR (OmpR-P)) and pH. Several OmpR binding sites (D1–D7) in the *cgsD* promoter were identified using a combination of response genetic studies and *in vitro* experiments (gel shifts and DNase I footprints) (Gerstel, Kolb, & Römling, 2006; Gerstel, Park, & Römling, 2003), showing similarities to the well-characterized *ompF* promoter (Huang & Igo, 1996). Further experiments showed that high affinity binding of OmpR-P to D1, directly upstream of the −35 box, is sufficient for transcriptional activation, while binding to the adjacent D2 and D3–D6 boxes, located further upstream, represses transcription. Binding of OmpR-P to D3–D6 represses transcription under aerobic condition and binding of OmpR-P to D7 represses transcription under microaerophilic conditions. Next to responding to osmolarity, Gerstel and Römling also showed *envZ* is involved in the ethanol induced *csgD* up-regulation (Gerstel & Römling, 2001).

IHF (integrating host factor) is a highly abundant histone-like, heterodimeric, key architectural protein involved in a variety of cellular processes (for a recent review see (Dillon & Dorman, 2010)). IHF, in contrast to H-NS, binds to consensus sequences and can facilitate activation or repression of transcription after DNA bending. Mutants in both IHF subunits *ihfA* and *ihfB* showed altered rdar morphotype expression on CR agar plates: reduced and abolished rdar morphotype expression in the semiconstitutive *csgD* promotor-up mutated strain and highly regulated *csgD* promoter strain, respectively (Gerstel et al., 2003). Since the same phenotypes were observed at different temperatures, IHF seems not to be involved in the temperature dependent rdar regulation (Gerstel & Römling, 2003). Under microaerophilic conditions, but not aerobic or high salt conditions, it was shown that IHF binds to one of the three identified IHF binding sites, IHF1, that overlaps with the above mentioned D3–D6 OmpR binding sites in the *csgD* promoter, and as such enhances *csgD* transcription (Gerstel et al., 2003, 2006). Binding sites IHF2 and IHF3 are both located in the coding sequence of *csgD*. Binding of IHF to IHF2 does not alter *csgD* transcription, whereas binding to IHF3 represses *csgD* transcription. Conceptually, evidence is emerging that transcription factors compete for overlapping binding sites forming different nucleo-protein complexes regulating *csgD* transcription in a fashion coupled with growth phase and environmental stimuli (Gerstel et al., 2006; Ogasawara, Yamada, Kori, Yamamoto, & Ishihama, 2010). An example of this can be found in the IHF/OmpR/oxygen tension interplay model as elaborated by Gerstel et al. (Gerstel et al., 2003; Römling et al., 2007).

The abundant, histone-like, nucleoid-structuring protein (H-NS) is a small DNA architectural protein that plays a key role for many Gram-negative bacteria by integrating a complex range of environmental signals such as temperature and osmolarity. It is involved in direct and indirect transcriptional regulation of many unrelated genes, predominantly acquired through horizontal gene transfer (Navarre et al., 2006). H-NS has no defined binding sites, but binds preferably to AT-rich, intrinsically bent DNA regions. In addition, non-specific DNA binding has also been observed (for reviews see (Dillon & Dorman, 2010; Dorman, 2004)). H-NS showed a complex role in regulating *csgD* expression, strongly dependent on strain background, suggesting that H-NS is not only important in direct regulation at the *csgD* promoter region, but also controls other parts in the complex network leading to *Salmonella* biofilm formation (Gerstel et al., 2003). Reduced *csgD* and rdar morphotype expression was noticed after *hns* gene inactivation in *S.* Typhimurium suggesting H-NS acts as a *csgD* activator. This activating effect, spatially located downstream of the transcriptional start site, seems to be beyond transcriptional regulation or indirect, since no direct H-NS binding was observed in this region. On the contrary, a repressive effect of H-NS was also identified and was attributed to its specific binding to AT-rich stretches in the intergenic *csgB–csgD* region (Gerstel et al., 2003), as could also be visualized by *in vivo* H-NS binding using a ChIP-on-chip (chromatin immunoprecipitation on microarray) approach (Lucchini et al., 2006). Recent evidence in *E. coli* supports the indirect role of H-NS in *csgD* regulation and points at parallels between RpoS and H-NS regulation at every level of the curli control cascade (Weber, Pesavento, Possling, Tischendorf, & Hengge, 2006).

CpxR is a part of the Cpx stress response system that is able to respond to environmental stimuli with envelope stress being the best studied. Although for *Salmonella* no direct role between this system and biofilm formation has been proven, its direct role (through cooperative binding at multiple sites in the *csgB–csgD* intergenic region) and indirect role in *E. coli* biofilm formation, mostly in response to changes in osmolarity, has been shown (reviewed by (Dorel, Lejeune, & Rodrigue, 2006) and further elaborated by others (Ma & Wood, 2009)). The expression of *cpxRA* and hence the Cpx regulon (Price & Raivio, 2009) is highly regulated by different factors such as RpoS, and CpxR-P acts as a biofilm repressor. For example, an increased *cpxRA* expression at the end of the exponential growth phase via RpoS was noticed (De Wulf, Kwon, & Lin, 1999). Since RpoS is also an important regulator of *Salmonella* biofilm formation (see previous section), a potential link between *Salmonella* biofilm formation and the *cpx* system seems reasonable. Interestingly and further confirming this link, in a recent study using the differential fluorescence induction (DFI) single cell approach to study *S.* Typhimurium biofilm formation we identified *cpxP*, the alkaline-induced periplasmic chaperone that inhibits the CpxA autokinase function (Danese & Silhavy, 1998), as being up-regulated under *Salmonella* biofilm formation conditions (Hermans et al., 2011).

MlrA (MerR-like regulator), identified in a cosmid complementation screen to recover *E. coli* and *S.* Typhimurium curli expression, is another *trans*-acting regulator that acts as a positive regulator of *csgD* expression (Brown et al., 2001). Unlike the other identified general transcription regulators, MlrA acts highly specifically on the transcription of the *csgDEFG* operon. Proteins of the MerR family consist of an N-terminal DNA-binding domain containing a HTH motif and a C-terminal receiver domain that responds to environmental stimuli such as metals (as reviewed by Brown, Stoyanov, Kidd, & Hobman, 2003). Unfortunately, the environmental trigger leading directly or indirectly to *mlrA* activation and the mechanism of MlrA-mediated *csgD* expression have not yet been identified. Recent evidence in *E. coli*, based on both *in vivo* and *in vitro* analyses, identified direct MlrA binding to a 33 bp palindromic sequence between the IHF and OmpR binding sites upstream of the *csgD* promoter (Ogasawara, Yamamoto, & Ishihama, 2010). The three positive regulators (MlrA, IHF and OmpR) binding to the same part of the *csgD* promoter region were found to function independently, without showing strong cooperation. Based on activation mechanisms of other MerR activators, Ogasawara et al. suggested MlrA to induce DNA curvature changes influenced by the activity of neighbouring DNA and by the molecular interplay between *csgD* promoter-bound transcription factors (Ogasawara, Yamamoto, et al., 2010). Further, they identified the MlrA-box sequence in the promoter region of some other *E. coli* genes, some of which encoding transcription factors, putting MlrA higher in the transcription factor network hierarchy.

Inactivation of *mlrA* in wild-type *S.* Typhimurium resulted in a loss of curli production and rdar/rugose phenotype expression, while these properties were not affected in a temperature-and RpoS-independent *csgD* promoter-up mutant strain (Brown et al., 2001). Garcia et al. also identified the *S.* Typhimurium ATCC14028 *mlrA* as a gene with the capacity to initiate cellulose production and biofilm formation in LB complex medium in *S.* Typhimurium SL1344, a strain that has lost the ability to synthesize this extracellular matrix compound due to domestication (Garcia et al., 2004). More specifically, it was noticed that SL1344 showed an *mlrA* transcription deficiency that provoked a deficit in cellular AdrA protein levels resulting in absence of cellulose synthesis and a biofilm-negative phenotype. A *S.* Typhimurium ATCC14028 *mlrA* mutant was also deficient in cellulose production and biofilm formation under LB conditions. Together, these data demonstrated that MlrA and AdrA (because of its activation through MlrA-dependent *csgD* activation) are crucial for biofilm formation under LB conditions. Remarkably, SL1344 was also the strain used by Brown et al. to initially determine the role of *mlrA* and they did not notice any matrix production defects in wild-type SL1344 cells. This discrepancy can be explained by the fact that strain isolates from different laboratories might show different stages of domestication (Davidson et al., 2008; Li, Yue, Guan, & Qiao, 2008). In this context, White and Surette hypothesized that the high prevalence of *trans*-regulatory mutations in the SARC16 isolates might also be the result of domestication (White & Surette, 2006). Considering *mlrA*, they showed that four rdar-deficient SARC16 isolates could be restored to the rdar-proficient phenotype with *mlrA* overexpression, as identified during a plasmid-based recovery screen, although all four had normal wild-type levels of *mlrA* expression

(White & Surette, 2006). This result showed that overexpression of this *trans*-acting regulator can compensate for other *trans*-regulatory defects, resulting in increased *csgD* expression. Interestingly, they also observed adaptive divergence to pellicle formation in three saw SARC16 strains, previously unable to colonize the air–liquid interphase, after prolonged growth in rich LB medium (through *cis* mutations in the *csgD* promoter region or *trans*-mutations upstream of *csgD*), indicating domestication can indeed cause alterations in biofilm phenotype.

Next to these *trans*-acting regulators, a *cis*-acting sequence that plays an important role in stationary phase *csgD* activation is the 174 bp 5′UTR of the *csgD* mRNA (Gerstel et al., 2006). Recent evidence in *E. coli* shows that this region is important in post-transcriptional down-regulation of *csgD* by two redundant sRNAs, OmrA and OmrB (Holmqvist et al., 2010) and indicates that the corresponding region in *Salmonella* could serve the same purpose. However, this still requires experimental validation. Expression of OmrA and OmrB in exponentially growing cells is controlled by the EnvZ/OmpR system (Guillier, Gottesman, & Storz, 2006), a system also directly involved in *csgD* transcriptional regulation, further stressing the complexity and importance of the *csgD* regulatory system.

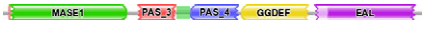
4.4. c-di-GMP

During recent years, it has become clear that bis-(3′–5′)-cyclic dimeric guanosine monophosphate or shortly c-di-GMP, originally discovered as an allosteric control factor of cellulose synthesis in *Gluconacetobacter xylinus* (Ross et al., 1987), is an important bacterial secondary messenger regulating multicellular behaviour, but also motility and virulence (Lamprokostopoulou, Monteiro, Rhen, & Römling, 2010), in response to a variety of extracellular signals (as recently reviewed by (Hengge, 2009)). C-di-GMP is synthesized by diguanylate cyclases (DGCs) (GGDEF domain proteins) starting from two molecules of guanosine triphosphate (GTP) and is degraded into 5′-phosphoguanyl-(3′–5′)-guanosine (pGpG), by specific phosphodiesterases (PDEs) (EAL- or HD-GYP domain proteins). pGpG can subsequently be converted into two molecules of guanosine monophosphate (GMP) by nonspecific PDEs. Many DGCs also contain an RxxD motif able to bind c-di-GMP and allosterically control the activity of these enzymes. Three other classes of c-di-GMP effectors are currently known with the PilZ domain proteins being the best studied. An abundance of these GGDEF, EAL and HD-GYP domain proteins were found to be encoded in the genomes of all kinds of bacteria, even in the deeply branching phyla (Galperin, Nikolskaya, & Koonin, 2001). The *S.* Typhimurium genome, for example, encodes 19 such proteins: 5 GGDEF, 7 EAL and 7 GGDEF/EAL domain proteins (Table 2). Since several of these proteins show modular structures with often cytoplasmic or sensory units in addition to the GGDEF and/or EAL domains, they are ideal candidates for the integration of different environmental signals into the cellular signalling cascade (Römling, 2005). For example, recently a heme-containing globin, with a GGDEF motif, has provided a direct link between oxygen sensing and c-di-GMP synthesis (Wan et al., 2009). Cellular c-di-GMP levels depend on the protein levels, their specific DGC and PDE activities and can vary intracellularly. In this context, it has recently been shown that *S.* Typhimurium exhibits asymmetric c-di-GMP distribution upon cell division, which might be caused by spatially restricted expression or activation of individual DGC and/or PDE enzymes within the progeny (Christen et al., 2010). The authors suggested this might be a general mechanism regulating and fine-tuning motility and other cellular properties. Generally, high c-di-GMP levels favour sessility, often by stimulation of extracellular matrix production, and repress motility and virulence (Hengge, 2009; Jenal & Malone, 2006; Kader, Simm, Gerstel, Morr, & Römling, 2006; Lamprokostopoulou et al., 2010; Römling & Amikam, 2006; Simm et al., 2004). As can be expected, the rdar morphotype, and more general, *Salmonella* biofilm formation, is highly regulated by c-di-GMP at various levels as will be discussed in this section and can be seen from Fig. 1.

AdrA was the first GGDEF domain protein identified to be important in *Salmonella* biofilm formation because of its regulatory role in cellulose biosynthesis (Zogaj et al., 2001). Subsequent studies, using CR/CF assays and cellular c-di-GMP quantification, showed that AdrA exerted its effect on the *bcs* genes, responsible for cellulose biosynthesis, post-transcriptionally through c-di-GMP, thus delivering the first evidence for the importance of this secondary messenger in *Salmonella* biofilm formation (Simm et al., 2004). The C-terminal domain of BcsA, the cellulose synthase catalytic subunit, contains a PilZ receptor domain that changes conformation after c-di-GMP binding, probably leading to BcsA activation (Ryjenkov, Simm, Römling, & Gomelsky, 2006). Simm et al. also found that the EAL domain proteins STM3611 (*yhjH*) and STM1827 are involved in down-regulation of cellular c-di-GMP levels leading to reduced cellulose production, rdar morphotype (curli and cellulose) expression and biofilm formation on the one hand, and enhanced motility (swimming and swarming) on the other hand (Simm et al., 2004). Furthermore, Garcia et al. reported that STM1987, a GGDEF domain protein not regulated by CsgD, is required for the activation of cellulose biosynthesis and hence biofilm formation under ATM conditions, a function that could not be fulfilled by AdrA (Garcia et al., 2004). Under LB conditions, however, STM1987 appeared not to be necessary for these processes, unless AdrA is absent, suggesting a certain degree of redundancy and task distribution in the c-di-GMP driven cellulose activation. Further investigating this redundancy, Garcia et al. showed that different GGDEF proteins (STM1987, STM4551, STM2123 (*yegE*) and STM3388), when expressed from a plasmid, are able to assume the role of AdrA under LB conditions, while another subset (STM4551, STM2123 (*yegE*), STM1283 (*yeaJ*) and STM3388) is able to complement STM1987 function under ATM biofilm conditions. One GGDEF protein (STM2672 (*yfiN*)) was found unable to restore cellulose synthesis in both media. STM1703 (*yciR*), a complex GGDEF/EAL domain protein, on the other hand, appeared to function as an EAL domain protein, down-regulating cellulose synthesis (and biofilm formation) via c-di-GMP degradation (Garcia et al., 2004), as recently confirmed (Simm et al., 2007). None of the corresponding GGDEF protein mutants, except for STM1987, AdrA and the PDE STM1703 showed effects on cellulose biosynthesis or biofilm formation, again pointing at the redundant character of c-di-GMP signalling. Moreover, transcription of none of these seven tested GGDEF proteins was found to be regulated by MlrA or CsgD, indicating they integrate different metabolic pathways and/or environmental stimuli into the system. More recently, Simm et al. showed that in addition to AdrA, only one other GGDEF protein, STM1987, is able to activate cellulose production when expressed at physiological conditions from its proper chromosomal location (Simm et al., 2007). Further elaborating on the redundancy and emphasizing the general occurrence of c-di-GMP signalling, functional interchangeability between similar GGDEF proteins from closely related bacteria was observed (Jonas et al., 2008; Rahman et al., 2007; Simm, Fetherston, Kader, Römling, & Perry, 2005). Together, these data clearly show the first level of c-di-GMP regulation in the *Salmonella* biofilm formation cascade: controlling cellulose biosynthesis.

Another level of c-di-GMP regulation became evident from research on the hierarchical involvement of GGDEF domain proteins in *S.* Typhimurium biofilm formation (Kader et al., 2006). It was shown that elevated c-di-GMP concentrations, next to activating cellulose biosynthesis, also enhance curli production through enhancing CsgD and CsgA expression transcriptionally and post-transcriptionally. Elevated c-di-GMP levels are also able to overcome the temperature regulation of the rdar morphotype (via AdrA overexpression or STM1703 or STM4264 mutation) (Kader et al., 2006; Simm et al., 2007), lead to monomodal CsgD-GFP expression (via STM1703

Table 2
Representation of all 19 *S.* Typhimurium GGDEF and EAL domain proteins.

Genome ID	Gene name	Domain organization[a]	GGDEF/EAL motif[b]	Enzymatic activity[c]	Cellular function	Regulation	Reference
STM0343			EAL	?	No reported function yet		
STM0385	*adrA*/*yaiC*		GGDEF	DGC	Stimulation of cellulose biosynthesis and rdar development; up-regulation of biofilm formation under LB (but not ATM) conditions; down-regulation of motility; restoring cellulose production in a c-di-GMP lacking strain	Direct up-regulation by *csgD*	(Garcia et al., 2004; Simm et al., 2004; Solano et al., 2009; Zakikhany et al., 2010; Zogaj et al., 2001)
STM1283	*yeaJ*		GGDEF	DGC	Assuming role of STM1987 under ATM conditions		
STM1344	*cdgR*		EII	No PDE	Indirect up-regulation of *csgD* and rdar expression; down-regulation of motility; role in virulence (survival in mice, antioxidant defense, macrophage killing)	Direct repression by CsrA	(Hisert et al., 2005; Jonas et al., 2010; Simm et al., 2007; Simm et al., 2009; Wozniak et al., 2009)
STM1697			EIT	?	Possible role in virulence, no obvious role in motility or biofilm formation		(Simm et al., 2007; Yoon, McDermott, Porwollik, McClelland, & Heffron, 2009)
STM1703	*yciR*		GGDEF/EAL	PDE	Strong down-regulation of *csgD* (temperature suppressive effect), *csgA* and rdar expression, biofilm formation and cellulose synthesis	Negatively regulated by STM1344 and CsrA	(Garcia et al., 2004; Simm et al., 2007; Simm et al., 2009)
STM1827			EAL	Putative PDE	Slight down-regulation of cellulose biosynthesis, *csgD*, rdar development and biofilm formation; very little up-regulation of swarming	Negatively regulated by CsrA	(Jonas et al., 2010; Simm et al., 2004; Simm et al., 2007)
STM1987			GGEEF	DGC	Up-regulation of cellulose production and biofilm formation under ATM conditions (independent of *csgD*); assume role of *adrA* under LB conditions; restoring cellulose production and ATM biofilm formation in a c-di-GMP lacking strain	Negatively regulated by CsrA	(Garcia et al., 2004; Jonas et al., 2010; Solano et al., 2009)
STM2123	*yegE*		GGDEF/WLV	DGC	Assuming role of *adrA* and STM1987 under LB and ATM conditions respectively; up-regulation of *csgD* and rdar expression; restoring cellulose production and ATM biofilm formation in a c-di-GMP lacking strain		(Garcia et al., 2004; Kader et al., 2006; Solano et al., 2009)
STM2215	*rtn*		EAL	?	Involved in fitness in mice		(Santiviago et al., 2009)
STM2410	*yfeA*		PGSEL/EAL	Probably PDE-like	Reinstating swimming in a c-di-GMP lacking strain		(Solano et al., 2009)
STM2503			SGHDL/EAL	Probably PDE-like	Reinstating swimming in a c-di-GMP lacking strain		(Solano et al., 2009)
STM2672	*yfiN*		GGDEF	DGC	Unable to assume role of *adrA* and STM1987 under LB and ATM conditions respectively; restoring cellulose production and ATM biofilm formation in a c-di-GMP lacking strain		(Garcia et al., 2004; Solano et al., 2009)
STM3375	*yhdA*/*csrD*		HRSDF/ELM	No PDE or DGC (in *E. coli*)	Up-regulation of motility (swimming) and biofilm formation (in liquid LB without salt); destabilizing activity of sRNA *csrB* and *csrC* along with RNase E, (in *E. coli*)	Negatively regulated by CsrA	(Jonas et al., 2010; Simm et al., 2007; Suzuki et al., 2006)
STM3388			GGDEF/EAL	DGC	Assuming role of *adrA* and STM1987 under LB and ATM conditions respectively; up-regulation of *csgD* and rdar expression		(Garcia et al., 2004; Kader et al., 2006; Solano et al., 2009)
STM3611	*yhjH*		ELL	PDE	Down-regulation of cellulose	Negatively	(Frye et al., 2006;

(*continued on next page*)

Table 2 (*continued*)

Genome ID	Gene name	Domain organization[a]	GGDEF/EAL motif[b]	Enzymatic activity[c]	Cellular function	Regulation	Reference
					biosynthesis, *csgD*, rdar and biofilm formation; up-regulation of motility (antagonized by the PilZ protein *ycgR*); involved in growth competition between different *S. enterica* strains	regulated by STM1344; positively regulated by CsrA and FlhDC via FliA	Jonas et al., 2010; Rychlik et al., 2002; Simm et al., 2004; Simm et al., 2007; Simm et al., 2009)
STM3615	*yhjK*		SGYDF/EAL	?	No reported function yet		
STM4264	*yjcC*		EAL	PDE	Strong down-regulation of *csgD* (slight temperature suppressive effect), *csgA* and rdar expression, biofilm formation and cellulose (also *csgD* independent) synthesis		(Kim & Wei, 2009; Simm et al., 2007; Simm et al., 2009)
STM4551			GGEEF	DGC	Assuming role of *adrA* and STM1987 under LB and ATM conditions respectively; regain motility, curli (rdar)production, virulence and long-term survival in a c-di-GMP lacking strain	Negatively regulated by CsrA	(Garcia et al., 2004; Jonas et al., 2010; Solano et al., 2009)

Some proteins only have one of both domains, while others harbour both. (a) Domain organization as retrieved from the pfam database (Finn et al., 2010). EAL domain: diguanylate phosphodiesterase; MASE2 domain: predicted integral membrane sensory domain; GGDEF domain: diguanylate cyclase activity; PAS domain: signal sensor domain, member of the PAS clan; MASE1 domain: predicted integral membrane sensory domain; PAS3 domain: signal sensor domain, member of the PAS clan; PAS4 domain: signal sensor domain, member of the PAS clan. (b) Alterations in the conservation pattern in GGDEF/EAL domains. (c) DGC (di-guanylate cyclase activity) and PDE (phosphodiesterase activity).

deletion) (Grantcharova et al., 2010) and even confer a positive feedback loop in the semiconstitutive rdar morphotype (higher *csgD* expression caused higher AdrA levels leading to elevated cytoplasmic c-di-GMP levels leading on its turn again to CsgD activation) (Kader et al., 2006). Although AdrA has a large impact on the generation of elevated cellular c-di-GMP levels, it only participates to a minor extent in CsgD activation (Kader et al., 2006). Considering DGCs, CsgD expression mainly appears to be activated by STM2123 and STM3388, complex GGDEF/EAL domain proteins, in an additive manner, transcriptionally as well as post-transcriptionally. While STM2123 is important for *csgD* expression in the beginning of the plate growth process, the positive effect of STM3388 occurs in a later stadium of rdar morphotype expression. Next to GGDEF domain proteins, 4 of the 15 *S.* Typhimurium EAL domain proteins (STM1703, STM1827, STM3611 and STM4264) are also involved in the regulation of *csgD* expression, as clarified by a reverse genetic approach (Simm et al., 2007). More specifically, mutants of these PDEs showed CsgD up-regulation, via up-regulated global c-di-GMP levels (except STM3611), and hence rdar morphotype expression, with STM1703 and STM4264 having the most pronounced effect. In addition, STM1703 and STM4264 mutants also enhance *csgA* expression. Since the levels of c-di-GMP and *csgD* expression do not fully coincide (*e.g.* an STM4264 mutant has the highest c-di-GMP up-regulation, but not the highest *csgD* expression) and since slight differences in rdar morphotype were noticed (*e.g.* between STM1703 and STM4264 mutants), it was suggested that different c-di-GMP pools serve different goals: (1) the c-di-GMP pool in a STM1703 mutant is to a higher extent dedicated to *csgD* up-regulation than the ones in STM1827 and STM4264 mutants; (2) a part of the c-di-GMP pool in a STM4264, and not STM1703 mutant is involved in a *csgD*-independent pathway of cellulose production. Furthermore it was found that only two PDEs (STM3375 and STM3611) are involved in motility and that of the four PDEs (STM1703, STM1827, STM3611 and STM4264) involved in rdar expression, only STM3611 is not involved in pellicle formation (Simm et al., 2007). Altogether, these data stress the complexity, partial redundancy and functional specificity of c-di-GMP signalling with task distribution between different GGDEF/EAL domain proteins and different, overlapping c-di-GMP pools serving different fates. Such a task distribution can involve a temporal and spatial component. Differences in output domain structures, however, can also lead to different stimuli and pathway dependency.

Recently, it was shown that STM3611 mutants require the PilZ domain protein, STM1798 (*ycgR*), for their motility inhibition, suggesting that STM3611 and STM1798 (both class 3 flagellar genes) have antagonistic functions (Paul, Nieto, Carlquist, Blair, & Harshey, 2010; Ryjenkov et al., 2006). The latter physically interacts with the flagellar motor proteins FliG and FliM in a c-di-GMP-mediated way via a backstop brake mechanism to inhibit motility and chemotaxis (Paul et al., 2010). As such, biofilm formation could be mediated (Girgis, Liu, Ryu, & Tavazoie, 2007; Paul et al., 2010; Pesavento et al., 2008). Since motor control is more rapid and reversible as compared to gene expression or assembly-based mechanisms, this could be a subtle way to keep cells ready to react more quickly when the biofilm disperses.

Next to DGC and PDE activity, some proteins (with degenerative GGDEF and EAL domains) in the c-di-GMP network also exhibit alternative functions. STM1344 (*ydiV*), for example, originally identified as important in virulence and antioxidant defense and renamed *cdgR* (Hisert et al., 2005), shows high similarity to STM3611 but can be seen as an unconventional EAL-domain protein since it does not possess PDE activity (Simm, Remminghorst, Ahmad, Zakikhany, & Römling, 2009). STM1344 was also shown to have an indirect role in c-di-GMP metabolism, able to alter c-di-GMP levels *in vivo* via direct or indirect upstream transcriptional repression of the PDEs STM1703 and STM3611. As such, it indirectly stimulates rdar and *csgD* expression (and hence biofilm formation) and represses swimming motility. STM1344 was also directly identified as a negative regulator of flagellar gene expression by acting on post class 1 flagellar genes (Wozniak, Lee, & Hughes, 2009).

Everything discussed concerning c-di-GMP regulation until now was inferred from studies with recombinant bacteria either overexpressing or lacking individual members of the c-di-GMP pathway. Since interference or compensation from other remaining members of the regulon is overlooked in such a situation, Solano et al. used a genetic reductionist approach to study the individual roles of *S.* Enteritidis GGDEF proteins within the c-di-GMP signalling network with respect to biofilm formation, motility and virulence (Solano et al., 2009). They confirmed that c-di-GMP is essential for biofilm formation (through curli and cellulose production via CsgD), virulence and motility, since a

strain lacking all GGDEF proteins does not show any of these phenotypes. Some interesting findings concerning biofilm formation were distilled using a c-di-GMP defective strain: (1) STM4451 is the only individual GGDEF protein able to reinstate curli production (CR binding); (2) none of the GGDEF proteins alone is able to restore biofilm formation under LB conditions; (3) STM1987, STM2123 or STM2672 are individually able to restore biofilm formation under ATM conditions, indicating their involvement in cellulose production; (4) individual AdrA, STM1987, STM2123, STM2672 or the heterologous *Yersinia pestis* HmsT protein stimulates cellulose production (CF binding); (5) swimming is only regained with STM2410 (*yfeA*), STM2503 or STM4551. Further research identified that STM4551 stimulates curli through direct or indirect activation of *csgD* transcription, a mechanism that does not involve c-di-GMP. In addition, cellulose biosynthesis appeared to be the only process totally dependent on c-di-GMP, without discrimination between the c-di-GMP sources, suggesting that the physiological c-di-GMP levels originating from different PDEs can regulate the same target. Altogether, GGDEF proteins, once activated, control cellular functions at two different levels: direct c-di-GMP-mediated control (through binding to and changing of protein activity) and gene expression level control (in which c-di-GMP-dependent and independent mechanisms can be intertwined).

4.5. BarA/SirA and Csr system

The BarA/SirA two component system, widely conserved within the gamma-proteobacteria, is an important global regulatory system involved in *Salmonella* virulence, motility and biofilm formation (Ahmer, van Reeuwijk, Timmers, Valentine, & Heffron, 1998; Jonas et al., 2010; Teplitski, Goodier, & Ahmer, 2003; Teplitski et al., 2006). SirA is a response regulator of the FixJ family (as is CsgD) that is phosphorylated by its cognate sensor kinase BarA (Altier, Suyemoto, Ruiz, Burnham, & Maurer, 2000; Pernestig, Melefors, & Georgellis, 2001) or by cellular acetyl phosphate (Lawhon, Maurer, Suyemoto, & Altier, 2002). Stimuli affecting the activation of this two-component system have not been fully elucidated but external pH and the metabolic end products formate and acetate were shown to be important in *E. coli* (Chavez, Alvarez, Romeo, & Georgellis, 2010; Mondragon et al., 2006; Pernestig et al., 2003), whereas in *Salmonella* bile salts and short-chain fatty acids seem to affect BarA/SirA mediated downstream responses (Lawhon et al., 2002; Prouty & Gunn, 2000). SirA transcriptionally activates the sRNAs *csrB* and *csrC*. Both sRNAs are part of the *Salmonella* Csr system and antagonize the activity of the RNA-binding protein CsrA. In *E. coli*, CsrA indirectly activates the transcription of its RNA antagonists *csrB* and *csrC* via BarA/SirA (Gudapaty, Suzuki, Wang, Babitzke, & Romeo, 2001; Suzuki et al., 2002). It has been suggested that a similar feed-back loop exists in *S.* Typhimurium (Jonas et al., 2010). This system is an important regulatory system controlling various phenotypes such as motility, carbon storage, virulence, secondary metabolism and biofilm formation (Jonas et al., 2010; Lapouge, Schubert, Allain, & Haas, 2008; Lucchetti-Miganeh, Burrowes, Baysse, & Ermel, 2008; Teplitski et al., 2006).

Teplitski et al. showed that *sirA* and *fimI* mutants as well as *csrB csrC* double mutants are defective in biofilm formation on plastic surfaces, while *flhDC* mutants show more biofilm formation (Teplitski et al., 2006). More specifically, they demonstrated the regulatory role of SirA (on transcriptional level) and the Csr system (on translational and/or message stabilization level) on the expression of flagellar and type I fimbrial genes, both contributing to biofilm formation through negative and positive regulation respectively, under their tested conditions. In this context, SirA-P transcriptionally activates *csrB* and *csrC*, the *fim* operon and *hilA* of the *Salmonella* pathogenicity island 1 (SPI-1). Up-regulated *csrB*/*csrC* levels inhibit CsrA activity. This reduced CsrA activity, in turn, leads to reduced FlhDC and HilA levels and, since both were shown to be biofilm-inhibiting factors (Teplitski et al., 2006), to up-regulated biofilm formation. Since CsrA also reduces the Fim levels, this reduced CsrA activity leads to more type I fimbriae and hence up-regulated biofilm formation.

After discovery of a link between the Csr system and c-di-GMP signalling in *E. coli* (Jonas et al., 2008), recently, a similar link has been identified in *S.* Typhimurium (Jonas et al., 2010). It was shown that CsrA directly and indirectly regulates the expression of at least eight genes involved in this network encoding GGDEF (STM1987 and STM4451), GGDEF/EAL (STM1703 and STM2275) as well as EAL (STM1687, STM1827, STM3611) domain proteins and the unconventional STM1344 (Simm et al., 2009). STM3611 was found to be the only positively regulated gene, while the rest was down-regulated by CsrA. Further experiments pointed at a complex regulation integrating the Csr, flagellar and c-di-GMP system to control biofilm formation. CsrA controls the switch between sessility and motility at multiple hierarchical levels, generally activating motility and inhibiting sessility. Firstly, by direct regulation of the master regulator FlhDC (class 1 flagellar gene), resulting in a *fliA* (class 2 flagellar gene)-mediated up-regulation of STM3611, STM1798 and class 3 flagellar genes (*fliC*, *fljB*, etc.). Secondly, CsrA directly represses STM1344. This unconventional EAL domain protein in turn transcriptionally represses STM1703 (Simm et al., 2009) and interferes with the flagella-cascade upstream of *fliA*, resulting in an up-regulation of STM3611, STM1798 and class 3 flagellar genes (*fliC*, *fljB*, etc.). Thirdly, by direct interaction with and stabilization of the STM3611 mRNA transcript. Fourthly, CsrA directly regulates STM3375 (*csrD*), a degenerative GGDEF/EAL domain protein with no apparent DGC/PDE activity, resulting in destabilization of CsrB and CsrC activity (in *E. coli*) (Jonas et al., 2008; Suzuki, Babitzke, Kushner, & Romeo, 2006). As such, CsrA controls its own activity through an autoregulatory loop. Finally, by direct inhibition of STM1697 and STM1987, and by indirect inhibition of STM1703, STM1827 and STM4551, of which the functions and integration into the network have been described above. Providing a direct link between these regulatory cascades and *Salmonella* multicellular behaviour, Solano et al. identified BarA to be essential for the formation of a tight pellicle under LB conditions (Solano et al., 2002). Surprisingly, however, a *S.* Typhimurium *csrA* mutant does not show increased biofilm formation (Teplitski et al., 2006), suggesting additional components are involved in this complex network.

4.6. PhoPQ–RstA

The PhoPQ system of *Salmonella* is a two-component system consisting of the cytoplasmic response regulator PhoP and the inner membrane located sensor kinase PhoQ (Kato & Groisman, 2008). Low Mg^{2+} concentration is the signal (Garcia Vescovi, Soncini, & Groisman, 1996) that, when detected by the periplasmic region of the sensor PhoQ, triggers autophosphorylation of PhoQ and transfer of the phosphate to the PhoP protein, resulting in PhoP activation (Castelli, Garcia Vescovi, & Soncini, 2000; Chamnongpol, Cromie, & Groisman, 2003; Shin & Groisman, 2005). It has been proposed that the PhoQ protein also senses antimicrobial peptides and low pH (Bader et al., 2005; Prost et al., 2007), however, this notion has been questioned (Groisman & Mouslim, 2006). Furthermore, a number of cellular regulators, such as H-NS (Song et al., 2008), SlyA (Navarre et al., 2005; Song et al., 2008), Rcs (Tierrez & Garcia-del Portillo, 2004), MgrB (Lippa & Goulian, 2009), and RstB (Nam, Choi, Kweon, & Shin, 2010) have been shown to regulate the PhoP/Q system at the transcriptional or post-transcriptional level. Upon activation, PhoP directly and indirectly controls the expression of more than 120 genes (Kato & Groisman, 2008), involved in several functions such as LPS modification, magnesium transport, invasion of epithelial cells and intramacrophage survival (Charles et al., 2009).

Prouty and Gunn reported that a *S.* Typhimurium *phoP* null-mutant is a better biofilm former on gallstones as compared to the wildtype (Prouty & Gunn, 2003). Consistently, the *phoP* null-mutant

exhibits enhanced biofilm formation on glass coverslips as compared to the wildtype, while a PhoP constitutive strain (mimicking continuous activation of the PhoP regulon) is unable to develop a biofilm (Prouty & Gunn, 2003). Together, these data clearly show that the PhoPQ system represses *S.* Typhimurium biofilm formation. A number of PhoP targets can be indicated as plausible candidates mediating the observed biofilm defect upon PhoP activation.

Firstly, Prouty and Gunn suggested *prgH*, a PhoPQ-repressed gene to be involved in the PhoPQ-dependent biofilm regulation (Prouty & Gunn, 2003). Indeed, it was found that a *prgH* mutant is unable to form a mature biofilm on gallstones and glass. Moreover the *prgH* mutation eliminates the phoP-null phenotype on gallstones and glass. Several hypotheses were formulated by the authors to explain how *prgH*, a gene which is involved in the formation of a functional *Salmonella* Pathogenicity Island 1 type III secretion apparatus (SPI-1-TTSS), could be linked to biofilm formation, including the possibility that the TTSS is utilized for EPS secretion or for cell-surface and/or cell–cell interactions.

A second factor possibly contributing to the PhoPQ dependency of biofilm regulation is the indirect regulation of the cellular RpoS levels by PhoPQ. As described in the previous sections, the stress sigma factor RpoS controls biofilm formation in a number of ways such as transcriptional up-regulation of the biofilm master regulator CsgD and motility genes. Tu et al. showed that PhoP stabilizes RpoS by acting as a transcriptional activator of *iraP*, which encodes a product that enhances RpoS stability by interacting with RssB, the protein that normally delivers RpoS to the ClpXP protease for degradation (Tu, Latifi, Bougdour, Gottesman, & Groisman, 2006). Contrastingly, PhoP has also been shown to activate the expression of RstA (Choi, Groisman, & Shin, 2009), a protein that induces RpoS degradation independently of the ClpXP-SsrB proteolytic pathway. RstA is the response regulator of the RstA/RstB two-component system (Cabeza, Aguirre, Soncini, & Vescovi, 2007). The opposing effects of IraP and RstA suggest that the cellular RpoS levels are fine-tuned depending on the extracellular signals encountered.

The activation of the RstA expression by PhoP could also contribute to the PhoPQ-dependent biofilm regulation in a number of alternative ways. Next to its effect on RpoS, RstA also affects the expression of *bapA* (Cabeza et al., 2007). Furthermore, in *E. coli* RstA overexpression has been shown to down-regulate *csgD* expression by direct binding to its promoter (Ogasawara et al., 2007). The presence of the RstA binding motif in the promoter of *Salmonella csgD* suggests that RstA also directly inhibits *csgD* expression in *Salmonella* (Cabeza et al., 2007). Consistently with the role of RstA in regulation of RpoS levels, *bapA* and *csgD* expression, RstA overexpression was found to reduce the phenotype of *Salmonella* biofilm formation on polystyrene surfaces (Cabeza et al., 2007).

A final possible link between PhoPQ and biofilm formation consists in the finding by Adams et al. showing that activation of PhoP results in decreased motility, decreased protein levels of phase 1 and phase 2 flagellin and decreased transcription of class 3 motility genes such as *fliC* (Adams et al., 2001). The influence of PhoP on the cellular RpoS levels could partially explain the effect of PhoP on motility, as RpoS was found to positively regulate the expression of flagellin and the transcription of class 3 motility genes (Adams et al., 2001). However, this may not be the full explanation since we observed that a PhoP constitutive strain is much more defective in motility than an *rpoS* mutant (Steenackers et al., unpublished data).

4.7. The Rcs system

The RcsC–RcsD–RcsB phosphorelay system consists of the sensor kinase RcsC, the intermediate phosphotransfer protein RcsD, the transcriptional regulator RcsB and the transcriptional co-regulator RcsA (Clarke, 2010). This system can be activated by high osmolarity, cell envelop stress (*e.g.* via *tolB* mutations, cationic antimicrobial peptides or iron challenge in a *pmrA* mutant) and overproduction of certain proteins such as DjlA (Mariscotti & Garcia-del Portillo, 2009). Certain point mutations in the *igaA* gene, such as a mutation (*igaA1* allele) that causes a non-conservative R188H amino acid change in the IgaA protein, have also been shown to result in an activation of the Rcs regulon by derepression of the RcsC kinase activity (Mariscotti & Garcia-del Portillo, 2009). Activation of the Rcs system results in a drastic induction of genes for colanic acid capsule synthesis and repression of flagellar synthesis genes and virulence genes (Majdalani & Gottesman, 2005; Wang, Zhao, McClelland, & Harshey, 2007). Consistent with this, an *igaA* mutant has been shown to be mucoid (Cano, Dominguez-Bernal, Tierrez, Garcia-Del Portillo, & Casadesus, 2002). These findings suggest the Rcs system to be in support of a sessile lifestyle within biofilms. This notion is further corroborated by the finding that activation of the Rcs system by the *igaA*1 mutation results in a repression of the transcription of *phoP* and several PhoP regulated genes, via a RpoS-mediated process (Tierrez & Garcia-del Portillo, 2004).

4.8. Biofilm formation coincides with a global metabolic shift

Next to the importance of well-defined regulators, a number of studies indicated that the switch from a free living state to the biofilm mode of growth coincides with a global change in metabolism (Hamilton et al., 2009; White et al., 2010).

Hamilton et al. studied the transcriptomic and proteomic profiles of *S.* Typhimurium biofilms (Hamilton et al., 2009). Several genes and proteins involved in bacterial attachment, motility, detection and response to oxygen availability, global gene regulation, transport, stress response and the SPI-2-TTSS system were found to be differentially expressed in biofilms as compared to planktonic cells. Interestingly, also several genes involved in amino acid metabolism were shown to be differentially expressed. At the proteomic level, AnsB, involved in asparagine metabolism, was found to be drastically up-regulated in the biofilm. At the transcriptomic level, genes involved in alanine (*dad*) and glutamine/glutamate (*gln*, *gltL*, *astE*, *nadE*, STM1795) metabolism and transport were found to be highly expressed in the biofilm as compared to the planktonic state. Furthermore, the biosynthetic genes of the *trp* operon, required for the synthesis of tryptophan, and the tryptophan-specific transporter *mtr* are up-regulated in well-established *S.* Typhimurium biofilms. To further investigate the importance of tryptophan synthesis and transport in *Salmonella* biofilm formation, *trpE* and *mtr* deletion mutants were tested for their ability to form biofilms. The *trpE* gene, a member of the *trp* operon, encodes anthranilate synthase component I, which catalyses the first reaction of the tryptophan pathway together with TrpD and is subject to feedback inhibition by tryptophan. While the *mtr* mutant did not appear to have an altered biofilm formation, the *trpE* mutant was found to form significantly less biofilm both on silicon rubber tubes and glass surfaces. CF/CR studies indicated that the reduced biofilm formation of the *trpE* mutant is related to reduced cellulose production. It was found that exogenous tryptophan or indole could restore the ability of the *trpE* mutant to form a biofilm, which suggests that the biofilm defect of the *trpE* mutant is the direct result of inability of the cells to synthesize or take up sufficient tryptophan or indole. Interestingly, Solano et al. previously already identified a link between *Salmonella* multicellular behaviour and amino acid metabolism since *carB*, encoding the carbamoyl phosphate synthase large subunit, and *cysE*, encoding serine acetyltransferase, insertion mutants are not able to form a tight pellicle under LB conditions (Solano et al., 2002). Mutants involved in the biosynthesis of aromatic amino acids (*aroA* and *aroD*), frequently used as live vaccines for domestic animals, also do not form biofilms on polystyrene surfaces. Malcova et al. showed that these *S.* Enteritidis mutants are altered in cellulose, curli, *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid-derived capsule biosynthesis, further

enhancing the intimate link between amino acid metabolism and biofilm formation (Malcova et al., 2009).

White et al. used a combined metabolomics and transcriptomics approach to compare extracellular matrix-embedded, wild-type *S.* Typhimurium and the matrix-deficient *csgD* mutant (White et al., 2010). Metabolite profiling of wild-type rdar colonies and saw *csgD* mutant colonies revealed that many compounds detected at higher levels in wild-type colonies are gluconeogenesis end products. In the *csgD* mutant, however, upper tricarboxylic acid (TCA) cycle intermediates are more abundant, which was explained by the hypothesis that a block in gluconeogenesis in *csgD* mutant cells is responsible for the accumulation of TCA cycle intermediates. Consistent with the metabolite profiles, it was found that several gluconeogenesis-specific enzymes (such as *ppsA*, encoding phosphoenolpyruvate synthase, and *pckA*, encoding phosphoenolpyruvate carboxylase) and enzymes that catalyze reversible steps in gluconeogenesis and glycolysis are up-regulated in wild-type cultures relative to *csgD* mutant cultures. Notably, the expression of these enzymes in wild-type cells displays a distinct temporal pattern of activation with peak expression occurring at the time that cell aggregation started. This indicates that the wild-type cells have a shift in central metabolism towards gluconeogenesis at the onset of aggregation. The finding that a *ppsA*/*pckA* mutant strain is unable to form rdar morphotype colonies and synthesize EPS or glycogen shows that the production of sugars from gluconeogenesis is an essential pathway for *S.* Typhimurium aggregation. In addition to a shift to gluconeogenesis, a number of stress-resistance adaptations coincide with aggregation. Several osmoprotectants were detected at high levels in rdar morphotype colonies and transcriptional analysis confirmed that systems for osmoprotectant synthesis and transport are induced. Furthermore, it was observed that wild-type cells have an increased capacity for reactive oxygen species (ROS) defense. These adaptations are expected to enhance survival under desiccation stress. Finally, also nutrient acquisition systems were found to be induced at the onset of biofilm formation. Interestingly, none of the genes involved in the synthesis of polyamines were found to be differentially expressed at the onset of biofilm formation. We, however, recently noticed that polyamine uptake and biosynthesis mutants have altered biofilm formation (Hermans et al., unpublished results). In addition, we also identified genes encoding an ABC transporter involved in putrescine uptake (*potFGHI*) to be up-regulated under *S.* Typhimurium biofilm conditions (Hermans et al., 2011). As the CsgD regulon did not reveal any gene targets linked to global carbon flux and relatively few related to stress resistance, the authors hypothesized that the primary role of CsgD is to control the aggregation process, while the induction of gluconeogenesis is a consequence of the metabolic demand of polysaccharide production at the onset of biofilm formation, rather than elaboration of a defined genetic program. Furthermore, the local microenvironment that results from being embedded in a self-produced matrix would result in the induction of numerous pathways associated with stress tolerance and nutrient scavenging. In addition to this hypothesis, we also identified *S.* Typhimurium genes involved in surface and transport properties to be up-regulated during biofilm growth (Hermans et al., 2011). Together, this suggests that homeostasis of the extracellular matrix is an important property during biofilm formation. Another example highlighting this can be found in recent data presented by Liu et al. They observed that the ability to synthesize osmoregulated periplasmic glucans (OPGs) (using *opgGH* mutants) is important for *S.* Typhimurium SL1344 to grow, form biofilms and compete in low-nutrient low-osmolarity environmental conditions such as leafy-green wash waters and during mouse organ colonization (Liu et al., 2009). The authors suggested a possible role of these OPGs in maintaining structural stability under these conditions, required for efficient nutrient uptake. Crawford et al. also provided a notion in this direction, with the identification of an *ompC* transposon mutant showing altered bile-mediated cholesterol biofilm formation, possibly by a direct role in adherence to or in modification of the cholesterol (Crawford, Reeve, et al., 2010).

4.9. Role of quorum sensing

Quorum sensing or bacterial cell-to-cell communication is a process by which bacteria sense and respond to their own population density (Fuqua, Winans, & Greenberg, 1996; Waters & Bassler, 2005). In several bacterial species, it has been reported that biofilm formation is partially regulated by quorum sensing (Dickschat, 2010). In this section, we describe the current understanding of the role of quorum sensing in the regulation of *Salmonella* biofilm formation. Three main types of quorum sensing systems, each represented in *Salmonella*, have been described: acyl-homoserine lactone (AHL), autoinducer-2 (AI-2) and autoinducer-3 (AI-3) signalling.

Firstly, *Salmonella* encodes a transcription factor of the LuxR family, named SdiA (Ahmer et al., 1998), which responds to AHLs produced by other bacterial species (Ahmer, 2004; Dyszel et al., 2010; Michael, Smith, Swift, Heffron, & Ahmer, 2001; J. L. Smith, Fratamico, & Yan, 2011; J. N. Smith & Ahmer, 2003; J. N. Smith et al., 2008). Dyszel et al. constructed a *S.* Typhimurium strain that is able to synthesize AHL. The observation that in this background *sdiA*$^+$ *S.* Typhimurium rapidly outcompetes the *sdiA* mutant during transit through mice indicates a function of SdiA in mice, although the exact mechanism by which *sdiA*$^+$ bacteria are more fit than *sdiA* mutant bacteria is not known (Dyszel et al., 2010). To date, SdiA is known to activate the expression of the *rck* operon and the *srgE* gene (Ahmer et al., 1998; J. N. Smith & Ahmer, 2003). In contrast to the well established function of SdiA in *E. coli* biofilm formation (Lee, Maeda, Hong, & Wood, 2009), no direct link between SdiA and *Salmonella* biofilms has been reported. However, the functions of the SdiA-regulated genes suggest a role for SdiA in *Salmonella* biofilm formation as well, since Rck has been shown to promote adherence to epithelial cells and extracellular matrix proteins next to its role in providing resistance to complement killing (Crago & Koronakis, 1999; Heffernan et al., 1992). Two other genes in the *rck* operon, *pef1* and *srgA,* appear to affect the expression and function of the *pef* operon, encoding plasmid-encoded fimbriae. *Pef1* is a regulator of the *pef* operon whereas *srgA* catalyzes the formation of a disulfide bond in the PefA fimbrial subunit (Bouwman et al., 2003; Nicholson & Low, 2000). As such, SdiA might indirectly affect the expression and assembly of plasmid-encoded fimbriae on the cell surface and influence biofilm formation.

The second quorum sensing system of *Salmonella* utilizes the LuxS enzyme for the synthesis of AI-2 (De Keersmaecker, Sonck, & Vanderleyden, 2006; Surette, Miller, & Bassler, 1999). The Lsr transport system is well-known to be involved in the detection and transport of AI-2 into the cell, while the *rbs* transporter has recently been suggested as an alternative AI-2 uptake system (Jesudhasan et al., 2010). A number of research groups reported that *Salmonella* biofilm formation is affected by mutating the *luxS* gene. Prouty et al. found that a *S.* Typhimurium *luxS* insertion mutant only showed scattered biofilm formation with little apparent EPS on the surface of gallstones after 4 days of incubation (Prouty et al., 2002). Jesudhasan et al. reported that a *S.* Typhimurium *luxS* deletion mutant is impaired in biofilm formation on polystyrene (Jesudhasan et al., 2010). Microarray analysis revealed that several motility genes and biofilm-related genes are down-regulated in the *luxS* deletion mutant as compared to the wildtype. The expression of some of these genes could be partially complemented by cell free supernatant of wild-type *S.* Typhimurium, suggesting that AI-2 might be involved in the regulation of these specific genes (Jesudhasan et al., 2010). De Keersmaecker et al. previously also showed that a *S.* Typhimurium *luxS* deletion mutant is impaired in biofilm formation on polystyrene (De Keersmaecker et al., 2005). However, although genetic complementation could be accomplished, the biofilm forming phenotype could not be rescued by addition of synthetic DPD, which non-catalytically is converted to AI-2.

This suggests that AI-2 is not the actual signal involved in *Salmonella* biofilm formation. To further unravel this, Kint et al. analyzed additional *luxS* mutants for their biofilm phenotype (Kint, De Coster, Marchal, Vanderleyden, & De Keersmaecker, 2010). Surprisingly, a *luxS* kanamycin insertion mutant and a partial deletion mutant, that only lacks 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 are unable to produce AI-2. Interference of the entire deletion of *luxS* with the expression of MicA, a sRNA molecule encoded just upstream of *luxS* on the opposite DNA strand, was raised as a possible explanation for the contrasting biofilm phenotypes of the different mutants. This hypothesis was corroborated by the finding that the entire deletion mutant contains significantly lower MicA levels compared to wild-type *Salmonella*, while the *luxS* insertion mutant and the partial deletion mutant have wild-type MicA expression levels. Moreover, it was shown that a tightly regulated balance of MicA expression is essential for proper *S.* Typhimurium biofilm formation. It can therefore be concluded that the *S.* Typhimurium biofilm formation phenotype, under the test conditions studied, is dependent on the sRNA molecule MicA, rather than on LuxS itself. However, further research is needed to assess whether this is also the case in other experimental setups.

The third quorum sensing system of *Salmonella* utilizes the two component system PreA/B (Bearson & Bearson, 2008; Merighi, Carroll-Portillo, Septer, Bhatiya, & Gunn, 2006; Moreira, Weinshenker, & Sperandio, 2010). This system is homologous to and functionally interchangeable with the *E. coli* QseB/C two component system (Merighi et al., 2006), which has been shown to sense the quorum signal AI-3 as well as the eukaryotic hormones epinephrine and norepinephrine (Clarke, Hughes, Zhu, Boedeker, & Sperandio, 2006; Sperandio, Torres, & Kaper, 2002). However, the signals activating this system in *Salmonella* are presently unknown. These activating signals are probably not present in LB medium, since it was found that PreB negatively affects PreA gene regulation during LB growth, likely acting as a phosphatase leaving PreA unphosphorylated and hence inactive (Merighi et al., 2006). PmrCAB, the PmrA and PhoP regulated genes *yibD, udg*, *cptA* and *pagP* and genes in the local region around *preA* were identified as possible targets of PreA/B in a genetic screen for activators of *pmrC* and microarray analysis (Merighi et al., 2006, 2009). Although not corroborated by microarray analysis. PreA/B also appears to play a role in motility as it was found that a *preB* mutant is drastically affected in motility (Bearson & Bearson, 2008; Bearson, Bearson, Lee, & Brunelle, 2010). Since motility and/or flagella are known to be important for *Salmonella* biofilm formation under certain conditions, this finding inspired us to study the effect of a *preB* mutation on the biofilm forming ability of *Salmonella*. As expected, the *preB* mutant was found to be defective in biofilm formation under certain conditions tested (Steenackers et al., unpublished data). Furthermore, it was found that a *preB* mutant displays decreased colonization of the gastrointestinal tract of swine as compared to the wild-type strain. This could possibly be explained in part by the observed biofilm defect of the *preB* mutant (Bearson & Bearson, 2008).

5. Resistance of *Salmonella* biofilms against stress factors

As already mentioned, *Salmonella* species display enhanced survival in non-host environments (Winfield & Groisman, 2003), which are fundamentally different from typical host environments (Mouslim & Groisman, 2003). Although *Salmonella* is an intestinal pathogen, it is profoundly adapted to live outside host organisms, with biofilm formation being a major adaptation. *Salmonella* biofilm formation has been shown to confer resistance against several stress factors encountered both in host and non-host environments.

5.1. Resistance against desiccation stress, heat, low pH and ionizing irradiation

White et al. demonstrated that *S.* Typhimurium cells in rdar colonies, that were peeled off from the agar surface and dried out in the plastic wells of a multiwell plate, have a survival rate of 68% after 3 months storage and 10% after 9 months storage (White et al., 2006). In contrast, cell numbers for *csgD*, *csgA* and *bcsA* mutant colonies are significantly reduced after the same period of time, indicating an important role for curli fimbriae, cellulose and other CsgD-regulated components in the protection against desiccation (White et al., 2006). O-Ag-capsule mutants (*yihO*, *yihQ*, and *yihP*) also have reduced survival levels, similar to the *csgD* mutant, demonstrating a role of O-Ag-capsule in desiccation tolerance (Gibson et al., 2006). As previously described, the observed increase in osmoprotectant synthesis and ROS defense capacity at the onset of biofilm formation, which is presumably a consequence of the microenvironment encountered within biofilms, is also likely to contribute to the resistance to desiccation stress (White et al., 2010). These results clearly indicate the important role of the rdar morphotype in enhanced long-term survival and persistence under desiccation conditions outside the host. A recent study showed that the number of viable cells recovered from rdar colonies after 30 months is similar to that obtained at the 9-month time point (approximately 10% survived) (Apel, White, Grassl, Finlay, & Surette, 2009). Live/dead staining and visualization with fluorescence microscopy revealed that even 60% of the rdar colonies are alive after 30 months. The discrepancy between results for cells recovered on nonselective media and with live/dead staining may represent “viable but nonculturable cells” or “active but nonculturable cells”.

In addition, the effect of other stress factors commonly encountered outside the host, such as heat, low pH and radiation, on *Salmonella* biofilms was determined. *S.* Typhimurium pellicle formation was not found to provide any benefit during heat or acidification (to pH 3) compared to stationary-phase planktonic cells (Scher, Römling, & Yaron, 2005). In line with this, no effect of varying the pH from 4.5 to 7.4 on the biofilm formation of *S.* Enteritidis on stainless steel could be observed (Giaouris, Chorianopoulos, & Nychas, 2005). However, it was shown that *Salmonella* association with lettuce leaves can enhance its acid tolerance and its persistence during storage (Kroupitski, Pinto, et al., 2009). No enhanced resistance against ionizing radiation of biofilms of different *S. enterica* serovars on glass could be observed as compared to planktonic cells (Niemira & Solomon, 2005).

5.2. Resistance against disinfectants

Several studies have been performed to compare the susceptibility between biofilm and planktonic *S.* Typhimurium cells against chemical disinfectants. In addition, the efficiency of different commonly used disinfectants against *Salmonella* biofilms has been compared (Arnold & Yates, 2009; Joseph et al., 2001; Korber, Choi, Wolfaardt, Ingham, & Caldwell, 1997; Mangalappalli-Illathu & Korber, 2006; Mangalappalli-Illathu, Vidovic, & Korber, 2008; Moretro et al., 2009; Ramesh et al., 2002; Somers, Schoeni, & Wong, 1994; Tabak et al., 2007; Wong, Townsend, Fenwick, Trengove, & O'Handley, 2010). Joseph et al. found that *Salmonella* biofilms on plastic, cement and stainless steel surfaces are much more resistant to the sanitizers chlorine and iodine as compared to planktonic cells (Joseph et al., 2001). Exposure to a solution of 100 ppm chlorine or 50 ppm iodine for at the least 15 min (depending on the surface) is needed to completely remove the biofilms, while planktonic cells are completely killed after exposure to a solution of 10 ppm of chlorine or iodine for 10 or 5 min, respectively. These results have been corroborated by Moretro et al., who found that disinfectants based on hypochlorite (approximately 400 ppm), glutaraldehyde and cationic tensides (alkylaminoacetate, didecylmethylammoniumchloride and benzalkonium chloride) do not show a sufficient effect on *Salmonella* biofilms on stainless steel surfaces at the

recommended user concentrations after 5 min of exposure, while they are effective against *Salmonella* in suspension (Moretro et al., 2009). Exposure to acidic peroxygen-based disinfectants and a product containing 70% ethanol, however, was found to completely eliminate the biofilms after 5 min. Wong et al. described that *Salmonella* biofilms on polystyrene pegs are also less susceptible to the disinfectants chlorhexidine gluconate, citric acid, benzalkonium chloride and other quaternary ammonium compounds, compared to planktonic cells (Wong et al., 2010). However, in contrast to the effect described by Moretro et al., sodium hypochlorite (at concentrations of approximately 1300 ppm) was found to completely eradicate biofilms on polystyrene pegs after 1 min of exposure, whereas 70% ethanol failed to eliminate the biofilms after 5 min of exposure. Consistent with the findings by Moretro et al., Ramesh and co-workers concluded from a comparative study of the effect of different classes of disinfectants (sodium hypochlorite, sodium chlorite, quaternary ammonium, iodine, enzymes, and phenol) on *Salmonella* biofilms on galvanized steel surfaces that a hypochlorite based disinfectant with a sodium hypochlorite concentration of 500 ppm is the most effective biofilm inhibitor (Ramesh et al., 2002).

Several studies have been performed in order to unravel the mechanistic basis of the increased resistance of *Salmonella* to disinfectants in biofilms as compared to planktonic cells. Solano et al. compared the influence of 30 ppm of sodium hypochlorite on the survival of biofilms of wild-type *S.* Enteritidis and cellulose mutants formed on glass (Solano et al., 2002). 75% of the wild-type cells survived a 20 min exposure to the disinfectant, while only 0.3% of the cellulose-deficient mutant cells survived, which clearly indicates the protective function of cellulose. In line with this finding, Scher et al. reported an enhanced resistance to hypochlorite of pellicle forming *S.* Typhimurium cells as compared to a *bcsA csgBA* double mutant (Scher et al., 2005). Cellulose and curli also seem to play a role in the protection of *S.* Typhimurium on parsley against chlorination. Other mechanisms such as the ability to penetrate the plant tissue or pre-existing biofilms and the production of different polysaccharides other than cellulose, possibly also provide and/or enhance protection against this treatment (Lapidot & Yaron, 2009; Lapidot et al., 2006). These results were further corroborated by White et al., who investigated the influence of 60 ppm of sodium chlorite on stationary phase planktonic cells and *S.* Typhimurium rdar colonies that had been stored for 3 months on plastic (White et al., 2006). Dried colonies of wild-type *S.* Typhimurium and a curli deficient *csgA* mutant strain were found to be highly resistant (less than 1-log reduction after treatment) as compared to planktonic cells (6-log reduction), while mucoid colonies of the cellulose deficient *bcsA* strain were found to be susceptible (4-log reduction). Remarkably, *csgD* colonies were even more susceptible (6-log reduction), indicating that next to cellulose, additional components regulated by CsgD, other than curli, confer protection against sodium hypochlorite. The finding of Stocki et al. that CsgD also mediates resistance of dried rdar colonies to a peroxygen based disinfectant, a quaternary ammonium sanitizer and chlorophenol, indicates that protection by CsgD regulated matrix components appears to be a general resistance mechanism (Stocki et al., 2007). Consistent results were found by Tabak et al. who studied the effect of the disinfectant triclosan on planktonic *Salmonella* (log and stationary phases), on biofilm-associated cells and on bacteria derived from disrupted biofilms (Tabak et al., 2007). While a strong effect of triclosan (1000 μg/mL) on log phase cells was observed, a smaller and identical effect was found on stationary phase and biofilm derived cells and only a weak effect was found on biofilm-associated cells. The higher resistance of biofilm-associated cells as compared to biofilm-derived cells suggests that the matrix also plays a significant role in the resistance against triclosan. This was further corroborated by the finding that deletions in the genes coding for curli and cellulose synthesis makes the biofilm more susceptible. Furthermore, resistance to triclosan was attributed to a biofilm-specific adaptive response which was obtained by an enhanced expression of (1) *acrAB* (encoding an efflux pump) and *marA* (activator of *acrAB*), resulting in an increased efflux of triclosan and (2) the cellulose synthesis genes *bcsA* and *bcsE*, resulting in enhanced EPS production. Mangalapalli-Illathu et al. found that adaptive resistance also plays a role in the resistance of *Salmonella* biofilms against benzalkonium chloride (Mangalappalli-Illathu & Korber, 2006; Mangalappalli-Illathu, Vidovic, et al., 2008). Indeed, biofilms adapted to benzalkonium chloride by exposure to subinhibitory concentrations over a certain time period, acquired the ability to survive a normally lethal exposure of benzalkonium chloride and then resume growth. Adaptation occurred concurrently with the up-regulation of key proteins involved in the cold shock response, stress response, detoxification and an overall increase in protein biosynthesis, explaining the mechanisms responsible for adaptive resistance.

5.3. Resistance against antibiotics

Next to an increased resistance against disinfectants, *Salmonella* biofilms also confer resistance to antibiotics. Olson et al. compared the effect of the antibiotics enrofloxacin, gentamicin, erythromycin, tilmicosin, ampicillin, oxytetracycline and trimethoprim-sulfadoxine on planktonic cells and on pre-established biofilms on polystyrene pegs of clinical *S.* Typhimurium and *S.* Bredeney isolates (Olson, Ceri, Morck, Buret, & Read, 2002). Planktonic populations were found to be sensitive (Minimal Inhibitory Concentration (MIC) <20 μg/mL for at least 1 of the isolates) to all antibiotics except for erythromycin and tilmicosin, whereas *Salmonella* biofilms are only sensitive to enrofloxacin and ampicillin (*Salmonella* Bredeney only). Furthermore, Tabak et al. reported that *S.* Typhimurium biofilms pre-formed on microplates are up to a 2000-fold more resistant to ciprofloxacin as compared to planktonic cells (Tabak, Scher, Chikindas, & Yaron, 2009). This is particularly concerning as ciprofloxacin, together with third-generation cephalosporins such as ceftriaxone and cefotaxime, is commonly used to treat non-typhoid *Salmonella* infections (Parry & Threlfall, 2008). In a different setup, Majtán et al. tested the effect of subinhibitory concentrations of gentamicin, ciprofloxacin and cefotaxime on the amount of biofilm formed on polystyrene microtiter plates by clinical *Salmonella* isolates (Majtan, Majtanova, Xu, & Majtan, 2008). While sub-MICs of gentamicin and ciprofloxacin reduced the amount of biofilm formed by all isolates tested, a significant increase in biofilm formation and EPS production was observed by cefotaxime at ½ MIC in three isolates. These results support the notion that antibiotics are not only bacterial weapons for fighting competitors, but also signalling molecules that may regulate microbial communities (Linares, Gustafsson, Baquero, & Martinez, 2006). Recently, Papavasileiou et al. investigated 194 *S. enterica* strains isolated from infected children, for their ability to form biofilms on silicone disks and compared the biofilms of the isolated strains to their corresponding planktonic forms with respect to susceptibility to 9 antimicrobial agents. About 56% of the strains were able to form biofilms. The biofilms showed increased antimicrobial resistance to all antibiotics as compared to the planktonic bacteria, with the highest resistance rates for gentamicin (90%) and ampicillin (84%) (Papavasileiou et al., 2010).

5.4. The rdar morphotype predominantly mediates survival outside the host

White et al. recently showed that rdar-expressing wild-type cells are outcompeted by curli-deficient *csgA* mutants during competitive infection experiments in mice. This finding, however, questions the pretended role of curli in invasion (Barnhart & Chapman, 2006). Moreover, it was shown that expression of curli genes (*csgB*) is turned off during *in vivo* infection, but turned on again once *Salmonella* is shed into the external environment (White et al., 2008). From this and

the above elaborated findings about *csgD* and rdar expression considering environmental regulation (*e.g.* temperature), the authors concluded that curli and hence aggregation via the rdar morphotype and biofilm formation are not a virulence adaptation in *S.* Typhimurium, but rather a survival strategy outside warm-blooded hosts. Cellulose, another important constituent of the extracellular matrix, was previously also shown not to be involved in virulence (Solano et al., 2002). A recent study underlining this notion was performed by Lamprokostopoulou et al. who found that activation of CsgD by high levels of c-di-GMP results in a reduced invasion of the gastrointestinal epithelial cell line HT-29 and a reduced pro-inflammatory response suggesting that transition between biofilm formation and virulence in *S.* Typhimurium at the epithelial cell lining is partly mediated by c-di-GMP signalling through CsgD (Lamprokostopoulou et al., 2010). Indeed, they showed that CsgD not only activates extracellular matrix production at the epithelial cell lining leading to steric hindrance interfering with TTSS-1 functionality, but also inhibits the secretion of FliC flagellin and the TTSS effector SopE2, both causing inflammation. Moreover, c-di-GMP itself is also directly involved in the regulatory cascade leading to the production of extracellular matrix compounds, some of which (cellulose and to a lesser extent O-Ag-capsule, but not curli and BapA) confer steric hindrance as just mentioned. However, *csgD* and *yihO* were found to be activated *in vivo* indicating that some rdar components (*e.g.* BapA was shown to contribute to invasion through the regular *Salmonella* infection route (Latasa et al., 2005)) could possibly have a role in virulence (White et al., 2008) which is in line with other previous studies (Latasa et al., 2005; Römling et al., 2000; Solano et al., 1998, 2001). Altogether, these data point at subtle links between biofilm formation, host colonization and virulence in general.

6. Inhibition of *Salmonella* biofilm formation

Given the resistance of *Salmonella* biofilms against commonly used disinfectants and antibiotics, a lot of effort is made to develop alternative strategies to interfere with *Salmonella* biofilm formation.

6.1. Biofilm-specific inhibitors: bottom-up

In the bottom-up approach, insight into regulatory and metabolic processes involved in biofilm formation as well as knowledge of structural features of biofilms is used to rationally design or identify biofilm inhibitors targeting certain biofilm-specific processes.

In this context, Mireles et al. discovered that the bacterial surfactant surfactin, as well as the chemical surfactant Tween 80, are able to disperse preformed *Salmonella* biofilms and to prevent biofilm formation in urethral catheters, without affecting planktonic cell growth (Mireles et al., 2001). The choice of testing surfactin as a biofilm inhibitor was inspired by the observation that mutants defective in LPS synthesis are affected in swarming motility (Toguchi, Siano, Burkart, & Harshey, 2000), while they contrastingly show an increased biofilm formation. The LPS mutants could be rescued for swarming by external addition of biosurfactant, suggesting that the LPS improves surface wettability, required for swarm colony expansion. Given the opposite effect of mutations in LPS synthesis with respect to swarming and biofilm formation, Mireles et al. correctly predicted that (bio)surfactants stimulating swarming motility, could reduce biofilm formation (Mireles et al., 2001).

As a second example of identification of biofilm inhibitors via the bottom-up approach, the identification of glucose as a *Salmonella* biofilm inhibitor is described. As previously mentioned, White et al. reported on an important role of gluconeogenesis in fulfilling the demands of EPS production at the onset of biofilm formation (White et al., 2010). It was assumed that, under the conditions investigated, the carbon flux is controlled by the catabolite repressor/activator (Cra) protein, which activates gluconeogenesis enzymes and represses sugar catabolism enzymes (Saier & Ramseier, 1996). The activity of Cra is known to be repressed by sugar catabolites, as they bind to Cra and displace it from the operator sites in the target operons (catabolite repression). Therefore the authors hypothesized that addition of glucose could inhibit gluconeogenesis and consequently biofilm formation. This hypothesis was confirmed by the finding that glucose leads to inhibition of the rdar morphotype, suggesting that gluconeogenesis may be a good target in the development of biofilm inhibitors against a wide variety of bacteria.

As described in the previous section, the role of quorum sensing (QS) in the regulation of *Salmonella* biofilm formation remains to be fully elucidated. Nevertheless, the well established significance of quorum sensing in pathogenic traits of other bacterial species and the possible role of quorum sensing in *Salmonella* virulence and biofilm formation, prompted a number of groups to investigate whether the activity of the *Salmonella* QS systems could be modulated by analogues of the natural signalling molecules (AHL, AI-2 and AI-3). Although the biofilm inhibitory activity of most of these analogues has not been tested yet, their potential role in biofilm inhibition justifies a short overview of these compounds in this section. Janssens et al. synthesized and screened a limited library of AHL analogues in order to obtain more information about the specificity of the ligand binding by SdiA, the AHL receptor of *Salmonella*, In this screening the *N*-(3-oxo-acyl)-homocysteine thiolactones (3O-AHTLs) and the *N*-(3-oxo-acyl)-trans-2-aminocyclohexanols were identified as two classes of analogues that are strong activators of SdiA (Janssens et al., 2007). In order to modulate the AI-2-dependent gene expression, Frezza et al. developed synthesis pathways towards an array of analogues of the AI-2 precursor 4,5-dihyroxy-2,3-pentanedione (DPD) such as chain-elongated analogues, 5-*O*-acylated derivatives, bis-(*O*)-acylated derivative and a trifluoromethyl analogue (Frezza, Soulere, Queneau, & Doutheau, 2005; Frezza et al., 2006, 2007). The bis-(*O*)-acylated derivative has been shown to be a stronger activator of the Lsr-regulated gene expression of *Salmonella* as compared to DPD, while the trifluoromethyl is a weaker activator than DPD. Similarly, Lowery et al. synthesized a series of C1-substituted analogues of DPD and found the propyl-substituted and butyl-substituted analogues to be potent inhibitors of the Lsr regulated gene expression (Lowery, Park, Kaufmann, & Janda, 2008; Lowery et al., 2009). Finally, Rasko et al. performed a screening of 150 000 small organic molecules to identify inhibitors of the AI-3/QseC-dependent virulence gene activation in EHEC (Enterohaemorrhagic *E. coli*) (Rasko et al., 2008). This screening and sequel structure–activity relationship studies resulted in compound LED209 (*N*-phenyl-4(((phenylamino)thioxomethyl)amino)-benzenesulfonamide). Besides its effect on *E. coli* QseC, this compound was also found to inhibit the QseC analogues of *Francisella tularensis* and *S.* Typhimurium (PreB). Furthermore, it was found that oral administration of LED209 to mice 3 h before and 3 h after intraperitoneal injection of a lethal dose of *S.* Typhimurium drastically prolonged the survival time of the mice, which is consistent with the observed defect of a *preB* mutant in colonization of the swine intestinal tract (previous section) and the observed attenuation of a *preB* mutant for systemic disease in mice.

6.2. Biofilm-specific inhibitors: top-down

In the top-down approach, libraries of chemical compounds and natural product analogues are screened for compounds that are able to prevent or eradicate biofilm formation. An advantage of this approach is that the possible targets of the inhibitors are not restricted to known biofilm related processes. On the other hand, this approach implicates a need for extensive, open approach research strategies to unravel the mode of action of the identified inhibitors.

Brominated furanones, a class of secondary metabolites originally isolated from the red alga *Delisea pulchra*, and their synthetic analogues have been shown to act as inhibitors of biofilm formation

for several bacterial species (De Nys, Givskov, Kumar, Kjelleberg, & Steinberg, 2006). To study their potential as inhibitors of *Salmonella* biofilm formation, Janssens et al. synthesized a small focused library of brominated furanones and tested their preventive effect against *S.* Typhimurium biofilm formation on polystyrene pegs (Janssens et al., 2008). Several furanones were found to inhibit the biofilm formation at non-growth-inhibiting concentrations, with (*Z*)-4-bromo-5-(bromomethylene)-5-alkyl-2(*5H*)-furanones with alkyl chain lengths of two to six carbon atoms being the most interesting compounds. In a sequel study, additional 3-alkyl-5-methylene-2(*5H*)-furanones were synthesized to extend the structure–activity relationship (Steenackers, Levin, et al., 2010). The bromination pattern of the furanone ring was found to have a large influence on the biofilm inhibitory activity, as dibrominated compounds with one bromine atom on the 4-position of the ring and one bromine atom on the methylene group were found to be much more active than the compounds with a dibrominated methylene group. Furthermore, introduction of a bromine atom on the first carbon atom of the alkyl side chain was shown to drastically improve the biofilm inhibitory activity, while introduction of an acetoxy function at this position in general did not improve the activity. Finally, the potential of bromoalkylmaleic anhydrides as a new and easily accessible class of biofilm inhibitors was also demonstrated. As interference with AHL- and AI-2-mediated quorum sensing systems has been proven as a mechanism for the biofilm inhibition by brominated furanones, at least in a subset of the bacterial species tested, the effect of the furanones on the expression of QS-controlled genes of *Salmonella* was investigated. Surprisingly, no evidence was found that furanones act on the currently known QS systems of *Salmonella*. Microarray analysis, however, revealed that the furanones interfere with *Salmonella* flagella synthesis (Janssens et al., 2008). Since it has been shown that functional flagella are needed for normal *Salmonella* biofilm formation under certain conditions (see previous sections), it is possible that this interference with the flagellar assembly causes the observed biofilm defect.

Grapefruit juice contains bioactive compounds such as furocoumarins, carotenoids, flavanoids, limonoids, pectin and vitamin C, which have shown to confer several health benefits. Girennavar et al. reported on the preventive effect of grapefruit juice and the isolated furocoumarins dihydroxybergamottin and bergamottin against *S.* Typhimurium biofilms formed on the bottom of polystyrene microtiter plates at non-growth-inhibiting concentrations (Girennavar et al., 2008). As the furocoumarins and the brominated furanones have a common furan moiety, it is possible that they have a similar mode of action.

Analogues of the sponge-derived 2-aminoimidazoles bromoageliferin and oroidin have previsously been shown to inhibit biofilm formation by *P. aeruginosa*, *Acinetobacter baumanii* and *Bordetella bronchiseptica* (Ballard, Richards, Wolfe, & Melander, 2008; Huigens, Rogers, Steinhauer, & Melander, 2009). Recently, it was shown that 4(5)-phenyl-2-amino-*1H*-imidazole, which has the 2-aminoimidazole scaffold in common with the bromoageliferin and oroidin analogues, is able to prevent the biofilm formation of *S.* Typhimurium (as well as *P. aeruginosa*) on polystyrene pegs (Ermolat'ev, Bariwal, Steenackers, De Keersmaecker, & Van der eycken, 2010). A broad structure activity relationship study revealed that either substitution of the (4)5-phenyl ring (*e.g.* with a chorine, fluorine, methyl or methoxy group) or introduction of medium length alkyl or cyclo-alkyl side chain at the *N1*-position could increase the activity of the compounds up to 30 fold (Steenackers, Ermolat'ev, et al., 2010). Furthermore, the potential of the *N1*-substituted imidazo[1,2-a]pyrimidinium salts, which are the chemical precursors of the *N1*-substituted 2-aminoimidazoles, as *Salmonella* biofilm inhibitors was demonstrated. A good correlation was found between the activity of the imidazo[1,2-a]pyrimidinium salts and their corresponding 2-aminoimidazoles, which could be explained by the hypothesis that the salts can also *in situ* be degraded to form the active 2-aminoimidazoles.

Salicylates and other nonsteroidal anti-inflammatory drugs are known to prevent bacterial adhesion on medical devices (Arciola, Montanaro, Caramazza, Sassoli, & Cavedagna, 1998). Rosenberg et al. found that the *S.* Typhimurium strain MAE52, which normally forms pellicles at the air-liquid interface, does not form pellicles when it was grown in growth medium containing a glass coverslip coated with a salicylate-based poly(anhdride ester) from which salicylic acid is released via surface erosion, while the planktonic growth of the bacteria is not reduced (Rosenberg, Carbone, Römling, Uhrich, & Chikindas, 2008). However, no difference was observed between the amount of biofilm formed by *S.* Typhimurium strain JSG210 on the surface of glass coverslips coated with salicylate-based poly(anhdride ester) and uncoated control coverslips. The authors concluded that the polymers may not interfere with the attachment of *Salmonella*, but rather affect another mechanism essential for biofilm formation by *Salmonella*.

An advantage of top-down screening is that stringent selection criteria regarding the range of application of the compounds (range of active concentrations, temperature range, range of substrate materials, preventive effect vs. destructive effect, etc.) can be applied. In this context, we screened a library of more than 20 000 very diverse compounds for molecules able to reduce the amount of *S.* Typhimurium biofilm formed on polystyrene pegs at 16 °C and 37 °C by more than 90% and 50%, respectively. Hits were identified with a rate of 0.7%. Dose–response relationship studies and structure–activity relationship studies of the identified hits, as well as studies of the potential of the compounds to eradicate pre-formed biofilms and inhibit biofilm formation by other bacterial species are currently ongoing, in an attempt to develop very broadly applicable *Salmonella* biofilm inhibitors (Robijns et al., unpublished data).

6.3. Combination therapy

A number of research groups studied the potential of the combined use of disinfectants, antibiotics and specific biofilm inhibitors as a strategy to combat *Salmonella* biofilms. A rationale behind this strategy is the emerging understanding that the decreased susceptibility of biofilms is linked to a process of phenotypic diversification, ongoing within the adherent populations (Drenkard & Ausubel, 2002; Lewis, 2007). This means that there are likely multiple cell types in single-species biofilms that ensure population survival when the biofilm is attacked by any single adversity. Therefore, combination therapy with chemically distinct compounds might be more effective.

Harrison et al. tested the effect of 4400 combinations of metals and biocides on pre-formed biofilms of *P. aeruginosa* on polystyrene pegs (Harrison et al., 2008). This screening revealed that copper and quaternary ammonium cations (such as benzalkonium chloride, cetalkonium chloride, cetylpyridinium chloride, myristalkonium chloride and polycide) exert a synergistic killing effect on *Pseudomonas* biofilms. Combinations of Cu^{2+} with polycide were found to be able to eradicate *Pseudomonas* biofilms at concentrations that are at least 128-fold lower than the sterilizing concentrations of either agent alone. Furthermore, this study indicated that the effect of combinations of Cu^{2+} and quaternary ammonium cations is not restricted to *P. aeruginosa*, as also biofilms of *E. coli*, *S. aureus* and *S.* Cholerasius were successfully eradicated. Tabak et al. reported on a synergistic effect between the disinfectant triclosan and the antibiotic ciprofloxacin (Tabak et al., 2009). While 500 μg/mL triclosan and 500 μ/mL ciprofloxacin alone were found to reduce the number of viable cells in pre-formed *Salmonella* biofilms on microplates only with 1.6 and 0.5 log, respectively, the sequential treatment of 500 μg/mL triclosan followed by ciprofloxacin resulted in 4.8 log reduction. This synergistic effect was suggested to be mediated by an improvement of the membrane permeability of ciprofloxacin after exposure to triclosan. Similarly, Janssens et al. showed that pre-treatment of *S.* Typhimurium biofilms with 60 μM of the biofilm-specific inhibitor (*Z*)-4-bromo-5-(bromomethylene)-5-hexyl-2(*5H*)-furanone (see previous section) resulted in a 50- to 2100-fold higher effectivity of

the antibiotics tetracyline, ciprofloxacin and cefotaxime (Janssens et al., 2008). Consistently, Vestby et al. described the ability of another brominated furanone, (*Z*)-5bromomethylene-2(*5H*)-furanone, to enhance the effect of the disinfectants hypochlorite and benzalkonium chloride on the biofilm formation of two *S.* Agona strains (Vestby, Lonn-Stensrud, et al., 2010).

6.4. Microemulsions and nanoemulsion

Fine emulsions have long been used in applications such as detergents, pharmaceuticals and cosmetics. However, recently microemulsions and nanoemulsions have been shown to hold great promise as antimicrobials and antifoulants (Al-Adham, Khalil, Al-Hmoud, Kierans, & Collier, 2000; Anderson, Caldwell, Beuchat, & Williams, 2003; Hamouda et al., 1999; Teixeira et al., 2007). Al-Adham et al. showed that the oil-in-water microemulsion TEOP is active against biofilms of *P. aeruginosa* (Al-Adham et al., 2000). Inspired by this finding, Teixeira et al. studied the effect of the soybean oil based nanoemulsion BCTP and the oil-in-water microemulsion TEOP on pre-formed biofilms of other bacterial pathogens, among which *S.* Typhimurium (Teixeira et al., 2007). Both emulsions were found to reduce the amount of *Salmonella* biofilm by approximately 60% after 30 min of treatment.

6.5. Mixed species biofilms

Most biofilms in their natural environments are likely to consist of consortia of species that influence each other synergistically or antagonistically (Wimpenny, Manz, & Szewzyk, 2000). Also the resistance against antimicrobial treatments has been shown to be influenced by the interactions between different species within mixed-species biofilms (Burmolle et al., 2006; Leriche, Briandet, & Carpentier, 2003). Knowles et al. tested the influence of carvacrol, on dual-species biofilm development by *S. aureus* and *S.* Typhimurium on stainless steel (J. R. Knowles, Roller, Murray, & Naidu, 2005). Carvacrol is a broad spectrum antimicrobial found in essential oils of herbs such as oregano, thyme and savory and has been shown to be effective against mono-species *S.* Typhimurium biofilms on stainless steel (J. Knowles & Roller, 2001). In steady state, the untreated duo-species biofilm was found to be constituted for approximately 99% of *S. aureus* while only a minor amount of *Salmonella* cells was present. Carvacrol was demonstrated to have inhibitory effects on both species in the duo-species biofilm, although the efficacy was species-dependent and dependent on the stage of biofilm formation, the applied concentration and the treatment regime (pulses vs. continuous treatment/prevention vs. eradication). Chorianopoulos et al. studied the effect of two acids (hydrochloric and lactic acid), sodium hydroxide, the essential oil of *Satureja thymbra* (containing carvacrol and thymol at 50% of its total volume) and two by-products of essential oil purification (decoction and hydrosol) against biofilms formed on stainless steel by five bacterial species, either as mono-species, or as mixed-culture of all species (*Staphylococcus simulans*, *Lactobacillus fermentum*, *Pseudomonas putida*, *S.* Enteritidis and *Listeria monocytogenes*) (Chorianopoulos, Giaouris, Skandamis, Haroutounian, & Nychas, 2008). Although identical inoculum sizes of the different bacterial species were used, the untreated biofilms mainly consisted of *P. putida* cells (97.8%), while *S.* Enteritidis and *L. monocytogenes* together represented only 2.2% of it. Surprisingly, *S. simulans* and *L. fermentum* were unable to form biofilms in the presence of the other three species. It was found that the essential oil and hydrosol of *S. thymbra* showed a strong antimicrobial action against both monospecies and mixed-culture biofilms, while the decoction fraction and the acid–base disinfectants were not adequate, although long treatment was applied.

The notion that individual species influence the biofilm formation of other species, prompted some research groups to investigate the influence of cell free culture supernatant (CFS) of bacterial species on the biofilm formation of other species. Chorianopoulos et al. demonstrated that compounds of *Hafnia alvei* cell-free culture supernatant negatively influence the early stage of biofilm formation by *S.* Enteritidis on stainless steel (Chorianopoulos, Giaouris, Kourkoutas, & Nychas, 2010). The nature of these compounds is presently unknown. However, these compounds are unlikely to be enzymes (as the CFS maintained activity after heating) or AHLs (as several AHLs were shown not to influence the *Salmonella* biofilm formation under the test conditions used) (Chorianopoulos et al., 2010). Dheilly et al. on the other hand reported the activity of CFS of *Pseudoalteromonas* sp. strain 3JS against biofilm formation by *S. enterica* and a broad spectrum of other Gram-negative bacteria, such as *Paracoccus*, *Vibrio*, *P. aeruginosa* and *E. coli* (Dheilly et al., 2010).

7. Conclusions and perspectives

Since bacteria residing in biofilms are better protected to different kinds of environmental stresses as compared to their planktonic counterparts and since biofilm formation seems to be the predominant mode of bacterial growth *in situ*, a direct link between contamination in food processing environments, bacterial biofilms and contamination of the end food products exists (*e.g.* (Olsen, Brown, Madsen, & Bisgaard, 2003; Rasschaert, Houf, & De Zutter, 2007)). Next to food-processing environments, *Salmonella* biofilms are often encountered in barns, kitchens and toilets, but also on produce (Teplitski et al., 2009) and gallstones (Crawford, Rosales-Reyes, et al., 2010), indicating that this link between *Salmonella* biofilm formation and subsequent product contamination can even be broadened. As such, every source of environmental *Salmonella* contamination and subsequent biofilm-bound persistence can be a potential health risk for society. This is reflected by the annual numbers of *Salmonella* infections as can be seen from yearly WHO publications and by recent large *Salmonella* outbreaks. A considerable body of work has been performed to get more genetic insight into *Salmonella* biofilms ranging from dedicated, low-throughput approaches to different, often complementary high-throughput techniques such as microarrays (Hamilton et al., 2009), proteomics (Hamilton et al., 2009; Mangalappalli-Illathu, Lawrence, Swerhone, & Korber, 2008), metabolomics (White et al., 2010), random (Kim & Wei, 2009) and directed (Hermans et al., unpublished results) mutagenesis and even single cell approaches such as DFI (Hermans et al., 2011). Although these studies have greatly enhanced our knowledge, we are only at the beginning of unraveling the intricacies of this complex process. Firstly, often genes with hypothetical or unknown functions were characterized (Barak et al., 2009; Hamilton et al., 2009) indicating the importance of these FUN genes in the *Salmonella* life cycle outside warm-blooded hosts and the fact that the genetic program for host-bound lifecycle (the one which has historically been best studied) differs significantly from non-host survival. Secondly, the developmental model of microbial biofilm formation, which has served as an important paradigm for biofilm research up to now, has recently been questioned (Monds & O'Toole, 2009). A finding that is strengthened by recent research from White and colleagues stating that common physiological biofilm properties (*e.g.* increased stress resistance) are not induced by a biofilm-specific gene expression program, but rather by differential expression of general metabolism pathways caused by extracellular matrix production (White et al., 2010). Thirdly, the knowledge-driven search for potent *Salmonella* biofilm inhibitors is lagging behind as can be seen from the fact that only few examples of biofilm inhibitors that have had their modes of action fully elucidated are available. Because of recent implementation of state-of-the-art high- and low-throughput microbiological techniques generating more knowledge important for the *Salmonella* biofilm research, a steady progress in the closure of these gaps is to be expected.

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