Salmonella biofilms

Greetje A.A. Castelijn

Thesis committee

Promotors
Prof. Dr Tjakko Abee
Personal chair at the laboratory of Food Microbiology
Wageningen University, The Netherlands

Prof. Dr Marcel H. Zwietering
Professor of Food Microbiology
Wageningen University, The Netherlands

Co-promotor
Dr *ir.* Roy Moezelaar
Senior researcher, Food and Biobased Research
Wageningen Univerity and Research Centre

Other members
Dr Romain Briandet, Institut National de la Recherche Agronomique and AgroParisTech, Micalis Institute, France
Prof. Dr Michiel Kleerebezem, Wageningen University
Prof. Dr Mari Smit, Wageningen University
Prof. Dr Bert Urlings, VION Food Group and Wageningen University

This research was conducted under the auspices of the Graduate School of advanced studies in Food Technology, Agrotechnology, Nutrition and Health Sciences(VLAG).

Salmonella biofilms

Greetje A.A. Castelijn

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 18 October 2013
at 4 p.m. in the Aula.

Greetje A.A. Castelijn
Salmonella biofilms, 171 pages

PhD thesis, Wageningen University, Wageningen, NL (2013)
With references and summaries in Dutch and English

ISBN 978-94-6173-733-5

Voor ons pa

TABLE OF CONTENTS

Chapter 1	General introduction	9
Chapter 2	Diversity in biofilm formation and production of curli fimbriae and cellulose of *Salmonella* Typhimurium strains of different origin in high and low nutrient medium	33
Chapter 3	Type 1 fimbriae are involved in the attachement of *Salmonella* Typhimurium to abiotic surfaces and biofilm formation	59
Chapter 4	Surface behaviour of *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.*Infantis	85
Chapter 5	Isolation and phenotypic characterization of resistant variants from *Salmonella* Typhimurium cell cultures treated with benzalkonium chloride	107
Chapter 6	General discussion, concluding remarks and future perspectives	133
Summary		153
Samenvatting		157
Acknowledgements		161
Personalia	Curriculum Vitae, List of publications and List of completed training activities	167

CHAPTER 1

General introduction

Greetje A.A. Castelijn

Human salmonellosis

Salmonella is a genus of Gram-negative, rod shaped, facultative anaerobic Enterobacteriaceae and constitutes over more than 2500 different serovars. Some *Salmonella* serovars are host-specific, such as *S.* Choleraesuis that primarily infects swine, but most serovars are able to infect a wide variety of cold and warm blooded hosts, including humans. A human *Salmonella* infection can result in diseases varying from mild diarrhoea to severe systemic infections, such as typhoid fever caused by *S.* Typhi and *S.* Paratyphi (de Jong *et al.* 2012). Nontyphoidal infections, i.e., salmonellosis, can be transferred between animals and humans. Salmonellosis is characterized by diarrhoea, abdominal cramps, fever and nausea. These symptoms are often mild and last for several days, though this can be variable between individuals. Especially for the very young, the elderly, the pregnant and the immunocompromised, symptoms can be more severe and the associated dehydration can be life threatening. Salmonellosis has also been related to systemic infections and long term or even chronic reactive arthritis (Hannu *et al.* 2006).

Human salmonellosis cases are generally caused by the consumption of contaminated food products from animal origin (RIVM 2011; EFSA 2011). These food products are also the source of most foodborne outbreaks caused by *Salmonella* (ECDC 2012). However, recent studies have identified fruits and vegetables to be a potential source of many *Salmonella* foodborne outbreaks as well (EFSA 2011; Berger *et al.* 2010).

Incidence of human salmonellosis

Nontyphoidal salmonellosis is one of the most common foodborne diseases. It is estimated that about 94 million (61,768,000, 5th percentile to 131,634,000, 95th percentile) cases of nontyphoidal salmonellosis occur annually worldwide (Majowicz *et al.* 2010). In the EU, a total of 99,020 confirmed cases were reported in 2010, and that year 2205 confirmed cases were reported in the Netherlands alone (ECDC 2012; RIVM 2011). However, it should be mentioned that the real frequency of salmonellosis is much higher than the reported cases since most cases are not confirmed and/or reported. It is estimated that the real incidence of salmonellosis is about 15 times higher than the cases reported (RIVM 2011).

Salmonella is also one of the most frequently reported causes of foodborne outbreaks (ECDC 2012). In the EU, it was the causative agent of 30.5% (±1700 cases) of all foodborne outbreaks in 2010 alone.

Pathogenesis of salmonellosis

Nontyphoidal salmonellosis is provoked through the consumption of contaminated food products and has an incubation time of 12 to 72 hours. After ingestion the *Salmonella* bacterial cells first encounter the acidic stomach after which they enter the intestine. In

the intestine the cells have to deal with reduced oxygen, bile salts, increased osmolarity, the hosts immune system, and competition with resident microorganisms for nutrient and space. Bacterial cells able to survive and adapt to these conditions attach to the intestinal tract (Rychlik and Barrow 2005). This attachment is mediated by protein structures on the bacterial cell surface, called fimbriae (Ledeboer *et al.* 2006). After attachment, a whole set of effector proteins are injected into the eukaryotic host cells via the type III secretion system (TTSS). The two most important TTSS are encoded by the *Salmonella* pathogenicity island 1 and 2 (SPI-1 and SPI-2). The effector proteins secreted by these TTSS's induce an immune response and cause the symptoms that characterize salmonellosis (de Jong *et al.* 2012; Srikanth *et al.* 2011). In some cases *Salmonella* manages not only to attach to, but also to invade the intestinal cells. The primary invasion side are the microfold cells (M cells) in the gut lumen. After invasion *Salmonella* can cause a severe systemic infection (de Jong *et al.* 2012).

Salmonella in pork

As mentioned earlier, *Salmonella* infections in humans are in most cases foodborne. Food from animal origin is the most important source of human salmonellosis (RIVM) 2011; EFSA 2011). Epidemiological studies of the European Food Safety Authority (EFSA) have indicated that about 15-20% of the reported human salmonellosis cases are associated with the consumption of pork, which is similar in the Netherlands where about 19% of the cases are associated with pork. EFSA studies also found that 7.8% of all foodborne outbreaks are attributable to pork (RIVM 2011; EFSA 2008b; 2011).

Salmonella is able to infect and colonize the gut of pigs, however often it causes no severe diseases. After infection, the pigs can become healthy carriers of *Salmonella* in the tonsils, the intestines or in the gut-associated lymphoid tissue. These so-called carriers are a potential source for infection of other animals, since they can shed *Salmonella* into the environment. They may also introduce *Salmonella* from the farm into the slaughterhouse environment (de Busser *et al.* 2011; Botteldoorn *et al.* 2003).

A surveillance study in the EU showed that at point of slaughter, 10.3% of the slaughter pigs are positive for *Salmonella* subspecies (EFSA 2008a). Therefore good slaughter hygiene is important to prevent carcasses contamination. Contamination of carcasses can occur in two ways. One way is directly by the content of the tonsils, the intestine or lymph nodes. The other way is indirectly by contact with dirty processing equipment, so-called cross-contamination. Cross-contamination greatly contributes to *Salmonella* contamination of carcasses within the slaughter process. It is responsible for about 29% of the total amount of contaminated carcasses (van Hoek *et al.* 2012; EFSA 2008a; Botteldoorn *et al.* 2003). Therefore,

the presence of *Salmonella* subspecies in the slaughterhouse environment is a significant problem for slaughterhouses. In addition, several studies have shown that *Salmonella* can survive and persist for a long period of time in slaughterhouses and that it can become a part of the house flora. The house flora is hard to eradicate completely and it is an important source of carcass contamination within a slaughterhouse (Smid *et al.* 2012; van Hoek *et al.* 2012; Hald *et al.* 2003).

Taxonomy of *Salmonella*

Salmonellae are a large group of bacteria that can be divided in two species: *S. bongori* and *S. enterica. S. enterica* can be further divided into six subspecies (spp); ssp. e*nterica* (subspecies I), spp. s*almonae* (subspecies II), ssp. *arizonae* (subspecies IIIa), ssp. *diarizonae* (subspecies IIIb), ssp. *houtenae* (subspecies IV) and spp. *indica* (subspecies VI). These subspecies comprise more than 2500 serovars of which 59% can be assigned to *S. enterica* ssp. *enterica*. Serovars belonging to this subspecies are responsible for nearly all *Salmonella* infections in humans and warm-blooded animals (Agbaje *et al.* 2011; Brenner *et al.* 2000; Su and Chiu 2007). Some well-known examples of serovars causing human *Salmonella* infections are *S.* Enteritidis, *S.* Typhimurium, *S.* Typhi and *S.* Paratyphi (ECDC 2012).

The *Salmonella* serovars are classified based on immunological reactivity of two surface structures, namely the O antigen and the H antigen, with specific antisera (Agbaje *et al.* 2011; Brenner *et al.* 2000; Su and Chiu 2007). Through diversity within the O and H antigen, different strains have a different antigenic code that is designated to a serovar name. This name normally refers to the geographic location where the serovar was first isolated. All recognized *Salmonella* serovars and the corresponding antigenic codes are listed in the Kauffman-White scheme. This scheme is yearly updated by the World Health Organisation (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute in Paris.

Microbial biofilms

Already in late seventeenth century, Antonie van Leeuwenhoek detected that microbes were attached to the tooth surface which can be considered as the first description of a microbial biofilm. However it was not until the 1930s that biofilm formation was described by Zobell and coworkers who reported that bacteria attached themselves to glass surfaces and formed sessile communities (Zobell 1943). Nowadays, microbial biofilms gain lots of attention since it is known that in nature bacteria tend to colonize surfaces and form biofilms instead of growing in suspension. Currently, a biofilm is defined as an accumulation of

bacterial cells immobilized on a surface that are embedded in an extracellular matrix (Fig. 1.1) (Costerton 1999; Shi and Zhu 2009).

Salmonella can form biofilms on abiotic surfaces, such as plastic, glass, cement, rubber and stainless steel (Arnold and Bailey 2000; Chia *et al.* 2009; Joseph *et al.* 2001; Stepanovi *et al.* 2004). These surfaces are commonly encountered in industrial settings and households and therefore biofilms on such surfaces can form a risk for (cross-)contamination of food products. Next to abiotic surfaces, *Salmonella* can also attach to and subsequently form a biofilm on biotic surfaces, such as epithelial cells within a host (Ledeboer *et al.* 2006; Misselwitz *et al.* 2011), and gall stones (Prouty and Gunn 2003). Additionally, recent *Salmonella* outbreaks are associated to *Salmonella* biofilms on plant surfaces, such as seeds, vegetables and fruits (Berger *et al.* 2010; Teplitski *et al.* 2009).

Biofilm formation can be divided in several stages. First the bacterial cells approach a surface, followed by attachment to the surface. This initial attachment is a physicochemical process driven by Van der Waals, electrostatic and steric forces between the bacterial cells and the surface. Moreover, several bacterial cell surface structures, such as fimbriae, flagella and extracellular polysaccharides are involved in the attachment of bacterial cells (Anriany *et al.* 2006; Barak *et al.* 2005; Kim and Wei 2009; Kumar and Anand 1998; Latasa *et al.* 2005; Prouty *et al.* 2002; van Houdt and Michiels 2010). After the initial attachment the bacterial cells start producing an extracellular matrix that firmly anchors the biofilm to the surface, and micro-colonies are formed. Through the continuous growth of the biofilm cells, the extracellular matrix production and the attachment of new cells, the biofilm grows and fully matures. The exact biofilm structure and composition differs between microorganisms, environmental conditions and depends on surface types to which the bacteria attach (Archer *et al.* 2011; Condell *et al.* 2012; Gerstel and Römling 2003; Kumar and Anand 1998; Shi and Zhu 2009). As the biofilm matures, cells can also detach and settle somewhere else where the biofilm forming process can start over again.

The extracellular matrix of *Salmonella* biofilms

Biofilm formation is a complex process and many environmental factors influence the formation of the extracellular matrix. The extracellular matrix is a dense network of protein structures, exopolysaccharides, lipopolysaccharides, extracellulair DNA (eDNA) and fatty acids. This matrix facilitates biofilm maturation, prevents desiccation of cells, and protects cells within the biofilm against diverse environmental stresses such as chemical disinfection treatments (Anriany *et al.* 2006; Gibson *et al.* 2006; Steenackers *et al.* 2012; White *et al.* 2006; Zogaj *et al.* 2001).

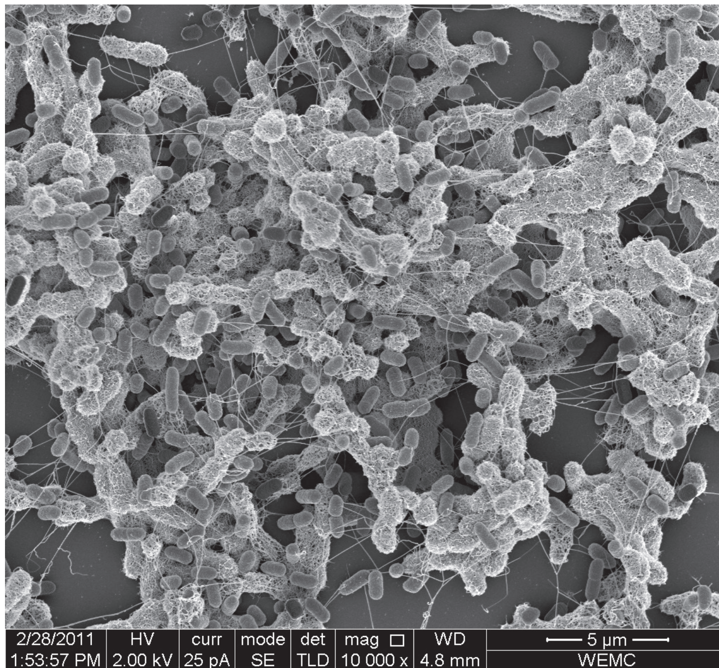


Figure 1.1: Scanning electron microscopy image of a *S.* Typhimurium biofilm. The cells within the biofilm are entrapped in an extracellular matrix.

Curli fimbriae and cellulose

The most studied structures of the extracellular matrix of *Salmonella* biofilms are curli fimbriae and cellulose. These structures are also the most important components of the rdar (red, dry and rough) morphotype formed during multicellular behaviour of *Salmonella* on agar plates. This morphotype is related to biofilm forming capacity (Gerstel and Römling 2003; Römling 2005; Römling *et al.* 1998).
Curli fimbriae and cellulose are widely expressed among Enterobacteriaceae, such as *Salmonella* ssp., *Escherichia coli*, *Shigella* spp., *Enterobacter* spp. and *Citrobacter* spp. (Bokranz *et al.* 2005; Gerstel and Römling 2003; Solomon *et al.* 2005). Co-expression of these structural components results in a hydrophobic network of tightly packed cells in a compact network (Fig. 1.1) (Zogaj *et al.* 2001).

Curli fimbriae

Curli fimbriae are extracellular protein structures with high adhesion properties that mediate adhesion to and biofilm formation on biotic surfaces, such as human and chicken epithelial cells and plant surfaces, but they also play a major role in biofilm formation on diverse

abiotic surfaces (Berger *et al.* 2010; Hermans *et al.* 2011; Ledeboer *et al.* 2006; Zogaj *et al.* 2001). Curli fimbriae share many biochemical and structural properties with the eukaryotic amyloid fibers. Like amyloid fibers, curli fimbriae are non-branching, β-sheet fibers that bind the dye congo red. Curli fimbriae are encoded by the two functional connected operons *csgBAC* and *csgDEFG* (Fig. 1.2). *CsgA* encodes for the major structural subunit, which is nucleated into fibers by CsgB outside the cell. CsgA and CsgB are secreted into the extracellular environment by the outer membrane protein CsgG. The exact role of periplasmic proteins CsgE and CsgF is not elucidated yet, but it is known that these proteins are also required for curli fimbriae assembly (Barnhart and Chapman 2006). Curli assembly is activated by CsgD. The regulation of *csgD* expression is very complex and under control of a large intergenic region located between the *csgBAC* and *csgDEFG* operons. This region is targeted by several regulatory proteins, such as RpoS, OmpR, H-NS, CpxA/CpxR, I-HF and MlrA. Binding of these regulatory proteins affects *csgD* expression (Fig. 1.2). A different set of regulatory proteins is activated by different environmental conditions and therefore expression of *csgD* is highly influenced by environmental cues (Barnhart and Chapman 2006; Gerstel and Römling 2003). Environmental conditions that induce *csgD* expression are ambient temperatures, low osmolarity, starvation and microaerophilic conditions (Gerstel and Römling 2003).

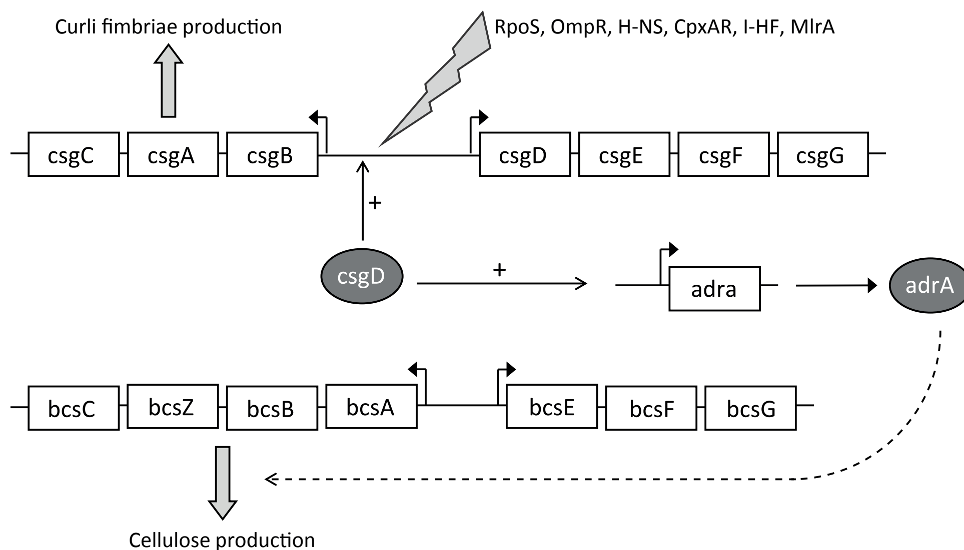


Figure 1.2: A schematic illustration of the role of CsgD in the expression of curli fimbriae, encoded by the *csg* operon. CsgD also indirectly influences cellulose production by increasing expression of *adrA*, and therefore increasing AdrA production that affects cellulose production at a posttranscriptional level. The transcription of *csgD* is influenced by different regulatory proteins that are activated and/or depressed by environmental cues.

Cellulose
Cellulose is a polysaccharide consisting of carbohydrate polymers from β(1→4)-linked D-glucose units. Like curli fimbriae, cellulose is involved in biofilm formation on diverse biotic and abiotic surfaces (Barak *et al.* 2007; Ledeboer *et al.* 2006; Zogaj *et al.* 2001). The proteins essential for cellulose biosynthesis are encoded by the *bcsABZC* and *bcsEFG* operons (Fig. 1.2). BcsA and BcsB form the cellulose synthase complex, in which BcsA is the catalytic subunit activated by the binding of BcsB, a cyclic-di(3'→5')-guanylic acid (c-di-GMP) binding protein (Zogaj *et al.* 2001). The genes encoding for these proteins are constitutively transcribed indicating that cellulose production is regulated on a post-transcriptional level. The post-transcriptional regulation of cellulose production is indirectly influenced by CsgD through the activation of *adrA* expression (Fig. 1.2). This gene encodes for di-guanylate cyclase which is involved in the production of c-di-GMP. c-di-GMP is an effector molecule that activates the cellulose synthase by binding to BcsB (Römling 2005; Zogaj *et al.* 2001).

Next to curli fimbriae and cellulose, CsgD also influences the expression of the biofilms extracellular matrix components BapA, a large secreted protein, and anionic O-antigen capsule, a high molecular weight capsular polysaccharide (Gibson *et al.* 2006; Latasa *et al.* 2005). So in conclusion CsgD is a key activator of multicellular behaviour of *Salmonella* on biotic and abiotic surfaces (Gerstel and Römling 2003; Römling 2005; Zakikhany *et al.* 2010; Zogaj *et al.* 2001).

Fimbriae of the chaperon-usher assembly class
Next to curli fimbriae, the genome of *S.* Typhimurium also possesses twelve other putative fimbrial gene clusters that are of the chaperon-usher assembly class, namely *fim*, *bcf*, *lpf*, *saf*, *stf, stb, stc*, *std*, *sth*, *sti*, and *stj*, and a plasmid-encoded fimbria (*pef*) (McClelland *et al.* 2001). However, two of these gene clusters miss one of the three genes encoding for the major structural subunit, the chaperone component or the porin component, which are needed for functional fimbriae assembly, which leaves 11 putative functional fimbriae gene clusters (Humphries *et al.* 2003). A phylogenomic analysis of all available *Salmonella* genomes (in April 2011) by Yue et al. (2012) revealed that the set of fimbrial gene clusters can differ between serovars and they identified a total of 35 fimbrial gene clusters along the analysed *Salmonella* strains. These identified fimbrial gene clusters could be divided in three groups. A group of core fimbrial gene clusters shared by more than 80% of the strains (*fim*, *bcf*, *stb, std*, *sth*, *saf* and *sti*), a second group of partially conserved fimbrial gene clusters shared by 40% to 80% of the strains, and a third group of so-called sporadic fimbrial gene clusters shared by less than 40% of the strains (Yue *et al.* 2012). The variation in fimbrial gene clusters among *Salmonella* serovars is believed to be a result of serovar adaption to specific hosts, niches or environments (Clayton *et al.* 2008; Yue *et al.* 2012).

Type 1 fimbriae

In this thesis we further focus on one of the core fimbrial gene clusters, namely the *fim* gene cluster that endoces for type 1 fimbriae (Fig. 1.3). This gene cluster contains the structural genes *fimAICDHF*, of which *fimA* encodes for the major structural subunit and *fimH* for the adhesin located at the tip of the fimbriae. Folding of the fimbriae in the periplasma is influenced by the FimC chaperone and the polymerization of the structural subunits on the outer membrane is catalysed by the FimD usher (Fronzes *et al.* 2008; McFarland *et al.* 2008; Yue *et al.* 2012). The expression of the structural genes is phase variable and under control of the *fimA* promoter. This promoter is activated by binding of the regulatory proteins FimZ and FimY, and repressed by the binding of FimZ and FimW. The fourth regulatory protein, FimU, is involved in the mRNA translation of *fimY* (Fig. 1.3) (McFarland *et al.* 2008). This complex regulatory mechanism enables fine-tuning of fimbriae biogenesis in response to different environmental cues (McFarland *et al.* 2008; Yue *et al.* 2012).

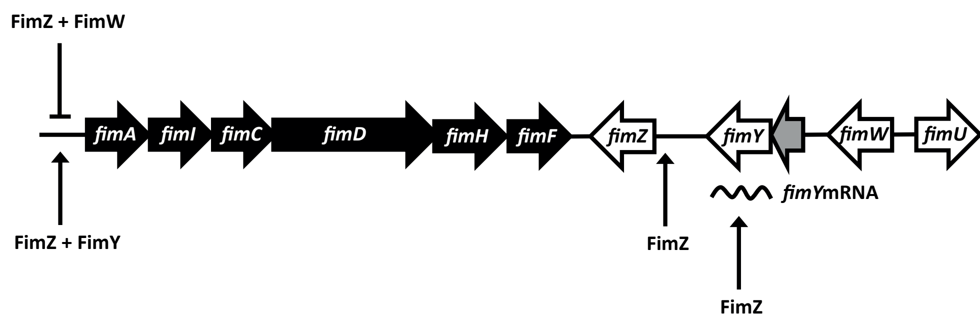


Figure 1.3: A schematic overview of the *fim* operon in *S.* Typhimurium and its regulation. Black arrows indicate structural genes and white arrows represent regulation genes. The structural genes are under control of the *fimA* promoter and expression is activated by FimZ+FimY and repressed by FimZ+FimW. This figure was kindly provided by the J Bacteriol, 2008 190(20):602-612 (McFarland *et al.* 2008).

Type 1 fimbriae are protein cell surface structures that are composed of ±1000 copies of the major structural subunit FimA, with FimH located on the tip. Up to 200-500 of these structures can be found on the outer membrane of fimbriated bacteria (Fronzes *et al.* 2008). They are involved in the initial attachment of *Salmonella* to intestinal epithelium where they mediate FimH-dependent mannose-sensitive adhesion to glycoconjugates on eukaryotic cells (Ledeboer *et al.* 2006; Kisiela *et al.* 2005; Boddicker *et al.* 2002). So far no studies describe a role of type 1 fimbriae in the adhesion to or biofilm formation on abiotic surfaces.

Implications of bacterial biofilms

Bacteria within a biofilm have a distinct phenotype from planktonic cells and generally show higher tolerance to antimicrobial agents, such as disinfectant treatments and antibiotics (Bridier *et al.* 2011a; Joseph *et al.* 2001; Mah and O'Toole 2001; Scher *et al.* 2005; van Houdt and Michiels 2010; Wong *et al.* 2010). Due to the increased tolerance against antimicrobial treatments, biofilms are hard to eradicate and they cause all kinds of problems in medical and industrial settings. In medicine, biofilms are found on different surfaces like catheters, biliary stents, artificial hips, teeth, contact lenses and in lungs of cystic fibrosis patients where they form a source of bacterial infections (Bjarnsholt *et al.* 2009; Stahlhut *et al.* 2012; van Merode *et al.* 2008). It has been estimated that up to 60% of all human bacterial infections are caused by biofilms (Spoering *et al.* 2006) *E coli.* But also in the food industry problems occur when bacterial cells manage to form biofilms on food contact surfaces like food processing equipment. Biofilms on food contact surfaces can cause equipment failure and they can become a persistent source of contamination resulting in food spoilage or food-borne illnesses (Shi and Zhu 2009; Simões *et al.* 2010; Vestby *et al.* 2009). Therefore biofilm formation forms a threat for human health and can lead to economics losses.

Mechanisms involved in biofilm resistance to antimicrobial agents

The increased resistance of biofilms against antimicrobial treatments compared to their planktonic counterpart can be explained by different mechanisms:

I) The extracellular matrix limits the penetration of antimicrobial agents into the biofilm. This is partly due to diffusion limitation caused by the 3-dimensional structure, but primarily because of absorption or reaction of the antimicrobial agent with extracellular matrix components. This takes place at the outer part of the biofilm and neutralizes the antimicrobial agent. Therefore the innermost bacterial cells of the biofilm are not reached by the antimicrobial agent and survive the treatment (Bridier *et al.* 2011b; de Beer *et al.* 1994).

II) The increased resistance can be caused by physiological changes of biofilm cells due to the expression of specific genes in response to reduced growth rate and/or cell attachment. These growth conditions result in the activation of stress responses protecting the biofilm cells from various environmental stresses including antimicrobial agents (Møretrø *et al.* 2012; van der Veen and Abee 2010). An example is the activation of the *Salmonella* general stress response regulator RpoS in slow growing cells, such as stationary and biofilm cells (Robbe-Saule *et al.* 2006).

III) The increased resistance of biofilms might also be attributable to the presence of a small population of stress-variants, also called persisters (Møretrø *et al.* 2012; Spoering

and Lewis 2001). These are cells that have increased resistance against specific or several stresses, caused by phenotypic or genetic changes (van Boeijen *et al.* 2010). The presence of stress variants in a bacterial population results in heterogeneity and a biphasic inactivation kinetic during a disinfection treatment, indicated by tailing of the inactivation curve (Fig. 1.4). Tailing of an inactivation curve indicates that longer treatment with an antimicrobial agent will have no effect (Spoering and Lewis 2001; van Boeijen *et al.* 2008).

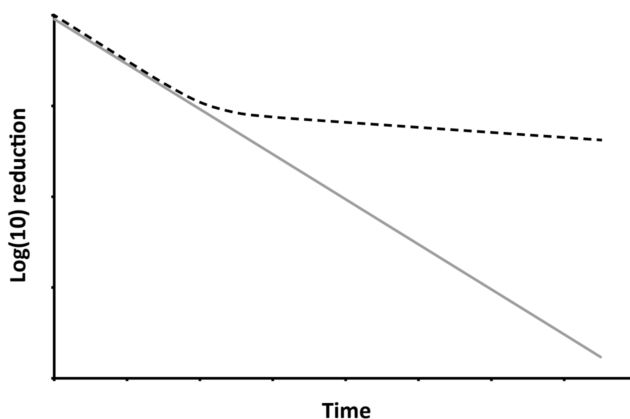


Figure 1.4: Inactivation kinetics described by a first-order also named log linear model (indicated by a grey line) and by a biphasic model (indicated by a black dotted line). Biphasic inactivation is indicated by tailing of the inactivation curve and due to the presence of a more resistant subpopulation.

Disinfection treatments

To control food quality and safety in food processing environments, it is important to clean and disinfect the food contact surfaces on a regular basis to inhibit accumulation of bacterial cells and possible biofilm formation (McDonnell and Russell 1999; Shi and Zhu 2009; van Houdt and Michiels 2010; Wirtanen and Salo 2003). The aim of the cleaning step is to remove residual organic matter and soil. Cleaning agents commonly used in the food industry are alkaline-based and acid-based compounds, which are most effective if applied in combination with mechanical forces and heat (Kumar and Anand 1998; van Houdt and Michiels 2010). After the cleaning step, the food contact surface is treated with a chemical disinfectant. A disinfectant is an agent used on inanimate objects, is not necessarily sporicidal, and reduces viable microbial cells to acceptable levels. A good disinfectant is safe to use, environmental friendly, cost efficient, not corrosive on the surface to which it is applied, stable during storage, active in a wide range of different environments (temperature, pH,

dilution, soiling) and it has a broad antimicrobial spectrum (Kumar and Anand 1998; McDonnell and Russell 1999; Møretrø *et al.* 2012). Obviously no single disinfectant meets all these requirements and for every different industrial setting another disinfectant might be the best choice. Moreover, one should also take into consideration that a disinfectant that kills planktonic cells might not be efficient in killing/reducing the more resistant biofilm cells.

A wide diversity of chemical disinfectants is available and it depends on the environmental conditions of the industrial setting and the target micro-organisms which disinfectant will be selected. The main groups of disinfectants used in the food industry and their mode of actions on Gram-negative bacteria are listed in table 1.1 (Ceragioli *et al.* 2010; McDonnell and Russell 1999). This thesis concentrates on two commonly used disinfectants, namely the quaternary ammonium compound benzalkonium chloride (BKC) and the oxidizing agent peracetic acid (PAA, $C_2H_4O_3$) (Fig. 1.5).

Table 1.1: An overview of chemical disinfectants commonly used in the food industry and their mode of action on Gram-negative bacteria.

Disinfection class	**Examples of disinfectants**	**Mode of action on Gram-negative bacteria**
Alcohols	Ethanol Isopropyl alcohol	Membrane damage and denaturation of proteins
Aldehydes	Formaldehyde Glutaradehyde	Cross-linking of proteins in the outer membrane, inhibition of transport and dehydrogenase activity, interaction with RNA and DNA synthesis
Halogens	Chlorine dioxide Sodium hypochlorite	Modification of DNA and proteins, especially enzymes with thiol or amino groups
Phenicols	Phenol Triclosan	Induction of cellular leakage and increasing cell membrane permeability
Oxidizing agents	Hydrogen peroxidize Peracetic acid	Formation of free radicals affecting DNA, proteins, lipids and inactivation of enzymes
Quaternary compouns	Benzalkonium chloride	Interaction with phopholipids of the inner and outer membrane resulting in loss of cell membrane integrity and cell leakage, degradation of proteins and DNA
Biguanides	Chlorhexidine	Affecting the inner cell membrane integrity resulting in cell leakage

Benzalkonium chloride

BCK is a mixture of alkylbenzyldimethylammonium chlorides of various alkyl chain lengths (Fig. 1.5A) that is widely used as surface disinfectant, antiseptic and preservative. It is a fast acting biocidal agent which is effective at non-toxic concentration, it is non-corrosive, non-irritating and it has no odor or flavor. BKC mechanism of action is dissociation of cellular membrane lipid bilayers, which increases the permeability and induces leakages of cellular content. It also reacts and deactivates enzymes that control a wide range of metabolic and respiratory activities (Mangalappalli-Illathu *et al.* 2008; McDonnell and Russell 1999).

Peracetic acid

PAA is an organic compound formed by the reaction of hydrogen peroxide (H_2O_2) with acetic acid ($C_2H_4O_2$) and it is a commonly used for disinfection, sterilization and antisepsis (Fig. 1.5B). It is active in a wide pH and temperature range and because it breaks down into water and oxygen it is also safe to use and environmental friendly. PAA kills microorganisms by formation of hydroxyl radicals (OH^-) that oxidize and affect enzymes, DNA and disrupt proteins and lipids the cell membrane (Ceragioli *et al.* 2010; McDonnell and Russell 1999).

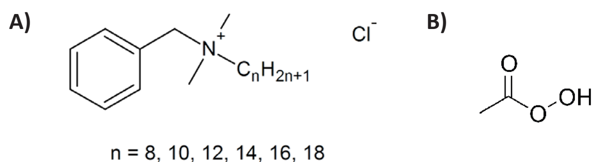


Figure 1.5: The structural formula of BKC (A) and PAA (B).

Stress responses of *Salmonella*

Salmonella is capable of growing and surviving in a wide variety of environments including, soil, foods and food processing plants, and in animal and humans. Many of these environments are far from the optimal growth conditions of *Salmonella* and are therefore a stress for the microbial cell. In order to survive stresses encountered, the cell must rapidly sense and respond appropriately to stresses. Stress responses in *Salmonella* are under control of various regulation factors, such as alternative sigma factors, two-component systems or transcriptional regulators. These regulation factors activate and/or repress sets of genes resulting in an increased resistance against a specific or a variety of stresses and facilitate survival (Poole 2012; Spector and Kenyon 2012). Various stress responses, e.g. starvation, acid, temperature, oxidative, osmotic, envelope, antimicrobial agent, iron and desiccation stress are extensively described in literature and for a detailed overview of these different stress responses we refer to reviews from Spector and Kenyon (2012) and Poole (2012).

Stress responses induced by antimicrobial agents

Exposure to antimicrobial agents induces stress responses in bacterial cells. Many antimicrobial agents generate reactive oxygen species (ROSs), and are therefore associated with the activation of the oxidative stress response (Bore *et al.* 2007; Ceragioli *et al.* 2010; Mangalappalli-Illathu *et al.* 2008; Poole 2012). Basically, *Salmonella* possesses two pathways dealing with oxidative stress. One is the transcriptional regulator OxyR, which is mainly activated upon exposure to H_2O_2 and induces genes involved in the conversion of H_2O_2, DNA protection, formation of disulfide bonds and reduction or oxidation of lipids (Poole 2012; Spector and Kenyon 2012). The other one is the two-component system SoxRS that is activated upon changes in the cellular redox balance. The SoxRS system activates maintenance of the reduced state of Fe-S clusters, DNA repair, increase of reducing power, efflux of redox-cycling compounds and replacement of redox-sensitive enzymes by redox-resistant enzymes (Bore *et al.* 2007; Poole 2012; Spector and Kenyon 2012). Both OxyR and SoxRS induce the expression of *fur*. Increased Fur levels prevent the formation of hydroxyl radicals via the Fenton reaction (Spector and Kenyon 2012).

SoxS also positively induces the expression of *acrAB*, which encodes for a part of the multidrug efflux pump AcrAB-TolC. Increased expression and activity of this efflux pump has frequently been associated with increased resistance against chemical and clinical antimicrobial agents (Braoudaki and Hilton 2004; Levy 2002; Poole 2012). Next to AcrAB-TolC, also other multidrug efflux pumps of the resistance nodulation division (RND) family are recognized as components of bacterial stress responses that facilitates increased resistance against diverse antimicrobial agents (Condell *et al.* 2012; Poole 2012).

Objective and outline of this thesis

The research undertaken in this thesis aims to gain more insight in the biofilm formation process and survival in industrial environment of various *Salmonella* serovars. More knowledge and understanding about these topics contributes to the development of better strategies for *Salmonella* control. Therefore biofilm formation was analysed at various environmental conditions and on different surfaces (Chapter 2 and 4). The influence of environmental conditions on the involvement of different mechanisms and bacterial cell surface structures in cell attachment and biofilm formation is described in Chapter 2 and 3. Also the correlation between biofilm forming capacity and survival in industrial settings was investigated (Chapter 4). Furthermore, the effect of repeated use of BKC was analysed and resistant variants with increased resistance for BKC were isolated to study the mechanisms that facilitate the increased resistance (Chapter 5).

Next to this introduction, this thesis consists of the following chapters:

Chapter 2 describes the effect of nutrient availability, temperature, and strains origin on the biofilm forming behaviour of *S.* Typhimurium. It was shown that curli fimbriae and cellulose specifically contribute to biofilm formation under low nutrient conditions at ambient temperature and that other components and or mechanisms are conceivably more important during biofilm formation at 37 °C and/or in nutrient-rich conditions. Therefore, in **Chapter 3**, the factors important during *S.* Typhimurium biofilm formation in nutrient-rich conditions at 37 °C were studied. The results indicated that type 1 fimbriae contribute to the initial attachment and biofilm formation of *S.* Typhimurium to abiotic surfaces. Furthermore it was shown that these fimbriae were expressed by a subpopulation of the cells only, which agreed with zeta potential measurements indicating two subpopulations in cultures of dense biofilm formers. After investigating the effect of environmental conditions on the biofilm forming process, the surface behaviour of different serovars which are frequently isolated in pork processing environments, viz. *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.* Infantis was examined in **Chapter 4**. This revealed diversity in surface behaviour among but also within the different serovars. Furthermore, it was shown that biofilm formation greatly contributed to the survival of *Salmonella* on dry stainless steel surfaces and no differences were observed between biofilm forming capacity on polystyrene and stainless steel. This means that results obtained with polystyrene, which is commonly used in the laboratory to study *Salmonella* biofilms, is representative for the surface behaviour on stainless steel.
In **Chapter 5** we studied the effect of repeated exposure of *S.* Typhimurium to BKC. The results showed that BKC exposure results in tailing of the inactivation curve indicating variation in BKC susceptibility within the initial population. Repeated exposure resulted in the enrichment of a more resistant population and variants with increased BKC resistance were isolated. Phenotypical characterization of these resistant variants showed loss of motility, reduced biofilm forming capacity and reduced cell membrane permeability pointing to modification of cell surface properties.

REFERENCES

Agbaje M, Begum RH, Oyekunle MA, Ojo OE, Adenubi OT. 2011. Evolution of *Salmonella* nomenclature: a critical note. Folia Microbiol 56(6):497-503.

Anriany Y, Sahu SN, Wessels KR, McCann LM, Joseph SW. 2006. Alteration of the rugose phenotype in *waaG* and *ddhC* mutants of *Salmonella enterica* serovar Typhimurium DT104 is associated with inverse production of curli and cellulose. Appl Environ Microbiol 72(7):5002-5012.

Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. 2011. *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. Virulence 2(5):445-459.

Arnold J, Bailey G. 2000. Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning electron and atomic force microscopy study. Poultry Sci 79(12):1839-1845.

Barak JD, Gorski L, Naraghi-Arani P, Charkowski AO. 2005. *Salmonella enterica* virulence genes are required for bacterial attachment to plant tissue. Appl Environ Microbiol 71(10):5685-5691.

Barak JD, Jahn CE, Gibson DL, Charkowski AO. 2007. The role of cellulose and O-antigen capsule in the colonization of plants by *Salmonella enterica*. Mol Plant Microbe Interact 20(9):1083-1091.

Barnhart MM, Chapman MR. 2006. Curli biogenesis and function. Ann Rev Microbiol 60(1):131-147.

Berger CN, Sodha SV, Shaw RK, Griffin PM, Pink D, Hand P, Frankel G. 2010. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environ Microbiol 12(9):2385-2397.

Bjarnsholt T, Jensen PO, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Hoiby N. 2009. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol 44(6):547-558.

Boddicker JD, Ledeboer NA, Jagnow J, Jones BD, Clegg S. 2002. Differential binding to and biofilm formation on HEp-2 cells by *Salmonella enterica* serovar Typhimurium is dependent upon allelic variation in the *fimH* gene of the *fim* gene cluster. Mol Microbiol 45(5):1255-1265.

Bokranz W, Wang X, Tschape H, Römling U. 2005. Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. J Med Microbiol 54(12):1171-1182.

Bore E, Hébraud M, Chafsey I, Chambon C, Skjæret C, Moen B, Møretrø T, Langsrud Ø, Rudi K, Langsrud S. 2007. Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses. Microbiol 153(4):935-946.

Botteldoorn N, Heyndrickx M, Rijpens N, Grijspeerdt K, Herman L. 2003. *Salmonella* on pig carcasses: positive pigs and cross contamination in the slaughterhouse. J Appl Microbiol

95(5):891-903.

Braoudaki M, Hilton AC. 2004. Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. J Clin Microbiol 42(1):73-78.

Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. 2000. *Salmonella* nomenclature. J Clin Microbiol 38(7):2465-2467.

Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F. 2011a. Resistance of bacterial biofilms to disinfectants: a review. Biofouling 27(9):1017-1032.

Bridier A, Dubois-Brissonnet F, Greub G, Thomas V, Briandet R. 2011b. Dynamics of the action of biocides in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemoth 55(6):2648-2654.

Ceragioli M, Mols M, Moezelaar R, Ghelardi E, Senesi S, Abee T. 2010. Comparative transcriptomic and phenotypic analysis of the responses of *Bacillus cereus* to various disinfectant treatments. Appl Environ Microbiol 76(10):3352-3360.

Chia TWR, Goulter RM, McMeekin T, Dykes GA, Fegan N. 2009. Attachment of different *Salmonella* serovars to materials commonly used in a poultry processing plant. Food Microbiol 26(8):853-859.

Clayton DJ, Bowen AJ, Hulme SD, Buckley AM, Deacon VL, Thomson NR, Barrow PA, Morgan E, Jones MA, Watson M. 2008. Analysis of the role of 13 major fimbrial subunits in colonisation of the chicken intestines by *Salmonella enterica* serovar Enteritidis reveals a role for a novel locus. BMC Microbiol 8(1):228.

Condell O, Sheridan Á, Power KA, Bonilla-Santiago R, Sergeant K, Renaut J, Burgess C, Fanning S, Nally JE. 2012. Comparative proteomic analysis of *Salmonella* tolerance to the biocide active agent triclosan. J Proteom 75(14):4505-4519.

Costerton JW. 1999. Introduction to biofilm. International J Antimicrob Agents 11(3-4):217-239.

de Beer D, Srinivasan R, Stewart PS. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Appl Environ Microbiol 60(12):4339-4344.

de Busser V, Maes D, Houf K, Dewulf J, Imberechts H, Bertrand S, De Zutter L. 2011. Detection and characterization of *Salmonella* in lairage, on pig carcasses and intestines in five slaughterhouses. International J Food Microbiol 145(1):279-286.

de Jong HK, Parry CM, van der Poll T, Wiersinga WJ. 2012. Host-pathogen interaction in invasive Salmonellosis. PLoS Pathog 8(10):4.

European Centre for Disease prevention and Control (ECDC). 2012. The European union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. EFSA J 10(3):442.

European Food Safety Authority (EFSA). 2008a. Report of the task force on zoonoses data collection on the analysis of the baseline survey on the prevalence of *Salmonella* in slaughter pigs, Part A. EFSA J 135:1-111.

EFSA. 2008b. Scientific opinion of the panel on biological hazards on a request from the European commission on a quantitative microbiological risk assessment on *Salmonella* in meat: Source attribution for human salmonellosis from meat. EFSA J 625:1-32.

EFSA. 2011. Estimation of the relative contribution of different food and animal sources to human *Salmonella* infections in the European Union.

Fronzes R, Remaut H, Waksman G. 2008. Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. European Molecular Biology Organization 27(17):2271-2280.

Gerstel U, Römling U. 2003. The *csgD* promoter, a control unit for biofilm formation in *Salmonella typhimurium*. Res Microbiol 154(10):659-667.

Gibson DL, White AP, Snyder SD, Martin S, Heiss C, Azadi P, Surette M, Kay WW. 2006. *Salmonella* produces an O-antigen capsule regulated by AgfD and important for environmental persistence. J Bacteriol 188(22):7722-7730.

Hald T, Wingstrand M, Swanenburg A, von Altrock, Thorberg B. 2003. The occurrence and epidemiology of *Salmonella* in European pig slaughterhouses. Epidemiol Infect 131(3):1187-1203.

Hannu T, Inman R, Granfors K, Leirisalo-Repo M. 2006. Reactive arthritis or post-infectious arthritis. Best Pract Res Clin Rheumatol 20(3):419-433.

Hermans K, Nguyen TL, Roberfroid S, Schoofs G, Verhoeven T, De Coster D, Vanderleyden J, De Keersmaecker SC. 2011. Gene expression analysis of monospecies *Salmonella typhimurium* biofilms using differential fluorescence induction. J Microbiol Methods 84(3):467-478.

Humphries AD, Raffatellu M, Winter S, Weening EH, Kingsley RA, Droleskey R, Zhang S, Figueiredo J, Khare S, Nunes J, Adams LG, Tsolis RM, Bäumler AJ. 2003. The use of flow cytometry to detect expression of subunits encoded by 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. Mol Microbiol 45(8):1357-1376.

Joseph B, Otta SK, Karunasagar I, Karunasagar I. 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. Int J Food Microbiol 64(3):367-372.

Kim SH, Wei CI. 2009. Molecular characterization of biofilm formation and attachment of *Salmonella enterica* serovar typhimurium DT104 on food contact surfaces. J Food Prot 72(9):1841-1847.

Kisiela D, Sapeta A, Kuczkowski M, Stefaniak T, Wieliczko A, Ugorski M. 2005. Characterization of FimH adhesins expressed by *Salmonella enterica* serovar Gallinarum biovars Gallinarum and Pullorum: reconstitution of mannose-binding properties by single amino acid substitution. Infect Immun 73(9):6187-6190.

Kumar CG, Anand SK. 1998. Significance of microbial biofilms in food industry: a review. Int J Food Microbiol 42(1-2):9-27.

Latasa C, Roux A, Toledo-Arana A, Ghigo J-M, Gamazo C, Penadés JR, Lasa I. 2005. BapA, a large secreted protein required for biofilm formation and host colonization of *Salmonella enterica* serovar Enteritidis. Mol Microbiol 58(5):1322-1339.

Ledeboer NA, Frye JG, McClelland M, Jones BD. 2006. *Salmonella enterica* serovar Typhimurium requires the Lpf, Pef, and Tafi fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. Infect Immun 74(6):3156-3169.

Levy SB. 2002. Factors impacting on the problem of antibiotic resistance. J Antimicrob Chemoth 49(1):25-30.

Mah TF, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9(1):34-39.

Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clin Infect Dis 50(6):882-889.

Mangalappalli-Illathu AK, Vidovic S, Korber DR. 2008. Differential adaptive response and survival of *Salmonella enterica* serovar enteritidis planktonic and biofilm cells exposed to benzalkonium chloride. Antimicrob Agents Chemother 52(10):3669-3680.

McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F. ***et al.*** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nat 413(6858):852-856.

McDonnell G, Russell AD. 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 12(1):147-179.

McFarland KA, Lucchini S, Hinton JCD, Dorman CJ. 2008. The leucine-responsive regulatory protein, Lrp, activates transcription of the fim operon in *Salmonella enterica* serovar Typhimurium via the fimZ regulatory gene. J Bacteriol 190(2):602-612.

Misselwitz B, Kreibich SK, Rout S, Stecher B, Periaswamy B, Hardt WD. 2011. *Salmonella enterica* serovar Typhimurium binds to HeLa cells via Fim-mediated reversible adhesion and irreversible type three secretion system 1-mediated docking. Infect Immun 79(1):330-341.

Møretrø T, Heir E, Nesse LL, Vestby LK, Langsrud S. 2012. Control of *Salmonella* in food related environments by chemical disinfection. Food Res Int 45(2):532-544.

Poole K. 2012. Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. Trends Microbiol 20(5):227-234.

Prouty AM, Gunn JS. 2003. Comparative analysis of *Salmonella enterica* serovar Typhimurium biofilm formation on gallstones and on glass. Infect Immun 71(12):7154-7158.

Prouty AM, Schwesinger WH, Gunn JS. 2002. Biofilm formation and interaction with the surfaces of gallstones by *Salmonella spp*. Infect Immun 70(5):2640-2649.

Rijks Instituut voor Volksgezondheid en Mileu (RIVM). 2011. Staat van zoönosen.

Robbe-Saule V, Jaumouille V, Prevost M-C, Guadagnini S, Talhouarne C, Mathout H, Kolb A, Norel F. 2006. Crl activates transcription initiation of RpoS-regulated genes involved in the multicellular behavior of *Salmonella enterica* serovar Typhimurium. J. Bacteriol 188(11):3983-3994.

Römling U. 2005. Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae. Cell Mol Life Sci 62(11).

Römling U, Sierralta WD, Eriksson K, Normark S. 1998. Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter. Mol Microbiol 28(2):249-264.

Rychlik I, Barrow PA. 2005. *Salmonella* stress management and its relevance to behaviour during

intestinal colonisation and infection. FEMS Microbiol Rev 29(5):1021-1040.

Scher K, Römling U, Yaron S. 2005. Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. Appl Environ Microbiol 71(3):1163-1168.

Shi X, Zhu X. 2009. Biofilm formation and food safety in food industries. Trends Food Sci Technol 20(9):407-413.

Simões M, Simões LC, Vieira MJ. 2010. A review of current and emergent biofilm control strategies. Food Sci Technol 43(4):573-583.

Smid JH, Heres L, Havelaar AH, Pielaat A. 2012. A biotracing model of *Salmonella* in the pork production chain. J Food Protect 75(2):270-280.

Solomon EB, Niemira BA, Sapers GM, Annous BA. 2005. Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. J Food Protect 68:906-912.

Spector MP, Kenyon WJ. 2012. Resistance and survival strategies of *Salmonella enterica* to environmental stresses. Food Res Int 45(2):455-481.

Spoering AL, Lewis K. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J Bacteriol 183(23):6746-6751.

Spoering AL, Vulic M, Lewis K. 2006. GlpD and PlsB participate in persister cell formation in *Escherichia coli*. J Bacteriol 188(14):5136-5144.

Srikanth CV, Mercado-Lubo R, Hallstrom K, McCormick BA. 2011. *Salmonella* effector proteins and host-cell responses. Cell Mol Life Sci 68(22):3687-3697.

Stahlhut SG, Struve C, Krogfelt KA, Reisner A. 2012. Biofilm formation of *Klebsiella pneumoniae* on urethral catheters requires either type 1 or type 3 fimbriae. FEMS Immunol Med Microbiol 65(2):350-359.

Steenackers H, Hermans K, Vanderleyden J, de Keersmaecker SCJ. 2012. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. Food Res Int 45(2):502-531.

Stepanovi S, Irkovi I, Ranin L, Svabi-Vlahovi M. 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol 38(5):428-432.

Su LH, Chiu CH. 2007. *Salmonella*: clinical importance and evolution of nomenclature. Chang Gung Med J 30(3):210-219.

Teplitski M, Barak JD, Schneider KR. 2009. Human enteric pathogens in produce: un-answered ecological questions with direct implications for food safety. Curr Opin Biotechnol 20(2):166-171.

van Boeijen IKH, Chavaroche AA, Valderrama WB, Moezelaar R, Zwietering MH, Abee T. 2010. Population diversity of *Listeria monocytogenes* LO28: phenotypic and genotypic characterization of variants resistant to high hydrostatic pressure. Appl Environ Microbiol 76(7):2225-2233.

van Boeijen IKH, Moezelaar R, Abee T, Zwietering MH. 2008. Inactivation kinetics of three *Listeria monocytogenes* strains under high hydrostatic pressure. J Food Protect 71(10):2007-2013.

van der Veen S, Abee T. 2010. Importance of SigB for *Listeria monocytogenes* static and continuous-flow biofilm formation and disinfectant resistance. Appl Environ Microbiol 76(23):7854-7860.

van Hoek AHAM, de Jonge R, van Overbeek WM, Bouw E, Pielaat A, Smid JH, Malorny B, Junker E, Löfström C, Pedersen K. 2012. A quantitative approach towards a better understanding of the dynamics of *Salmonella* spp. in a pork slaughter-line. Int J Food Microbiol 153(1–2):45-52.

van Houdt R, Michiels CW. 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. J Appl Microbiol 109(4):1117-1131.

van Merode AEJ, Duval JFL, van der Mei HC, Busscher HJ, Krom BP. 2008. Increased adhesion of *Enterococcus faecalis* strains with bimodal electrophoretic mobility distributions. Colloids and Surface B 64(2):302-306.

Vestby LK, Møretrø T, Ballance S, Langsrud S, Nesse LL. 2009. Survival potential of wild type cellulose deficient *Salmonella* from the feed industry. BMC Vet Res 5(1):43.

White AP, Gibson DL, Kim W, Kay WW, Surette MG. 2006. Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of *Salmonella*. J Bacteriol 188(9):3219-3227.

Wirtanen G, Salo S. 2003. Disinfection in food processing – Efficacy testing of disinfectants. Rev Environl Sci Biotechnol 2(2):293-306.

Wong H, Townsend K, Fenwick S, Trengove R, O'Handley R. 2010. Comparative susceptibility of planktonic and 3-day-old *Salmonella* Typhimurium biofilms to disinfectants. J Appl Microbiol 108(6):2222-2228.

Yue M, Rankin SC, Blanchet RT, Nulton JD, Edwards RA, Schifferli DM. 2012. Diversification of the *Salmonella* fimbriae: a model of macro- and microevolution. PLoS One 7(6):12.

Zakikhany K, Harrington CR, Nimtz M, Hinton JCD, Römling U. 2010. Unphosphorylated CsgD controls biofilm formation in *Salmonella enterica* serovar Typhimurium. Mol Microbiol 77(3):771-786.

Zobell CE. 1943. The effect of solid surfaces upon bacterial activity. J Bacteriol 46(1):39-56.

Zogaj X, Nimtz M, Rohde M, Bokranz W, Römling U. 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol Microbiol 39(6):1452-1463.

CHAPTER 2

Diversity in biofilm formation and production of curli fimbriae and cellulose by *Salmonella* Typhimurium strains of different origin in high and low nutrient medium

Greetje A.A. Castelijn, Stijn van der Veen, Marcel H. Zwietering, Roy Moezelaar and Tjakko Abee

Published in Biofouling 2012, 28:51-63

ABSTRACT

Biofilm forming behaviour of 51 *Salmonella* Typhimurium strains was determined in TSB and 20 times diluted TSB (1/20TSB) at 25 °C and 37 °C. The results indicated that biofilm forming behaviour is influenced by environmental conditions and associated with the origin of the strains. Clinical, outbreak-associated and retail product isolates showed dense biofilm formation in both media at 25 °C, and in TSB also at 37 °C. However, industrial isolates only showed dense biofilm formation in 1/20TSB at 25 °C. By enumeration of biofilm cells, LIVE/DEAD staining and SEM analysis of biofilms it was found that the ratio of cells and extracellular matrix is affected by environmental conditions. Indeed, genes involved in curli fimbriae and cellulose production are highly induced during biofilm formation at 25 °C in 1/20TSB. This indicates that these are important matrix components during biofilm formation in 1/20TSB at 25 °C and that other factors contribute to biofilm formation of clinical, outbreak-associated and retail product isolates at 37 °C and/or nutrient-rich conditions.

INTRODUCTION

Salmonella is one of the most common and widely distributed bacterial pathogens and the causative agent of salmonellosis. It is a major public health problem, and millions of human cases are reported worldwide every year. In 2007, 151,995 human cases of S*almonella* infections were reported in the EU (EFSA 2009). In the Netherlands, one of the predominant serovar causing human salmonellosis is *Salmonella enterica* supp. *enterica* serovar Typhimurium, associated with approximately 30% of the cases (Doorduyn *et al.* 2006). In general, *Salmonella* infections in humans are foodborne. In particular food products of animal origin, such as eggs, poultry and meat, are important sources of infection.

Salmonella is able to attach to various surfaces where it can form biofilms (Sinde and Carballo 2000; Prouty *et al.* 2002; Stepanovi *et al.* 2004; Ledeboer and Jones 2005). A biofilm is an accumulation of bacterial cells immobilized on a surface and embedded in an extracellular matrix (Costerton 1999). Bacteria within a biofilm have a distinct phenotype from planktonic cells and generally show higher resistance to disinfectants and antibiotics (Kumar and Anand 1998; Scher *et al.* 2005). Proposed mechanisms for the increased resistance of biofilms against disinfectants and antibiotics are activation of biofilm specific defense mechanisms and changes in the physiology of the bacteria induced by attachment and limited availability of key nutrients. Furthermore, the extracellular matrix contributes to the increased resistance of biofilm cells against disinfection treatments (Solano *et al.* 2002; White *et al.* 2006; Vestby *et al.* 2009b). The extracellular matrix limits the diffusion of chemicals to the inside of the biofilm and the outer layer of the biofilm might quench disinfectants or antibiotics (Gilbert et al. 2002). The ability of *Salmonella* to form biofilms on food processing equipment poses a major problem for the food industry (Swanenburg *et al.* 2001; Vestby *et al.* 2009b). Also in medical settings problems occur due to *Salmonella* biofilm formation (Prouty *et al.* 2002). Although the environmental conditions differ between industrial and medical settings, so far, no differences in biofilm forming capacity between *S.* Typhimurium strains isolated in industrial or medical settings were found (Stepanovi *et al.* 2004; Solomon *et al.* 2005; Vestby *et al.* 2009a).

Several studies have shown that curli fimbriae and cellulose are major components of the extracellular matrix of *S*. Typhimurium biofilms (Römling *et al.* 1998; Jonas *et al.* 2007). These components are widely expressed among the Enterobacteriaceae species (Zogaj *et al.* 2003; Bokranz *et al.* 2005; Solomon *et al.* 2005). Curli fimbriae are proteinaceous extracellular fibers that promote surface colonization and cell-cell interactions. Production of curli fimbriae is dependent on the proteins transcribed by the *csgBAC* and *csgDEFG* operons. *CsgBA* encodes for the structural proteins, while *csgEFG* encodes for accessory proteins required for curli assembly (Barnhart and Chapman 2006). Both these operons are positively regulated by the autoregulator CsgD. CsgD is also indirectly involved in cellulose production

by activating transcription of *adrA*. *AdrA* expression is required for the synthesis of cellulose, even though the *bcs* genes, encoding for proteins essential for biosynthesis of cellulose, are constitutively expressed (Zogaj *et al.* 2001). The AdrA protein contains a GGDEF domain which has diguanylase cyclase activity. Therefore, AdrA can mediate the production of cyclic-di-GMP, an effector molecule involved in the activation of cellulose production (Zogaj *et al.* 2001; Römling 2005).

The promoter region of the *csgD* promoter is a large intergenic region to which several regulatory proteins can bind directly. Different environmental conditions activate a different set of regulatory proteins. By binding to the intergenic region of the *csgD* promoter, they affect the expression of *csgD* (Römling *et al.* 1998; Gerstel and Römling 2003). Thus, environmental conditions can influence the production of the extracellular matrix components curli fimbriae and cellulose, and the question arises whether differences in biofilm forming behaviour could be linked to differences in the activation of these components, and whether this can be linked to the strain's origin. Therefore, the biofilm forming capacity of 51 *S.* Typhimurium strains from different origin at ambient (25 °C) and body (37 °C) temperature in nutrient-rich (TSB) and nutrient-low (1/20TSB) conditions was analysed. These media were chosen since TSB is frequently used in biofilm research and 1/20TSB is used in several other studies to mimic food industry conditions (Stepanovi *et al.* 2004; Solomon *et al.* 2005; Kroupitski *et al.* 2009). The biofilm forming capacity was investigated with both the crystal violet (CV) assay and enumeration of biofilm cells by plate counts. Furthermore, biofilms grown under different conditions were examined by LIVE/DEAD staining, SEM analysis and they were stained with calcofluor to determine the cellulose content. In addition, the expression of key regulators of the extracellular matrix components curli fimbriae and cellulose was analysed by Q-PCR.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 51 *Salmonella enterica* ssp. *enterica* serovar Typhimurium strains of different origin was used in this study to investigate diversity in biofilm forming capacity. The strain collection contained strains isolated in industrial environments, clinical isolates, outbreak-associated isolates, and strains isolated from retail products. The industrial isolates were all recently obtained in multiple meat processing plants and were isolated from equipment surfaces, food products, raw materials and the processing environment. Also the completely sequenced *S.* Typhimurium strain LT-2 was included. Due to a mutation in the *rpoS* gene, the LT-2 strain has an attenuated RpoS function. It has previously been shown that RpoS is a positive regulator of biofilm formation (Adams and McLean 1999; Römling et al. 1998; Robbe-Saule et al. 2006). Therefore the *rpoS* deletion mutant of the LT-2 strain from the *Sal*

Table 2.1: *Salmonella enterica* ssp. *enterica* serovar Typhimurium strains used in this study.

Strain number	Isolation source	Reference	Biofilm former group
C1	Feces	(Berk *et al.* 2005)	A
OA1	Retail product, helva	(Berk *et al.* 2005)	
C2	Feces	(Berk *et al.* 2005)	
C3	Human, feces	(Berk *et al.* 2005)	
C4	Human, feces	(Berk *et al.* 2005)	
OA2	Retail product, tahini	(Berk *et al.* 2005)	
C5	Human, feces	(Berk *et al.* 2005)	
OA3	Retail product, helva	(Berk *et al.* 2005)	
OA4	Retail product, tahini	(Berk *et al.* 2005)	
C6	Feces	(Berk *et al.* 2005)	
C7	Human, feces	(Berk *et al.* 2005)	
C8	Human, blood	(Berk *et al.* 2005)	
R1	Retail product, meat	(Berk *et al.* 2005)	
C9	Human, blood	(Berk *et al.* 2005)	
C10	Human, feces	(Berk *et al.* 2005)	
R2	Retail product, meat	(Berk *et al.* 2005)	
R3	Retail product, meat	(Berk *et al.* 2005)	
R4	Retail product, meat	(Berk *et al.* 2005)	
R5	Retail product, cocoa	(Berk *et al.* 2005)	
R6	Retail product, nuds	(Berk *et al.* 2005)	
C11	Human, blood	This thesis	
C12	Human, blood	This thesis	
C13	Human, feces	(Berk *et al.* 2005)	
I1	Food industry	This thesis	
I2	Foodindustry	This thesis	
C14	Feces	(Berk *et al.* 2005)	
I3	Food industry	This thesis	B
I4	Food industry	This thesis	
I5	Food industry	This thesis	
I6	Food industry	This thesis	
I7	Food industry	This thesis	
I8	Food industry	This thesis	
C15	Human, blood	This thesis	
I9	Food industry	This thesis	
I10	Food industry	This thesis	
C16	Human, blood	This thesis	
I11	Food industry	This thesis	
I12	Food industry	This thesis	
I13	Food industry	This thesis	
I14	Food industry	This thesis	
I15	Food industry	This thesis	
I16	Food industry	This thesis	
I17	Food industry	This thesis	
I18	Food industry	This thesis	
I19	Food industry	This thesis	
I20	Food industry	This thesis	
I21	Food industry	This thesis	
I22	Food industry	This thesis	C
I23	Food industry	This thesis	
L1	SGSC 1412(LT -2)	McClelland *et al.* (2001)	
L2	SGSC 2618	Fang *et al.* (1992)	

monella Genetic Stock Centre, was also included (Table 2.1). Stocks were stored at -80 °C in Brain Hearth Infusion broth (BHI, Becton Dickson) supplemented with 15% glycerol (Sigma). Single colonies were used to inoculate Luria Bertani broth (LB, Merck) and cultures were incubated for 18 h at 37 °C with agitation (200 rpm), after which the bacteria were used in the different experiments.

Biofilm formation

Biofilm formation was analysed in Tryptone Soya Broth (TSB, Oxoid) and 20 times diluted TSB (1/20TSB) in flat bottom 96 well polystyrene microtiter plates (Greiner Bio-one), using the crystal violet (CV) assay (Djordjevic *et al.* 2002; Wijman *et al.* 2007) and plate counting. Wells were filled with 200 µl medium, inoculated with 1.5% (vol/vol) overnight grown cultures, and incubated statically for 24 h at 25 °C or 37 °C. In the CV assay, the wells were washed two times with 250 µl water. Subsequently, the biofilms were stained for 30 min with 225 µl of 0.1% (wt/vol) CV (Merck) and washed three times with 250 µl water. The attached CV was solubilized with 250 µl 96% ethanol and the absorbance was measured at 595 nm (SpectraMax, Molecular Devices).

For enumeration by plate counting, the wells were washed four times with 250 µl of peptone physiological salt solution (pps) to remove unbound cells. Subsequently the wells were swabbed to detach the biofilm cells. The swab and the cell suspension were vortexed with glass beads (≤106 µm, Sigma) in order to remove biofilm cells from the swab and to obtain single cells. An appropriate dilution was made in pps and the cells were enumerated by plating on TSA. The plates were incubated overnight at 37 °C and the colonies were counted. Both biofilm formation assays were performed in three biologically independent experiments.

Modeling the relationship between microtiter plate assay and plate counts

To investigate the relation between the CV assay and the amount of attached biofilm cells, the following model was used to fit the data for each culture condition:

$$A_{595} = a \cdot N + b \qquad (1)$$

where A_{595} is the absorbance of the solubilized CV after biofilm formation, *N* is the number of viable attached biofilm cells (CFU/well), *b* the background signal (three times the standard deviation above the mean A_{595} of the negative control) and *a* is the proportionality constant between CV staining and cell counts. The parameters *a* and b were estimated by fitting equation 1 to the data points in Tablecurve 2D (Windows v. 2.03). The fitted parameters were verified in Microsoft Excel by using Excel Solver add-in.

Morphotype identification on congo red and calcofluor agar plates

The ability to produce curli fimbriae and cellulose was analyzed on LB without NaCl agar plates supplemented with 40 µg/ml congo red (Sigma) and 20 µg/ml coomassie brilliant blue (Merck). Cellulose production was further characterized on agar plates containing 40 µg/ml calcofluor (Fluorescent Brightner 28, Sigma) (Römling *et al.* 2003). After 48 h of incubation at 25 °C or 37 °C, the colony morphology was examined.

Evaluation of biofilms with fluorescent microscopy

For the microscopic analysis, biofilms were grown on polystyrene coupons (18 mm x 22 mm) placed in a 12 well plate. The wells were half filled with 3 ml of TSB or 1/20TSB and inoculated with 1.5% (vol/vol) overnight grown cultures. To analyse the cellulose content of a biofilm, the media were supplemented with 40 µg/ml calcofluor. After 24 h incubation at 25 °C or 37 °C the coupons were washed four times with peptone buffered saline (PBS) and examined with an Olympus BX41 microscope. To visualize the calcofluor stain a U-MWU-2 fluorescence filter (Olympus) was used. To determine cell viability the coupons were stained with the LIVE/DEAD *Bac*Light bacterial viability staining kit according to manufacturer's protocol. Fluorescence of the LIVE/DEAD probes was observed using U-MNBV (SYTO9) and U-MWIG (PI) fluorescence filters. A Dialux 20 microscope (Leica) was used to make images at 1000x magnification. In total, about 25 microscopic images were examined for each sample.

Scanning electron microscopy

Biofilms were grown as described above. After the coupons were washed 4 times with PBS, the samples were fixed with 3% glutaraldehyde in 0.1 M phosphatebuffer pH 7.2 for 1 h at room temperature. Subsequently, they were rinsed three times during 45 minutes with buffer and treated with 1% OsO4 for 1 h at room temperature. Next, the samples were washed with water and dehydrated in an acetone series (10%, 30%, 50%, 70%, 90%, 100 %, each step 15 minutes). Then, they were critical point dried (CPD) with carbon dioxide (CPD 030, BalTec, Liechtenstein). For SEM imaging the samples were attached on a sample holder by carbon adhesive tabs (EMS Washington USA) and sputter coated with 5 nm tungsten (MED 020, Leica, Vienna, Austria). Samples were analysed at 2 kV at room temperature in a Field Emission Scanning Electron Microscope (Magellan 400, FEI, Eindhoven, the Netherlands).

RNA isolation and cDNA synthesis

Total RNA was isolated from planktonic cells, grown statically in tubes containing 10 ml of media, and from biofilm cells, grown as described above. Both planktonic and biofilm cells were cultured in TSB or 1/20TSB at 25 °C or 37 °C for 24 h. Planktonic cells and biofilms were quenched in RNAprotect Bacteria Reagent (QIAGEN) and harvested by centrifugation (planktonic cells) or scraping (biofilm cells). Total RNA was isolated using the RNeasy kit (QIAGEN) with an on-column DNase treatment according to manufacturer's protocol. For

each sample, 300 ng total RNA was used for cDNA synthesis using the SuperScript III reverse transcriptase kit (Invitrogen) according to manufacturers' protocol. RNA samples were checked for genomic DNA contamination by omitting the Superscript III reverse transcriptase.

Quantitative real-time PCR

Quantitative real time PCR (Q-PCR) was performed using the 2x SYBR® Green PCR Master Mix (Applied Biosystems) in a final volume of 25 µl. Forward and reverse primers (Table 2.2) were designed with an amplicon length of approximately 100 bp using *PRIMER 3* (free software, http://frodo.wi.mit.edu/primer3/). Primers were added in a concentration of 250 nM. Reactions were run on an ABI Prism 7000 Sequence Detection System with an initial step of 10 min at 95 °C, and 40 cycli of 15 s at 95 °C and 1 min at 59 °C. To check for specificity of the amplification, a melting curve was added. Furthermore a standard curve was derived for each primer set to determine the efficiency. The relative expression levels of the genes of interest between conditions and strains were compared using the housekeeping gene 16S rRNA for normalization. Q-PCR was performed on three biological independent experiments using two replicates each. Significant differences in expression levels were identified by using a two-tailed student's t-test in Excel ($p<0.05$, t-test).

Table 2.2: Primers used for Q-PCR to analyse expression of *csgD*, *csgA*, *bcsA* and *adrA*.

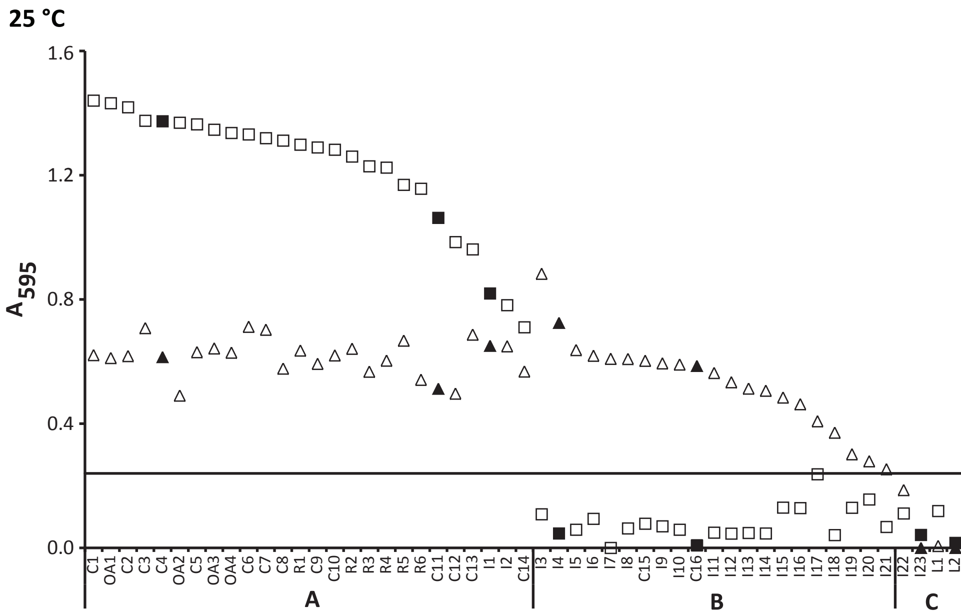
Gene	Forward primer 5´- 3´	Reverse primer 5´- 3´
csgD	CCTGACGATTATCCCTACCG	CCTGACGATTATCCCTACCG
csgA	GGATTCCACGTTGAGCA	TAACGCTCTGGGTAATGG
bscA	ATTCTGGGCGTTATCGTGAC	CCACAGCAACAGCAGAAAGA
adraA	GTCGGCTGGTCTTTTGTCTG	GATCGCATCCACTTTCAGGT
16S *rRNA*	GTCGGCTGGTCTTTTGTCTG	GTGCAATATTCCCCACTGCT

RESULTS

Diversity in biofilm forming capacity

Biofilm forming capacity of 51 *S.* Typhimurium strains was determined under various culture conditions using the CV assay. The biofilm forming capacity was dependent on medium and temperature, but also on the origin of the strain (Fig.2.1 and Table 2.1). All *S.* Typhimurium strains exhibiting dense biofilm formation, showed most CV binding to the wells at the liquid-air interface (data not shown). Notably, the formation of pellicles was not observed under the tested conditions.

At 25 °C, the biofilm forming behaviour of the complete collection of *S.* Typhimurium strains could be divided into three different groups, group A, B and C (Fig. 2.1). Group A strains


25 °C
A595
1.6
1.2
0.8
0.4
0.0
C1 OA1 C2 C3 C4 OA2 C5 OA3 OA4 C6 C7 C8 R1 C9 C10 R2 R3 R4 R5 R6 C11 C12 C13 I1 I2 C14 I3 I4 I5 I6 I7 I8 C15 I9 I10 C16 I11 I12 I13 I14 I15 I16 I17 I18 I19 I20 I21 I22 I23 L1 L2
A
B
C

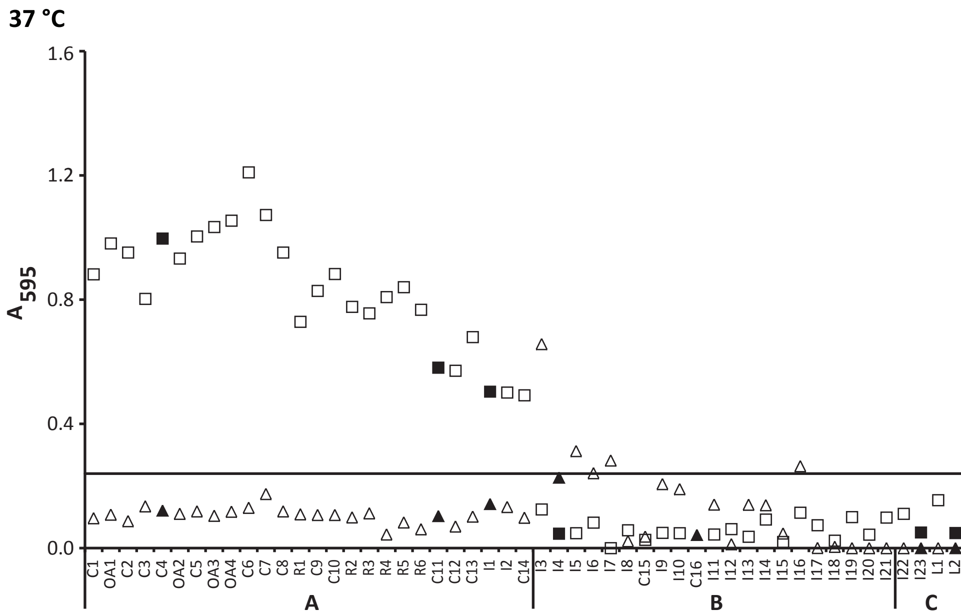

37 °C
A595
1.6
1.2
0.8
0.4
0.0
C1 OA1 C2 C3 C4 OA2 C5 OA3 OA4 C6 C7 C8 R1 C9 C10 R2 R3 R4 R5 R6 C11 C12 C13 I1 I2 C14 I3 I4 I5 I6 I7 I8 C15 I9 I10 C16 I11 I12 I13 I14 I15 I16 I17 I18 I19 I20 I21 I22 I23 L1 L2
A
B
C

Figure 2.1. Biofilm forming capacity of 51 *S.* Typhimurium strains analysed with the CV assay. The graphs show biofilm formation at 25 °C and 37 °C in TSB (■) and 1/20TSB (▲). The three different strain groups, A, B and C are indicated under the strain numbers. The level of dense biofilm formation (three times the background level) is indicated with a solid line and the seven selected strains are indicated by black symbols.

produced dense biofilms in both media at 25 °C, and in TSB also at 37 °C. A dense biofilm was defined at an A_{595} ≥ 0.24, which is three times the background signal. The background signal was defined as three times the standard deviation above the mean A_{595} of the negative control. The group B strains only formed dense biofilms in the 1/20TSB medium at 25 °C and the group C strains showed little to no biofilm formation at all tested culture conditions. At 37 °C, the strains generally showed less biofilm formation than at 25 °C (Fig. 2.1). This was not related with differences in growth capabilities, since higher cell densities were reached after growth in the 96 well plates at 37 °C (data not shown). These data indicate that biofilm formation is influenced by environmental conditions like medium composition, oxygen and temperature, and that biofilm forming behaviour is associated with strains origin.

In general, the clinical, outbreak-associated and retail product isolates were group A strains and most industrial isolates belong to the group B strains. Only two of the 16 clinical isolates, strain C15 and C16, assigned to group B, and two of the 23 industrial isolates, strain I1 and I2, assigned to group A. The group C strains comprises two industrial isolates, strain I22 and I23, and the reference strain LT-2 (L1) and its *rpoS* mutant (L2) (Table 2.1 and Fig. 2.1). Of the *S.* Typhimurium strains, three group A, two group B, and two group C strains were selected for further analyses. The selected strains are marked by a closed symbol in Figure 2.1. One of the selected group C strains (strain L2) is a *rpoS* mutant of the completely sequenced *S.* Typhimurium LT2 strain (McClelland et al. 2001). It has previously been shown that *rpoS* is a positive regulator of biofilm formation (Adams and McLean 1999; Römling et al. 1998; Robbe-Saule et al. 2006). Sequence analysis verified the presence of an intact *rpoS* gene for all selected strains, except for strain L2. Furthermore, no mutations in the upstream promoter regions were found (data not shown).

Enumeration and viability of biofilm cells

The CV assay is generally used to analyse biofilm forming capacity of bacteria. However, not much is known about the contribution of the number of biofilm cells, and the extracellular matrix to the total amount of CV binding. Therefore, it is important to determine how the results of the CV assay correlate with the number of biofilm cells. First the viability of cells within the biofilm was determined using a LIVE/DEAD staining. In TSB, ± 90-95% (Fig. 2.2B) and in 1/20TSB ± 95-100% (Fig. 2.2C) of the biofilm cells grown at 25 °C were stained green, indicating that most cells were viable. Similar data on viability were obtained with cells from biofilms grown at 37 °C (data not shown). Thus, in all tested culture conditions no notew-

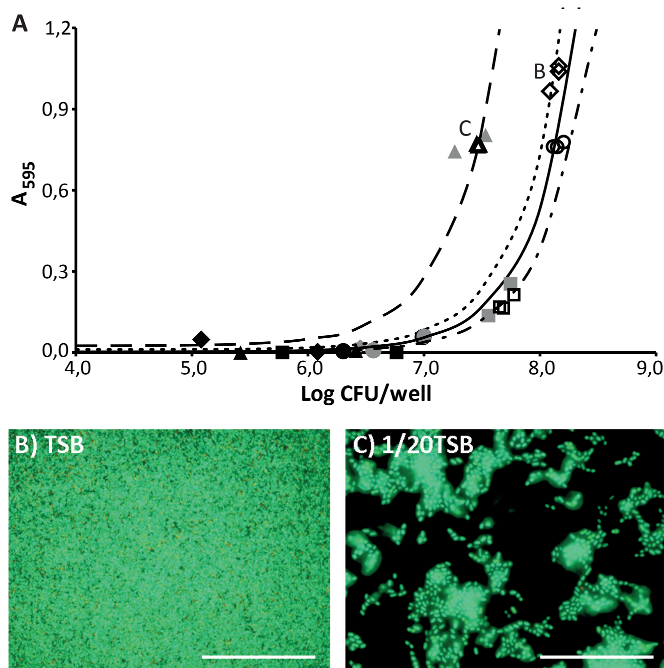


Figure 2.2: Enumeration and viability of biofilm cells. A) The relation between the CV assay and plate counts of *S.* Typhimurium biofilms grown in different culture conditions. Data from the CV assays (A_{595}) is plotted against the log CFU/well. Also the corresponding curves that are fitted with the linear model are shown in this graph. The biofilms were grown in TSB at 25 °C (--♦--), TSB at 37 °C (—●—), 1/20TSB at 25 °C (—▲—), and 1/20TSB at 37 °C (—■— -). The open symbols represent the group A strains, the grey symbols represent the group B strains, and the black symbols represent the group C strains. B and C) Representative fluorescent images of LIVE/DEAD stained biofilms grown in TSB (B) or 1/20TSB (C) at 25 °C of a group A strain (strain I1). The scalebar represents 20 µm. The letters in the graph correspond with the letters of the images. Both images were made at 1000x magnification.

orthy contribution of dead cells to 24 h old biofilms was observed. Next we quantified the amount of viable biofilm cells by plate counts. The biofilms were detached by swabbing after which cell suspensions were vortexed with glass beads to separate the biofilm cells. Microscopic evaluation revealed that the method used to obtain single cells is sufficient for biofilms grown in TSB or 1/20TSB at 25 °C and 37 °C. In figure 2.2A, the relation between the OD values obtained in the CV assay and the number of biofilm cells for the different culture conditions is shown. In TSB, the group A strains yielded approximately 8.1 log CFU/well at both 25 °C and 37 °C after 24 h, while the group B and C strains showed less than 7 log CFU/well under these conditions. Interestingly, for the group A strains no significant difference in CFU/well was detected between 25 °C and 37 °C, even though the CV assay

showed a significant difference. In 1/20TSB, the number of biofilm cells for the group A and B strains was comparable, which is in agreement with the results of the CV assay. However, in this medium there are slightly, but significantly, more biofilm cells for these strains at 37 °C (7.6-7.8 log CFU/well) than at 25 °C (7.3-7.5 log CFU/well) (p<0.05, t-test), even though CV binding is considerably higher at 25 °C than at 37 °C.

To enable a comparison of the data obtained, the data set for each culture condition was fitted with a linear model (equation 1). This model contains a factor (a) that represents a proportional constant between the CV staining and cell counts (Fig. 2.2A). Factor a was higher for the fitted model of the results in 1/20TSB at 25 °C ($a = 2.54 \cdot 10^{-8}$) compared with the other culture conditions ($a = 7.22 \cdot 10^{-9}$ for TSB at 25 °C, $a = 5.30 \cdot 10^{-9}$ for TSB at 37 °C, and $a = 4.11 \cdot 10^{-9}$ for 1/20TSB at 37 °C). These results suggest that in 1/20TSB at 25 °C, the extracellular matrix production is increased compared with the other tested culture conditions. Therefore the influence of environmental conditions on the formation of the biofilm extracellular matrix was further investigated.

Curli fimbriae and cellulose production on agar plates

Curli fimbriae and cellulose production was analysed on agar plates containing congo red or calcofluor. At 25 °C, both the group A and B strains expressed the red, dry, and rough (rdar) morphotype (Table 2.3), which correlates with production of curli fimbriae and cellulose (Römling et al. 2003; Jonas et al. 2007; Malcova et al. 2008). The group C strains expressed the smooth and white (saw) morphotype at 25 °C, which relates to absence of curli fimbriae and cellulose production (Römling et al. 1998). At 37 °C, all strains expressed the saw morphotype except for L2 that expressed a smooth and red (sar) morphotype. This indicates that congo red was capable of binding to this strain under this culture condition.

Table 2.3: Curli fimbriae and cellulose production on congo red and calcofluor agar plates of the selected *S.* Typhimurium strains. The morphotypes expressed on congo red agar plates: rdar (red, dry and rough), saw (smooth and white) and sar (smooth and red) are comparable to those found in other studies (Barnhart and Chapman 2006; Römling *et al.* 1998; Jones *et al.* 2007). Fluorescence intensities on the calcofluor agar plates are indicated by - = no fluorescence, + = fluorescence and ++ = intense flourescence.

Strain	Biofilm former group	25 °C		37 °C	
C4	A	rdar	++	saw	-
I1		rdar	++	saw	-
C11		rdar	++	saw	-
I4	B	rdar	++	saw	-
C16		rdar	++	saw	-
I23	C	saw	-	saw	-
L2		saw	-	sar	-

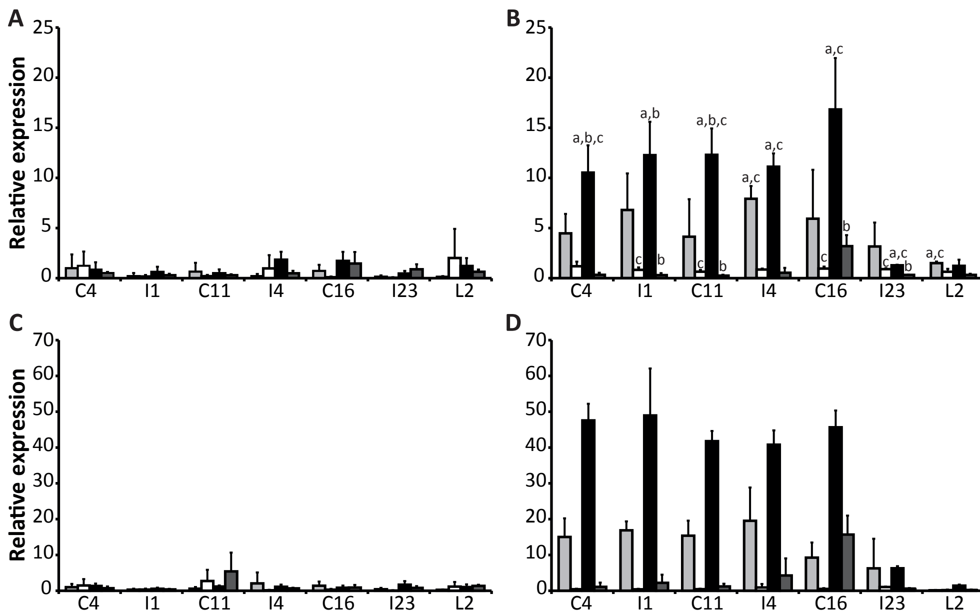


Figure 2.3: Expression of *csgD* and *csgA* in planktonic and biofilm cells of selected *S.* Typhimurium strains. The graphs show the expression of *csgD* in TSB (A), *csgD* in 1/20TSB (B), *csgA* in TSB (C) and *csgA* in 1/20TSB (D) of planktonic cells grown at 25 °C (light grey) or 37 °C (white) and in biofilm cells grown at 25 °C (black) or 37 °C (dark grey). The expression values are relative to the expression of strain C4, a group A strains, grown planktonically in TSB at 25 °C. The bars denote standard deviations of three biological repeats. [a] Expression in planktonic or biofilm cells at 25 °C is significantly different from 37 °C ($p < 0.05$, *t*-test). [b] Expression in biofilm cells grown at 25 °C or 37 °C is significantly different from planktonic cells ($p < 0.05$, *t*-test). [c] Expression in 1/20TSB is significantly different from TSB ($p < 0.05$, *t*-test).

The cellulose production was further characterized on agar plates supplemented with calcofluor (Table 2.3). At 25 °C, the group A and B strains showed intense fluorescence, whereas for the group C strains no fluorescence was detected. At 37 °C, no fluorescence was observed for the strains that expressed a saw morphotype on the congo red agar plates. For strain L2, which expressed a sar morphotype on the congo red agar plates, some fluorescence was detected at the calcofluor agar plate. This indicates that strain L2 produced cellulose at 37 °C, although the fluorescence was less compared with the group A and B strains at 25 °C. In conlcusion, the results of these plate assays indicate that the group A and B strains have the capacity to produce curli fimbriae and cellulose at 25 °C.

Curli fimbriae expression

Curli fimbriae are known to be an important component of the biofilm extracellular matrix (Römling et al. 1998; Jonas et al. 2007). Therefore, the expression of curli fimbriae was analyzed by Q-PCR in planktonic and biofilm cells, in TSB and 1/20TSB, at 25 °C and 37 °C (Fig. 2.3). The expression of both the gene encoding the transcriptional regulator of the *csg* operons, *csgD*, and the gene encoding the major structural subunit of curli fimbriae, *csgA*, was investigated. Both genes showed comparable expression patterns. In TSB, no relevant differences in *csgD* and *csgA* expression between the different culture conditions or strains were detected (Fig. 2.3A and 2.3C). However in 1/20TSB, significant differences in expression were observed (Fig. 2.3B and 2.3D). In general, both genes were higher expressed in 1/20TSB at 25 °C. Expression of *csgD* and *csgA* was significantly increased in biofilm cells grown in 1/20TSB at 25 °C compared with 37 °C or TSB (p<0.05, t-test), except for strain L2. In addition, the expression of both genes was considerable higher for the group A and B strains than for the group C strains at this culture condition (p<0.05, t-test). Furthermore in 1/20TSB at 25 °C, the expression of *csgA* in group A and B strains was induced more in biofilm cells compared with planktonic cells (p<0.05, t-test). These results are consistent with the biofilm forming capacity of these strains in 1/20TSB at 25 °C. This suggests that curli fimbriae are important for biofilm formation and that they form an important component of the biofilm extracellular matrix under this culture condition. Presumably, they might not be as important for biofilm formation at 37 °C and/or in a nutrient-rich environment.

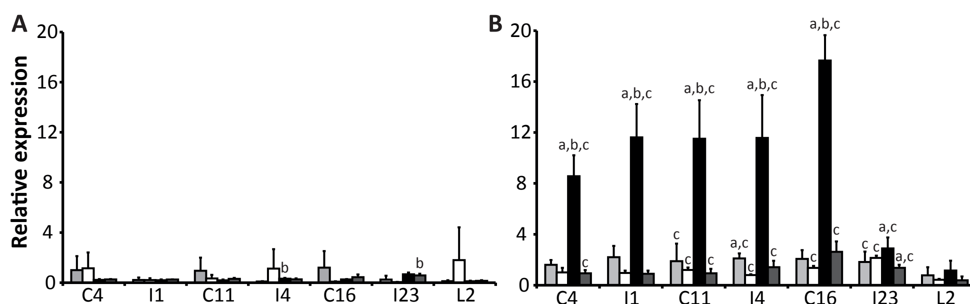


Figure 2.4: Expression of *adrA* in planktonic and biofilm cells of selected *S.* Typhimurium strains. The graphs show the expression in TSB (A) and 1/20TSB (B) in planktonic cells grown at 25 °C (light grey) or 37 °C (white) and biofilm cells grown at 25 °C (black) or 37 °C (dark grey). The expression values are relative to the expression of strain C4 grown planktonically in TSB at 25 °C. The bars denote standard deviations of three biological repeats. [a] Expression in planktonic or biofilm cells at 25 °C is significantly different from 37 °C ($p < 0.05$, *t*-test). [b] Expression in biofilm cells grown at 25 °C or 37 °C is significantly different from planktonic cells ($p < 0.05$, *t*-test). [c] Expression in 1/20TSB is significantly different from TSB ($p < 0.05$, *t*-test).

Cellulose expression

Besides curli fimbriae, cellulose may also contribute to the biofilm extracellular matrix. Therefore the expression of *bcsA*, which encodes the cellulose synthase, and *adrA*, which encodes a protein proposed to be required for cellulose synthesis (Zogaj et al. 2001), were analysed in planktonic and biofilm cells grown in TSB and 1/20TSB at 25 °C and 37 °C. No significant differences in *bcsA* expression between any of the tested culture conditions or between the different strains, was detected (data not shown). Thus the expression of *bcsA* was not affected by environmental conditions. However, significant differences were observed in *adrA* expression. The expression of this gene was higher in biofilm cells grown in 1/20TSB compared with biofilm cells grown in TSB (p<0.05, t-test), except for strain L2 (Fig. 2.4). In addition, biofilm cells of group A and B strains show increased expression of *adrA* in 1/20TSB at 25 °C compared with planktonic cells (p<0.05, t-test) (Fig. 2.4B). These results are consistent with the biofilm forming capacity of these strains and with the induced expression of curli fimbriae in 1/20TSB at this temperature. In TSB, no relevant differences in *adrA* expression between the different culture conditions or the different strain groups were detected (Fig. 2.4A).

The expression analysis of *csgA*, *csgD* and *adrA*, suggest that both curli fimbriae and cellulose act as matrix components during biofilm formation in 1/20TSB at 25 °C. Interestingly, the group A strains did show dense biofilm formation in TSB at 25 °C and 37 °C, as well (Fig. 2.1). Therefore, it is possible that other features contribute to biofilm formation in a nutrient-rich medium.

Microscopic evaluation of *S.* Typhimurium biofilms

Biofilms grown on coupons of the selected strains were microscopically examined to obtain more information on the morphology of *S.* Typhimurium biofilms. In addition the biofilms were stained with calcofluor to analyse the cellulose content of the biofilms, and to investigate if increased *adrA* expression is associated with induced cellulose production. Representative pictures of group A, B, and C strains per culture conditions are shown in Figure 2.5. In TSB, the group A strains showed at 25 °C and 37 °C a dense layer of bacteria, in which small spots of calcofluor were visible (Fig. 2.5). These results might suggest that cellulose was expressed only by some small clusters of cells. For the group B and C strains, small cell clusters and single attached cells were observed (Fig. 2.5). In biofilms from the group B strains also a small subpopulation of cells exhibited some cellulose production in TSB. On the other hand, in 1/20TSB at 25 °C, large cell clusters with high level calcofluor staining were observed for the group A and B strains (Fig 2.5), which implies large amounts of cellulose to be present in the extracellular matrix of these biofilms. The same strains showed smaller cell clusters stained with calcofluor at 37 °C, but the fluorescence intensity was substantial lower than at 25 °C. Less biofilm cells were found for the group C strains, but also these biofilms displayed some cellulose production around the microcolonies and single attached cells in 1/20TSB (Fig 2.5).

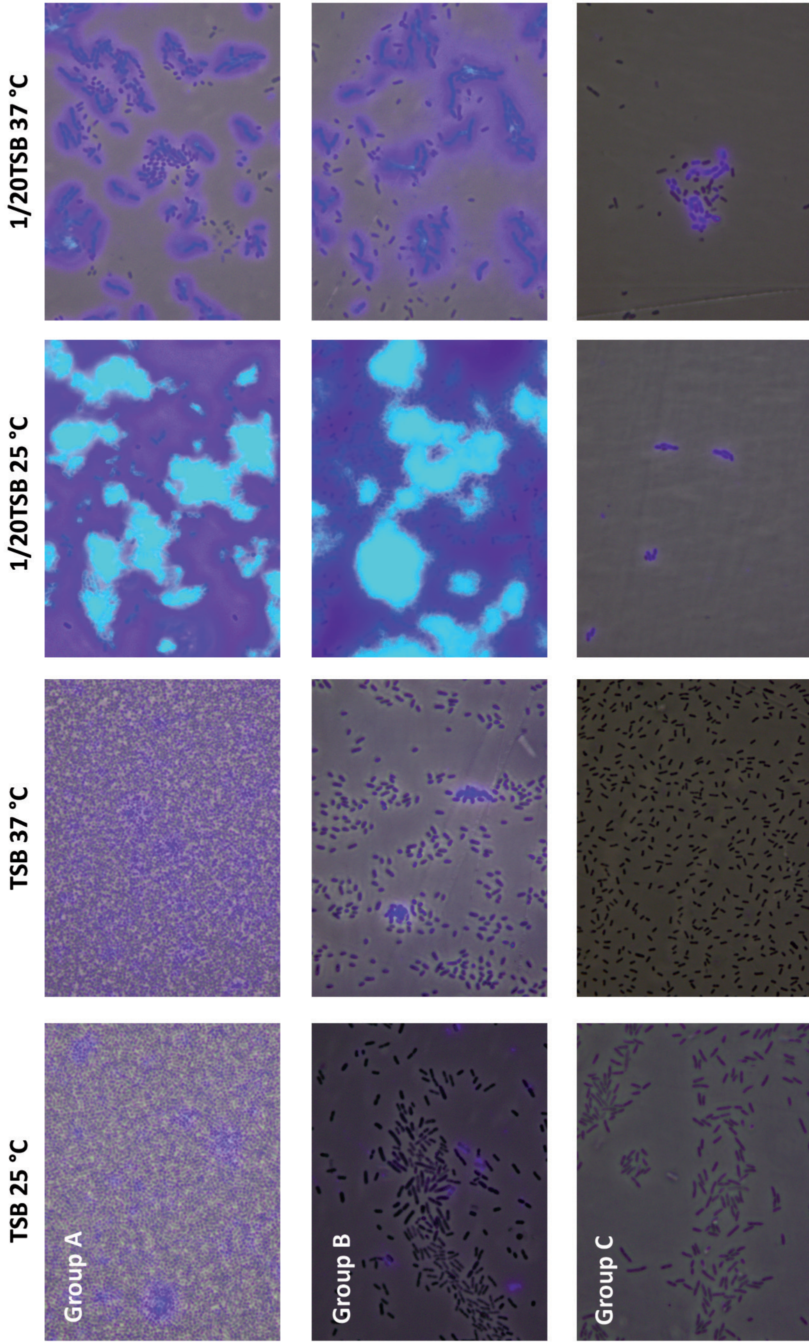


Figure 2.5: Representative images of calcofluor stained biofilms of a group A strain (strain I1), a group B strain (strain I4) and a group C strain (strain I23) grown in TSB or 1/20TSB at 25 °C or 37 °C. All images were taken at a magnification of 1000x and the scale bar represents 20 µm.

The Q-PCR data and the calcofluor stained biofilms showed that curli fimbriae and cellulose are specifically induced during biofilm formation in 1/20TSB at 25 °C, which is also in agreement with the results of CV staining and cell counts, that suggested increased extracellular matrix production at this culture condition.

Scanning electron microscopy

With SEM more detailed images of biofilms of group A strains grown in TSB or 1/20TSB at 25 °C were made (Fig 2.6). The SEM images confirmed the conclusion that biofilms grown in 1/20TSB produce more extracellular matrix than biofilms grown in TSB. In TSB, a dense layer of cells with almost no extracellular matrix was observed. In 1/20TSB, clusters of cells were found of which a subpopulation of the cells was completely encapsulated. Although the cells are encapsulated, by vortexing with beads we were still able to obtain single cells. Presumably these cells are encapsulated with cellulose, since high levels of calcofluor bind to biofilms grown in 1/20TSB at 25 °C.

DISCUSSION

In this study, by combining the CV assay with LIVE/DEAD staining and enumeration of the biofilm cells, we demonstrated that the number of attached biofilm cells and the extracellular matrix were influenced by environmental conditions, like temperature and nutrient availability. Further analysis of the expression and production of curli fimbriae and cellulose showed that these components particularly contribute to the total amount of biofilm formation in low nutrient conditions (1/20TSB) at ambient temperatures and that these components might not be as important during biofilm formation at 37 °C or in nutrient-rich conditions such as TSB (Fig 2.7).

By screening the collection of 51 *S.* Typhimurium strains with the CV assay, we found that the industrial isolates generally exhibited dense biofilm formation in 1/20TSB at 25 °C only. However, the clinical, outbreak-associated and retail product isolates also showed dense biofilm formation in TSB at 25 °C and 37 °C. These results are in contradiction with previous studies using a range of clinical, animal, outbreak-associated and retail product isolates, that showed no relation between biofilm forming behavior and strain origin (Stepanovi et al. 2004; Solomon et al. 2005). However, in these studies the biofilm forming capacity was tested at relatively high temperatures only, i.e., at 30 and 35 °C, and this may have affected the outcome of the latter studies, since our results indicate that temperature greatly influences biofilm formation.

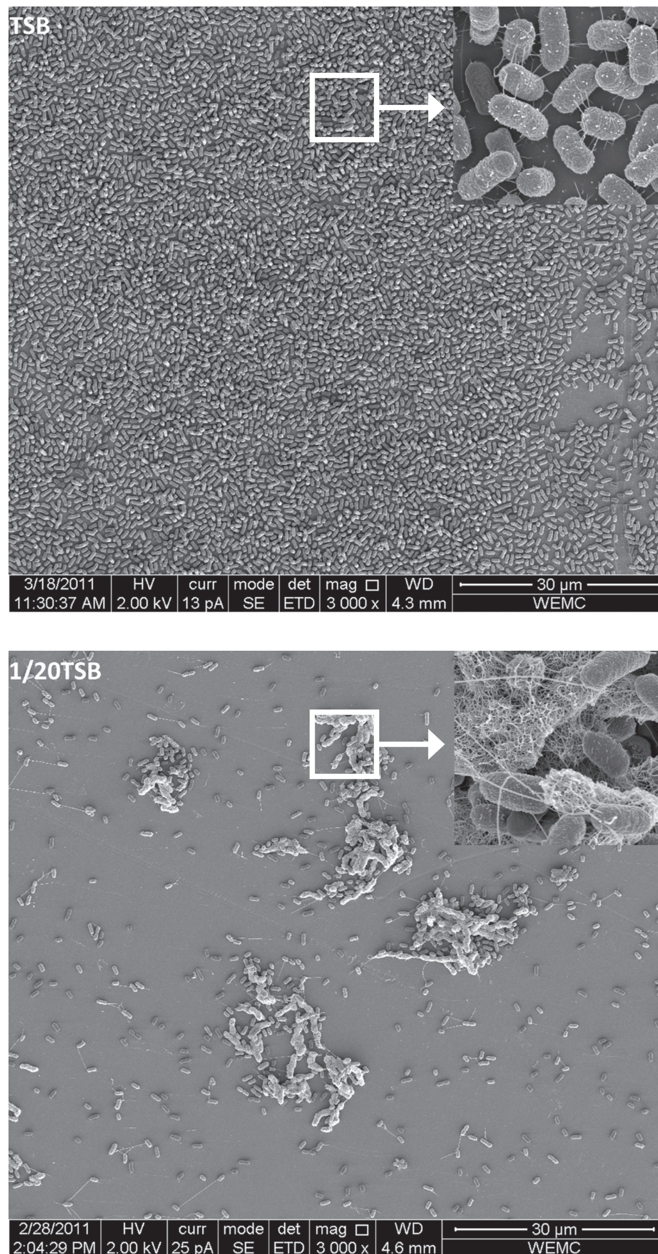


Figure 2.6: Representative images made by SEM of biofilms from a group A strain (strain I2) cultured in TSB or 1/20TSB at 25 °C.

Furthermore, we observed highest biofilm formation by the group A strains in the nutrient-rich TSB medium, while the group B strains only showed dense biofilm formation in 1/20TSB. So biofilm forming capacity was also influenced by the culture medium. Efficient biofilm formation of the group B strains in 1/20TSB might be associated with limitation of a specific component or combinations thereof, but alternatively, biofilm formation might be inhibited by one or more components that are present in higher levels in TSB. To test the latter hypothesis, a range of amino acids and carbon sources were separately added to 1/20TSB to concentrations comparable to that in TSB, and the effect on biofilm formation was analysed. However, none of the tested components showed inhibition of biofilm formation of the group B strains (data not shown). Alternatively, efficient biofilm formation of group A strains in nutrient-rich conditions could be linked to specific features that are either absent or not induced in the group B and C strains in such conditions. The putative factor(s) involved remain to be elucidated.

Analysis of biofilm forming capacity with the widely used CV assay does not supply information on the relative contribution of biofilm cells and the extracellular matrix. Our results of the combined CV and enumeration assays, revealed that the lowest boundary for the CV assay is approximately 6.8 log CFU/well, which indicates that this assay is not very sensitive and may fail to detect low levels of biofilm formation. Furthermore, we showed that the contribution of the extracellular matrix is variable between different environmental conditions. Notably, in none of the conditions significant amounts of dead cells were found to contribute to the biofilm, indicating that in particular at 25 °C in 1/20TSB the extracellular matrix is an important component of the biofilms formed. Subsequent, Q-PCR analysis indeed showed that the expression of genes with roles in the production of curli fimbriae (*csgD* and *csgA*) and cellulose (*adrA*) was particularly induced in the biofilm cells of the group A and B strains in 1/20TSB at 25 °C. This suggests that curli fimbriae and cellulose are specifically involved in biofilm formation in a low nutrient medium at ambient temperatures, and that other factors might be involved in biofilm formation at body temperature and/or in a nutrient-rich environment (Fig. 2.7). Furthermore, a correlation in expression patterns between *csgD* and *adrA* was observed, i.e., increased *csgD* expression is associated with up-regulation of *adrA*. Recently, Zakikhany et al. (2010) presented evidence that CsgD interacts with DNA directly and activates transcription of *adrA*. In addition, in our studies, no significant differences were detected in the expression of *bcsA* between the different culture conditions or strains, while microscopic images of the biofilms showed intense calcofluor stained biofilm cells for the group A and B strains in 1/20TSB at 25 °C, only. These results support the previously postulated hypothesis that AdrA positively affects cellulose production at a posttranscriptional level (Zogaj et al. 2001; Römling 2005; Zakikhany et al. 2010). However, to confirm this interaction, more research is needed.

Further microscopic analyses of the calcofluor stained biofilms and SEM analysis revealed heterogeneity in cellulose production. For example, the SEM images show that a subpopulation of biofilm cells in 1/20TSB is encapsulated by extracellular matrix, conceivably cellulose. Heterogeneity in cellulose production in biofilms has not been described before for *Salmonella*, but our observation is in line with observations made by Grantcharova et al. (2010). They reported single-cell expression analysis which revealed that c*sgD* is expressed in a bistable manner during biofilm development. The subpopulation of cells expressing large amounts of c*sgD* is engaged in cellulose production indicating that bistability at the level of c*sgD* expression leads to a corresponding pattern of task distribution in *S.* Typhimurium biofilms (Grantcharova et al. 2010). Notably, in earlier studies on *Salmonella* biofilm formation the analysis of cellulose production included detachment of cellulose from the cell wall

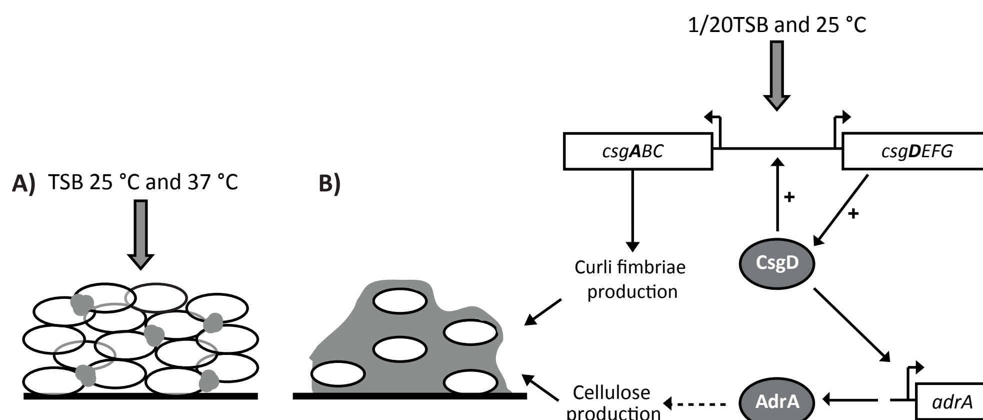


Figure 2.7: A) A schematic illustration of a biofilm produced by the group A strains in TSB at 25 °C or 37 °C, adapted from Jonas *et al.* (2007) and Zakikhany et al. (2010). Mechanisms involved in the formation of biofilms under these environmental conditions are not known yet. B) A schematic illustration of a biofilm produced by the group A or B strains in 1/20TSB at 25 °C, adapted from Jonas et al. (2007) and Zakikhany et al. (2010). This latter culture condition conceivably activates CsgD, which positively regulates the expression of the *csg* operons (curli fimbriae) and *adrA* (Zakikhany et. al. 2010). The putative activation of cellulose production involving AdrA is indicated by a dotted arrow. See text for details.

followed by hydrolysis and quantification of the sugar monomers by GS-MS (Bokranz et al. 2005). However, in this way it is not possible to analyse the distribution of cellulose in biofilms. Moreover, in the current study we provide evidence that environmental conditions affect both the extracellular matrix and biofilm morphology. In the rich TSB medium, biofilms of the group A strains showed a dense layer of bacterial cells, while in 1/20TSB group A and B strains showed cell clusters imbedded in cellulose.

The results indicate that both group A strains, which are mainly clinical, out-break associated and retail product isolates, and group B strains, which are mainly industrial isolates, are able to express curli fimbriae and cellulose. However, the expression of these components in both group A and B strains was particularly induced in 1/20TSB at 25 °C, which appeared to be related with the expression of *csgD*. Previously, it was shown that expression of *csgD* is regulated by a variety of environmental signals (Gerstel and Römling 2003). Our results suggest that CsgD is highly activated under low nutrient conditions at ambient temperatures, which results in the production of curli fimbriae and cellulose. Apparently, under nutrient-rich conditions biofilm formation of the group A strains is dependent on other features that are either absent or not induced in the group B and C strains under this environmental condition (Fig 2.7). This suggests that the majority of our clinical, outbreak-associated and retail product isolates (group A strains) have adapted to and/or been selected for their capacity produce dense biofilms under nutrient-rich conditions at ambient and body temperature. Whether this has provided a selective advantage for the clinical isolates in colonization of the host remains to be elucidated.

It is conceivable that biofilms containing large amounts of extracellular matrix might be more difficult to eradicate during disinfection treatments due to the protective effect of the extracellular matrix (Solano et al. 2002; White et al. 2008; Vestby et al. 2009a). On the other hand, biofilm cells that are not imbedded in a dense extracellular matrix might disperse more easily from the biofilm, and subsequently contaminate food products more easily. Apparently, more information about *in situ* biofilm formation and their characteristics is required before these questions can be answered.

In conclusion, we demonstrated that biofilm forming behavior is affected by environmental conditions like temperature and medium, but also that it is associated by strain origin. In addition, it was shown that curli fimbriae and cellulose contribute specifically to biofilm production under low nutrient conditions at ambient temperatures and that other components are conceivably more important during biofilm formation at 37 °C and/or in nutrient-rich conditions. However, further research is needed to identify these components. In addition, our findings on the extracellular matrix production provide good opportunities to study the effects of the absence or presence of extracellular matrix on the survival of *S.* Typhimurium biofilms during disinfection treatments and may therefore contribute to the development of more efficient disinfection strategies.

Acknowledgement
The authors would like to thank Tiny Franssen-Verehijen of the Wageningen Electron Microsopy Centre (WEMC) for technical support with the SEM analysis.

REFERENCES

Adams JL, McLean RJC. 1999. Impact of *rpoS* Deletion on *Escherichia coli* Biofilms. Appl Environ Microbiol 65(9):4285-4287.

Barnhart MM, Chapman MR. 2006. Curli Biogenesis and Function. Ann Rev Microbiol 60(1):131-147.

Berk PA, Jonge R, Zwietering MH, Abee T, Kieboom J. 2005. Acid resistance variability among isolates of *Salmonella enterica* serovar Typhimurium DT104. J Appl Microbiol 99(4):859-866.

Bokranz W, Wang X, Tschape H, Römling U. 2005. Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. J Med Microbiol 54(12):1171-1182.

Costerton JW. 1999. Introduction to biofilm. International J Antimicrob Agents 11(3-4):217-239.

Djordjevic D, Wiedmann M, McLandsborough LA. 2002. Microtiter Plate Assay for Assessment of *Listeria monocytogenes* Biofilm Formation. Appl Environ Microbiol 68(6):2950-2958.

Doorduyn Y, van den Brandhof WE, van Duynhoven YTHP, Wannet WJB, van Pelt W. 2006. Risk factors for *Salmonella* Enteritidis and Typhimurium (DT104 and non-DT104) infections in The Netherlands: predominant roles for raw eggs in Enteritidis and sandboxes in Typhimurium infections. Epidemiol Infect 134(3):617-626.

European Food Safety Authority (EFSA). 2009. The Community Summary Report on Trends and Sources of Zoonoses and Zoonotic Agents in the European Union in 2007. EFSA J 223.

Gerstel U, Römling U. 2001. Oxygen tension and nutrient starvation are major signals that regulate *agfD* promoter activity and expression of the multicellular morphotype in *Salmonella typhimurium*. Environ Microbiol 3(10):638-648.

Gerstel U, Römling U. 2003. The *csgD* promoter, a control unit for biofilm formation in *Salmonella typhimurium*. Res Microbiol 154(10):659-667.

Gilbert P, Maira-Litran T, McBain AJ, Rickard AH, Whyte FW. 2002. The physiology and collective recalcitrance of microbial biofilm communities. Adv in Microb Physiol 46:203-256.

Grantcharova N, Peters V, Monteiro C, Zakikhany K, Römling U. 2010. Bistable Expression of CsgD in Biofilm Development of *Salmonella enterica* Serovar Typhimurium. J Bacteriol 192(2):456-466.

Jonas K, Tomenius H, Kader A, Normark S, Römling U, Belova LM, Melefors O. 2007. Roles of curli, cellulose and BapA in *Salmonella* biofilm morphology studied by atomic force microscopy. BMC Microbiol 7(1):70.

Kroupitski Y, Pinto R, Brandl MT, Belausov E, Sela S. 2009. Interactions of *Salmonella enterica* with lettuce leaves. J Appl Microbiol 106(6):1876-1885.

Kumar CG, Anand SK. 1998. Significance of microbial biofilms in food industry: a review. Int J Food Microbiol 42(1-2):9-27.

Ledeboer NA, Jones BD. 2005. Exopolysaccharide Sugars Contribute to Biofilm Formation by *Salmonella enterica* Serovar Typhimurium on HEp-2 Cells and Chicken Intestinal Epithe-

lium. J Bacteriol 187(9):3214-3226.

Malcova M, Hradecka H, Karpiskova R, Rychlik I. 2008. Biofilm formation in field strains of *Salmonella enterica* serovar Typhimurium: Identification of a new colony morphology type and the role of SGI1 in biofilm formation. Vet Microbiol 129(3-4):360-366.

McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F. ***et al.*** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nat 413(6858):852-856.

Prouty AM, Schwesinger WH, Gunn JS. 2002. Biofilm Formation and Interaction with the Surfaces of Gallstones by *Salmonella* spp. Infect Immun 70(5):2640-2649.

Robbe-Saule V, Jaumouille V, Prevost M-C, Guadagnini S, Talhouarne C, Mathout H, Kolb A, Norel F. 2006. Crl Activates Transcription Initiation of RpoS-Regulated Genes Involved in the Multicellular Behavior of Salmonella enterica Serovar Typhimurium. J Bacteriol 188(11):3983-3994.

Römling U. 2005. Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae. Cellular and molecular life sciences 62(11).

Römling U, Bokranz W, Rabsch W, Zogaj X, Nimtz M, Tschäpe H. 2003. Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. Int J Med Microbiol 293(4):273-285.

Römling U, Sierralta WD, Eriksson K, Normark S. 1998. Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter. Mol Microbiol 28(2):249-264.

Scher K, Römling U, Yaron S. 2005. Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. Appl Environ Microbiol 71(3):1163-1168.

Sinde E, Carballo J. 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. Food Microbiol 17(4):439-447.

Solano C, García B, Valle J, Berasain C, Ghigo J-M, Gamazo C, Lasa I. 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. Mol Microbiol 43(3):793-808.

Solomon EB, Niemira BA, Sapers GM, Annous BA. 2005. Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. J Food Protect 68:906-912.

Stepanovi S, irkovi I, Ranin L, Svabi-Vlahovi M. 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol 38(5):428-432.

Swanenburg M, Urlings HAP, Snijders JMA, Keuzenkamp DA, van Knapen F. 2001. *Salmonella* in slaughter pigs: prevalence, serotypes and critical control points during slaughter in two slaughterhouses. Int J Food Microbiol 70(3):243-254.

Vestby LK, Moretro T, Ballance S, Langsrud S, Nesse LL. 2009a. Survival potential of wild type cellulose deficient *Salmonella* from the feed industry. BMC Vet Res 5(1):43.

Vestby LK, Moretro T, Langsrud S, Heir E, Nesse LL. 2009b. Biofilm forming abilities of *Salmonella*

are correlated with persistence in fish meal- and feed factories. BMC Vet Res 5(1):20.

White AP, Gibson DL, Grassl GA, Kay WW, Finlay BB, Vallance BA, Surette MG. 2008. Aggregation via the red, dry, and rough morphotype is not a virulence adaptation in *Salmonella enterica* Serovar Typhimurium. Infect Immun 76(3):1048-1058.

White AP, Gibson DL, Kim W, Kay WW, Surette MG. 2006. Thin Aggregative Fimbriae and Cellulose Enhance Long-Term Survival and Persistence of *Salmonella*. J Bacteriol 188(9):3219-3227.

Wijman JGE, de Leeuw PPLA, Moezelaar R, Zwietering MH, Abee T. 2007. Air-liquid interface bofilms of *Bacillus cereus*: formation, sporulation, and dispersion. Appl Environ Microbiol 73(5):1481-1488.

Zakikhany K, Harrington CR, Nimtz M, Hinton JCD, Römling U. 2010. Unphosphorylated CsgD controls biofilm formation in *Salmonella enterica* serovar Typhimurium. Mol Microbiol 77(3):771-786.

Zogaj X, Bokranz W, Nimtz M, Römling U. 2003. Production of cellulose and curli fimbriae by members of the family Enterobacteriaceae isolated from the human gastrointestinal ract. Infect Immun 71(7):4151-4158.

Zogaj X, Nimtz M, Rohde M, Bokranz W, Römling U. 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol Microbiol 39(6):1452-1463.

CHAPTER 3

Type 1 fimbriae are involved in the attachment of *Salmonella* Typhimurium cells to abiotic surfaces

Greetje A.A. Castelijn, Stijn van der Veen, Bastiaan P. Krom,
Marcel H. Zwietering, Roy Moezelaar and Tjakko Abee

Submitted for publication

ABSTRACT

This study analysed the role of fimbriae during the initial attachment and subsequent biofilm formation of *Salmonella* Typhimurium strains to abiotic surfaces. An expression analysis of eleven putative functional fimbriae clusters revealed that only type 1 fimbriae were significantly higher expressed in strains showing dense biofilm formation compared to strains showing little to no biofilm formation. The presence of type 1 fimbriae was confirmed by a hemagglutination assay, transmission and scanning electron microscopy and was observed for a subpopulation of cells only. This was in agreement with zeta potential measurements that revealed a heterogeneous apparent zeta potential distribution for cell cultures of dense biofilm formers. The higher adhesion capacity of dense biofilm forming strains appeared to be mannose-sensitive since addition of mannose resulted in less attachment. These results indicate that type 1 fimbriae contribute to the initial attachment of *S.* Typhimurium to abiotic surfaces ultimately leading to dense biofilm formation.

INTRODUCTION

Salmonella is one of the leading causes of foodborne illness worldwide. It is the causative agent of salmonellosis which is characterized by abdominal pain, diarrhea, the onset of fever, nausea and sometimes vomiting. One of the predominant serovars causing human salmonellosis in the EU is *Salmonella enterica* serovar Typhimurium (EFSA 2012). *S.* Typhimurium is able to attach and form biofilms on a variety of surfaces. These surfaces can be biotic like epithelial cells in the intestinal tract, or abiotic like processing equipment in the food industry (Ledeboer *et al.* 2006; Joseph *et al.* 2001; Sinde and Carballo 2000). In both medicine and the food industry, biofilms cause problems such as chronic infections and food product contamination respectively, especially since biofilms are hard to eradicate due to increased resistance against antimicrobial agents (Wong *et al.* 2010; Scher *et al.* 2005).

Biofilm formation is a complex process that starts with the attachment of cells to a surface. This initial attachment and subsequent biofilm formation is influenced by environmental conditions, the physicochemical cell surface properties, surface characteristics and the presence of cell surface structures (van Merode *et al.* 2006; Cowan *et al.* 1992a; Cowan *et al.* 1992b). *S.* Typhimurium can express a variety of cell surface structures that have been associated with biofilm formation on different types of surfaces (Steenackers *et al.* 2012). In a previous study we provided evidence that the surface structures curli fimbriae, encoded by the *csg* fimbrial gene cluster, and cellulose are involved in the biofilm formation on abiotic surfaces at ambient temperatures and low-nutrient conditions (Castelijn *et al.* 2012). The factors involved in dense biofilm formation at 37 °C and nutrient-rich conditions remained to be elucidated in that study. Therefore this current study analysed if one of the other putative functional fimbrial gene clusters of *S.* Typhimurium play a role in the initial attachment and subsequent biofilm formation on abiotic surfaces in nutrient-rich conditions at 37 °C. This temperature is relevant for medical settings, e.g. biofilm formation on implants, but also and in meat processing settings, since temperatures up to 40 °C have been reported, e.g. in slaughter plants (Skandamis *et al.* 2009; Botteldoorn *et al.* 2003).

Type 1 fimbriae are encoded by the *fim* gene cluster (Tinker and Clegg 2000), one of the putative functional fimbrial gene clusters found in the genome of *S.* Typhimurium (McClelland *et al.* 2001). Type 1 fimbriae play an important role in the initial attachment and subsequent biofilm formation on human and animal epithelial cells (Ledeboer *et al.* 2006; Boddicker *et al.* 2002). We hypothesized that these fimbriae could also play a role in initial attachment and subsequent biofilm formation of *S.* Typhimurium strains on abiotic surfaces. Dependent on growth history and media used, type 1 fimbriae can be found on the cell surface of most members of the Enterobacteriaceae (Tinker *et al.* 2001). They are assembled by the chaper-

one-usher system and are composed of ±1000 copies of the major structural subunit FimA, with FimH located on the tip. Up to 200-500 of these structures can be found on the outer membrane of fimbriated bacteria (Fronzes *et al.* 2008).

This study shows that type 1 fimbriae are important for attachment of *S.* Typhimurium to abiotic surfaces in a rich medium (TSB) at 37 °C. Under these conditions, increased *fimA* expression by planktonic cells was associated with fimbriae on the cell surface of a subpopulation of cells only. Cell cultures with a subpopulation of cells expressing type 1 fimbriae showed increased initial attachment that was found to be mannose sensitive. Whether type 1 fimbriae are involved directly in interactions of bacterial cells with the surface, or indirectly by affecting the physicochemical cell surface properties, or a combination of these two, remains to be elucidated.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Seven *Salmonella enterica* ssp. *enterica* serovar Typhimurium isolates were used in this study; C4, C11, C16, I1, I4, I23 and L2 (Table 1) (Castelijn *et al.* 2012). Stocks were stored at -80 °C in Brain Hearth Infusion broth (BHI, Becton Dickson) supplemented with 15% glycerol (Sigma). Prior to the experiments, strains were cultured from the frozen stocks on Tryptone Soy Agar (TSA, Oxoid) at 37 °C. Subsequently, single colonies were used to inoculate 10 ml Luria Bertani broth (LB, Merck) and cultures were incubated overnight at 37 °C with agitation (200 rpm) after which they were used in the different experiments.

Biofilm formation and quantification of initial attachment

Biofilm formation was quantified in flat bottom 96 well polystyrene plates (Greiner Bio-one) using the crystal violet (CV) assay and by enumeration of the biofilm cells by plate counting as described previously (Castelijn *et al.* 2012). In short, the wells were filled with 200 µl of Tryptone Soy Broth (TSB, Oxoid) medium or TSB + 3% mannose and inoculated with 1.5% (vol/vol) overnight grown cell cultures. Then the biofilms were statically grown for 1h or 24 h at 37 °C.

For the CV assay, the biofilms were washed twice with water and stained with 0.1% CV (Merck) for 30 min. Subsequently the biofilms were washed three times and the attached CV was dissolved with 96% ethanol (Merck). The absorbance was measured at 595 nm (SpectraMax, Molecular Devices).

For enumeration of biofilm cells after 1 h and 24 h, the biofilms were washed three times with peptone physiological salt (pps). Next the biofilm cells were detached by swabbing and the cell suspension and swabs were transferred to a tube containing 1.5 ml of pps and 0.3 g glass beads (≤106 µm, Sigma). In order to detach the biofilms and obtain single cells the

tube was vortexed for 1 min. Then, appropriate dilutions were made in pps and the cells were enumerated by plating on TSA. After overnight incubation at 37 °C, the colonies were counted. The CV assay and the enumeration of biofilm cells were performed in three biologically independent experiments.

Microscopic evaluation of SYTO-9 stained biofilms

For the microscopic analysis, biofilms were grown on polystyrene coupons (18 mm x 22 mm) placed vertically in a 12 well plate. The wells were filled with 3 ml of TSB and inoculated with 1.5% (vol/vol) overnight grown cultures. Then the plates with coupons were incubated for 24 h at 37 °C. After incubation the coupons were washed three times with peptone buffered saline (pbs) and stained with SYTO 9 (Invitrogen) for 15 min, according to manufacturer's protocol. Subsequently, the coupons were washed again twice with pbs and the biofilms were visualized with an Olympus BX41 microscope and a U-MWU-2 fluorescence filter (Olympus). This experiment was performed in duplicate and in total about 25 images was examined for each sample.

RNA isolation and cDNA synthesis

Total RNA was isolated from planktonic cells grown static for 24 h in TSB at 37 °C. The RNA in the cells was stabilized with RNAprotect Bacteria Reagent (Qiagen), and RNA was isolated by use of the RNeasy Kit (Qiagen) according to manufacturer's protocol. To prevent DNA contamination, an on-column DNase treatment (Qiagen) was performed.

The SuperScript III reverse transcriptase kit (Invitrogen) was used according to manufacturer's protocol to convert 300 ng of total RNA into cDNA. RNA samples were controlled for genomic DNA contamination by omitting the Superscript III reverse transcriptase.

Quantitative real-time PCR

Primer 3 (available from: http://frodo.wi.mit.edu/primer3/) was used to design primers for use in quantitative real-time PCR (Q-PCR). Primers used in this study are listed in Table S3.1 provided as supplementary. For the Q-PCR reaction on *fimA* the forward primer 5' TGTCGATCAGACCGTGACAT 3' (FWfimA) and reverse primer 5' CGCAGTCTTCCAGTTTGATG 3' (RVfimA) (250 nM each) were added to 5 µL cDNA (300 ng/sample) and 12.5 µL 2x SYBR® Green PCR Master Mix (Applied Biosystems). Then sterile water was added to a final volume of 25 µL. The reactions (10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 59 °C) were carried out on an ABI Prism 7000 Sequence Detection System. To verify single product formation a melting curve was included. The housekeeping genes 16S *rRNA*, *rpoD* and *gmk* were used for normalization and a standard curve was derived for each primer set to determine the efficiency. Q-PCR was performed on three biological independent experiments using two technical replicates each.

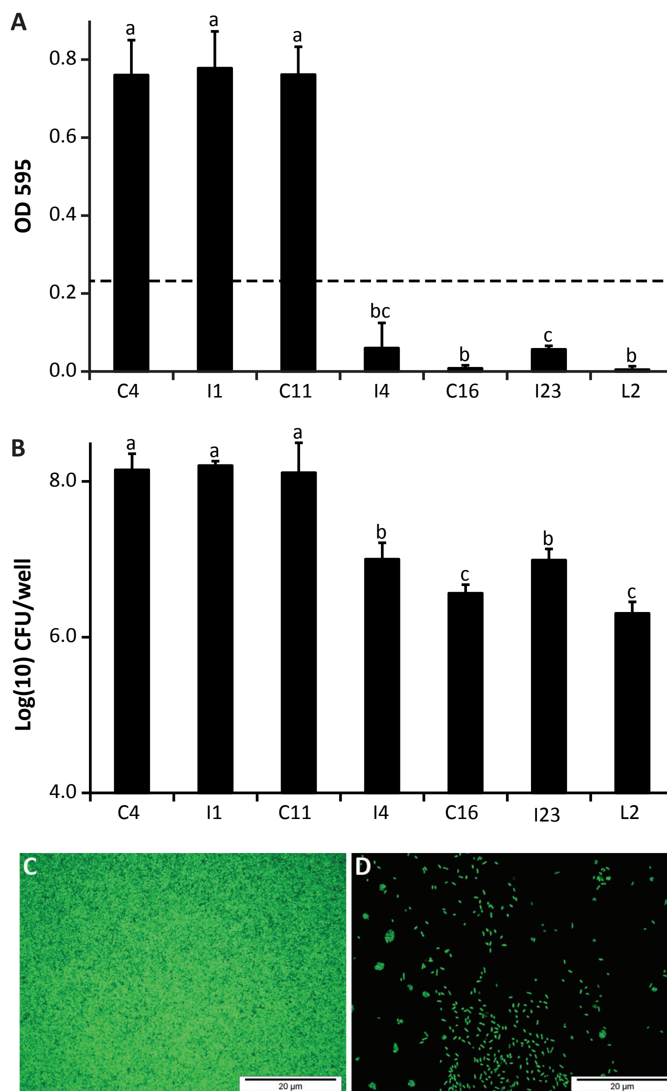


Figure 3.1: Biofilm forming capacity in TSB at 37 °C after 24 h based on optical density following crystal violet staining (A) and by enumeration of the biofilm cells (B). The bars denote the SDs of three biological repeats and significant differences are indicated by different letters ($p < 0.05$, *t*-test). The level of dense biofilm formation in the CV assay (three times the background level) is indicated by a dotted line. C) Representative image of a SYTO-9 stained 24 h old biofilm of a dense biofilm former grown in TSB 37 °C (strain I1). D) Representative image of a SYTO-9 stained 24 h old biofilm of a poor biofilm former grown in TSB 37 °C (strain I4). All images were taken at a magnification of 400x and the scale bar represents 20 µm.

Hemagglutination assay

For the hemagglutination assay the strains were grown overnight in 20 ml TSB in a 100 ml Erlenmeyer flask, under static conditions at 37 °C. Of these cultures, aliquots of 50 µl were mixed with 50 µl of 5% (vol/vol) guinea pig erythrocytes suspended in pbs, on a glass slide. After 5 minutes, the samples were scored positive or negative for agglutination. Mannose sensitivity was determined by mixing the bacterial cell cultures with 5% (vol/vol) guinea pig erythrocytes suspended in pbs containing 3% D-(+)-mannose (wt/vol) (Acros). This assay was performed in three biologically independent experiments.

Transmission electron microscopy

For transmission electron microscopy (TEM) the strains were statically grown in TSB at 37 °C for 24 h. Of these overnight cell cultures 1 ml was centrifuged (3 min, 5000*g*) and the cell pellet was resuspended in sterile demineralized water. Subsequently, the cells were negatively stained with 1% phosphotungstic acid for 30 s and put on a copper grid coated with a formvar film (Wageningen Electron Microscopy Centre, WEMC). The grids were air dried and evaluated with a JEOL 1011 transmission electron microscope.

Scanning electron microscopy

The strains were examined with scanning electron microscopy (SEM) to analyse the cell surface morphology in more detail. For this, cell cultures grown statistically overnight at 37 °C were deposited on a 0.22 µm pore size filter (Millipore) using negative pressure. Subsequently the samples were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 1 h at room temperature and further processed as described by Castelijn *et al.* 2012. The cells were analysed at 2 kV at room temperature in a Field Emission Scanning Electron Microscope (Magellan 400, FEI, Eindhoven, the Netherlands).

Zeta potential distribution

The zeta potential distribution was determined by particulate micro-electrophoresis. The electrophoretic mobility of individual bacteria is determined and converted to an apparent zeta potential. The strains were grown overnight in 20 ml TSB in a 100 ml Erlenmeyer flask, under static conditions at 37 °C. The cells were harvested by centrifugation (6500x *g*, 5 min) and washed with 10 mM potassium phosphate (pH 7.0). The apparent zeta potential was determined in 10 mM potassium phosphate (pH 7.0), with a Lazer Zee Meter 501 (PenKem) as described previously (van Merode *et al.* 2006). The zeta potential measurements were performed in three biological independent experiments. For each experiment, a minimum of 100 cells was analyzed.

RESULTS

Biofilm formation

Based on our previous study (Castelijn *et al.* 2012) in which 51 *S.* Typhimurium strains were screened for biofilm formation under various conditions, seven strains were selected of which three (C4, I1 and C11) showed dense biofilm formation and four (I4, C16, I23 and L2) showed poor biofilm formation in TSB at 37 °C (Fig. 3.1A). The differences in biofilm formation were not related to differences in growth capabilities, since the strains show similar growth rates (Table 3.1) and final OD600 nm values reached in TSB at 37 °C (data not shown). The different biofilms were further investigated by enumeration of biofilm cells and microscopic analyses (Fig. 3.1B-D). The biofilms of the dense biofilm formers consisted of approximately 8.1 log CFU/well, which was significantly higher than that of the poor biofilm formers that contained approximately 6.3 to 7.0 log CFU/well (p<0.05, t-test) (Fig. 3.1B). These results were in agreement with the microscopy analyses of the biofilms, which showed that the biofilms of the dense biofilm formers consisted of a dense layer of cells (Fig. 3.1C), while for the poor biofilm formers only small cell clusters and single attached cells were detected (Fig. 3.1D).

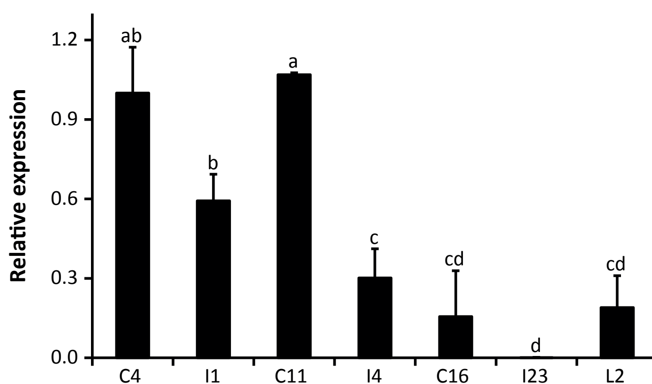


Figure 3.2: Expression of *fimA* by *S.* Typhimurium planktonic cells cultures grown in TSB at 37 °C for 24 h. The expression values are relative to the expression of *fimA* by C4. The bars denote the SDs of three biological replicates and significant differences are indicated by different letters ($p < 0.05$, *t*-test).

FimA expression and production on the cell surface

Several studies have shown that fimbriae can play a role in initial attachment and subsequent biofilm formation of bacteria on biotic surfaces (Clayton *et al.* 2008; Ledeboer *et al.* 2006; Weening *et al.* 2005). The genome of *S.* Typhimurium possess 13 fimbrial gene clusters, but two of these gene clusters miss one of the three genes (encoding for the major

structural subunit, the chaperone component or porin component) minimally needed for functional fimbriae assembly (Humpries *et al.* 2003). Therefore the expression of only the eleven putative functional fimbrial gene clusters present in the *S.* Typhimurium genome (*bcf*, *csg*, *fim*, *lpf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, and *pef*) was analysed for cells grown in TSB at 37 °C (Fig. 3.2 and Fig. S3.1). This revealed that *fimA*, encoding for the major structural subunit of type 1 fimbriae, was significantly higher expressed than the other genes in planktonic cultures. Furthermore, *fimA* was also significantly higher expressed by the dense biofilm formers compared to the poor biofilm formers. This pointed to a specific role for type 1 fimbriae in the initial attachment and subsequent dense biofilm formation on abiotic surfaces in TSB. Interestingly no significant differences in *fimA* expression were observed for biofilm cells (data not shown).

Table 3.1: Growth performance and hemagglutination assays with *S.* Typhimurium cell cultures grown at 37 °C in TSB for 24 h. Hemagglutination of guinea pig red blood cells was assayed in TSB without and with 3% mannose. (-) No agglutination was observed, (+) agglutination was observed.

Strain	TSB	TSB + 3% mannose	Growth rate μ_{max} (h^{-1})	Isolation source	Reference
C4	+	-	0.40	Human feces	Berk *et al.* 2005
I1	+	-	0.41	Food industry	Castelijn *et al.* 2012
C11	+	-	0.40	Human, blood	Berk *et al.* 2005
I4	-	-	0.40	Food industry	Castelijn *et al.* 2012
C16	-	-	0.44	Human, blood	Berk *et al.* 2005
I23	-	-	0.36	Food industry	Castelijn *et al.* 2012
L2	-	-	0.43	SGSC 2618	Fang *et al.* 1992

To verify that a higher expression of *fimA* is related with increased presence of type 1 fimbriae on the bacterial cell surface, a hemagglutination assay and TEM analysis were performed (Fig. 3.3). The hemagglutination assay is frequently used to detect type 1 fimbriae. The tip of type 1 fimbriae, i.e., FimH, binds to mannose residues on eukaryotic erythrocytes which results in agglutination (Kisiela *et al.* 2005; Duguid *et al.* 1966). This agglutination reaction can be blocked by addition of excess D-mannose (Tinker *et al.* 2001; Old 1972). Of the seven *S.* Typhimurium strains, agglutination was observed only for the three dense biofilm forming strains, while the other strains did not show agglutination (Table 3.1 and Fig. 3.3A and B). Furthermore, the agglutination reaction of the three dense biofilm formers appeared to be blocked in the presence of D-mannose confirming the putative role of type 1 fimbriae.

The presence of fimbriae on the cell surface of the dense biofilm formers was also evaluated using TEM (Fig. 3.3C and Fig. S3.2). Indeed, fimbriae-like-structures were observed on the surface of the dense biofilm formers that showed positive in the agglutination assay (Fig. 3.3C arrow 1). The observed surface structures showed similarity with those in other studies that also used TEM for type 1 fimbriae detection and identification (Chuang *et al.* 2008; Humphries *et al.* 2003; Hancox *et al.* 1997; Duguid *et al.* 1966). Notably, fimbriae were ob served on the cell surface of a subpopulation only, with the largest population composed of

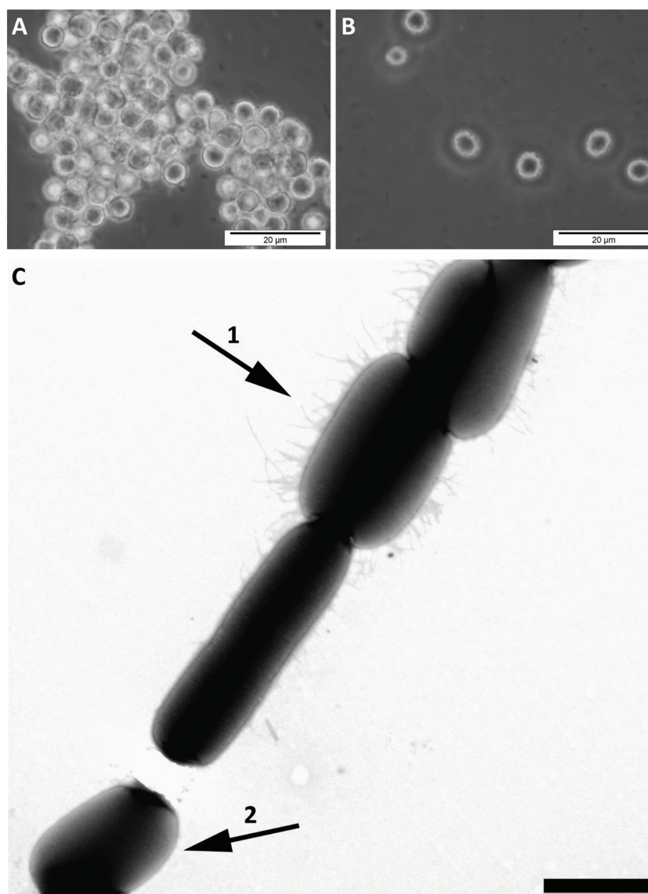


Figure 3.3: The presence of type 1 fimbriae was analysed for planktonic cells grown for 24 h in TSB at 37 °C with the hemagglutination assay (A and B) and with TEM (C). A and B) Microscopy images of a positive (strain I1) and a negative hemagglutination assay reaction (strain I4), respectively. The scale bars indicate 20 µm. C) Representative TEM image of a cell culture form a dense biofilm former (strain I1) containing fimbriated (arrow 1) and non-fimbriated cells (arrow 2). The scale bar indicates 1 µm.

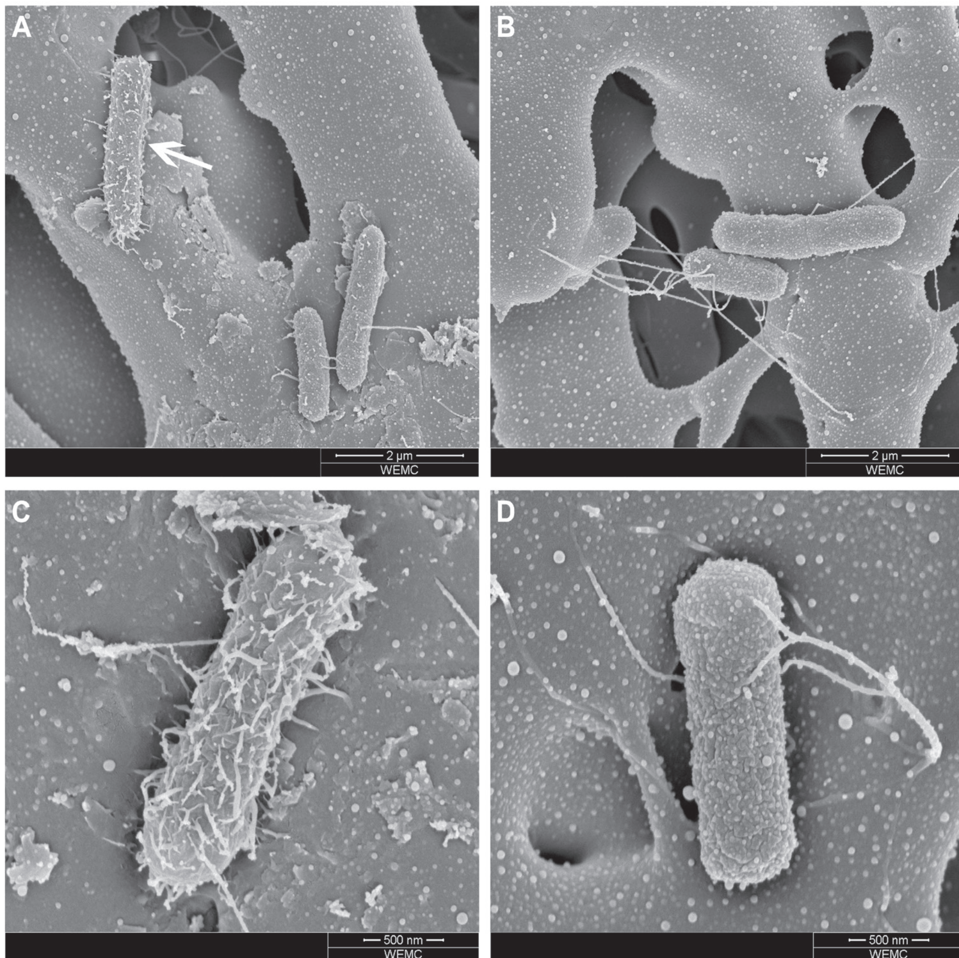


Figure 3.4: SEM images of planktonic cells grown for 24 h in TSB at 37 °C on a 0.22 µm filter which is visible as the irregular background. A) Representative image of a cell culture form a dense biofilm former containing non-fimbriated and fimbriated cells (strain I1). The cell indicated by an arrow is completely covered with fimbriae-like-structures. B) Representative image of a cell culture from a poor biofilm former containing non-fimbriated cells only (strain I4). C) A magnification of a completely fimbriated cell (strain I1). D) A magnification of a non-fimbriated cells, with some long tubular filaments attached to the surface (strain I4).

non-fimbriated cells (Fig. 3.3C arrow 2). For the poor biofilm formers only non-fimbriated cells were observed. Thus, Q-PCR data, hemagglutination results and TEM analysis point to expression of type 1 fimbriae in a subpopulation of TSB grown cells in cultures derived from strain showing dense biofilm formation.

SEM analysis of the bacterial surface

With SEM the cell morphology can be analysed in more detail. The SEM analysis confirmed the TEM analysis including heterogeneity in cell cultures from the dense biofilm formers. For these strains, subpopulations of non-fimbriated and fully fimbriated cells (approximately 25% of the cells) were observed (Fig. 3.4A and C). Cell cultures from the poor biofilm formers were found to be homogeneous and contained only non-fimbriated cells (Fig. 3.4B and D). SEM analysis also revealed the presence of morphologically distinct filaments that were less abundant, much longer and found on the surface of both dense and poor biofilm formers. These filamentsare comparable to flagella observed in other SEM studies (Sampaio *et al.* 2009; Girón *et al.* 2002; Bertin *et al.* 1994).

Zeta potential distribution

Type 1 fimbriae are assembled on the cell surface and can therefore influence the physicochemical properties of the cells (Cowan *et al.* 1992a; Cowan *et al.* 1992b). To test this hypothesis the distribution of the apparent zeta potential of planktonic cells was determined by analysing the electrophoretic mobility of individual bacteria (Fig. 3.5). This showed that cell cultures of the poor biofilm formers displayed a homogenous apparent zeta potential of about -2 mV. Conversely, cell cultures derived from dense biofilm formers showed a heterogeneous apparent zeta potential distribution. For these strains a subpopulation of about 20% of the cells displayed a more negative apparent zeta potential of -12 mV, in addition to a population with an apparent zeta potential of -2 mV (Fig. 3.5). These results are in line with the SEM analysis of the dense biofilm formers, which revealed that a subpopulation of approximately 25% of the cells was completely fimbriated. Therefore these data suggest that the presence of type 1 fimbriae on the cell surface influences the electrophoretic mobility.

Mannose-sensitive initial attachment

The experiments discussed above suggest that only a subpopulation of planktonic cultures from dense biofilm formers expresses type 1 fimbriae. To assess the impact on initial attachment, the number of attached cells after 1 h of incubation in TSB at 37 °C was determined. This revealed that the dense biofilm formers show approximately ten-fold higher initial attachment than the poor biofilm formers grown in TSB (Fig. 3.6), which resulted in significantly higher biofilm formation after 24 h (Fig. 3.1). Mannose was added to the TSB medium to verify the role of type 1 fimbriae in the initial attachment. This indeed significantly reduced the initial attachment (Fig. 3.6) and the subsequent total biofilm formation of the dense biofilm formers to levels comparable to the poor biofilm formers (Fig. S3.3). These data strongly suggest that the expression of type 1 fimbriae by a subpopulation of planktonic cells increases the initial attachment supporting dense biofilm formation.

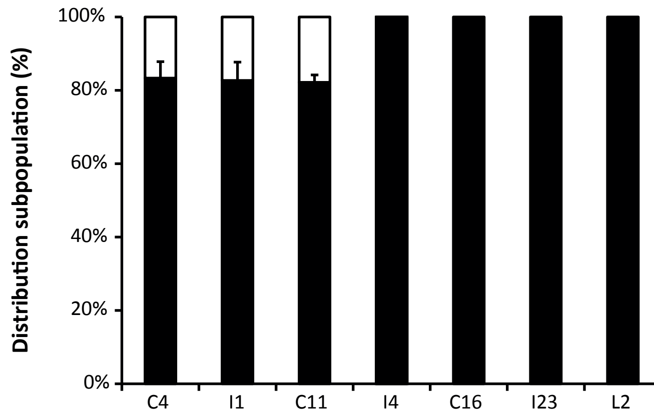


Figure 3.5: Distribution of subpopulations of *S.* Typhimurium cell cultures grown in TSB at 37 °C. Cell cultures from the dense biofilm formers contain a subpopulation with a more negative apparent zeta potential of -12 mV ± 2 (indicated in white), in addition to a population with an apparent zeta potential of -2 mV ± 2 (indicated in black). The bars denote the SDs of the apparent zeta potential fractions of three biological replicates.

DISCUSSION

S. Typhimurium biofilm formation is influenced by environmental cues that activate a variety of parameters contributing to this process (Gerstel and Römling 2003) In our previous study it was shown that curli fimbriae and cellulose are involved in biofilm formation on abiotic surfaces in nutrient-low media at ambient temperatures (Castelijn *et al.* 2012). The current study analysed which other fimbriae are involved in biofilm formation in a nutrient-rich environment (TSB) at 37 °C and revealed a role for type 1 fimbria in the initial attachment and subsequent biofilm formation of *S.* Typhimurium on abiotic surfaces.

The seven strains used in this study were selected based on a biofilm forming capacity screening in our previous study (Castelijn *et al.* 2012). Three of the selected strains, C4, I1 and C11, showed dense biofilm formation in the rich TSB medium. Differences in total biofilm formation between the dense and poor biofilm formers appeared to be mainly attributed to differences in cell numbers, since no significant contribution of extracellular matrix components was observed in previous studies (Castelijn *et al.* 2012). Attachment of *S.* Typhimurium cells to biotic surfaces such as epithelial cells is known to be affected by fimbriae (Clayton *et al.* 2008; Ledeboer *et al.* 2006; Weening *et al.* 2005). In addition, Teplitski *et al.* (2006) reported that the *S.* Typhimurium ATCC 14028 *fimI* mutant grown in LB medium was slightly deficient in biofilm formation in polystyrene microtitre plates compared to the wild type. Therefore in our study we analysed if fimbriae also play a role in the attachment

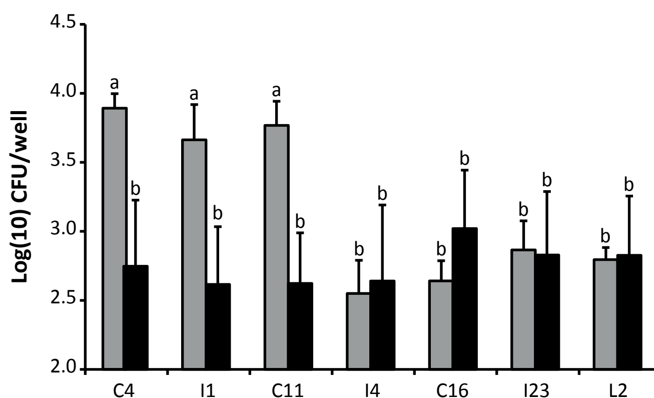


Figure 3.6: Initial attachment after 1 h of incubation at 37 °C in TSB (grey bars) and TSB + 3% mannose (black bars). The bars denote the SDs of three biological repeats and significant differences are indicated by different letters (p < 0.05, t-test).

of *S.* Typhimurium isolates to abiotic surfaces. An expression analysis of all putative functional fimbriae gene clusters in cell cultures of the selected *S.* Typhimurium strains showed an increased *fimA* expression at 37 °C for the dense biofilm formers compared to the other fimbriae and to the *fimA* expression of the poor biofilm formers (Fig. 3.2, Fig. S3.1).

Interestingly, *fimA* expression was also observed in biofilms grown for 24 h in TSB at 37 °C, but no significant differences were observed between the poor and dense biofilm formers (data not shown). This suggests that type 1 fimbriae mainly enhance the initial attachment of dense biofilm former cells. It is conceivable that FimH, located on the tip of the fimbriae, binds to mannose residues originating from the TSB medium (Hsiao *et al.* 2006) and deposited on the surface. This is supported by the observation that addition of D-mannose to the growth medium resulted in decreased attachment. In addition, no differences were detected between initial attachment of dense and poor biofilm formers in potassium buffer with or without mannose (data not shown). Taken these results together suggests that type 1 fimbriae are involved in initial attachment to mannose-coated surfaces and that their role in subsequent biofilm formation is limited. This is in line with a study by Ledeboer *et al.* (2006), that showed that type 1 fimbriae are required for the attachment to epithelial cells, where FimH binds to mannose carbohydrates present on the surface of the epithelial cells (Borowsky *et al.* 2009, Kisiela *et al.* 2005, Althouse *et al.* 2003).

The presence of type 1 fimbriae on the cell surface of dense biofilm formers was verified by a hemagglutination assay and TEM. Both these methods are frequently used in literature and proven to be successful to detect type 1 fimbriae on the bacterial cell surface (Chuang *et al.* 2008; Humphries *et al.* 2003; Boddicker *et al.* 2002; Tinker *et al.* 2001; Duguid *et al.* 1966) .

Type 1 fimbriae expression by cell cultures of the dense biofilm formers was found to be heterogeneous (Fig.3.3 and 3.4A). This phenomenon was previously described for *S.* Typhimurium planktonic cell cultures grown at 37 °C in Luria Broth (LB) medium or Nutrient Broth (NB) and is hypothesized to result from phase variable expression (on-off expression) of the *fim* operon (Humphries *et al.* 2003; Duguid *et al.* 1966). Phase variable expression is also observed for several other fimbriae such as curli fimbriae (Grantcharova *et al.* 2010) and long polar fimbriae (Kingsley *et al.* 2002; Norris *et al.* 1998). The ability to switch from fimbriated to non-fimbriated growth modes may contribute to survival capacity of bacteria in diverse environments and it helps to evade the immune system of a host (Ledeboer *et al.* 2006; Tinker and Clegg 2000; Duguid *et al.* 1966).

The heterogeneous type 1 fimbriae expression conceivably resulted in a heterogeneous apparent zeta potential distribution (Fig. 3.5). The zeta potential is commonly used to measure the bacterial surface charge and is subjected to the charges on the cell surface as well as the presence of cell surface structures such as fimbriae. An analysis of the net charge of the primary amino acid sequence of FimA, which composes the largest part of type 1 fimbriae, by Protein Calculator version 3.3 (available from: http://www.scripps.edu/~cdputnam/protcalc.html) revealed that FimA is negatively charged at pH 7-7.5 (pH TSB ±7.3). The net charge of the primary amino acid sequence of FimH, which composes only the tip of type 1 fimbriae, was neutral to slightly negative at pH 7-7.5. These results together with the zeta potential measurements showed that the dense biofilm formers contained a subpopulation of ±20% with a more negative apparent zeta potential (Fig. 3.5), which suggest that the assembly of type 1 fimbriae on the cell surface of a subpopulation of cell cultures of the dense biofilm formers is responsible for the heterogeneous zeta potential distribution, conceivably by introducing negative charges on the surface.

A relation between the expression of fimbriae structures and the apparent zeta potential has been described before for *Actinobacillus actinomycetemcomitans* (Cowan *et al.* 1992b) and *Enterococcus faecalis* (Tariq *et al.* 2012). For *E. faecalis* it was recently shown that deletion or overexpression of the fimbriae structure resulted in a cell culture with a homogenous apparent zeta potential distribution instead of a heterogeneous one as was found for the wild-type strain (Tariq *et al.* 2012).

The initial attachment of bacterial cells is subjected to the physicochemical cell surface properties (Cowan *et al.* 1992a). Previous observations by van Merode *et al.* (2008 and 2006) with *E. faecalis* showed that cell cultures with a heterogeneous zeta potential distribution exhibit more initial attachment than corresponding homogeneous cell cultures. However, the effect of D-mannose on the initial attachment indicates that initial attachment is conceivable affected by the type 1 fimbriae directly.

Combination of the results indicates that although type 1 fimbriae are expressed by a sub-population of cells only (20-25%), they positively contributes to the initial attachment and subsequent total biofilm formation of *S.* Typhimurium on abiotic surfaces when grown in a nutrient-rich medium. Whether type 1 fimbriae are involved directly in interactions of bacterial cells with the surface, or indirectly by affecting the physicochemical cell surface properties, or a combination of these two, remains to be elucidated.

Acknowledgements
The authors would like to thank Tiny Franssen-Verhijen and Adriaan C. van Aelst of the Wageningen Electron Microscopy Centre (WEMC) for technical support with the TEM and SEM analysis.

REFERENCES

Althouse C, Patterson S, Fedorka-Cray P, Isaacson RE. 2003. Type 1 fimbriae of *Salmonella enterica* serovar Typhimurium bind to enterocytes and contribute to colonization of swine in vivo. Infect Immun 71(11):6446-6452.

Berk PA, Jonge R, Zwietering MH, Abee T, Kieboom J. 2005. Acid resistance variability among isolates of *Salmonella enterica* serovar Typhimurium DT104. J Appl Microbiol 99(4):859-866.

Bertin P, Terao E, Lee EH, Lejeune P, Colson C, Danchin A, Collatz E. 1994. The H-NS protein is involved in the biogenesis of flagella in *Escherichia coli*. J Bacteriol 176(17):5537-5540.

Boddicker JD, Ledeboer NA, Jagnow J, Jones BD, Clegg S. 2002. Differential binding to and biofilm formation on, HEp-2 cells by *Salmonella enterica* Serovar Typhimurium is dependent upon allelic variation in the *fimH* gene of the *fim* gene cluster. Mol Microbiol 45(5):1255-1265.

Borowsky L, Corção G, Cardoso M. 2009. Mannanoligosaccharide agglutination by *Salmlonella enteria* strains isolated from carrier pigs. Braz J Microbiol 40:458-464.

Botteldoorn N, Heyndrickx M, Rijpens N, Grijspeerdt K, Herman L. 2003. *Salmonella* on pig carcasses: positive pigs and cross contamination in the slaughterhouse. J Appl Microbiol 95(5):891-903.

Castelijn GAA, van der Veen S, Zwietering MH, Moezelaar R, Abee T. 2012. Diversity in biofilm formation and production of curli fimbriae and cellulose of *Salmonella* Typhimurium strains of different origin in high and low nutrient medium. Biofouling 28(1):51-63.

Chia TWR, McMeekin TA, Fegan N, Dykes GA. 2011. Significance of the rdar and bdar morphotypes in the hydrophobicity and attachment to abiotic surfaces of *Salmonella* Sofia and other poultry-associated *Salmonella* serovars. Lett Appl Microbiol 53(5):581-584.

Chuang YC, Wang KC, Chen YT, Yang CH, Men SC, Fan CC, Chang LH, Yeh KS. 2008. Identification of the genetic determinants of *Salmonella enterica* serotype Typhimurium that may regulate the expression of the type 1 fimbriae in response to solid agar and static broth culture conditions. BMC Microbiol 8(1):126.

Clayton DJ, Bowen AJ, Hulme SD, Buckley AM, Deacon VL, Thomson NR, Barrow PA, Morgan E, Jones MA, Watson M, Stevens MP. 2008. Analysis of the role of 13 major fimbrial subunits in colonisation of the chicken intestines by *Salmonella enterica* serovar Enteritidis reveals a role for a novel locus. BMC Microbiol 8(1):228.

Condell O, Sheridan Á, Power KA, Bonilla-Santiago R, Sergeant K, Renaut J, Burgess C, Fanning S, Nally JE. 2012. Comparative proteomic analysis of *Salmonella* tolerance to the biocide active agent triclosan. J Proteom 75(14):4505-4519.

C**owan MM, Van der Mei HC, Rouxhet PG, Busscher HJ.** 1992a. Physicochemical and structural investigation of the surfaces of some anaerobic subgingival bacteria. Appl Environ Microbiol 58(4):1326-1334.

Cowan MM, Van der Mei HC, Stokroos I, Busscher HJ. 1992b. Heterogeneity of surfaces of subgingival bacteria as detected by zeta potential measurements. J Dental Res 71(11):1803-1806.

Duguid JP, Anderson ES, Campbell I. 1966. Fimbriae and adhesive properties in Salmonellae. J Pathol Bacteriol 92(1).

European Food Safety Authority (EFSA). 2012. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. EFSA J 10(3):442.

Fang, FC, Libby SJ, Buchmeier NA, Loewen PC, SwitalaJ , HarwoodJ, Guiney GD. 1992. The alternative sigma factor katF (*rpoS*) regulates *Salmonella* virulence. Proc Nati Acad Sci U.S.A. 89(24):11978-11982.

Fronzes R, Remaut H, Waksman G. 2008. Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. EMBO J 27(17):2271-2280.

Gerstel U, Römling U. 2003. The *csgD* promoter, a control unit for biofilm formation in *Salmonella typhimurium*. Res Microbiol 154(10):659-667.

Girón JA, Torres AG, Freer E, Kaper JB. 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. Mol Microbiol 44(2):361-379.

Grantcharova N, Peters V, Monteiro C, Zakikhany K, Römling U. 2010. Bistable expression of *csgD* in biofilm development of *Salmonella enterica* serovar Typhimurium. J Bacteriol 192(2):456-466.

Hancox LS, Yeh KS, Clegg S. 1997. Construction and characterization of type 1 non-fimbriate and non-adhesive mutants of *Salmonella typhimurium*. FEMS Immunol Med Microbiol 19(4):289-296.

Hsiao HY, Anderson DM, Dale NM. 2006. Levels of β-mannan in soybean meal. Poultry Sci 85:1430-1432.

Humphries AD, Raffatellu M, Winter S, Weening EH, Kingsley RA, Droleskey R, Zhang S, Figueiredo J, Khare S, Nunes J. 2003. The use of flow cytometry to detect expression of subunits encoded by 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. Mol Microbiol 48(5):1357-1376.

Joseph B, Otta SK, Karunasagar I, Karunasagar I. 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. Int J Food Microbiol 64(3):367-372.

Kingsley RA, Weening EH, Keestra AM, Bäumler AJ. 2002. Population heterogeneity of *Salmonella enterica* serotype Typhimurium resulting from phase variation of the *lpf* operon In Vitro and In Vivo. J Bacteriol 184(9):2352-2359.

Kisiela D, Sapeta A, Kuczkowski M, Stefaniak T, Wieliczko A, Ugorski M. 2005. Characterization of FimH adhesins expressed by *Salmonella enterica* serovar Gallinarum biovars Gallinarum and Pullorum: reconstitution of mannose-binding properties by single amino acid substitution. Infect Immun 73(9):6187-6190.

Ledeboer NA, Frye JG, McClelland M, Jones BD. 2006. *Salmonella enterica* serovar Typhimurium requires the Lpf, Pef, and Tafi Fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. Infect Immun 74(6):3156-3169.

McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nat 413(6858):852-856.

Norris TL, Kingsley RA, Bäumler AJ. 1998. Expression and transcriptional control of the *Salmonella typhimurium lpf* fimbrial operon by phase variation. Mol Microbiol 29(1):311-320.

Old DC. 1972. Inhibition of the interaction between fimbrial haemagglutinins and erythrocytes by D-mannose and other carbohydrates. J Gen Microbiol 71:149-157.

Sampaio SCF, Gomes TAT, Pichon C, du Merle L, Guadagnini S, Abe CM, Sampaio JLM, Le Bouguénec C. 2009. The flagella of an atypical enteropathogenic *Escherichia coli* strain are required for efficient interaction with and stimulation of interleukin-8 production by enterocytes *in vitro*. Infect Immun 77(10):4406-4413.

Scher K, Römling U, Yaron S. 2005. Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. Appl Environ Microbiol 71(3):1163-1168.

Sinde E, Carballo J. 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. Food Microbiol 17(4):439-447.

Skandamis PN, Stopforth JD, Ashton LV, Geornaras I, Kendall PA, Sofos JN. 2009. *Escherichia coli* O157:H7 survival, biofilm formation and acid tolerance under simulated slaughter plant moist and dry conditions. Food Microbiol 26(1):112-119.

Steenackers H, Hermans K, Vanderleyden J, De Keersmaecker SCJ. 2012. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. Food Res Int 45(2):502-531.

Tariq M, Bruijs C, Kok J, Krom BP. 2012. Link between culture zeta potential homogeneity and Ebp in *Enterococcus faecalis*. Appl Environ Microbiol 78(7):2282-2288.

Teplitski M, Al-Agely A, Ahmer BMM. 2006. Contribution of the SirA regulon to biofilm formation in *Salmonella enterica* serovar Typhimurium. Microbiol 152:3411-3423.

Tinker JK, Hancox LS, Clegg S. 2001. FimW is a negative regulator affecting type 1 fimbrial expression in *Salmonella enterica* serovar Typhimurium. J Bacteriol 183(2):435-442.

Tinker JK, Clegg S. 2000. Characterization of FimY as a coactivator of type 1 fimbrial expression in *Salmonella enterica* serovar Typhimurium. Infect Immun 68(6):3305-3313.

van Merode AEJ, Duval JFL, van der Mei HC, Busscher HJ, Krom BP. 2008. Increased adhesion of *Enterococcus faecalis* strains with bimodal electrophoretic mobility distributions. Colloid Surface B 64(2):302-306.

van Merode AEJ, van der Mei HC, Busscher HJ, Waar K, Krom BP. 2006. *Enterococcus faecalis* strains show culture heterogeneity in cell surface charge. Microbiol 152(3):807-814.

Weening EH, Barker JD, Laarakker MC, Humphries AD, Tsolis RM, Bäumler AJ. 2005. The *Salmonella enterica* Serotype Typhimurium *lpf, bcf, stb, stc, std,* and *sth* fimbrial operons are

required for intestinal persistence in mice. Infect Immun 73(6):3358-3366.

Wong H, Townsend K, Fenwick S, Trengove R, O'Handley R. 2010. Comparative susceptibility of planktonic and 3-day-old *Salmonella* Typhimurium biofilms to disinfectants. J Appl Microbiol 108(6):2222-2228.

SUPPLEMENTARY MATERIAL

Table S3.1: Primers used for the expression analysis of the 11 putative functional fimbriae gene clusters

Fimbriae	**Forward primer 5' → 3'**	**Reverse primer 5' → 3'**
bcf	TGTCGATCAGACCGTGACAT	CGCAGTCTTCCAGTTTGATG
csg	GGATTCCACGTTGAGCA	TAACCGCTCTGGGTAATGG
fim	TGTCGATCAGACCGTGACAT	CGCAGTCTTCCAGTTTGATG
lpf	ACGGCACCATTAAATTCACC	AACCTGACCCAGCACAACTT
pef	TTGAAAGCGTGAACTCCAAA	ACCTTTCAGCTTGGCTTTGA
saf	GCCGCAGAAAAACTTCAGAC	GATAAGTCACCCAGCCTTGC
stb	AATCAGGCTAAACCCGTCGT	GGACAGCCCAATATCAAACG
stc	GAGACGGGGGTGACACTAGA	TCACCAGAAGCAACATCACC
std	CATCACCAACTCACCCTGTG	GCATTGGCGGTATTCAGTTT
stf	TTCTCTGATGGGCGGTAAAG	GGCAGAGAAAACGGTAGACG
sti	AACACTCACTGTGGCACTGG	TACCGTACAGGCGTTTTGCT
16S *rRNA*	CGATCCCTAGCTGGTCTGAG	GTGCAATATTCCCCACTGCT

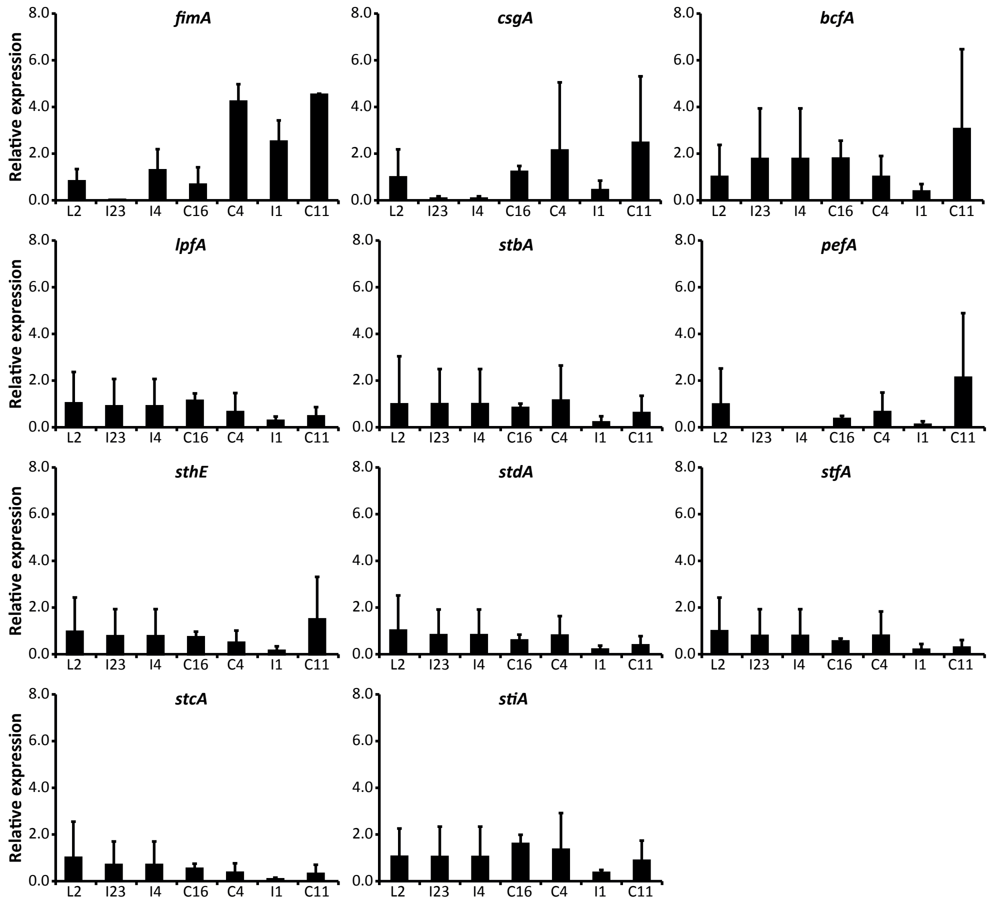


Figure S3.1: Expression of *fimA, bcfA*, *csgA*, *lpfA*, *stbA*, *stcA*, *stdA*, *stfA*, *sthE*, *stiA*, and *pefA* by *S.* Typhimurium planktonic cells cultured in grown TSB at 37 °C. The expression values are relative to the expression of the L2 strain. The bars denote the SDs of three biological repeats.

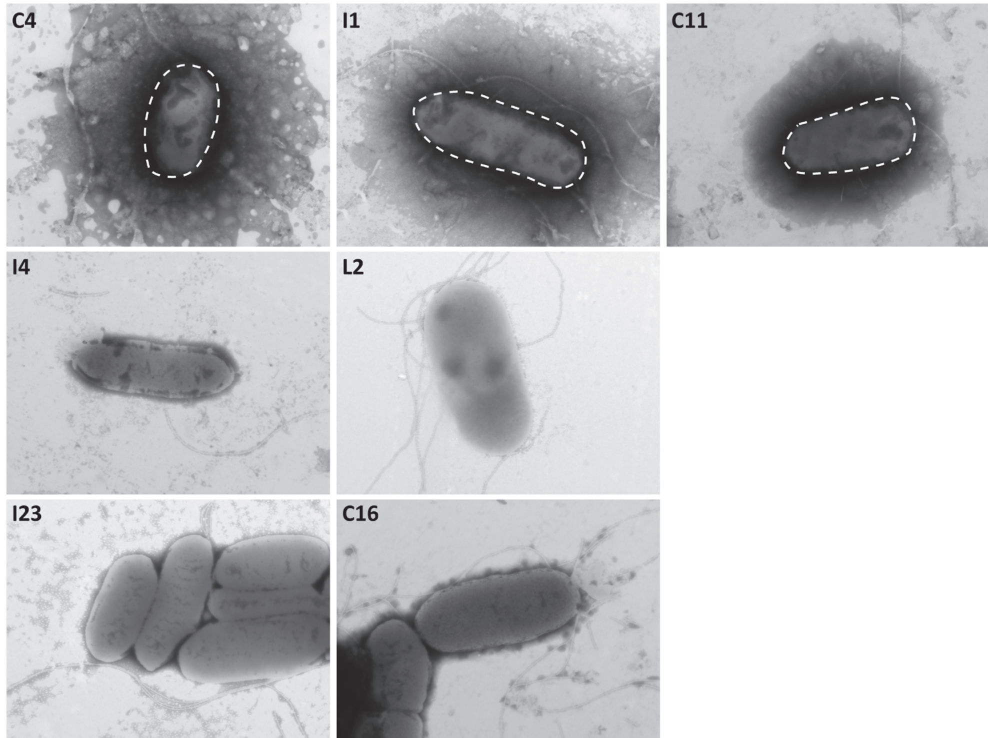


Figure S3.2: The presence of type 1 fimbriae was analysed with TEM for cell cultures from all strains used in this study grown for 24 h in TSB at 37 °C. The strain number is indicated in the figure. The staining is darker compared to figure 3.3 due to the use of higher dye concentrations. Since the dye is bound to and captured by the fimbriae expressed by strain C4, I1 and C11 the cells are not clearly visible. Therefore the outside of the cell is indicated by a white dotted line to illustrate the cell outer membrane.

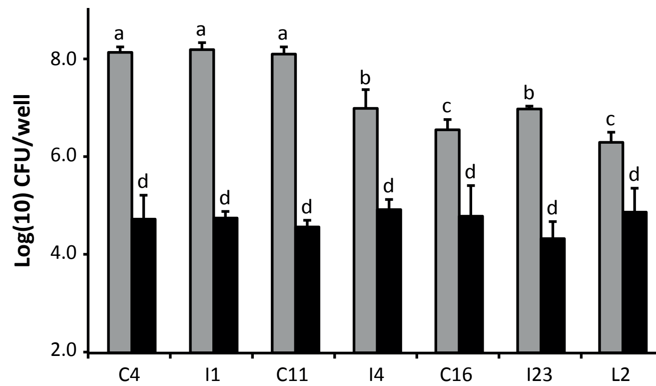


Figure S3.3: Amount of biofilm cells after 24 h of incubation at 37 °C in TSB (grey bars) and TSB + 3% mannose (black bars). Biofilm forming capacity is similar for of selected isolates in TSB + 3% mannose after 24 h. For comparison, data obtained in TSB without mannose are shown (taken from Fig. 3.2). The bars denote the SDs of three biological repeats and significant differences are indicated bij different letters (p < 0,05, t-test).

CHAPTER 4

Surface behaviour of *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.* Infantis

Greetje A.A. Castelijn, Jo-Ann Parabirsing, Marcel H. Zwietering, Roy Moezelaar and Tjakko Abee

Published in Veterinary Microbiology 2013, 161:305-314

ABSTRACT

Cross-contamination due to *Salmonella* on the surface of processing equipment greatly contributes to contamination of pork products. Therefore, a clear understanding of surface and survival behaviour of relevant *Salmonella* serovars in pork processing environments is needed to develop better strategies for *Salmonella* control. Within this study the biofilm forming behaviour *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.* Infantis isolates was analyzed using the crystal violet assay. This assay, commonly used to analyse total biofilm formation, revealed variation in biofilm forming capacity between and within serovars. This has not been shown before for *S.* Derby, *S.* Brandenburg and *S.* Infantis. From each serovar, isolates with different biofilm forming capacity were selected to analyse biofilm formation on stainless steel. This revealed no significant differences between biofilm formation on polystyrene compared to stainless steel. Furthermore a relation was observed between biofilm forming capacity of an isolate and survival on stainless steel surfaces. On such surfaces, biofilms showed greater and longer survival than planktonic cells, and they were less susceptible to peracetic acid disinfection treatments. However, the latter effect was marginal and only observed in the presence of organic material, which drastically decreased the activity of peracetic acid. With the obtained results also a hierarchical cluster was performed to identify differences and similarities between the four different serovars. This indicated that the surface behaviour of *S.* Typhimurium was more comparable to *S.* Infantis than to *S.* Derby or *S.* Brandenburg.

INTRODUCTION

Salmonella infections in humans are in general foodborne, with an important source of risk being food from animal origin. The most frequently implicated food sources are eggs, poultry meat and pork (EFSA, 2011). As shown by epidemiological studies in the European Union (EU), consumption of pork is associated with 15-20% of all human cases of salmonellosis and with 7.8% of all foodborne outbreaks (ECDC, 2011; Botteldoorn *et al.*, 2003)

Within the EU, the prevalence of *Salmonella* on slaughter pigs and in pork processing environments is well monitored. This reveals that at point of slaughter on average 10.3% of the slaughter pigs and after the slaughter process 8.3% of the carcasses are positive for *Salmonella* (EFSA, 2008). *Salmonella* serovars that are repeatedly found on farm animals within the pork processing environments and on carcasses at the end of the slaughter process are *S.* Typhimurium, *S.* Derby, *S.* Infantis and *S.* Brandenburg (De Busser *et al.*, 2011; Volf *et al.*, 2010; EFSA, 2008; Botteldoorn *et al.*, 2003).

Contamination of carcasses during the slaughter process may occur in two ways. One route is direct contamination by the contents of the intestine or lymph nodes. The other one is indirect by contact with dirty processing equipment, so-called cross-contamination. Cross-contamination is common within the slaughter process, accounting for about 29% of the total amount of contaminated carcasses (Van Hoek *et al.*, 2012; BIOHAZ, 2010; EFSA, 2008a; Botteldoorn *et al.*, 2003). This indicates that the presence of *Salmonella* on surfaces of processing equipment is a problem in pork processing environments and that better strategies for *Salmonella* control require a clear understanding of the survival and surface behaviour of *Salmonella*. Since *Salmonella* is exposed to a variety of different conditions within the processing environment different characteristics contribute to the survival and persistence of *Salmonella* in slaughterhouses. In this study the biofilm forming capacity and its relation with survival of *Salmonella* on stainless steel surfaces and susceptibility for peracid acid (PAA) were analysed and compared for *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.* Infantis. Also the influence of organic matter (3% BSA) on the effectiveness of PAA was investigated.

MATERIALS AND METHODS

Bacterial isolates and media

A total of 23 *S.* Typhimurium, 13 *S.* Derby, 29 *S.* Brandenburg and 55 *S.* Infantis isolates were used in this study. All isolates were obtained from multiple pork processing plants and were isolated from processing equipment surfaces, food products, raw materials or the processing environment. Isolates from different origin within a pork processing environment were

used to get a broad view of *Salmonella* isolates present is these settings. Stock cultures of these isolates were stored at –80 °C in Brain Hearth Infusion Broth (BHI, Becton Dickson) supplemented with 15% glycerol (Sigma). Isolates were cultivated in Luria Bertani broth (LB, Merck) for 18 h at 37 °C with agitation (200 rpm), after which they were used in the different experiments. To mimic the conditions in a pork processing environment, the experiments were performed at 25 °C in the meat-based medium BHI.

Preparation of surfaces

Coupons (18 mm x 22 mm) of polystyrene (PS) and stainless steel (SS) (AISI type 304L) were used. Before use, the PS coupons were soaked for >2 h in 70% ethanol, after which they were washed three times with sterile deionized water and dried in a safety hood. The SS coupons were soaked in 1M NaOH (Sigma) for 30 min at 50 °C, after which they were rinsed with water. Subsequently, the SS coupons were soaked in acetone (BDH, Prolabo) for 15 min at room temperature, washed four times with deionized water, autoclaved and dried overnight at 50 °C.

Crystal violet assay

Biofilm forming capacity was examined using the crystal violet (CV) assay for analysis of biofilms in polystyrene 96 well microtiter plates (Greiner Bio-one) or on PS and SS coupons. For the microtiter plate CV assay, wells were filled with 200 µl BHI medium. For the coupon CV assay, a coupon was vertically placed in a well from a 12 well plate (Greiner Bio-one) filled with 3 ml of BHI medium. For both CV assays, the medium was inoculated with 1.5% (vol/vol) overnight cultures and the plates were incubated for 24 h at 25 °C. After incubation, biofilms were washed twice with water and stained with 0.1% (wt/vol) CV (Merck) for 30 min. Subsequently, the biofilms were washed three times with water and the attached CV was dissolved in 250 µl per well for the microtiter plate and 5 ml for the coupon CV assay of 96% ethanol. The absorbance was measured at 595 nm (SpectraMax, Molecular Devices). Both CV assays were performed in at least three biologically independent experiments.

Enumeration of biofilm cells

For enumeration of biofilm cells by plate counting, biofilms were grown as described above on PS and SS coupons. After incubation, the biofilms on the coupons were washed four times with peptone physiological salt solution (PPS, 0.8% NaCl and 0.1% neutralised bacteriological peptone (Oxoid) to remove unbound cells. Then each coupon was transferred to a tube containing 5 ml of PPS and glass beads (≤106 µm, Sigma) and vortexed for 1 min to detach the biofilm and obtain single cells. Next, appropriate dilutions were made in PPS and the cells were enumerated by plating on tryptone soy agar (TSA, Oxoid). After overnight incubation at 37 °C, the colonies were counted.

Curli fimbriae and cellulose production

Curli fimbriae and cellulose production was analysed as described previously (Römling *et al.*, 2003) on LB without NaCl agar plates supplemented with 40 µg/ml congo red (Sigma) and 20 µg/ml Coomassie brilliant blue (Merck) or with 40 µg/ml calcofluor (Fluorescent Brightner 28, Sigma). After 48 h of incubation at 25 °C, the colony morphology on the agar plates was examined.

Survival of *Salmonella* on dry SS surfaces

Survival on dry SS surfaces was examined for both planktonic cells and biofilms. For analysis of planktonic cells, the isolates were cultured statically in tubes containing 10 ml of BHI for 24 h 25 °C. Of this overnight culture 30 µl (~8.0 log CFU) was applied to a SS coupon which was subsequently dried in a safety hood for 30 min. For analysis of biofilms, SS coupons were used to grow biofilms as described above, after which the coupons were washed three time with phosphate buffered saline (PBS), dried for 30 min in a safety hood and placed in a 12 well plate. After incubation for 1, 2, 5, 7 or 14 days at 25 °C at 35-65% RH, the coupons were transferred to tubes containing 5 ml of PPS and glass beads (≤106 µm) and cells were detached and enumerated as describes above.

Peracetic acid disinfection treatment

Susceptibility for PAA (Sigma) was analysed for both planktonic cells and biofilms. For disinfection treatments of planktonic cells, the selected isolates were statically cultured in BHI for 24 h at 25 °C. Aliquots of 1 ml of these overnight cultures (~8.1 log CFU) were centrifuged for 3 min at 5000 *g* and the cells were resuspended in 1 ml sterile deionized water with or without 3% bovine serum albumin (BSA, Sigma). To these cell suspensions, PAA was added to a final concentration of 100 ppm. After 2 min exposure, the samples were diluted ten times in neutralizer Dey-Engley Neutralizing medium (Becton Dickison). For disinfection treatments of biofilms, biofilms grown on SS coupons as described above were washed three times with PBS and transferred to a well with 100 ppm PAA in deionized water with or without 3% BSA. After 2 min exposure, the coupons were transferred to tubes containing 5 ml of Dey-Engley Neutralizing medium and glass beads (≤106 µm), which were vortexed for 1 min. After neutralization of the disinfectant the planktonic and biofilms cells were enumerated as described above. The detection limits of these PAA disinfection treatments were 1.2 log CFU/ml for the planktonic cells and 1.9 log CFU/coupon for biofilms.

Statistical analysis

Each experiment was repeated three times at different days. The biological replicates were averaged and numbers were, when necessary, $\log_{10}$-transformed for statistical analysis. Statistical differences were analysed by variance analyses using Microsoft Excel for Windows (Data analysis) in which a $p < 0.05$ was considered as statistically significant.

To determine possible correlations between the different parameters tested, the Pearson

correlation coefficient *r* was calculated using IBM SPSS Statistics version 19 for Windows. For this purpose a data matrix of all tested parameters was made and the morphologies on congo red agar plates rdar, sap and saw, were scored respectively 1, 0.5 and 0, and the results of the calcofluor agar plates, +, +/- and - were scored respectively 1, 0.5 and 0. Furthermore this data matrix was used for a hierarchical clustering to identify isolates showing similar behaviour. All tested parameters were clustered using the average linkage method and the Euclidian distance matrix (IBM SPSS Statistics version 19). Since the different parameters were measured on a different scales, all values were set between 0 and 1.

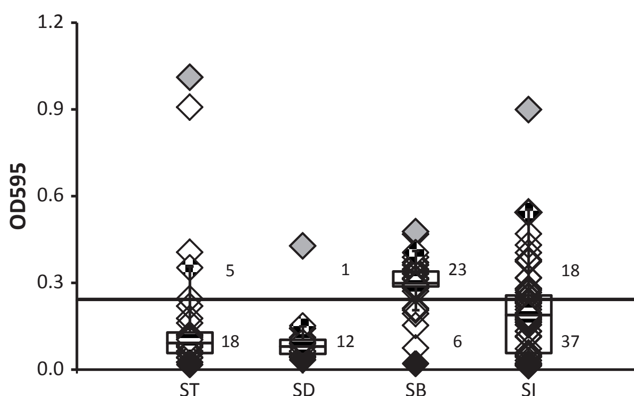


Figure 4.1: The biofilm forming capacity of *S.* Typhimurium (ST n=23), *S.* Derby (SD n=13), *S.* Brandenburg (SB n=29) and *S.* Infantis (SI n=55) analysed with the CV microtiter plate assay. Each diamond denotes the average of five biological replicates of one isolate. The boxes from the box-and-whiskers plots range from the 25th and 75th percentile and are intersected by the median. The whiskers extend from the lower and upper 1.5 interquartile range (IQR). The selected isolates are indicated with a grey check (extreme biofilm former), a dotted check (maximum biofilm former), a striped check (average biofilm former) or a black check (minimal biofilm former). The level of dense biofilm formation (three times the background level) is indicated by a solid line. Number of isolates grouped as dense and low level biofilm formers are also indicated in the figure.

RESULTS

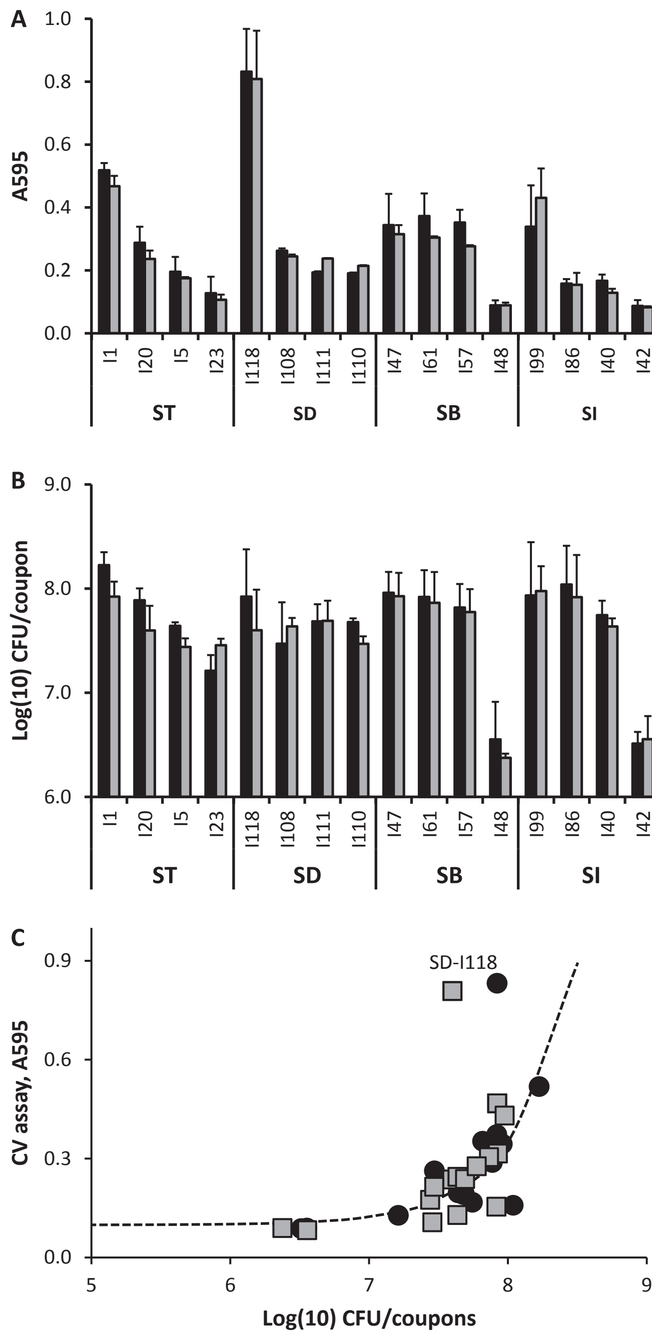
Screening of biofilm formation with the microtiter plate CV assay

Biofilm forming capacity of 120 *Salmonella* isolates in BHI at 25 °C was analysed using the microtiter plate CV assay. A dense biofilm was defined at an $A_{595} \geq 0.24$, which is three times the background signal. The background signal was defined as three times the standard deviation above the mean A_{595} of the negative control.

Biofilm forming capacity varied both between and within serovars (Fig. 4.1). Greatest variation was observed within the serovars *S.* Typhimurium and *S.* Infantis with some isolates exhibiting dense biofilm formation with A_{595} values up to 1.0, yet most isolates showing little to no biofilm formation, 18 out of the 23 *S.* Typhimurium and 37 out of the 55 *S.* Infantis isolates. Poor biofilm forming capacity was (with the exception of isolate SD-I118 which formed dense biofilms) also observed for *S.* Derby. In contrast to the other serovars, dense biofilms were observed for 23 of the 29 *S.* Brandenburg isolates. So the percentage of *S.* Brandenburg isolates exhibiting dense biofilm formation (80%) is much higher than for the other serovars (*S.* Typhimurium 22%, *S.* Infantis 33% and *S.* Derby 8%). Based on the results of the microtiter plate CV assay 16 isolates (4 isolates per serovar, Table 4.1), were selected for further analysis. For each serovar, the isolate showing the extreme, average and minimal biofilm forming capacity, and the isolate that had the highest biofilm forming capacity but still within 1.5 interquartile range (1.5 IQR) of the upper quartile (maximum) (Fig. 4.1) was selected. These isolates with different biofilm forming capacity were selected in order to get a broad view of the different serovars.

Table 4.1: The *Salmonella* isolates that were selected in this study for further research and their biofilm forming behaviour. The morphologies expressed on congo red (CR) agar plates: rdar (red, dry and rough), sap (smooth and pink) and saw (smooth and white). And fluorescence observed at the calcofluor agar plates: + = intense fluorescence, +/- = moderate fluorescence and - = no fluorescence.

Serovar	Isolate number	Biofilm forming behaviour	Congo red agar plates	Calcofluor agar plates
Typhimurium	ST-I1	Extreme	rdar	+
Typhimurium	ST-I20	Maximum	rdar	+
Typhimurium	ST-I15	Average	rdar	+
Typhimuirum	ST-I23	Minimal	saw	-
Derby	SD-118	Extreme	sap	+/-
Derby	SD-108	Maximum	rdar	+
Derby	SD-I111	Average	rdar	+
Derby	SD-I110	Minimal	saw	-
Brandenburg	SB-I47	Extreme	rdar	+
Brandenburg	SB-I61	Maximum	rdar	+
Brandenburg	SB-I57	Average	rdar	+
Brandenburg	SB-I48	Minimal	saw	-
Infantis	SI-I99	Extreme	rdar	+
Infantis	SI-I86	Maximem	rdar	+
Infantis	SI-I40	Average	saw	-
Infantis	SI-I42	Minimal	saw	-


A
1.0
0.8
0.6
0.4
0.2
0.0
A595
I1
I20
I5
I23
I118
I108
I111
I110
I47
I61
I57
I48
I99
I86
I40
I42
ST
SD
SB
SI
B
9.0
8.0
7.0
6.0
Log(10) CFU/coupon
C
0.9
0.6
0.3
0.0
CV assay, A595
SD-I118
5
6
7
8
9
Log(10) CFU/coupons

Biofilm formation on different substrata

The biofilm forming capacity of the selected isolates was analysed on both PS and SS coupons. Most processing equipment in pork processing environments is made from SS and to enable a good comparison between biofilm forming capacity on SS and PS, biofilm formation was also analysed on PS coupons.

With a CV assay the total amount of biofilm formation is analysed, since CV binds to the extracellular matrix and to the biofilm cells. The number of biofilm cells were enumerated by plate counts (Fig. 4.2B). This revealed that biofilms of the extreme biofilm formers contained significant more cells than the biofilms of the minimal biofilm formers, except for *S.* Derby. For this serovar no significant differences were detected between the four selected isolates. This is remarkable since isolate SD-I118 showed considerable more CV binding (Fig. 4.2A). This isolate was also an outlier when the data of the coupon CV assay was plotted against the log CFU counts of the biofilm enumeration (Fig. 4.2C). Additionally, this graph showed a relation between the coupon CV assay and the amount of biofilm cells which can be described by a linear model $A_{595} = a \cdot N + b$ (where A_{595} is the absorbance of the dissolved CV, a is the proportionality constant between the CV staining and the cell counts, N is the number of attached biofilm cells and b is the background signal) as defined by Castelijn *et al.* (2012). The relation between the coupon CV assay and the CFU counts was further confirmed with a Pearson correlation analysis, with a correlation coefficient of 0.975 (significant at the level 0.01, 2-tailed) for PS coupons and 0.941 (significant at the level 0.01, 2-tailed) for SS coupons.

Plotting CV and CFU data obtained with PS and SS coupons revealed no significant differences between the biofilm forming capacity on these surfaces. Especially since the data corresponds well with the model $y = x$ (Fig. 4.3).

Curli fimbriae and cellulose production capacity

The ability to produce curli fimbriae and cellulose was assessed on congo red and calcofluor agar plates. After 48 h incubation at 25 °C, on both the congo red (rdar, sap and saw) and calcofluor (+, +/- and -) agar plates, three different morphotypes were observed (Table 4.1). The saw morphotype and no calcofluor binding (-) was observed for the minimal biofilm

---

Figure 4.2: Biofilm formation of the selected isolates on PS (black bars) and SS (grey bars) coupons analysed with the coupon CV assay (A) or by enumeration of biofilm cells (B). The serovars *S.* Typhimurium (ST), *S.* Derby (SD), *S.* Brandenburg (SB), *S.* Infantis (SI) and the isolate numbers are indicated on the *x*-axis. The bars denote the mean of three biological replicates with the standard deviates of the mean. C) The data from the CV assays (A_{595}) are plotted against the log CFU/coupon for both the biofilms grown on PS coupons (black circles) and SS coupons (grey squares). The data is fitted with the linear model $A_{595} = a \cdot N + b$ (dotted line). Data of isolate SD-I118 was not included in the model, since this was an outlier.

expressing this morphotype generally exhibit low biofilm forming capacity (Castelijn *et al.*, former of each serovar and for the average biofilm former of *S.* Infantis (SI-I40). Isolates 2012; Malcova *et al.*, 2008). The other isolates expressed the rdar morphotype and calco fluor binding. Although in several studies this has been related to high biofilm forming capacity (Malcova *et al.*, 2008; Römling *et al.*, 2003), not all of the isolates expressing the rdar morphotype showed dense biofilm formation under the tested conditions (Fig. 4.1). The extreme biofilm former of *S.* Derby, isolate SD-I118, exhibited a sap morphotype with moderate calcofluor binding (+/-) (Table 4.1). Interestingly, compared to the other isolates, this isolate showed extensive CV binding in the coupon CV assay (Fig. 4.2A).

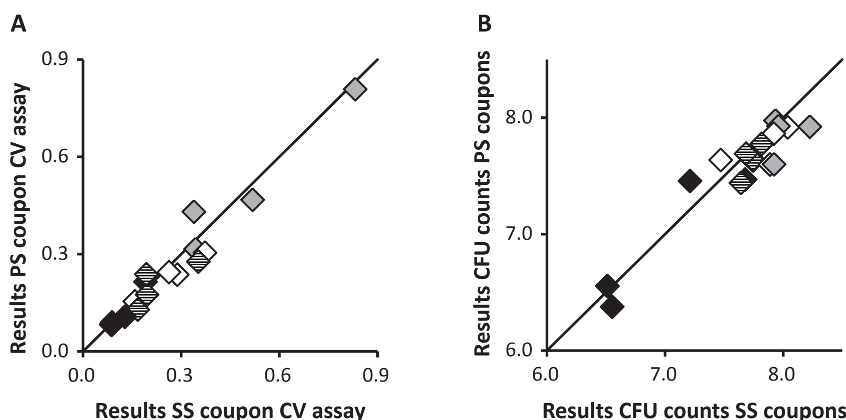


Figure 4.3: Correlation between biofilm formation on PS and SS analysed with the CV assay (A) and by enumeration of biofilm cells (B). For the different serovars, the extreme biofilm former is indicated by a grey diamond, the maximum biofilm former by a white diamond, the average biofilm former by a striped diamond and a minimal biofilm former by a black diamond. The linear model y = x is indicated by a solid line.

Survival of planktonic cells and biofilms on dry stainless steel surfaces

Survival of planktonic cells on dry stainless steel surfaces is presented in Fig. 4.4. The most rapid decrease in survival, about 3.9 ± 0.6 log CFU/coupon, was observed in the first 24 h. However, until 48 h viable cells could be detected for all isolates. After another 3 days of incubation, divergence in survival between and within serovars was observed. At day 5, all four *S.* Infantis isolates and three *S.* Typhimurium isolates were below the detection limit. Longer survival was found for *S.* Brandenburg and *S.* Derby, with two and three isolates respectively, showing survival even up to 14 days. None of the tested isolates showed a relation between survival of planktonic cells on dry SS surfaces and biofilm forming capacity.

The survival of biofilms from the selected isolates was also examined (Fig. 4.5). Almost all isolates showed most reduction in the first two days, after which the reduction nearly stagnated. However, for the isolates with the lowest biofilm forming capacity reduction was also observed after another day of incubation. Furthermore, in contrast to the other isolates, hardly any reduction was found for biofilms of isolates SD-I118 and SD-I111. For SD-I111, even after 14 days, only 1.3 log CFU reduction and for SD-I118 1.7 log CFU reduction was detected.

For all serovars, enhanced and prolonged survival was observed for cells within a biofilm compared to planktonic cells, (Fig. 4.4 and 4.5). Furthermore, survival of biofilms was related to the biofilm forming capacity on PS and SS coupons of an isolate, This relation was also confirmed by a Pearson correlation analysis. These data suggest that biofilm forming capacity contributes to survival of *Salmonella* on dry SS surfaces.

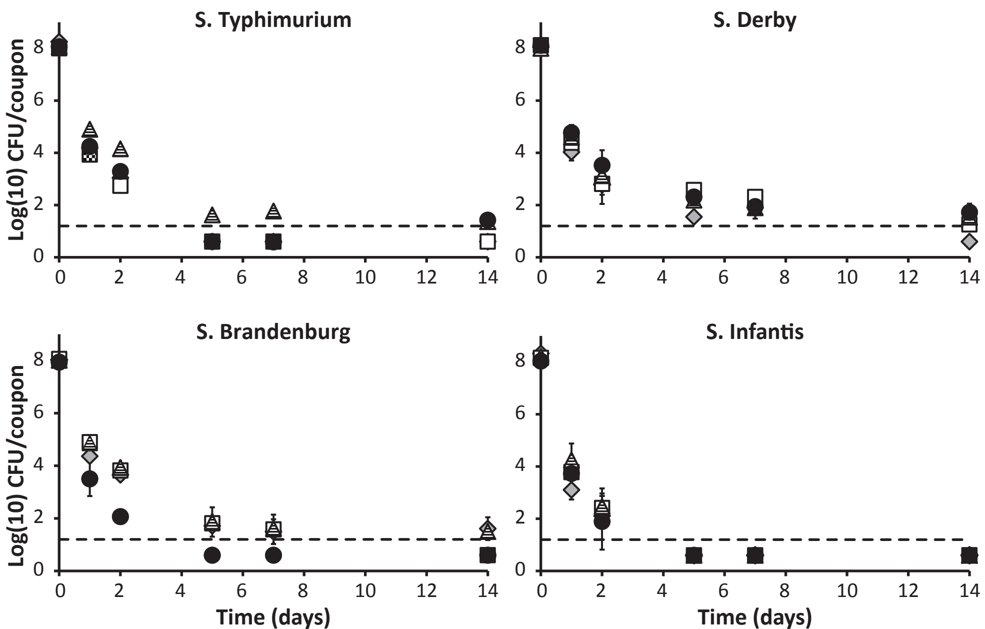


Figure 4.4: Survival of planktonic cells on dry SS surfaces at 25 °C. For the different serovars, the extreme biofilm former is indicated by a grey diamond, the maximum biofilm former by a white square, the average biofilm former by a striped triangle and a minimal biofilm former by a black circle. Each symbol denotes the average of three biological triplicates with the corresponding standard deviations (sometimes not visible). Data below the detection limit were set to one-half of the detecting limit which is indicated by a dotted line.

PAA disinfection treatments

Since processing equipment and the industrial environment are daily disinfected, reduced susceptibility for disinfection treatments could also contribute to survival of bacterial cells in industrial settings. Therefore, the susceptibility to PAA, a disinfectant commonly used in the food industry (Mueller-Doblies *et al.*, 2010; Wirtanen and Salo, 2003), was analysed for both planktonic cells and biofilms (Fig. 4.6). Since in industrial settings organic waste is abundantly present on processing equipment, susceptibility was also analysed in the presence of 3% BSA. This revelaed that the activity of PAA drastically decreased in the presence of BSA (Fig. 4.6). At this condition, a two minute exposure resulted in approximately 1 log CFU reduction only for the planktonic cells. Compared to the planktonic cells, biofilms were slightly less susceptible in the presence of BSA. However, no significant differences were observed between planktonic cells and biofilms if BSA was absent. At this condition, for all isolates, two minute exposure to 100 ppm PAA led to about 5.7 log reduction (Fig. 4.6).

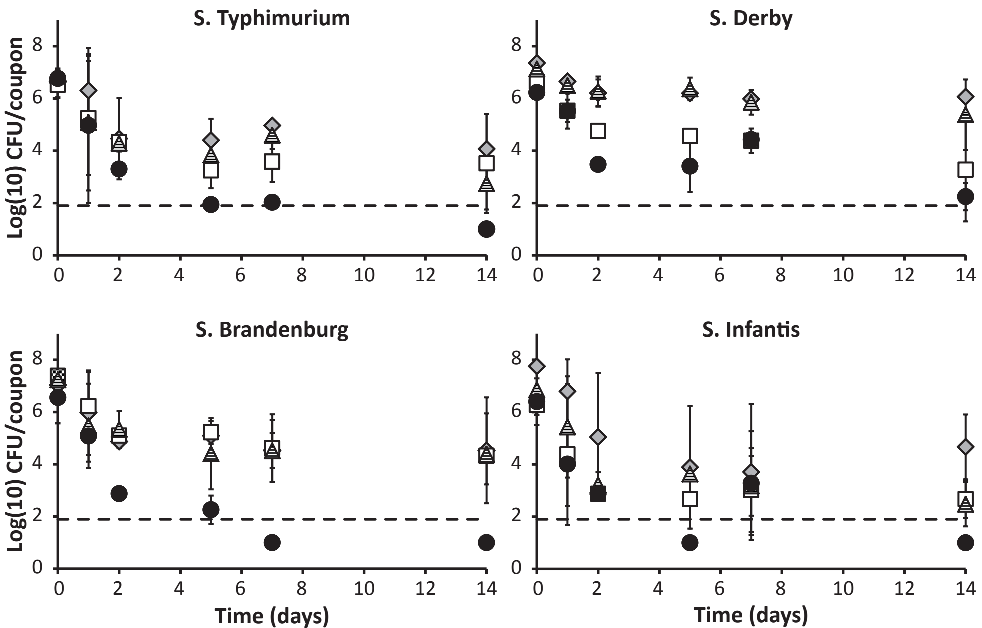


Figure 4.5: Survival of biofilms on dry SS surfaces at 25 °C. For the different serovars the extreme biofilm former is indicated by a grey diamond, the maximum biofilm former by a whit square, the average biofilm former by a striped triangle and a minimal biofilm former by a black circle. Each symbol denotes the average of three biological triplicates with the corresponding standard deviations (sometimes not visible). Data below the detection limit were set to one-half of the detecting limit that is indicated by a dotted line.

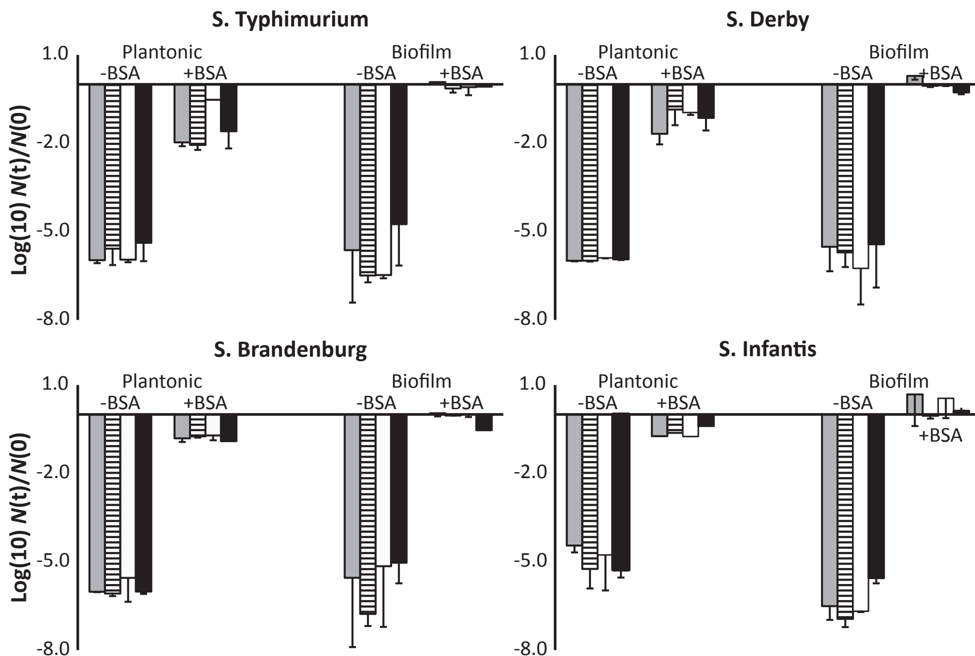


Figure 4.6: Disinfection treatment of planktonic cells and biofilms for 2 min with 100 ppm PAA with BSA (+BSA) and without BSA (-BSA). The extreme biofilm former is indicated by grey bars, the maximum biofilm former by striped bars, the average biofilm former by white bars and the minimal biofilm former by black bars. The bars denote the means and corresponding standard deviations of three biological replicates.

Furthermore, the results showed no significant differences in PAA susceptibility within or between the serovars and no relation was observed between the biofilm forming capacity and resistance against PAA.

Cluster analysis of the selected isolates

A hierarchical clusters analysis was performed to identify similarities and differences between the surface behaviour of the different isolates (Fig. 4.7). For this purpose all tested parameters were clustered using the average linkage method and the Euclidian distance matrix. This revealed five different clusters, of which two clusters were only comprised of one isolate. One cluster was comprised of the minimal biofilm former of *S.* Derby, isolate SD-I110, which was next to the second cluster that contained the average biofilm former of *S.* Infantis and the minimal biofilm formers of the other serovars. A third cluster was formed by the *S.* Typhimurium and *S.* Infantis isolates which exhibited extreme and maximum biofilm forming capacity. The fourth cluster was formed by the isolates of the other two serovars, *S.*

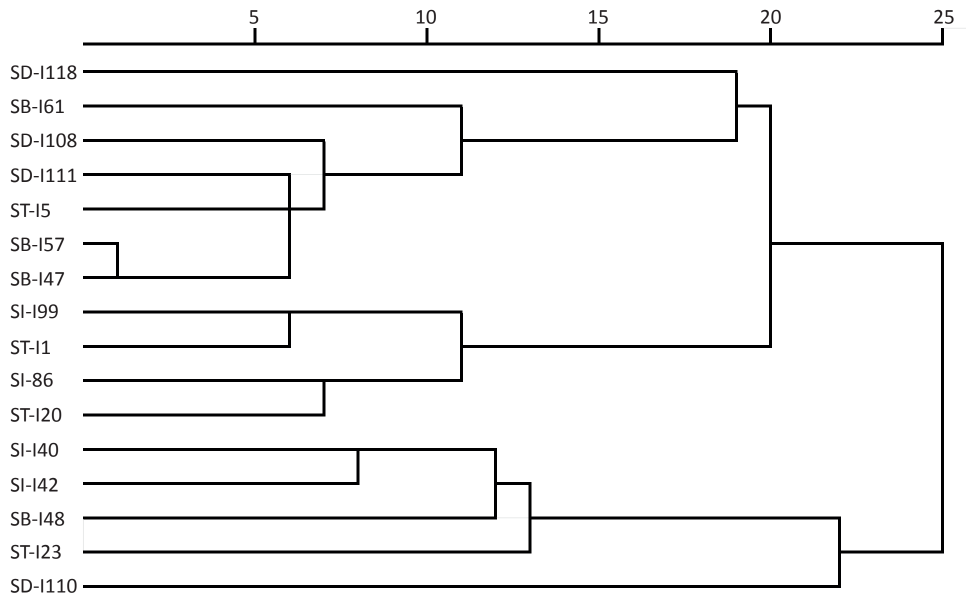


Figure 4.7: Hierarchial cluster analysis of the tested parameters using the average linkage method and the Euclidian distance matrix.

Brandenburg and *S.* Derby. Next to the latter cluster was the fifth cluster comprised of only isolate SD-I118. Compared to the other isolates, this isolate exhibited extensive CV binding in the coupon CV assay, expressed a divergent morphotype on the congo red and calcofluor agar plates and showed prolonged survival during desiccation.

DISCUSSION

The slaughter process is a critical step in the pig meat chain with respect to pork and carcass contamination. During the slaughter process cross-contamination due to contaminated equipment is the main route of *Salmonella* transmission (De Busser *et al.*, 2011; BIOHAZ, 2010; Botteldoorn *et al.*, 2003). So the presence of *Salmonella* on food contact surfaces can lead to food quality and safety problems. Therefore there is an interest in gaining more knowledge on the survival behaviour of *Salmonella* to develop better strategies for control in processing lines. Within the processing environment *Salmonella* is exposed to a variety of different conditions and therefore different characteristics can contribute to the survival and persistence of *Salmonella* in these environments. In this study, the biofilm forming capacity and its relation with survival on stainless steel surfaces and PAA resistance were analysed and compared for *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.* Infantis isolates. Also the influence of organic material (BSA) on the effectiveness of PAA was investigated.

By screening the *Salmonella* isolate collection with the microtiter plate CV-assay, diversity in the biofilm forming capacity between and within the serovars was found (Fig. 4.1). This phenomenon was observed before for *S.* Typhimurium (Castelijn *et al.*, 2012; Vestby *et al.*, 2009; Solomon *et al.*, 2005). However, so far this had not yet been described for *S.* Derby, *S.* Brandenburg and *S.* Infantis. The screening also revealed that *S.* Brandenburg isolates exhibited in general higher biofilm forming capacity than *S.* Typhimurium and *S.* Derby. This is remarkably since latter two serovars are more frequently encountered in clinical cases of pig *Salmonella* infections and it has been shown that the ability of biofilm formation contributes to the pathogenesis of *Salmonella* (Foley *et al.*, 2008; Boddicker *et al.*, 2002).

In terms of biofilm forming capacity no noteworthy differences were observed between PS and SS surfaces (Fig. 4.3). So this study showed that results obtained with PS, which is commonly used in the laboratory to study *Salmonella* biofilms, can be representative for the surface behaviour on SS, a substratum generally encountered in slaughterhouses and processing lines. In literature similar findings were reported (Park *et al.*,2012; Allan *et al.*, 2004) although some authors described differential attachment to PS and SS (Chia *et al.*, 2009; Joseph *et al.*, 2001). This contradiction concerning the biofilm forming capacity of *Salmonella* on different surfaces can be explained by the fact that the different studies used different culture conditions and *Salmonella* isolates. Initial attachment and subsequent biofilm formation depends on the properties of both the substratum and the bacterial cell surface which are influenced by environmental conditions such as temperature, humidity, pH, and the presence of organic material and cell surface structures (Chia *et al.*, 2009; Shi and Zhu, 2009; Tresse *et al.*, 2007; Hood and Zottola, 1997) .

An analysis of the ability to produce curli fimbriae and cellulose, revealed a relation between the expression of a rdar morphotype on congo red agar plates and biofilm forming capacity. Although this has been shown before for *S.* Typhimurium (Castelijn *et al.*, 2012; Malcova *et al.*, 2008; Solomon *et al.*, 2005; Römling *et al.*, 2003), in this study this relation was also observed for *S.* Brandenburg, *S.* Infantis and *S.* Derby, except for isolate SD-I118, which expressed the distinct sap morphotype.

Expression of the rdar morphotype on the LB agar plates does not necessarily correspond to curli and/or cellulose production during biofilm formation. Therefore the cellulose content of biofilms from the selected isolates was analysed by calcofluor staining (a dye binding to cellulose). This revealed little to no calcofluor binding by *Salmonella* biofilms grown in BHI (data not shown). These results are in agreement with a previous study by Castelijn *et al.* (2012), that showed *Salmonella* biofilms grown in a rich medium to be composed of multilayers of cells and lack of excessive amounts of cellulose.
Survival on dry SS surfaces was also examined, since this parameter also contributes to the survival of *Salmonella* in pork processing environments (Vestby *et al.*, 2009; Møretrø

et al., 2003). The data indicated diversity in the survival among the different serovars (Fig. 4). Longer survival was observed for *S.* Derby and *S.* Brandenburg than for *S.* Typhimurium and *S.* Infantis. Contrary to our results, a number of studies found no variation in the desiccation tolerance of different *Salmonella* serovars (Knudsen *et al.*, 2011; Hiramatsu *et al.*, 2005;Møretrø *et al.*, 2003). However, in these studies different *Salmonella* serovars were studied and survival was examined at other temperatures.

Analysis of survival of planktonic cells on dry SS surfaces indicated that *S.* Derby and *S.* Brandenburg isolates could survive up to 14 days (Fig. 4.4). This is consistent with other studies that also found long term survival of *Salmonella* planktonic cells (Møretrø *et al.*, 2010; Hiramatsu *et al.*, 2005; Allan *et al.*, 2004; De Cesare *et al.*, 2003; Kusumaningrum *et al.*, 2003). For nearly all isolates, biofilm survival in terms of quantity and duration was, with the exception of isolate SI-I42, greater than for planktonic cells (Fig. 4.4 and 4.5). Increased desiccation tolerance of cells within a biofilm has been related to the protective properties of matrix components (Espinal *et al.*, 2012; Iibuchi *et al.*, 2010). Since biofilms grown in selected conditions used in our study did not produce significant amounts of cellulose (data not shown and Castelijn *et al.* 2012), other factors including phenotypic changes related to attachment or the presence of persisters/stress-variants within a biofilm may contribute to the long term survival of the cells within the biofilms (Møretrø *et al.*, 2012; Scher *et al.*, 2005). Nevertheless, the presence of other extracellular matrix structures besides cellulose was not investigated and remains to be elucidated. Also serovar and strain-specific parameters contributing to long-term survival were not identified.

The data obtained by survival analysis of planktonic cells and biofilms expresses the need for sufficient cleaning and disinfection in pork processing environments, because the surviving cells might form a risk for contamination of products. Especially given the fact that cells survive even longer in the presence of organic material that might be present on processing equipment (Møretrø *et al.*, 2010; Allan *et al.*, 2004; De Cesare *et al.*, 2003). Furthermore, exposure to desiccation may result in cross-protection to several disinfection treatments (Gruzdev *et al.*, 2011; Kieboom *et al.*, 2006).

A disinfectant is considered to be effective if it reduces a microbial population by 5 log in suspension and by 3 log for microbial cells attached to a surface (Surdeau *et al.*, 2006; Wirtanen and Salo, 2003). A disinfectant commonly used in the food industry, PAA (Mueller-Doblies *et al.*, 2010; Wirtanen and Salo, 2003), was shown by our results to meet these directives for both planktonic cells and biofilms in the absence of BSA (Fig. 4.6). PAA or commercial disinfectants containing PAA have been shown previously to be efficient in reducing *Salmonella* grown in suspension or biofilms (Møretrø *et al.*, 2003; Wirtanen and Salo, 2003). Disinfectant exposure showed only marginal differences between the susceptibility for PAA of planktonic cells and biofilms (Fig. 4.6). This was unexpected, since it is commonly report-

ed that biofilms are less sensitive for disinfection treatments than planktonic cells (Wong *et al.*, 2010; Scher *et al.*, 2005; Joseph *et al.*, 2001). This phenomenon has been reported for PAA, however only for microorganisms other than *Salmonella* (der Veen and Abee, 2011; Surdeau *et al.*, 2006; Van Stopforth *et al.*, 2002). Similar sensitivity to PAA of planktonic cells and biofilms in our studies are best explained by lack of a dense cellulose matrix in biofilms (see above).

In pork processing environments, despite the cleaning step, organic material is likely to be present during the disinfection treatment. Notably, in the presence of 3% BSA, simulating the presence of organic material, activity of PAA was almost fully blocked. This is in line with previous studies using only suspension tests (Møretrø *et al.*, 2010; Allan *et al.*, 2004; De Cesare *et al.*, 2003; Wirtanen and Salo, 2003), and these data indicate that the presence of organic material may enhance the survival of *Salmonella* in pork processing environments.

Conclusion

This study showed variation in biofilm forming capacity between and within the four tested serovars, but no differences were observed between their biofilm formation on PS and SS surfaces. On such surfaces, biofilms showed greater and longer survival than planktonic cells, and they were less susceptible to peracetic acid disinfection treatments. However, the latter effect was marginal and only observed in the presence of organic material, which drastically decreased the activity of PAA. Taken these results together indicates that biofilm formation contributes to the survival of *Salmonella* in pork processing environments. The findings of this study might support the development of better strategies for control in order to reduce the transfer of *Salmonella* to pork products during processing thus minimizing the risk of cross contamination.

Acknowledgements

The authors would like to thank Linda McPhee for editing the paper and for her advice on organization and style.

REFERENCES

Allan J, Yan Z, Kornack, J. 2004., Surface material, temperature, and soil effects on the survival of selected foodborne pathogens in the presence of condensate. J Food Protect 67:2666-2670.

Boddicker JD, Ledeboer, NA, Jagnow J, Jones, BD, Clegg, S. 2002. Differential binding to and biofilm formation on, HEp-2 cells by *Salmonella enterica* serovar Typhimurium is dependent upon allelic variation in the *fimH* gene of the *fim* gene cluster. Mol Microbiol 45:1255-1265.

Botteldoorn N, Heyndrickx M, Rijpens N, Grijspeerdt K, Herman L. 2003. *Salmonella* on pig carcasses: positive pigs and cross contamination in the slaughterhouse. J Appl Microbiol 95:891-903.

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

Chia TWR, Goulter RM, McMeekin T, Dykes GA, Fegan N. 2009. Attachment of different *Salmonella* serovars to materials commonly used in a poultry processing plant. Food Microbiol 26:853-859.

De Busser V, Maes D, Houf K, Dewulf J, Imberechts H, Bertrand S, De Zutter L. 2011. Detection and characterization of *Salmonella* in lairage, on pig carcasses and intestines in five slaughterhouses. Int J Food Microbiol 145:279-286.

De Cesare A, Sheldon BW, Smith KS, Jaykus LA. 2003. Survival and persistence of *Campylobacter* and *Salmonella* species under various organic loads on food contact surfaces. J Food Protect 66:1587-1594.

European Food Safety Authority (EFSA). 2008. Report of the task force on zoonoses data collection on the analysis of the baseline survey on the prevalence of *Salmonella* in slaughter pigs, Part A. EFSA J 135:1-111.

EFSA, European Centre for Disease and Control, (ECDC). 2011. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. EFSA J 2011 9(3):2090.

EFSA. 2011. Estimation of the relative contribution of different food and animal sources to human *Salmonella* infections in the European Union.

EFSA panel of Biological Hazards (BIOHAZ). 2010. Scientific opinion on a quantitative microbiological risk assessment of *Salmonella* in slaughter and breeder pigs. EFSA J 8:1-80.

Espinal P, Martí S, Vila J. 2012. Effect of biofilm formation on the survival of *Acinetobacter baumannii* on dry surfaces. J Hospital Infect 80:56-60.

Foley SL, Lynne AM, Nayak R. 2008. *Salmonella* challenges: Prevalence in swine and poultry and potential pathogenicity of such isolates. J Animal Sci 86:E149-E162.

Gruzdev N, Pinto R, Sela S. 2011. Effect of desiccation on tolerance of *Salmonella enterica* to multiple stresses. Appl Environ Microbiol 77:1667-1673.

Hiramatsu R, Matsumoto M, Sakae K, Miyazaki Y. 2005. Ability of Shiga toxin-producing *Escherichia coli* and *Salmonella spp*. to survive in a desiccation model system and in dry foods. Appl Environ Microbiol 71:6657-6663.

Hood SK, Zottola EA. 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. Int J Food Microbiol 37:145-153.

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

Joseph B, Otta SK, Karunasagar I, Karunasagar I. 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. Int J Food Microbiol 64:367-372.

Kieboom J, Kusumaningrum HD, Tempelaars MH, Hazeleger WC, Abee T, Beumer RR. 2006. Survival, elongation, and elevated tolerance of *Salmonella* enterica serovar Enteritidis at reduced water activity. J Food Protect 69:2681-2686.

Knudsen GM, Sommer HM, Sørensen ND, Olsen JE, Aabo S. 2011. Survival of *Salmonella* on cuts of beef carcasses subjected to dry aging. J Appl Microbiol 111:848-854.

Kusumaningrum HD, Riboldi G, Hazeleger WC, Beumer RR. 2003. Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. Int J Food Microbiol 8:227-236.

Malcova M, Hradecka H, Karpiskova R, Rychlik I. 2008. Biofilm formation in field strains of *Salmonella enterica* serovar Typhimurium: Identification of a new colony morphology type and the role of SGI1 in biofilm formation. Vet Microbiol 129:360-366.

Møretrø T, Heir E, Mo KR, Habimana O, Abdelgani A, Langsrud S. 2010. Factors affecting survival of Shigatoxin-producing *Escherichia coli* on abiotic surfaces. Int J Food Microbiol 138:71-77.

Møretrø T, Heir E, Nesse LL, Vestby LK, Langsrud S. 2012. Control of *Salmonella* in food related environments by chemical disinfection. Food Res Int 45:532-544.

Møretrø T, Midtgaard ES, Nesse LL, Langsrud S. 2003. Susceptibility of *Salmonella* isolated from fish feed factories to disinfectants and air-drying at surfaces. Vet Microbiol 94:207-217.

Mueller-Doblies D, Carrique-Mas JJ, Sayers AR, Davies RH. 2010. A comparison of the efficacy of different disinfection methods in eliminating *Salmonella* contamination from turkey houses. J Appl Microbiol 109:471-479.

Römling U, Bokranz W, Rabsch W, Zogaj X, Nimtz M, Tschäpe H. 2003. Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. Int J Med Microbiol 293:273-285.

Scher K, Römling U, Yaron S. 2005. Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. Appl Environ Microbiol 71:1163-1168.

Shi X, Zhu X. 2009. Biofilm formation and food safety in food industries. Trends in Food Sci Technol 20:407-413.

Solomon EB, Niemira, BA, Sapers GM, Annous BA. 2005. Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical

sources. J Food Protect 68:906-912.

Stopforth JD, Samelis J, Sofos JN, Kendall PA, Smith GC. 2002. Biofilm formation by acid-adapted and nonadapted *Listeria monocytogenes* in fresh beef decontamination washings and its subsequent inactivation with sanitizers. J Food Protect 65:1717-1727.

Surdeau N, Laurent-Maquin D, Bouthors S, Gellé MP. 2006. Sensitivity of bacterial biofilms and planktonic cells to a new antimicrobial agent, Oxsil® 320N. J Hospital Infect 62:487-493.

Tresse, O., Shannon, K., Pinon, A., Malle, P., Vialette, M., le, Midelet-Bourdin, G., 2007, Variable adhesion of *Listeria monocytogenes* isolates from food-processing facilities and clinical cases to Inert surfaces. J Food Protect 70, 1569-1578.

Van der Veen S, Abee T. 2011. Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. Int J Food Microbiol 144:421-431.

Van Hoek AHAM, de Jonge R, van Overbeek WM, Bouw E, Pielaat A, Smid JH, Malorny B, Junker E, Löfström C, Pedersen K, Aarts HJM, Heres L. 2012. A quantitative approach towards a better understanding of the dynamics of *Salmonella spp*. in a pork slaughter-line. Int J Food Microbiol 153:45-52.

Vestby L, Møretrø T, Langsrud S, Heir E, Nesse L. 2009. Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal- and feed factories. BMC Vet Res 5:20.

Volf J, Havlickova H, Hradecka H, Ondrackova P, Matiasovic J, Faldyna M, Rychlik I. 2010. Epidemiology and interaction of *Salmonella enterica* serovar Derby, Infantis and Typhimurium with porcine alveolar macrophages. Vet Microbiol 146, 105-110.

Wirtanen G, Salo S. 2003. Disinfection in food processing – Efficacy testing of disinfectants. Rev Environ Sci Biotechnol 2:293-306.

Wong HS, Townsend KM, Fenwick SG, Trengove RD, O'Handley RM. 2010. Comparative susceptibility of planktonic and 3-day-old *Salmonella* Typhimurium biofilms to disinfectants. J Appl Microbiol 108:2222-2228.

CHAPTER 5

Isolation and characterization of resistant variants from *Salmonella* Typhimurium cell cultures treated with benzalkonium chloride

Greetje A.A. Castelijn, Stijn van der Veen, Marcel H. Zwietering, Roy Moezelaar and Tjakko Abee

Submitted for publication

ABSTRACT

An efficient disinfection strategy within the food industry is needed to ensure food safety and prevent the spreading of foodborne pathogens such as *Salmonella* Typhimurium. However, several factors affect the efficiency of disinfection treatments resulting in low level exposure of the bacterial flora facilitating survival of bacterial cells. Therefore, this study analysed the effect of repeated sub-lethal BKC exposure of *S.* Typhimurium cells. One single BKC exposure showed tailing of the inactivation curve indicating variation in BKC susceptibility within the initial population. Repeated exposure resulted in the enrichment of a more resistant population and variants with increased BKC resistance were isolated. Phenotypical characterization of these resistant variants showed loss of motility, reduced biofilm forming capacity and reduced cell membrane permeability pointing to modification of cell surface properties. In conclusion, repeated exposure of *S.* Typhimurium to sub-lethal BKC concentrations rapidly selects for resistant variants. Occurrence and persistence of such variants in food processing environments requires further study.

INTRODUCTION

To ensure food safety and prevent spreading of bacterial pathogens, processing equipment and the production environment in the food industry are frequently disinfected. A disinfectant commonly used in the food industry is benzalkonium chloride (BKC) (Møretrø *et al.* 2012; Gradel *et al.* 2005). It is a quaternary ammonium compound consisting of a mixture of alkylbenzyldimethylammonium chlorides of various alkyl chain lengths. The bactericidal activity of BKC is primarily due to the interaction of the agent with the cytoplasmic membrane. This results in membrane disorganization that increases the permeability and induces leakages of cellular content leading finally to cell lysis. Furthermore, BKC causes dissociation of other biomolecular complexes such as enzymes that control a wide range of metabolic and respiratory activities (Joynson *et al.* 2002; McDonnell and Russell 1999).

The effectiveness of a disinfectant treatment depends on biocide concentration and contact time with the bacterial flora. These two parameters might be far from optimal within industrial settings due to lack of time, incorrect use or the presence of organic material (Castelijn *et al.* 2013; Møretrø *et al.* 2012). Also biofilm formation can result in insufficient disinfection, since large quantities of extracellular matrix limit the penetration of a biocide into the biofilm which results in low level (sub-lethal) exposure of the cells in the deeper region (Bridier *et al.* 2011; Davison *et al.* 2010). Due to these different factors a disinfection treatment is not always sufficient in removing and killing the complete bacterial flora present. Therefore residual bacteria are repeatedly exposed and this might lead to the generation and/or selection of bacterial cells with increased resistance, so-called resistant variants. The increased resistance of the variants could facilitate survival of these cells and forms therefore a risk for public health (Gradel *et al.* 2005), especially since adaptation to chemical disinfectants can result in cross-protection against clinical antibiotics (Condell *et al.* 2012; Copitch *et al.* 2010; Braoudaki and Hilton 2004; Levy 2002).

Several studies show that prolonged exposure of pathogenic bacteria to sub-lethal BKC concentrations result in adaptation and increased resistance (Mangalappalli-Illathu *et al.* 2008; Bore *et al.* 2007; Langsrud *et al.* 2004; Joynson *et al.* 2002; Loughlin *et al.* 2002). This phenomenon is also described for the emerging foodborne pathogen *Salmonella* Typhimurium (Condell *et al.* 2012; Gradel *et al.* 2005; Karatzas *et al.* 2007; Braoudaki and Hilton 2004). These studies were performed *in vitro*, but there are concerns that adaptation to BKC also occurs in practice.

Various mechanisms are reported to be involved in adaptation to BKC. Among them adjustment of the outer membrane by modification of the LPS and/or fatty acid composition (Karatzas *et al.* 2008; Mangalappalli-Illathu *et al.* 2008; Loughlin *et al.* 2002), increase in the total lipid content by membrane blebbing (Joynson *et al.* 2002) or alterations in the

outer membrane porin proteins (Hu *et al.* 2011; Ishikawa *et al.* 2002). Another mechanism that is most frequently associated with increased BKC resistance is overexpression and elevated efflux pump activity (Karatzas *et al.* 2008; Braoudaki and Hilton 2005; Gradel *et al.* 2005; Langsrud *et al.* 2004). Efflux pumps are trans-membrane proteins that actively export substances from the cell interior to the extracellular environment (Piddock 2006). Furthermore, activation of different stress responses, alteration of the cytoplasmic membrane, and changing the cell surface charge or hydrophobicity have been reported to contribute to BKC resistance (Møretrø *et al.* 2012; Karatzas *et al.* 2008; Bore *et al.* 2007; Braoudaki and Hilton 2005; Loughlin *et al.* 2002).

So far, studies on BKC resistance of *Salmonella* concerned adaptation to sub-lethal concentrations of BKC obtained by constant exposure. However within industrial settings bacterial cells are not constantly exposed to a biocide since the processing equipment is periodically disinfected. Therefore this study investigated the effect of repeated BKC exposure of *S.* Typhimurium cells. A sub-lethal BKC concentration (75 ppm) was used to mimic inadequate disinfection treatments in food processing environments. The obtained resistant variants were phenotypically characterized to identify the mechanisms contributing to the enhanced BKC tolerance.

MATERIAL AND METHODS

Bacterial strains and growth conditions

In this study *S.* Typhimurium strain I4 was used. This strain was also used in previous studies (Castelijn *et al.* 2012) and will be referred to as WT-I4 in the current study. The strain was kept at -80 °C in Brain Hearth Infusion broth (BHI, Becton Dickson) supplemented with 15% glycerol (Sigma). From the bacterial -80 °C stocks, the strain was streaked on Tryptone Soya Agar (TSA, Oxoid) and incubated overnight at 25 °C. Next, single colonies were inoculated in 10 ml of Tryptone Soya Broth (TSB, Oxoid) and the cultures were statically incubated at 25 °C for 24 h after which they were used in the different experiments which were all performed in triplicate.

Susceptibility to benzalkonium chloride

To determine the inactivation kinetics due to BKC, aliquots of a 24h-old TSB cell culture containing ~8.0 log CFU were centrifuged (3 min at 5000x *g*) and the pellet was washed once with 1 ml of sterile deionized water. After the washing step, the cells were suspended in 1 ml of sterile deionized water and from this cell suspension 100 µl was used as t=0. Next BKC (Merck) was added to the cell suspension to a final concentration of 75 ppm BKC and the cells were exposed for 1, 3, 6, 9, 12, 15 or 30 min. After exposure, all samples from the different time points were diluted ten times in Dey-Engley Neutralizing medium (Becton Dick-

ison), serial diluted in physiological salt solution (pps) and plated on TSA plates. The plates were incubated for 48 h at 25 °C after which the colonies were enumerated. After 15 and 30 min of exposure, also the concentration of BKC was determined by use of QUANTOFIX® QUAT test strips (Macherey-Nagel), 0 to 1000 mg/L BKC.

Isolation of resistant variants

For isolation of resistant variants, aliquots of a 24h-old TSB cell culture containing ~8.0 log CFU were centrifuged (3 min at 5000x *g*) and the pellet was washed once with 1 ml of sterile deionized water. Then the samples were exposed for 15 min to 75 ppm BKC and subsequently ten times diluted in Dey-Engley Neutralizing medium. After 5 min incubation in Dey-Engley Neutralizing medium, the samples were centrifuged (3 min at 5000x *g*) and washed once with 1 ml of TSB. Next, the samples were re-suspended in 1 ml of TSB and 100 µl of this bacterial suspension was used to inoculate 10 ml of TSB. After 24 h of incubation at 25 °C, the cell culture was exposed to 75 ppm BKC again as described in the procedure above. In total this procedure was repeated four times. After four times BKC exposure, the 24h-old TSB cell culture was spread on TSA and incubated 24 h at 25 °C. Then, 75 single colonies were randomly picked to inoculate 10 ml of TSB. After 24 h of incubation at 25 °C, -80 °C bacterial stocks were made from each cell culture. Next, the susceptibility for BKC (75 ppm) was analysed for every selected variant.

Fitting of the inactivation data

The inactivation data was fitted with the biphasic linear model (equation 1). This model represents the inactivation of two populations with different resistance (van Boeijen *et al.* 2008; Cerf and Metro 1977). The biphasic linear model can be formulated as followed:

$$\log_{10} N(t) = \log_{10} N(0) + \log_{10} [\ (1 - f)\ .\ 10^{-t/D_{sens}} + f\ .\ 10^{t/D_{res}}\ ] \qquad (1)$$

were (1 f) is the sensitive and f is the resistant fraction of the population. The values D_{sens} and D_{res} are the decimal reduction time, which is the time needed for 1 log reduction of the population, of the sensitive and resistant population, respectively.

Growth kinetics

Growth kinetics at 25 °C were measured for cultures grown in TSB. The optical density at 600 nm was measured in time and the maximum specific growth rate (μ_{max} in h^{-1}) was calculated with the modified Gompertz equation (Zwietering *et al.* 1990) using the TableCurve 2D software package (version 2.03; Jandel Scientific, San Rafael, CA).

Biofilm formation

The biofilm formation was analysed with the CV-assay in polystyrene 96 microtiter plates (Greiner Bio-one) as describes previously by Castelijn *et al.* (2012a). Biofilms were grown in TSB and 20 times diluted TSB (1/20TSB) at 25 °C for 24 h.

Motility test

The motility of the variants was analysed in semisolid medium containing 0.4% (wt/vol) agar (BBL Motility Test Medium, Becton Dickinson) supplemented with 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich Chemie). A tube containing 10 ml of semisolid medium was inoculated by stabbing and incubated for 48 h at 25 °C, after which the tubes were examined for motility.

Antibiotic susceptibility

Antibiotic susceptibility was analysed using Etest strips (BioMérieux) according to the manufacturer's protocol. Susceptibility was determined for amikacin, amiocillin/clavulanic acid, ampicillin, cefoxitin, ceftriaxone, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, tetracyclin, trimethoprim and vancomycin. These antibiotics comprise different antimicrobial classes defined by the Clinical and Laboratory Standards Institute (CLSI).

Hydrophobicity of the bacterial cell surface

Cell surface hydrophobicity was analysed with the microbial adhesion to hydrocarbon (MATH) method. For this purpose, a 24h-old TSB cell culture was centrifuged (3 min 5000x g), washed once with phosphate buffered saline (pbs) and the pellet was re-suspsended in 10 ml pbs. The OD_{600} of this cell suspension (A_0) was measured and 2.5 ml was added to 0.5 ml hexadecane and vortexed for 1 min. After separation of the water (polar) and n-hexadecane (apolar) phase the OD_{600} of the water phase (A) was measured again in order to calculated the percentage of cells in the apolar phase (equation 2).

$$\% \text{ bounded cells} = 100 \cdot 1 - A/A_0 \qquad (2)$$

Permeability assay

Uptake of the fluorescent N-phenyl-naphthalene (NPN) probe was applied to determine permeability changes of the outer membrane of the the resistant variants versus the wild-type (Langsrud *et al.* 2004; Alkomi *et al.* 2003). NPN gives a fluorescent signal in the hydrophobic environment of the bacterial inner membrane in Gram-negative bacteria. An intact outer membrane is a permeability barrier and excludes hydrophobic substances, such as NPN, resulting in low fluorescence, but once damaged it can allow the entry of NPN to the inner phospholipid bilayer, resulting in prominent fluorescence. The access of NPN to the the inner phospholipid bilayer is enhanced by the activity of membrane-active compounds

like EDTA and BKC. Exposure to BKC results in different cell counts between the wild-type and the selected resistant variants. Therefore, to enable a good comparison between the wild-type and the resistant variants, only the effect of EDTA on cell membrane permeability was analysed.
For the assay, 2 ml of a 24h-old TSB culture was centrifuged (3 min at 5000x *g*), washed once with 1 ml of 5mM HEPES (pH 7) and the pellet was re-suspended in 2 ml of 5mM HEPES. Aliquots of 100 µl of this cell suspension were transferred to a black polystyrene microtiter plate (Greiner Bio-one) containing 10 µM NPN, 0.1mM, 1mM EDTA or HEPES buffer (as a control) to make a final volume of 200 µl. Fluorescence (excitation 340 nm bandwidth 6 nm and emission at 400 nm bandwidth 10 nm) was measured for 15 min on an Infinite®F500 (Tecan) plate reader.

Efflux pump activity
The transport of ethidium bromide (EtBr) was analysed as a measure of efflux pump activity as described by Langsrud et al. (2004). Briefly, 10 ml of a 24h-old TSB cell culture was centrifuged (3 min at 5000x g) and washed once with 1.5 ml of 20 mM HEPES (pH 7). The washed cells were re-suspended in 1.5 ml 20 mM HEPES (pH7) and 20 µl of this cell suspension was transferred to a black polystyrene microtiter plate (Greiner Bio-one) containing 160 µl of 20 mM HEPES (pH 7) and 10 µl of 100 µM EtBr (Bio-Rad). Then the fluorescence (excitation 520 nm bandwidth 6 nm and emission at 590 nm bandwidth 10 nm) was measured for 5 min with an Infinite®F500 (Tecan) plate reader after which 10 µl of 1% glucose (Oxoid) was added to initiate the efflux activity and the fluorescence measurement was continued.

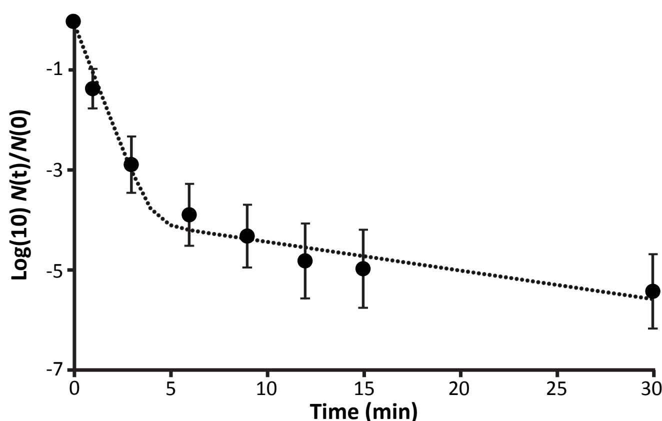


Figure 5.1: Inactivation kinetics of WT-I4 exposed to 75 ppm BKC for 30 min (indicated by circles). The data points represent the average of five biological replicates with the corresponding standard deviation. The inactivation kinetics is fitted with the biphasic linear model (indicated by the dotted line).

RNA isolation and cDNA synthesis

Total RNA was isolated from WT-I4 and the selected resistant variants at t=0 and t=3 min during the BKC exposure (as described above). After quenching BKC, the cells were quenched with RNAprotect Bacteria Reagent (Qiagen) and harvested by centrifugation (3 min 5000x *g*). Next total RNA was isolated using the RNeasy kit (Qiagen) with an on-column DNase treatment (Qiagen) according to the manufacturer's protocol. Then for each sample 500 ng of total RNA was converted into cDNA using the SuperScript III reverse transcriptase kit (Invitrogen) according to the manufacturer's protocol. For each sample a non-reverse transcriptase sample was included to check for genomic DNA (gDNA) contamination.

Quantitative real-time PCR

For Q-PCR, 5 µl of ten times diluted cDNA sample and 1 µl of both the forward and reverse primer (10 µM) were added to 12.5 µl of 2x SYBR® Green PCR Master Mix (Applied Biosystems) and demineralized water was added to a final volume of 25 µl. The Q-PCR reactions were run on an ABI Prism 7000 Sequence Detection System with an initial step of 10 min at 95 °C, and 40 cycli of 15 s at 95 °C and 1 min at 60 °C. Furthermore a melting curve was added to check for amplification specificity, and a standard curve was derived for each primer set to determine the amplification efficiency. The primers of the reference (16S *rRNA* and *rpoD*) and target genes are listed in Table S5.1. Q-PCR was performed on three biological independent experiments using two technical replicates.

RESULTS

Inactivation kinetics of WT-I4

The S. Typhimurium strain WT-I4 was exposed to 75 ppm BKC for 30 min. The inactivation curve reveals two different phases of inactivation resulting in tailing after approximately 5 min (Fig. 5.1). This phenomenon was not caused by a decreasing level of BKC, since the BKC concentration was still 75 ppm after 30 min of exposure.

Tailing inactivation can be described by a biphasic linear model (Fig. 5.1 and Boeijen *et al.* 2008) and indicates the presence of two sub-populations within the initial population with different susceptibility. The difference in susceptibility of these two sub-populations was reflected by a difference in the *D*-values (Table 5.1). The D-value is the time needed for 1 $\log_{10}$ reduction in number of cells and was about 17 times higher for the more resistant sub-population (so-called resistant population, D_{res}) compared to the more sensitive sub-population (so-called sensitive population D_{sen}).

Table 5.1: The parameters of the fitted biphasic linear model of WT-I4 and the selected resistant variants I4-2, I4-3 and I4-59, and the parameters from the fitted log linear model of I4-3 and I4-59.

	Biphasic model		Log linear model
	D_{sens}	D_{res}	D
WT-I4	0.993	17.42	
I4-2	1.400	26.67	
I4-3	$1 \cdot 10^{-10}$	21.32	20.36
I4-59	$1 \cdot 10^{-10}$	48.07	41.95

Effect of repeated BKC exposure and isolation of resistant variants

The results in figure 5.1 illustrate the inactivation kinetics of one single BKC exposure. However, in practice food processing equipment is repeatedly disinfected and therefore cells that survive one disinfection treatment, like the survivors in the tail, are repeatedly exposed. The effect of repeated exposure to the same BKC concentration during 15 min was analysed by re-exposure of the sub-population that survived one BKC exposure step (the survivors in the tail). This revealed that repeated exposure resulted in a reduction of the total inactivation (Fig. 5.2). After four exposure steps, or more, the susceptibility for BKC did not decrease any further and remained at 1 log CFU reduction after 15 min exposure. Therefore these results reveal that repeated exposure to the same BKC concentration leads to an enrichment of the resistant sub-population.

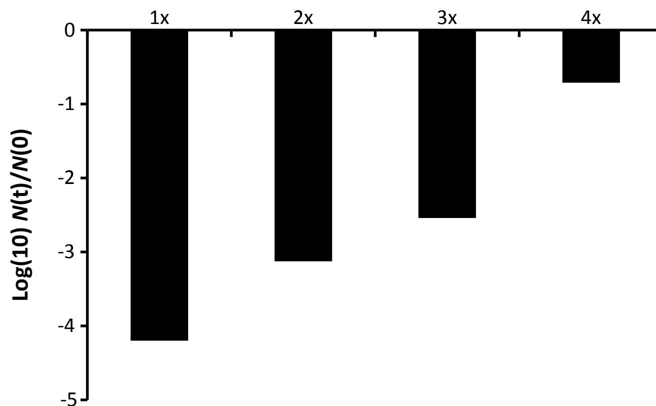


Figure 5.2: Enrichment for resistant variants by repeated exposure to 75 ppm BKC. Times of 15 min exposure to BKC is indicated on the *x*-axis.

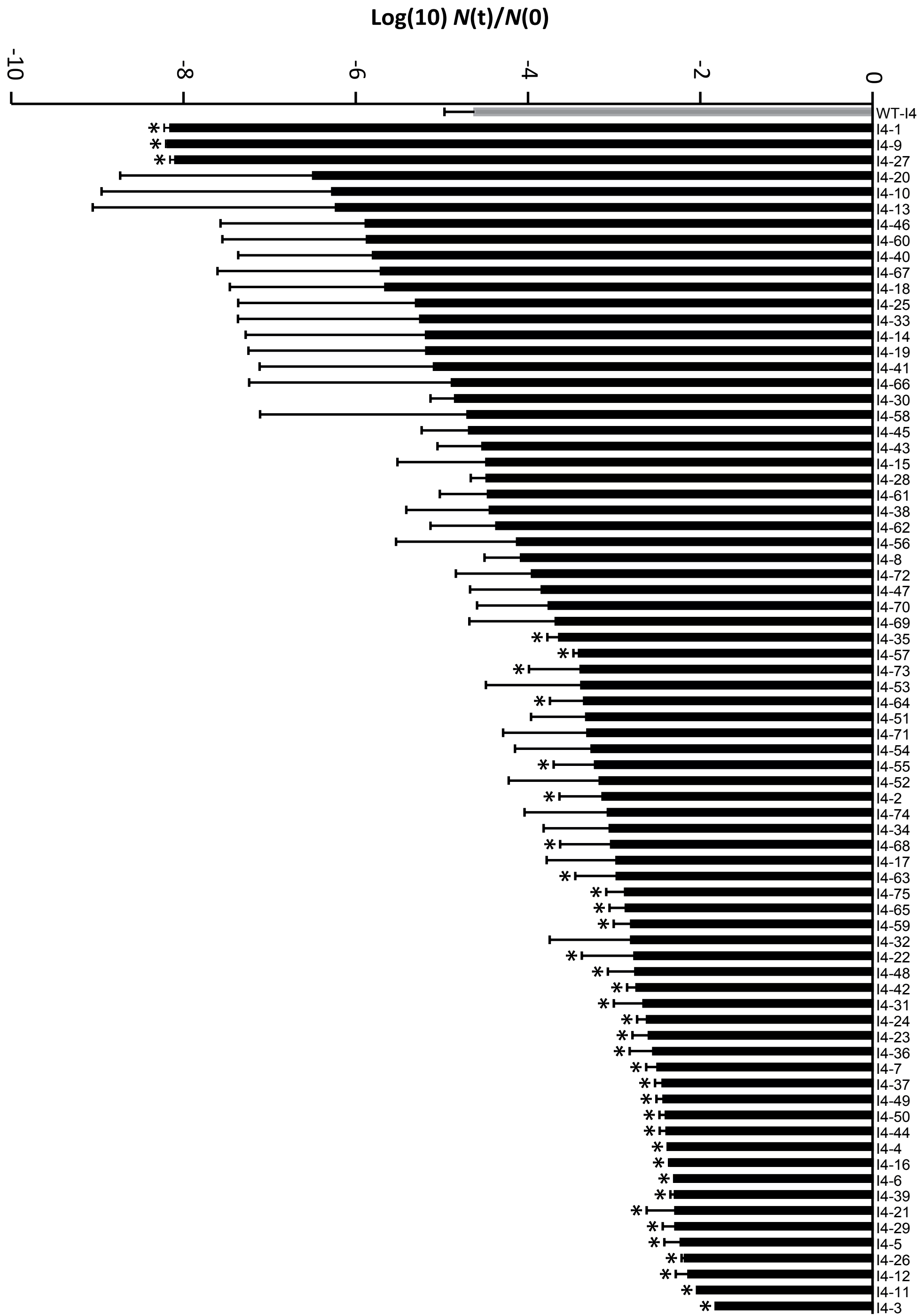
To assess whether the increased resistance is stable and which features are responsible for the increased resistance, resistant variants were isolated. For this purpose, the cell culture that was exposed four times (Fig. 5.2) was grown for 24 h and spread on agar plates (without BKC) to obtain single colonies. Next, single colonies were randomly picked, grown to 24h-old cell cultures and their BKC susceptibility was analysed as described in the material and method section. This revealed that 41 out of 75 (55%) cell cultures from the randomly picked colonies had no significantly increased BKC resistance compared to the wild-type (Fig. 5.3). Three of them were even significantly less resistant suggesting temporary increased resistance due to phenotypic switching (Pennell *et al.* 2008; Van Boeijen *et al.* 2008; Levin 2004). However, 34 out of the 75 (45%) cell cultures from the randomly picked colonies were significantly more resistant than the wild-type ($p < 0.05$, t-test) and were considered to be resistant variants.

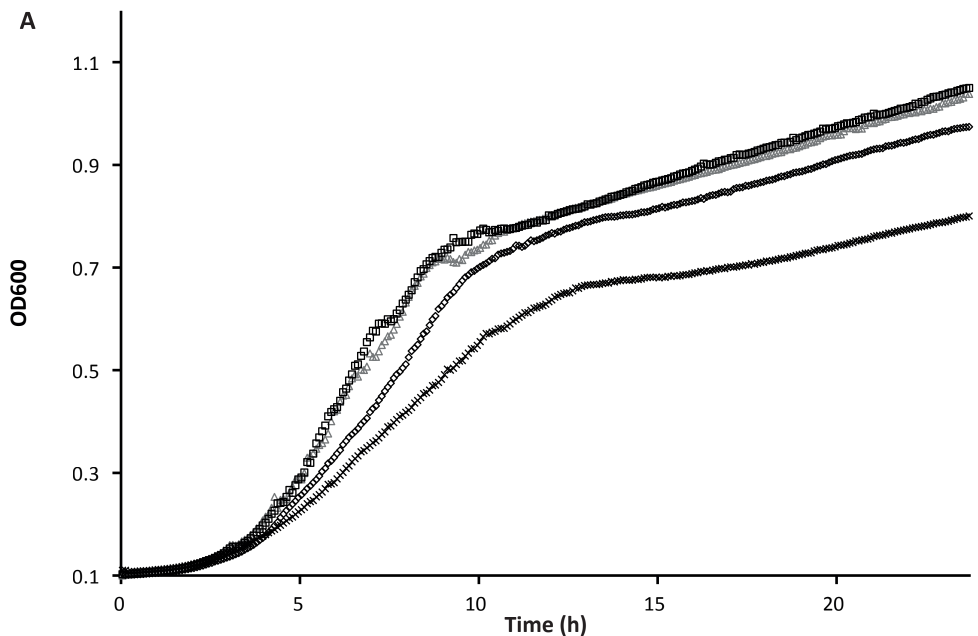
Phenotypic characterization of the resistant variants

The resistant variants were phenotypically characterized by analyzing their growth kinetics, biofilm forming capacity and motility. Their growth kinetics at 25 °C were comparable to WT-I4, except for resistant variant I4-59 that showed a reduced growth rate (Fig. 5.4A). In addition, I4-59 showed significant less total biofilm formation in 1/20TSB (Fig. 5.4B). Therefore this resistant variant is phenotypically different from the WT-I4 and the other resistant variants. But the phenotypic characterization revealed more differences among the resistant variants. Three resistant variants (I4- 35, I4-2 and I4-64) showed similar biofilm forming capacity in 1/20TSB and motility to the wild-type. The other 30 resistant variants exhibited significant less biofilm forming capacity in 1/20TSB, which was correlated to reduced motility (Fig. 5.4B).

Based on the BKC resistance after 15 min exposure, and the phenotypic characterization described above, three resistant variants with different phenotypes were selected for further research: I4-2, I4-3 and I4-59.

Figure 5.3: Exposure of the variants selected after the enrichment by four times exposure to 75 ppm BKC for 15 min. The inactivation of WT-I4 is indicated by a grey bar, and the inactivation of the variants are indicated by black bars. The data points represent the average of three biological replicates with the corresponding standard deviation. Significant different values ($p < 0.05$, t-test) compared with the wild-type are indicated by an asterisk.


Log(10) N(t)/N(0)
-10
-8
-6
-4
-2
0
WT-I4
I4-1
I4-9
I4-27
I4-20
I4-10
I4-13
I4-46
I4-60
I4-40
I4-67
I4-18
I4-25
I4-33
I4-14
I4-19
I4-41
I4-66
I4-30
I4-58
I4-45
I4-43
I4-15
I4-28
I4-61
I4-38
I4-62
I4-56
I4-8
I4-72
I4-47
I4-70
I4-69
I4-35
I4-57
I4-73
I4-53
I4-64
I4-51
I4-71
I4-54
I4-55
I4-52
I4-2
I4-74
I4-34
I4-68
I4-17
I4-63
I4-75
I4-65
I4-59
I4-32
I4-22
I4-48
I4-42
I4-31
I4-24
I4-23
I4-36
I4-7
I4-37
I4-49
I4-50
I4-44
I4-4
I4-16
I4-6
I4-39
I4-21
I4-29
I4-5
I4-26
I4-12
I4-11
I4-3


A
OD600
1.1
0.9
0.7
0.5
0.3
0.1
0
5
10
15
20
Time (h)

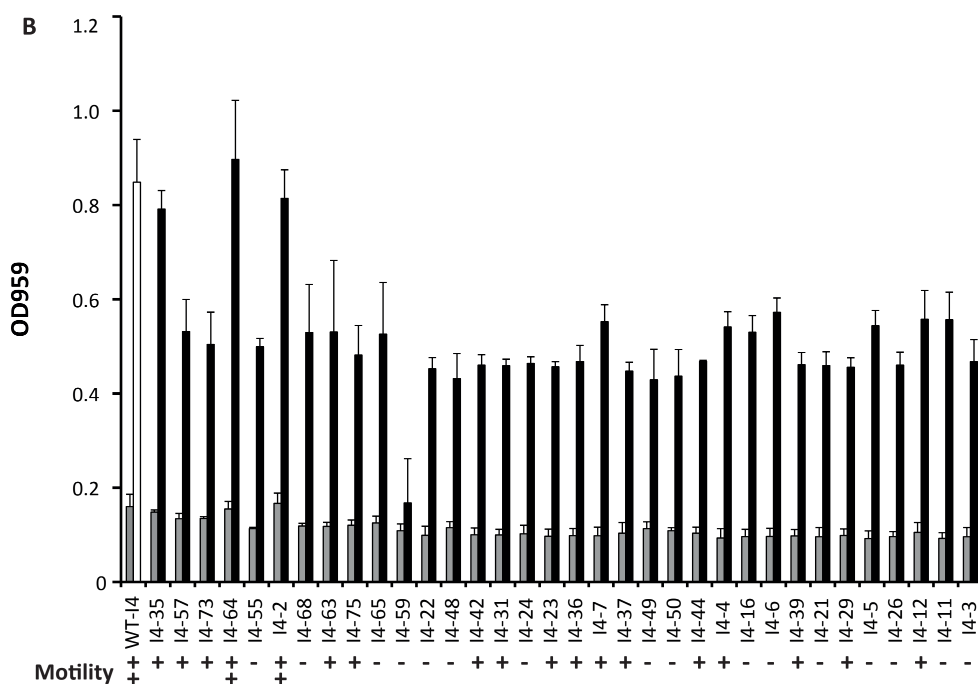

B
OD959
1.2
1.0
0.8
0.6
0.4
0.2
0
WT-I4
I4-35
I4-57
I4-73
I4-64
I4-55
I4-2
I4-68
I4-63
I4-75
I4-65
I4-59
I4-22
I4-48
I4-42
I4-31
I4-24
I4-23
I4-36
I4-7
I4-37
I4-49
I4-50
I4-44
I4-4
I4-16
I4-6
I4-39
I4-21
I4-29
I4-5
I4-26
I4-12
I4-11
I4-3
Motility
++ + + + ++ - ++ - + + - - - - + + - + + + + - - + + - - + - + - - + - -

Figure 5.4: A) Growth performance in TSB at 25 °C of WT-I4 (grey triangles), I4-2 (black squares), I4-3 (black diamonds) and I4-59 (black cross). For cleartity only the growth kinetics of these varinats are shown. B) Biofilm formation in TSB (grey bars) and 1/20TSB (black bars) and motility of WT-I4 and of the generated resistant variants. The motility is indicated on the *x*-axis.

Inactivation kinetics of the selected resistant variants

The selected resistant variants were exposed to 75 ppm BKC for 30 min (Fig. 5.5), and the inactivation data was fitted with the biphasic linear model (Table 5.1). This revealed that the inactivation curve of I4-2 could be described by this model as the inactivation curve showed tailing after approximately 5 min which was comparable to the wild-type (Fig. 5.1). However the *D*-value of the resistant sub-population of I4-2 was 1.5 times higher compared to WT-I4, meaning that the resistant subpopulation of I4-2 was more resistant than the one of the wild-type strain.

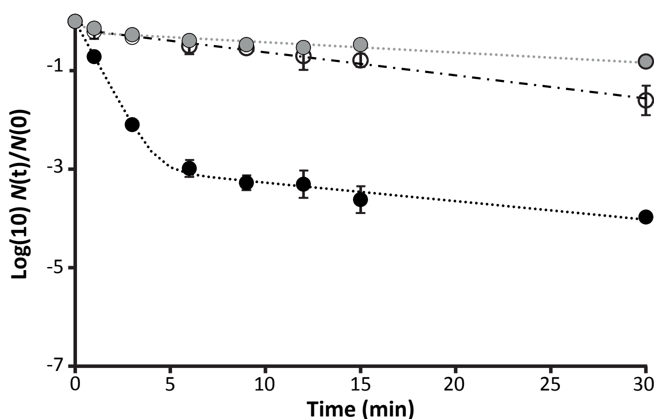


Figure 5.5: Inactivation kinetics of the selected resistant variants, I4-2 (black circles), I4-3 (open circles) and I4-59 (grey circles) during 30 min exposure to 75ppm BKC. The inactivation data of I4-3 and I4-59 was fitted with a log linear model (indicated by a dotted line) and the inactivation data of I4-2 was fitted with the biphasic linear model (indicated by a dotted line).

The inactivation curve of the resistant variants I4-3 and I4-59 showed that only time zero diverged from the other time points (Fig. 5.4). Therefore the inactivation data fitted well with the biphasic linear model, but due to the fact that the first phase only includes two data points (t=0 and t=1) this model was not used to describe the inactivation kinetics of these two resistant variants. This could also be concluded form the parameters obtained by fitting the biphasic model (Table 5,1). These parameters suggest that ~30% of the initial population

will be inactivated at an extremely high rate, namely 1 $\log_{10}$ inactivation in 1.10^{-10} minute. Since this is very unlikely, the inactivation data of I4-3 and I4-59 were fitted with a log linear model ($\log_{10} N(t) = \log_{10} N(0)-t/D$), meaning that the inactivation kinetics of these resistant variants were not comparable to the wild-type strain. Furthermore, fitting the inactivation data of the I4-3 and I4-59 with the log linear model (Table 5.1) revealed that the *D*-values of strains I4-3 and I4-59 are 21 and 42 times higher, respectively, compared to the D_{sens} of WT-I4 and 1.7 and 2.4 times higher compared to the D_{res} of WT-I4.

Cross-protection and efflux pump expression and activity

Increased BKC resistance is often correlated with elevated expression levels and activity of the multidrug resistant efflux pump AcrAB-TolC, resulting in cross-protection to clinical antibiotics and other chemical disinfectants (Li *et al.* 2011; Karatzas *et al.* 2008; Braoudaki and Hilton 2004; Levy 2002). However, in this study the selected resistant variants did not exhibit cross-protection to clinical important antibiotics (Table S5.2), including known substrates of the AcrAb-TolC efflux pump (amikacin, ampicillin, cefoxitin, chloramphenicol, ciprofloxacin, and tetracycline). In addition, no increased tolerance was observed for other chemical disinfectants; sodium hypochlorite, peracetic acid and also not for another quaternary compound, cetylpyridinium chloride (Table S5.2). In conclusion, no cross-protection was observed against other antimicrobial agents than BKC. In relation to this, no increased efflux pump activity was observed in resistant variants compared to the wild-type by analysing the transport of EtBr (data not shown). Furthermore, the expression level of *tolC*, encoding the outer membrane protein of the efflux pump, was not increased for the resistant variants compared to WT-I4, at both t=0 and t=3 min exposure to BKC, though, *tolC* expression was increased for all four isolates at t=3 compared to t=0 (Fig. 5.6). Besides the expression of *tolC*, the expression of *acrB*, the transporter protein located in the inner membrane, was analysed. This revealed that the expression was slightly higher for resistant variant I4-59 compared to WT-I4 at t=0. However, after 3 min exposure to BKC expression increased for both these strains and no significant difference could be detected between WT-I4 and the selected variants (Fig. 6). Taken these results together, it can be concluded that the increased BKC resistance of I4-2, I4-3 and I4-59 cannot be explained by increased expression, activity and/or activation of the AcrAB-TolC efflux pump (Table S5.2 and Fig. 5.6).

Condition of the bacterial cell envelope

Alterations in the outer membrane could be responsible for the increased BKC resistance of the resistant variants. Therefore the cell surface hydrophobicity and the outer membrane permeability were analysed. Hydrophobicity of the cell surface was analysed with the MATH assay and showed no differences between WT-I4 and the selected resistant variants (data not shown). So the increased BKC resistance of the resistant variants is not a result of alteration of the cell surface hydrophobicity.

To investigate the outer membrane permeability, NPN fluorescence was measured in the absence and presence of the outer membrane damaging agent EDTA (Fig. 5.7). This revealed that the resistant variants were more resistant to EDTA, since they showed a significant reducted NPN fluorescence, i.e., a reduced NPN-uptake compared to WT-I4. Higher resistance to EDTA-induced outer membrane permeability was observed for I4-3 and I4-59. The BKC resistance of these two resistant variants was also higher compared to the other resistant variant I4-2. These results suggest that alterations in the outer membrane may contribute to increased BKC tolerance of the resistant variants.

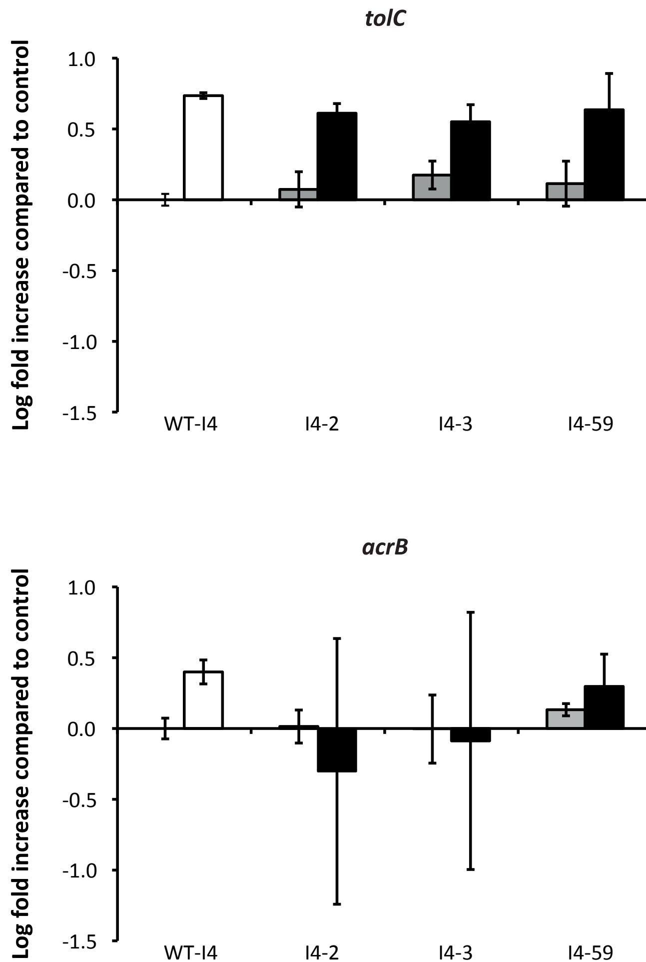


Figure 5.6: Expression of *acrB* and *tolC* of WT-I4 (white bars) and the selected resistant variants at t=0 (grey bars) and after 3 min exposure to 75 ppm BKC (black bars).

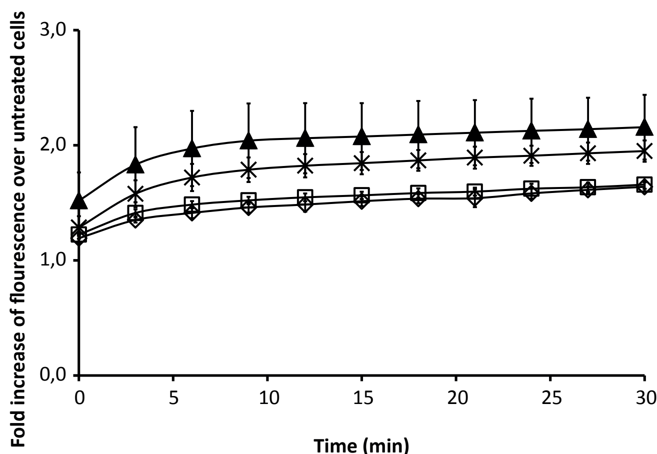


Figure 5.7: Fold increase of NPN into WT-I4 (black triangles), I4-2 (crosses), I4-3 (open squares) and I4-59 (open diamonds) upon treatment with 0.1 mM EDTA for 30 min. Similar results were obtained upon treatment with 1 mM EDTA (data not shown).

DISCUSSION

Previous studies have shown that constant exposure of bacterial cell cultures to gradually increasing sub-lethal BKC concentrations results in development of enhanced resistance (Karatzas *et al.* 2008; Mangalappalli-Illathu *et al.* 2008; Bore *et al.* 2007; Braoudaki and Hilton 2005; Langsrud *et al.* 2004; Joynson *et al.* 2002; Loughlin *et al.* 2002). However, in food processing environments persistent bacteria are not constantly exposed to disinfection chemicals, but periodically. This present study showed that also repeated exposure of *S.* Typhimurium cell cultures to the same sub-lethal BKC concentration rapidly results in enhanced resistance and selection of resistant variants. This indicates that inadequate disinfection treatments causing sub-lethal exposure of the bacterial flora results in the selection for resistant variants. The increased resistance against the disinfectant used might contribute to the persistence of *Salmonella* in food processing environments and the resistant variants could even become part of the house flora within a food processing environment (Gradel *et al.* 2005; Davison *et al.* 2003; Møretrø *et al.* 2003).

The present study revealed tailing of the inactivation curve of the industrial isolate WT-I4, indicating the presence of two sub-populations with different BKC susceptibility. Occurrence of sub-populations indicates that it is important to study the inactivation kinetics of a specific biocide in detail before selecting a disinfection strategy, since deviations from the gen-

erally assumed first-order kinetics apparently take place. In the present study, extrapolation of the inactivation data supposing first order kinetics suggests that 8 min of BKC treatment would be sufficient to kill 8 log CFU. Hence, in real-life about 4.3 log CFU was not eradicated, causing significant safety risks.

Repeated exposure of a *S.* Typhimurium cell culture from WT-I4 resulted in the enrichment of the resistant sub-population and so resistant variants could be isolated. Phenotypic analysis of these resistant variants showed loss of motility and reduced biofilm forming capacity compared to the wild-type. Notably, the reduced biofilm formation capacity in selected variants in our study was not caused by impaired curli fimbriae and cellulose production, as this was comparable to the wild-type strain on congo red agar plates (data not shown). A link between motility and biofilm forming capacity has been observed before, as deletion of genes involved in flagella biosynthesis and/or function (motility) resulted in decreased biofilm forming capacity (Kim and Wei 2009; Stafford and Hughes 2007; Prouty and Gunn 2003; Prouty *et al.* 2002). In addition, loss of motility has also been associated with adaptation to antimicrobial agents (Bailey *et al.* 2009; Karatzas *et al.* 2008; Webber *et al.* 2008). However, this was at the same time also associated with higher energy requirements due to increased efflux pump activity that results via a cascade of events in elevated intracellular acetate that causes impaired motility (Karatzas *et al.* 2008). In the present study no increased efflux pump activity was observed, but it is likely that other energy requiring mechanisms are activated in the resistant variants.

A correlation between reduced biofilm forming capacity and increased biocide resistance as observed in the present study, has not been reported before. On the contrary, Pagedar et al. (2011) showed that environmental *Pseudomonas* isolates with high biofilm forming potential exhibit higher BKC resistance compared to isolates that have low biofilm forming potential. In a follow-up study in *E. coli*, adaptation to BKC or ciprofloxacin was shown to be correlated to an increased biofilm forming potential (Pagedar *et al.* 2012). The exact mechanisms underlying these phenomena remain to be elucidated.

Within the present study it was attempted to identify the mechanism(s) causing the increased BKC resistance. There are several reports that associated increased BKC resistance to elevated activity and/or overexpression of the multi-drug efflux pump AcrAB-TolC (Karatzas *et al.* 2008; Karatzas *et al.* 2007; Bore *et al.* 2007; Braoudaki and Hilton 2005; Gradel *et al.* 2005), which was often correlated to increased MIC values for clinical antibiotics (Li *et al.* 2011; Karatzas *et al.* 2008; Braoudaki and Hilton 2004; Levy 2002). Interestingly, the resistant *S.* Typhimurium variants did not exhibit elevated activity or overexpression of the AcrAB-TolC efflux pump (Fig. 5.6). Furthermore, no cross-protection to antibiotics and other chemical biocides was observed (Table S5.2). Therefore it is conceivable that other mechanisms are responsible for the increased BKC resistance of the variants.

The outer membrane is the primary site of action for BKC (McDonnell and Russell 1999) and therefore changes in the outer membrane composition and characteristics may affect the susceptibility for BKC of Gram-negative bacteria, as shown for *Pseudomonas aeruginosa* (Joynson *et al.* 2002; Loughlin *et al.* 2002). Also for *S.* Typhimurium a correlation between gradual adaptation to chemical biocides and alterations in the outer membrane has been reported. Previous studies revealed that changes in the hydrophobicity (Braoudaki and Hilton 2005), alterations of the fatty acids (Mangalappalli-Illathu *et al.* 2008; Webber *et al.* 2008) or a decreased amount of porins (Karatzas *et al.* 2008) was associated with gradual adaptation to BKC. Nonetheless, also repeated exposure seems to be correlated to alterations of the outer membrane, as the resistant variants obtained in the present study exhibited reduced outer membrane permeability compared to the wild-type strain. This phenomenon was less pronounced for I4-2 compared to I4-3 and I4-59, which were also less susceptible for BKC than I4-2. Interestingly, the alterations of the outer membrane did not facilitate increased tolerance for other antimicrobial agents, not even for cetylpyridinium chloride, also a membrane active quaternary compound. This indicates that the mechanisms underlying the alterations of the outer membrane resulting in decreased permeability are specific for BKC and to not result in cross-protection against other membrane active compounds. Furthermore, the alterations of the outer membrane did not affect the overall cell surface hydrophobicity, as this was comparable between the resistant variants and WT-I4.

The BKC resistance of the variants was stable, since it was not lost during growth without BKC. Furthermore, the resistant variants were already more resistant at the start of exposure as indicated by the inactivation curves (Fig. 5.5). Taken these results together suggest that the increased tolerance was inheritable and that the resistant variants are genetically different from the wild-type strain. Nevertheless, the exact mechanisms remain to be elucidated. Furthermore, the results imply that the mechanisms underlying the increased resistance potentially differ between the selected resistant variants, as they exhibit different phenotypes. This supports the hypothesis of Loughlin *et al.* (2002) that each isolate has the potential to develop a unique spectrum of resistance, although they are placed in the same environment. Comparative genome sequence analysis, combined with transcriptome and proteome studies may support elucidation of underlying BKC resistance mechanisms in the selected variants.

In conclusion, repeated exposure of *S.* Typhimurium to sub-lethal BKC concentrations rapidly selects for resistant variants. This indicates that inadequate disinfection treatments causing sub-lethal exposure of the bacterial flora can result in the selection for such variants. The relative contribution of the *S.* Typhimurium cellular parameters affected including increased BKC resistance, reduced motility and biofilm formation, will determine whether such variants can persist in food processing environments and become part of the so-called house flora.

REFERENCES

Alakomi, HL, Saarela M, Helander, IM. 2003. Effect of EDTA on *Salmonella enterica* serovar Typhimurium involves a component not assignable to lipopolysaccharide release. Microbiol 149(8):2015-2021.

Bailey AM, Constantinidou C, Ivens A, Garvey MI, Webber MA, Coldham N, Hobman JL, Wain J, Woodward MJ, Piddock LJV. 2009. Exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification. J Antimicrob Chemoth 64(5):973-985.

Bore E, Hébraud M, Chafsey I, Chambon C, Skjæret C, Moen B, Møretrø T, Langsrud Ø, Rudi K, Langsrud S. 2007. Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses. Microbiol 153(4):935-946.

Braoudaki M, Hilton AC. 2004. Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. J Clin Microbiol 42(1):73-78.

Braoudaki M, Hilton AC. 2005. Mechanisms of resistance in *Salmonella enterica* adapted to erythromycin, benzalkonium chloride and triclosan. Int J Antimicrob Agents 25(1):31-37.

Bridier A, Dubois-Brissonnet F, Greub G, Thomas V, Briandet R. 2011. Dynamics of the action of biocides in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemoth 55(6):2648-2654.

Castelijn GAA, Parabirsing J-A, Zwietering MH, Moezelaar R, Abee T. 2013. Surface behaviour of *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.* Infantis. Vet Microbiol 161(3–4):305-314.

Castelijn GAA, van der Veen S, Zwietering MH, Moezelaar R, Abee T. 2012. Diversity in biofilm formation and production of curli fimbriae and cellulose of *Salmonella* Typhimurium strains of different origin in high and low nutrient medium. Biofouling 28(1):51-63.

Cerf O, Metro F. 1977. Tailing of survival curves of *Bacillus licheniformis* spores treated with hydrogen peroxide. J Appl Bacteriol 42(3):405-415.

Condell O, Sheridan Á, Power KA, Bonilla-Santiago R, Sergeant K, Renaut J, Burgess C, Fanning S, Nally JE. 2012. Comparative proteomic analysis of *Salmonella* tolerance to the biocide active agent triclosan. J Proteom 75(14):4505-4519.

Copitch JL, Whitehead RN, Webber MA. 2010. Prevalence of decreased susceptibility to triclosan in *Salmonella enterica* isolates from animals and humans and association with multiple drug resistance. Int J Antimicrob Agents 36(3):247-251.

Davison S, Benson CE, Munro DS, Rankin SC, Ziegler AE, Eckroade RJ. 2003. The role of disinfectant resistance of *Salmonella enterica* serotype enteritidis in recurring infections in Pennsylvania egg quality assurance program monitored flocks. Avian Dis 47(1):143-148.

Davison WM, Pitts B, Stewart PS. 2010. Spatial and temporal patterns of biocide action against *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemoth 54(7):2920-2927.

Gradel KO, Randall L, Sayers AR, Davies RH. 2005. Possible associations between *Salmonella* persistence in poultry houses and resistance to commonly used disinfectants and a putative role of mar. Vet Microbiol 107(1–2):127-138.

Hu WS, Chen HW, Zhang RY, Huang CY, Shen CF. 2011. The expression levels of outer membrane proteins STM1530 and OmpD, which are influenced by the CpxAR and BaeSR two-component systems, play important roles in the ceftriaxone resistance of *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemoth 55(8):3829-3837.

Ishikawa S, Matsumura Y, Yoshizako F, Tsuchido T. 2002. Characterization of a cationic surfactant-resistant mutant isolated spontaneously from *Escherichia coli*. J Appl Microbiol 92(2):261-268.

Joynson JA, Forbes B, Lambert RJW. 2002. Adaptive resistance to benzalkonium chloride, amikacin and tobramycin: the effect on susceptibility to other antimicrobials. J Appl Microbiol 93(1):96-107.

Karatzas KAG, Randall LP, Webber M, Piddock LJV, Humphrey TJ, Woodward MJ, Coldham NG. 2008. Phenotypic and proteomic characterization of multiply antibiotic-resistant variants of *Salmonella enterica* serovar Typhimurium selected following exposure to disinfectants. Appl Environ Microbiol 74(5):1508-1516.

Karatzas KAG, Webber MA, Jorgensen F, Woodward MJ, Piddock LJV, Humphrey TJ. 2007. Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. J Antimicrob Chemoth 60(5):947-955.

Kim SH, Wei CI. 2009. Molecular characterization of biofilm formation and attachment of *Salmonella enterica* serovar Typhimurium DT104 on food contact surfaces. J Food Prot 72(9):1841-1847.

Langsrud S, Sundheim G, Holck AL. 2004. Cross-resistance to antibiotics of *Escherichia coli* adapted to benzalkonium chloride or exposed to stress-inducers. J Appl Microbiol 96(1):201-208.

Levin BR. 2004. Noninherited resistance to antibiotics. Science 305(5690):1578-1579.

Levy SB. 2002. Factors impacting on the problem of antibiotic resistance. J Antimicrob Chemoth 49(1):25-30.

Li B, Yao Q, Pan XC, Wang N, Zhang R, Li J, Ding G, Liu X, Wu C, Ran D. 2011. Artesunate enhances the antibacterial effect of {beta}-lactam antibiotics against *Escherichia coli* by increasing antibiotic accumulation via inhibition of the multidrug efflux pump system AcrAB-TolC. J Antimicrob Chemother 66(4):769-777.

Loughlin MF, Jones MV, Lambert PA. 2002. *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. J Antimicrob Chemoth 49(4):631-639.

Mangalappalli-Illathu AK, Vidovic S, Korber DR. 2008. Differential adaptive response and survival of *Salmonella enterica* serovar enteritidis planktonic and biofilm cells exposed to benzalkonium chloride. Antimicrob Agents Chemoth 52(10):3669-3680.

McDonnell G, Russell AD. 1999. Antiseptics and disinfectants: activity, action, and resistance.

Clin Microbiol Rev 12(1):147-179.

Møretrø T, Heir E, Nesse LL, Vestby LK, Langsrud S. 2012. Control of *Salmonella* in food related environments by chemical disinfection. Food Res Int 45(2):532-544.

Møretrø T, Midtgaard ES, Nesse LL, Langsrud S. 2003. Susceptibility of *Salmonella* isolated from fish feed factories to disinfectants and air-drying at surfaces. Vet Microbiol 94(3):207-217.

Pagedar A, Singh J, Batish VK. 2012. Adaptation to benzalkonium chloride and ciprofloxacin affects biofilm formation potential, efflux pump and haemolysin activity of *Escherichia coli* of dairy origin. J Dairy Res 79(04):383-389.

Pennell KG, Aronson AI, Blatchley ER. 2008. Phenotypic persistence and external shielding ultraviolet radiation inactivation kinetic model. J Appl Microbiol 104(4):1192-1202.

Piddock LJV. 2006. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev 4(8):629-636.

Prouty AM, Gunn JS. 2003. Comparative analysis of *Salmonella enterica* serovar Typhimurium biofilm formation on gallstones and on glass. Infect Immun 71(12):7154-7158.

Prouty AM, Schwesinger WH, Gunn JS. 2002. Biofilm formation and interaction with the surfaces of gallstones by *Salmonella* spp. Infect Immun 70(5):2640-2649.

Stafford GP, Hughes C. 2007. *Salmonella* typhimurium *flhE*, a conserved flagellar regulon gene required for swarming. Microbiol 153(2):541-547.

Van Boeijen IKH, Moezelaar R, Abee T, Zwietering MH. 2008. Inactivation kinetics of three *Listeria monocytogenes* strains under high hydrostatic pressure. J Food Prot 71(10):2007-2013.

Webber MA, Coldham NG, Woodward MJ, Piddock LJV. 2008. Proteomic analysis of triclosan resistance in *Salmonella enterica* serovar Typhimurium. J Antimicrob Chemoth 62(1):92-97.

Zwietering MH, Jongenburger I, Rombouts FM, van 't Riet K. 1990. Modeling of the bacterial growth curve. Appl Environl Microbiol 56(6):1875-1881.

SUPPLEMENTARY MATERIAL

Table S5.1: Primers used for the Q-PCR in this study.

Gene	Forward primer 5´- 3´	Reverse primer 5´- 3´
acrB	ACCTCCAACGGATGAGTTTGG	TCGCAATGACGTCGTAGTTC
tolC	TGGGTTTAGGTGCCGACTAC	GCTTTTTCTTGCAGGGTGAG
rpoD	GGGATCAACCAGGTTCAATG	GGACAAACGAGCCTCTTCAG
16S *rRNA*	CGATCCCTAGCTGGTCTGAG	GTGCAATATTCCCCACTGCT

Table S5.2: MICs (μg/ml) of amikacin, amiocillin/clavulanic acid, ampicillin, cefoxitin, ceftriaxone, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, tetracyclin, trimethoprim and vancomycin analysed of WT-I4, I4-2, I4-3 and I4-59, analysed by use of E-strips. MICs of sodium hypochlorite, peracetic acid, cyclohexane and another quaternary compound cetylpyridinium chloride analysed by the broth dilution method.

Antibiotic-desinfectant	**WT-I4**	**I4-2**	**I4-3**	**I4-59**
Amikacin	4	3	4	4
Amioxicillin/clavulanic acid	3	3	3	3
Ampicilline	>256	>256	>256	>256
Cefoxtine	1.5	1.5	1	1
Ceftriaxone	0.094	0.094	0.047	0.064
Chloramphenicol	4	4	4	3
Ciprofloxacin	0.016	0.016	0.012	0.006
Clidamycin	>256	>256	>256	>256
Erythromycin	16	12	16	16
Gentamycin	1.5	1.5	1.5	2
Tetracyclin	>256	>256	>256	>256
Trimethoprim	0.19	0.19	0.064	0.064
Vancomycin	>256	>256	>256	>256
Sodium hypochlorite	150	150	150	150
Peracetic acid	75	75	75	75
Cetylpyridinium chloride	19	19	19	19

CHAPTER 6

General discussion, concluding remarks and future perspectives

Greetje A.A. Castelijn

Outline

Salmonellosis is one of the most common foodborne diseases worldwide. It is a major public health burden and worldwide billion cases of human salmonellosis are reported every year, and the disease results in millions of deaths (ECDC 2011; ECDC 2010). Salmonellosis is contracted through the consumption of food contaminated with *Salmonella* species. In general, food products from animal origin are the source of a *Salmonella* infection, but also other foods such as green vegetables are implicated in the transmission of *Salmonella* (Berger *et al.* 2010). Food products can get contaminated with *Salmonella* on production sites, in processing plants or in households through contact with surfaces contaminated with *Salmonella* biofilms, so-called cross-contamination. Cross-contamination greatly contributes to the transmission of *Salmonella* through the food chain (Hermans *et al.* 2011; Botteldoorn *et al.* 2003). To prevent cross-contamination and develop better strategies for *Salmonella* control, it is important to understand the surface behaviour of *Salmonella* and the mechanisms of biofilm formation. In this study, cell surface structures involved in *Salmonella* biofilm formation at different environmental conditions were analysed, with *S.* Typhimurium as a model (chapter 2 and 3). Furthermore, biofilm formation was determined for different *Salmonella* serovars relevant for the pork industry. Therefore biofilm formation was analysed on stainless steel and its contribution to survival of *Salmonella* in conditions mimicking industrial settings was investigated (chapter 4). Within industrial settings processing equipment is disinfected on regular basis to control food quality and safety within the food industry. The effect of repeated disinfection on *Salmonella* cell was also examined (chapter 5).

Methods for biofilm analysis

Within this study different methods were used to analyse biofilm formation by *Salmonella*. One of these methods is the crystal violet (CV) assay, which gives an indication about the total biomass of the biofilm and it is commonly used to screen biofilm formation of large strain collections (Díez-García *et al.* 2012; Kroupitski *et al.* 2009; Malcova *et al.* 2008; Bokranz *et al.* 2005; Stepanovi et al. 2004; Borucki *et al.* 2003; Solomon *et al.* 2005). Also in this thesis the CV assay was used to screen the biofilm formation of strain collections (chapter 2 and 4). Based on the results of a CV assay, bacterial strain collections are often divided into strong/dense, moderate/average or weak/poor biofilm formers (Bhowmick *et al.* 2011). However, the CV assay results should be interpreted with caution, since it is not a very sensitive method and might fail to detect low levels of biofilm formation as was shown in chapter 2 and 4. These studies revealed that strains normally classified as no or weak/poor biofilm formers, still exhibit substantial amounts of cells attached. Therefore the term no or weak/poor biofilm former can be misleading since it suggests that the strain does not adhere to a surface, though it can still cause problems due to cell attachment and subsequent biofilm formation. It is therefore better to combine the CV assay with other methods such as enumeration of biofilm cells or microscopic evaluation to analyse biofilm forming behaviour. An additional advantage of combining methods is that it also provides information on the composition of the biofilm.

Biofilm forming behaviour at different environmental conditions

Biofilm formation is a complex process which is influenced by many environmental factors, such as temperature, nutrient availability, oxygen, pH and osmolarity. Environmental factors affect the regulatory network involved in the expression and production of different structural components involved in *Salmonella* biofilm formation (Kroupitski *et al.* 2009; Solomon *et al.* 2005; Stepanovi *et al.* 2004; Gerstel and Römling 2003). Consequently different environmental conditions trigger different biofilm forming behaviour. Within food processing environments, *Salmonella* is exposed to a variety of conditions and therefore the effect of environmental conditions on biofilm forming behaviour, composition and structure was analysed in this thesis (Fig. 6.1).

Curli fimbriae and cellulose

In chapter 2 biofilm forming behaviour was analysed at different environmental conditions. This revealed that temperature and culture medium greatly affected the biofilm forming behaviour, structure and composition. In addition it was shown that curli fimbriae and cellulose specifically contribute to biofilm formation at ambient temperatures under nutrient-low conditions. Nutrient-low conditions were simulated by the use of 1/20TSB, but dilutions

of other nutrient-rich medium, such as BHI → 1/20BHI, gave the same results (data not shown). This indicates that the phenomenon of rich versus diluted medium, resulting in biofilm consisting of cell clusters encapsulated in an extracellular matrix for nutrient-low conditions, was not specific for TSB.

The results described above and in chapter 2 were obtained for *S.* Typhimurium. However, problems in the food industry due to biofilm formation are also caused by other serovars. In chapter 4 we focussed on serovars that are frequently isolated within pork processing environments, namely *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.* Infantis (de Busser *et al.* 2011; EFSA 2008; Botteldoorn *et al.* 2003). The biofilm forming behaviour of all these serovars was found to be affected by medium composition (Fig. 6.2, unpublished data). Moreover, the TSB versus 1/20TSB phenomenon was observed for *S.* Derby and *S.* Brandenburg as well. For these two serovars the percentage of isolates showing dense biofilm formation was substantial higher in 1/20TSB compared to TSB (77% in 1/20TSB versus 18% in TSB for *S.* Derby, and 97% in 1/20TSB versus 62% in TSB for *S.* Brandenburg). Dense biofilm formation in 1/20TSB could be explained by extensive curli fimbriae and cellulose production as was shown for *S.* Typhimurium (chapter 2) and therefore these mechanisms might not be a specific feature of *S.* Typhimurium. However, it is not applicable to all *Salmonella* species, as illustrated in figure 6.2. This figure shows that only 2 out of 55 (3.6%) *S.* Infantis isolates exhibited dense biofilm formation in the nutrient-low medium. Moreover, these results reveal different biofilm forming behaviour along different *Salmonella* serovars which is in line with previous studies (Díez-García *et al.* 2012; Vestby *et al.* 2009a)

Type 1 fimbriae

Type 1 fimbriae were found to be involved in the initial surface attachment of the clinical, outbreak-related and retail product S. Typhimurium isolates in the rich TSB medium at 37 °C (chapter 3). However, these isolates also exhibited dense biofilm formation in a rich medium at 25 °C (Fig. 6.1), but the factors involved in dense biofilm formation at this condition remain to be elucidated. The expression analysis of all putative functional fimbriae operons of S. Typhimurium as described in chapter 3 gave no indication that fimbriae were involved, which suggests that other structures, such as BapA (Latasa et al. 2005), capsular polysaccharides (Gibson et al. 2006; de Rezende et al. 2005) or lipopolysaccharides (Anriany et al. 2006) could be important structures during biofilm formation in nutrient-rich environments at ambient temperatures.

The fimbria expression analysis described in chapter 3 exposed that the expression of type 1 fimbriae by *Salmonella* is influenced by temperature, since *fimA* expression was significantly lower at 25 °C compared to 37 °C. Furthermore, *S.* Typhimurium cell cultures grown at 25 °C exhibited no agglutination in the hemagglutination assay and also by TEM no type 1 fimbriae-like-structures were detected on the cell surface (data not shown). Taken these

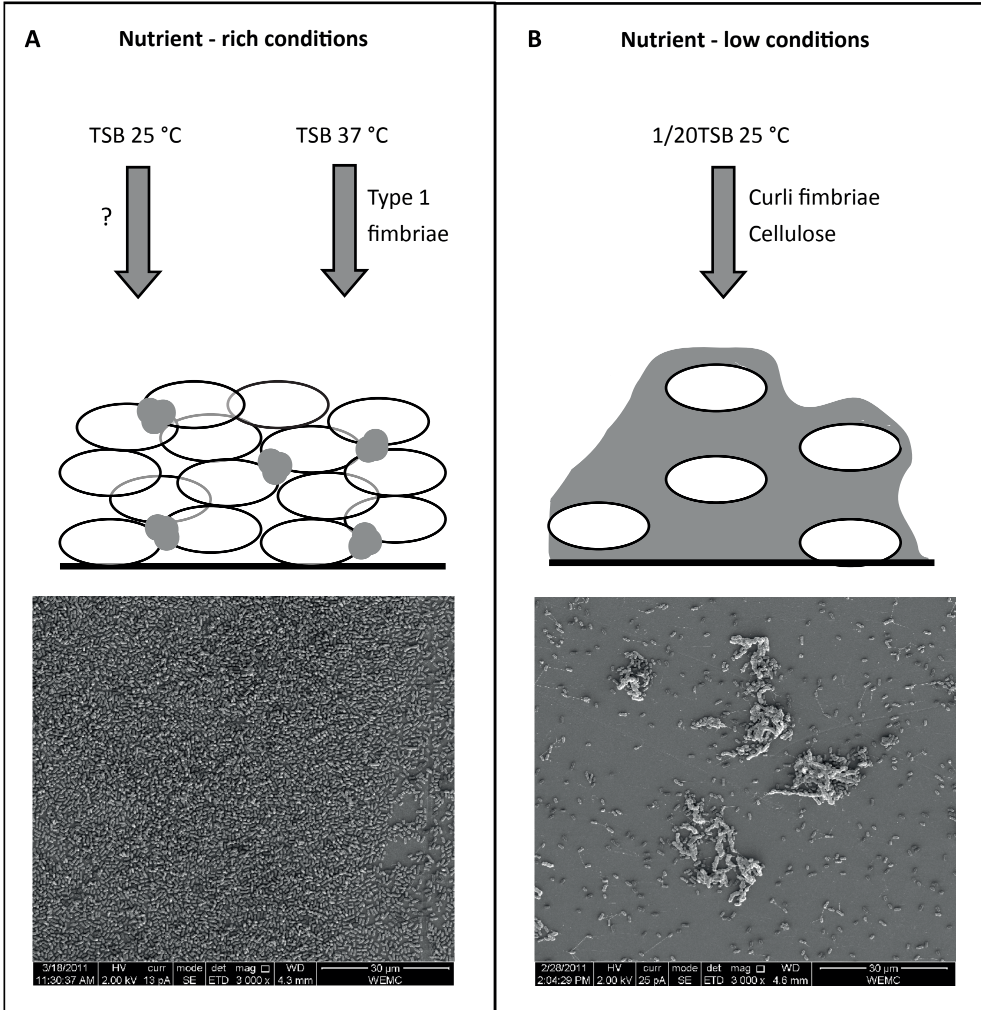


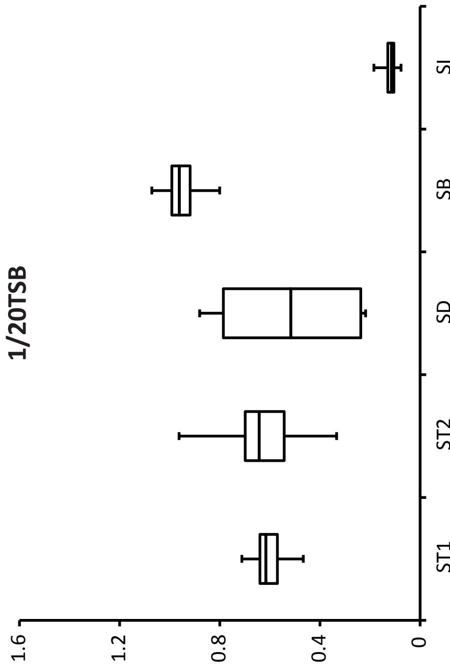
Figure 6.1: A schematic illustration of the environmental conditions analysed in this thesis that resulted in dense biofilm formation. A) Nutrient-rich conditions result in biofilms consisting of a layer of cells with no significant amounts of extracellular matrix at both 25 °C and 37 °C. At 37 °C, type 1 fimbriae expressed by a subpopulation of cells only, are involved in the attachment of cells. The components and/or mechanisms involved in dense biofilm formation at 25 °C remain to be elucidated. B) Under nutrient-low conditions, dense biofilm formation was observed at 25 °C only, and was associated with the expression of curli fimbriae and cellulose resulting in biofilms consisting of cell clusters encapsulated in an extracellular matrix.

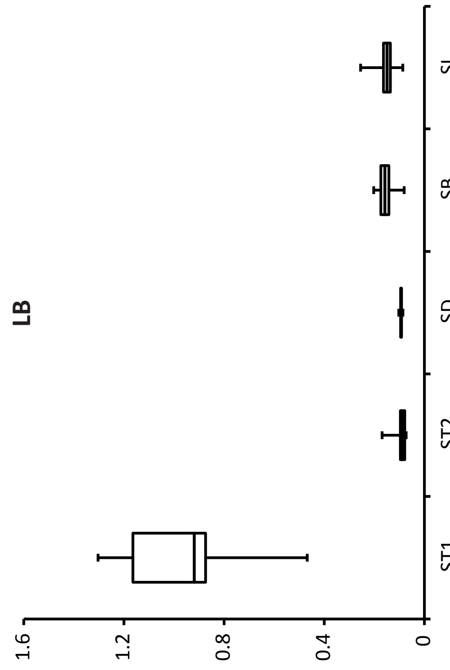
data together implies that type 1 fimbriae are not produced at ambient temperatures. This is the first study to describe a temperature effect on the regulation of the *fim* gene cluster in *S.* Typhimurium. However, for *Escherichia coli* temperature dependent phase variable expression of type 1 fimbriae was already described. Temperatures other than 37 °C repress the OFF→ON switching in this organism (Gally *et al.* 1993). Regulation of the *fim* gene cluster in *E. coli* however differs from that in *S.* Typhimurium (Tinker and Clegg 2000; Clegg *et al.* 1996). In *E. coli*, fimbrial phase variation is associated with the inversion of a 314-bp DNA element upstream of *fimA*. Inversion of this DNA element requires the recombinases *fimB* and *fimE* (Freitag *et al.* 1985; Gally *et al.* 1993). No homologues to *fimB* and *fimE* are found in the *fim* gene cluster of *Salmonella*. In addition, the *S.* Typhimurium *fim* promoter is not active in *E. coli* (Clegg *et al.* 1996; Yeh *et al.* 1995). In *S.* Typhimurium the expression of type 1 fimbriae is also phase variable and activated at 37 °C, but not controlled by the inversion of a DNA element. Its expression is regulated at the transcriptional level by three regulatory proteins FimW, FimY and FimZ. FimY and FimZ activate the *fim* promoter and FimW is the negative regulator that represses the *fim* gene expression (Saini *et al.* 2009; Tinker and Clegg 2000). However, how these regulation systems are affected by temperature is not known yet.

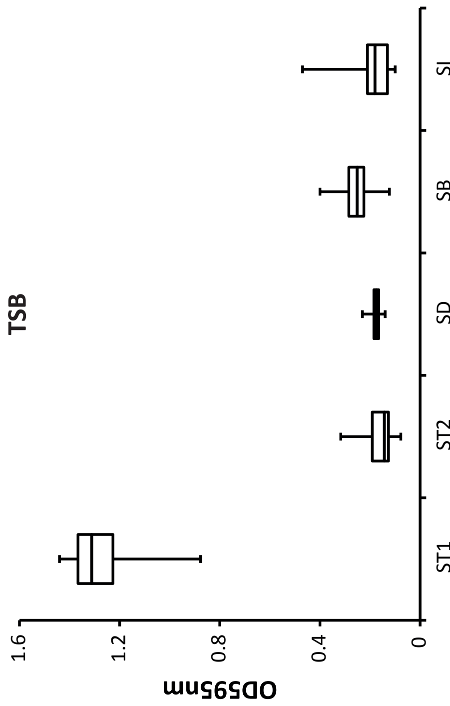
The proposed mechanism in chapter 3 is that type 1 fimbriae are involved in initial attachment to mannose carbohydrate-coated surfaces and that this determines the subsequent level of biofilm formation The strains able to form dense biofilms in TSB at 37 °C, also showed dense biofilm formation in BHI and LB medium (Fig. 6.2). These two nutrient-rich media also contain mannose carbohydrates, as BHI is derived from boiled bovine and porcine hearts and brains and LB contains yeast extract. Therefore these observations support the proposed mechanism of mannose-sensitive binding of type 1 fimbriated *S.* Typhimurium cells. In addition, dilution of TSB (chapter 2) and BHI, with conceivably concomitant reduction in deposition of mannose carbohydrate residues on the surface, revealed that other mechanisms than type 1 fimbriae play a role in dense biofilm formation under these nutrient-low conditions.

Reducing human salmonellosis by a mannanoligosaccharide-rich diet on the farm

To reduce the health problems due to consumption of *Salmonella* contaminated pork, control measures can be implemented on several levels throughout the farm-to-fork chain. The main focus has been on reducing the prevalence of *Salmonella* on the farm to achieve a lower number of carrier pigs. One of the methods to reduce the number of carrier pigs is the use of prebiotics that decrease *Salmonella* infection in animals (Berge and Wierup 2011; Borowsky *et al.* 2009; Yang *et al.* 2007; Gibson *et al.* 2005). The prebiotic mannanoligosaccharides (MOS), complex carbohydrates derived from the cell wall of *Sacharomyces cerevisiae*, can adhere to *Salmonella* type 1 fimbriae, i.e., the FimH protein, thereby inhibiting attachment of cells expressing the mannose-sensitive fimbriae to host epithelial cells. This


1/20TSB
1.6
1.2
0.8
0.4
0
ST1
ST2
SD
SB
SI


LB
1.6
1.2
0.8
0.4
0
ST1
ST2
SD
SB
SI


TSB
OD595nm
1.6
1.2
0.8
0.4
0
ST1
ST2
SD
SB
SI

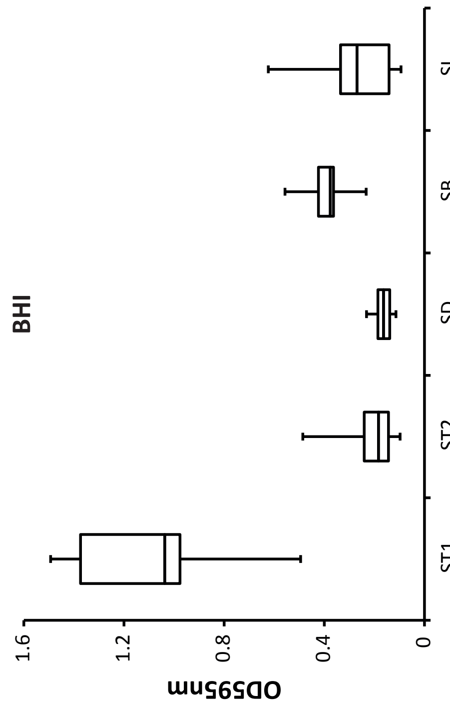

BHI
OD595nm
1.6
1.2
0.8
0.4
0
ST1
ST2
SD
SB
SI

Figure 6.2: Biofilm forming capacity of 51 *S.* Typhimurium (ST1 and ST2), 13 *S.* Derby (SD), 29 *S.* Brandenburg (SB) and 55 *S.* Infantis (SI) isolates in TSB, 1/20TSB, BHI and LB analysed with the CV assay. The *S.* Typhimurium isolates are divided in two groups according to strain origin (ST1; clinical, outbreak-related and retail products isolates and ST2; industrial isolates). The represented data is the average of five independent biological assays using three technical replicates each.

was shown by Borowsky *et al.* (2009) for a variety of porcine *Salmonella* strains and the mechanism is possibly similar to that observed in chapter 3 where addition of mannose to the growth medium reduces initial attachment of *S.* Typhimurium cell cultures that contained a fimbriated subpopulation. The use of MOS to control *Salmonella* infections in vivo needs further research, but it is of great interest since it could replace the use of antibiotics on the farm (Jacela *et al.* 2010).

Biofilm forming behaviour is associated with strain origin

In chapter 2 it was shown that *Salmonella* Typhimurium isolates from different origin display different biofilm forming behaviour in the rich TSB medium. Clinical, outbreak-related and retail product isolates formed dense biofilms consisting of a monolayer of cells, which was distinct compared to biofilms of industrial isolates in this medium (chapter 2). This trend was however not specific for TSB, as was shown by screening the biofilm forming behaviour of the same *S.* Typhimurium isolates in two other rich media, namely BHI and LB (figure 6.2). Also in these media, more total biofilm formation was observed for the clinical, outbreak-related and retail product isolates compared to *S.* Typhimurium industrial isolates, and also compared to industrial isolates from other *Salmonella* serovars. Taken these results together shows that it is a general phenomenon that clinical, outbreak-related and retail product isolates exhibit higher biofilm forming capacity in nutrient-rich conditions compared to industrial *Salmonella* isolates.

The clinical isolates used in this study were isolated from faeces or blood of patients that suffered from a *Salmonella* infection. The outbreak-related and retail product isolates were isolated from food products that were a suspicious source of a human *Salmonella* infection. Herein, these isolates differ from the industrial isolates that were isolated in a processing plant during a surveillance study and were therefore not linked to a human *Salmonella* infection. The ability to cause an infection in the nutrient-rich intestinal tract seems to be correlated to the biofilm forming capacity. This hypothesis is supported by studies that showed that deletion of genes involved in the virulence of *Salmonella* strains resulted in reduced biofilm forming capacity on both biotic and abiotic surfaces (Lu et al. 2012; Dong Lu et al. 2011; Ledeboer *et al.* 2006). Based on fimH-mediated binding to mannose residues, diver-

sity in biofilm formation under nutrient-rich conditions for the different *Salmonella* strains may be linked to single nucleotide polymorphisms of *fimH* (Dwyer *et al.* 2011). This issue will be discussed below.

Diversity in biofilm forming capacity along *Salmonella* serovars and strains

The differences observed in biofilm forming behaviour in this thesis could be linked to the expression and production of different structural components, including curli fimbriae, cellulose and type 1 fimbriae. Notably, significant differences were observed in biofilm formation of the selected *Salmonella* serovars, *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.* Infantis. *S.* Infantis did not show dense biofilm formation in 1/20TSB and on average only the clinical, outbreak-related and retail product *S.* Typhimurium isolates exhibited dense biofilm formation in nutrient-rich media (Fig. 6.2). It is conceivable that these differences are not associated with the absence of the genes encoding these structural components, since the *csg* and *fim* operons appeared to be present in all genomes of *Salmonella* strains sequenced so far (Yue *et al.* 2012; Clayton *et al.* 2008). This was also confirmed for the strains used in this current thesis by a PCR on the genomic DNA, which also established the presence of the *bcs* operon and the *adrA* gene (data not shown). These data suggest that differences in biofilm forming capacity at the tested culture conditions could occur at the level of gene expression. It is possible that the genes encoding regulatory factors are absent or that modifications in the promoter region have occurred. Next to this, polymorphism of FimH has been shown to affect binding to mannose carbohydrates and hoist epithelial cells. This topic is addressed in the next section.

Polymorphism of FimH affects binding capacity of type 1 fimbriated *Salmonella* cells

The observed variation in biofilm forming capacity among *S.* Typhimurium strains in TSB at 37 °C (chapter 2) might be, next to possible differences on regulatory level, a result of polymorphism in the gene encoding FimH. FimH is located on the tip of type 1 fimbriae where it mediates binding to mannose-containing structures on epithelial cells (Dwyer *et al.* 2011; Kisiela *et al.* 2005). Polymorphism in the *fimH* gene have been described for *Salmonella* serovars, including *S.* Typhimurium (Dwyer *et al.* 2011; Boddicker *et al.* 2002). A single point mutation within *fimH* can result in amino acid substitution(s) leading to differences and even loss of the mannose binding properties of type 1 fimbriae, as shown by the hemagglutination assay, binding to mannosylated proteins and binding abilities to epithelial cells both in vitro and in vivo (Dwyer *et al.* 2011; Guo *et al.* 2009; Kisiela *et al.* 2005). In conclusion, variation in adhesion and biofilm formation capacity in TSB at 37 °C between the *Salmonella* strains and serovars used in this thesis may be related to polymorphisms in *fimH* sequences.

Extracellular matrix and disinfection treatments

Within food processing environments, *Salmonella* is exposed to a variety of environmental conditions, which results in the formation of biofilms with different structure and extracellular matrix composition. The extracellular matrix plays a major role in the resistance, or better said tolerance, of biofilms to disinfection treatments. It limits the penetration of antimicrobial agents into the biofilm. This is partly due to diffusion limitation caused by the 3-dimensional structure, but primarily because of absorption or inactivation of the antimicrobial agent in the extracellular matrix components (Bridier *et al.* 2011b; Habimana *et al.* 2011; Vestby *et al.* 2009b; White *et al.* 2008; Solano *et al.* 2002; Stewart 1998).

The penetration and reactivity of biocides within a native biofilm structure can be monitored by the recently developed real-time method confocal laser scanning microscopy (CLSM) (Bridier *et al.* 2011b; Davison *et al.* 2010; Takenaka *et al.* 2008). This method demonstrated that penetration into *Staphylococcus epidermidis* biofilms was depent on the molecular weight of the biocide and the diameter of biofilm clusters (Davison *et al.* 2010). Similar results were described for *Pseudomonas aeruginosa* biofilms by Bridier *et al.* (2011). They showed that inactivation dynamics differed between biocides and that these were also influenced by the composition and density of the biofilm extracellular matrix. Large quantities of extracellular matrix resulted in slower penetration of the biocide into the biofilm (Bridier *et al.* 2011b). A protective effect by the extracellular matrix is also described by Vestby *et al.* (2009b), White *et al.* (2008) and Solano *et al.* (2002). Taken these observations together implies that *S.* Typhimurium biofilms formed under nutrient-low conditions at ambient temperature might be more difficult to eradicate than biofilms formed under nutrient-rich conditions, due to the difference in extracellular matrix composition and quantity. This hypothesis is supported by the data generated in chapter 3, where the susceptibility of biofilms grown in a rich medium (BHI) that showed no significant extracellular matrix, was comparable to planktonic cells. In a broader perspective, these data indicate that different disinfection treatments might be needed in food processing environments to eradicate the different types of biofilms that can be present.

Factors contributing to survival of *Salmonella* at production sides

Salmonella is constantly introduced into food processing environments via animals acting as carriers. The slaughter process is a critical step in the pig meat chain with respect to pork and carcass contamination. During the slaughter process cross-contamination due to contaminated equipment is the main route of *Salmonella* transmission (de Busser *et al.* 2011; van Hoek *et al.* 2012; EFSA panel on Biological Hazards 2010; EFSA 2008; Botteldoorn

et al. 2003). Therefore great effort is taken to ensure food safety and eradicate *Salmonella* in processing environments. However, several studies indicated that *Salmonella*, despite all hygienic measures taken, is still able to survive within slaughterhouses and has become a part of the house flora (van Hoek *et al.* 2012; EFSA panel on Biological Hazards 2010).

Within a slaughterhouse, bacteria are faced with many different environments and therefore different characteristics can contribute to the survival of *Salmonella* in such environments. In this thesis it was shown that biofilm formation, and therefore biofilm forming capacity, contributes to the survival of *Salmonella* at conditions as found in pork processing environments (chapter 4). This was also supported by other studies that showed increased desiccation tolerance of cells within a biofilm (Espinal *et al.* 2012; Iibuchi *et al.* 2010). However these studies analysed the survival of biofilm cells entrapped in extracellular matrix which was different from the biofilms analysed in this thesis since these did not contain significant amounts of extracellular matrix. This implies that other factors including phenotypic changes related to attachment, or growth within a biofilm contribute to the long term survival of the cells within the biofilms (Møretrø *et al.* 2012; Bridier *et al.* 2011a; Scher *et al.* 2005). Some examples of phenotypic adaption induced by growth within a biofilm include activation of stress responses (Hamilton *et al.* 2009) and modification of membrane composition due to decreased growth and metabolic activity (Coenye 2010; Werner *et al.* 2004). The phenotypic changes induced by growth in biofilms might also result in cross-protection for diverse conditions to which bacterial cells can be exposed within a production side, such as disinfection treatments (Espinal *et al.* 2012; Gruzdev *et al.* 2011; Hiramatsu *et al.* 2005).

Increased biocide resistance

Besides biofilm formation, increased biocide tolerance also could facilitate the survival of *Salmonella* and contributes to the persistence of a strain in a processing environment. During the cleaning and subsequent disinfection procedure within a food processing environment, the bacterial population might not always be exposed to the recommended effective biocide concentration, but to sub-lethal concentrations. This is due to the presence of residual organic matter, insufficient biocide use, or biofilm formation that limits the penetration of a biocide to the deeper regions of the biofilm (Bridier *et al.* 2011b; van Houdt and Michiels 2010). Exposure of a range of pathogens to sub-lethal biocide concentrations has been shown to result in increased biocide tolerance (Karatzas *et al.* 2008; Mangalappalli-Illathu *et al.* 2008; Bore *et al.* 2007; Gradel *et al.* 2005; Loughlin *et al.* 2002). Since literature has focused on adaption during constant exposure, we evaluated the effect of repeated exposure to sub-lethal concentrations of active biocide components (chapter 5). This revealed that repeated exposure to membrane targeting BKC results in rapid selection of resistant variants. However repeated exposure to the oxidative agent peracetic acid did not result in

the selection or generation of resistant stress-variants (tested concentrations were 5, 10, 20, 50 and 100 ppm PAA; data not shown). This is in line with previous studies that reported differences in stress adaptation capacity during constant exposure to gradually increasing concentrations of BKC and PAA (Condell *et al.* 2012). These results suggest that *Salmonella* strains can adapt more efficiently to lethal concentrations of membrane active compounds such as BKC, than to oxidizing agents such as PAA. This information may provide leads for the development of more efficient (combination) disinfection treatments.

Mechanisms involved in increased BKC resistance

The outermost layers of microbial cells can have a significant effect on the susceptibility to antiseptics and disinfectants. It is therefore not surprising that in Gram-negative bacteria many reported mechanisms contributing to increased biocide tolerance are related to the inner and outer cell membrane (Hu *et al.* 2011; Karatzas *et al.* 2008; Mangalappalli-Illathu *et al.* 2008; Braoudaki and Hilton 2005; Joynson *et al.* 2002; Loughlin *et al.* 2002). Also in this thesis, alterations of the cell membrane seemed to be involved in the increased resistance of the resistant variants, as significant differences in outer membrane permeability were observed between wild-type and resistant variants. The exact mechanisms causing decreased permeability and sensitivity to membrane-active compounds in the selected *Salmonella* resistant variants remain to be elucidated. Previous studies suggested a relation between increased expression of *fabI* and *fabB*, resulting in elevated fatty acid biosynthesis and increased tolerance for the membrane active agent triclosan (Webber *et al.* 2008). In addition, altered fatty acid profiles have also been correlated to triclosan and BKC adaption in *Salmonella* (Karatzas *et al.* 2008; Mangalappalli-Illathu *et al.* 2008; Loughlin *et al.* 2002). Besides alterations in fatty acid biosynthesis, also differences in the LPS composition in the outer membrane between wild-type and biocide adapted strains have been reported (Karatzas *et al.* 2008; Loughlin *et al.* 2002). However, Broudaki et al. (2005) concluded that adaptation to BKC did not result in significant changes in the LPS composition. Next to fatty acids and LPS (outer membrane), bacterial membranes also contain a wide diversity of membrane proteins involved in energy transducing reactions and transport of ions, vitamins and nutrients. Notably, several membrane proteins have also been related to increased biocide tolerance acting as so-called efflux-pumps (Karatzas *et al.* 2008; Braoudaki and Hilton 2005; Gradel *et al.* 2005; Langsrud *et al.* 2004). However, the results in this thesis showed no correlation between efflux-pump expression and activity with the increased resistance of the isolated variants. In Gram-negative bacteria, presence and activity of outer membrane-associated porins has also been related to increased tolerance (Hu *et al.* 2011; Ishikawa *et al.* 2002). Down-regulation of large porins, such as OmpF, results in reduced access of specific biocides and increased resistance (Bore *et al.* 2007). Also blebbing of the outer membrane contributes to increased resistance. Blebbing results in a larger total cell surface which can act as

a quencher for membrane active compounds (Joynson *et al.* 2002). Based on observations in these previous studies, it is conceivable that increased resistance of the resistant variants selected in this study (chapter 5) may be associated with modifications in the inner and/or outer membrane of the cells. Future studies, combining comparative genome sequence analysis, transcriptome and proteome studies of wild-type and resistant variants may provide more insight in mechanisms underlying the generation and/or selection of variants and corresponding resistant phenotypes.

Concluding remarks and future perspectives

Within this study different characteristics contributing to survival of *Salmonella* in industrial settings were analysed, with the main focus on surface adhesion, biofilm formation and generation of stable disinfectant resistant variants. In an initial screening, biofilm formation of a collection of 148 *Salmonella* strains with diverse origin was tested under a range of environmental conditions. This revealed a large variation in biofilm formation capacity between different *Salmonella* serovars, but also within serovars. Furthermore, for the serovars Typhimurium it was shown that strains from different origin have different biofilm forming behaviour and that biofilm formation was greatly affected by environmental conditions. Rich-nutrient conditions at both ambient and body temperature result in biofilms consisting of a monolayer of cells with little to no extracellular matrix, while low-nutrient conditions at ambient temperature result in biofilms consisting of cell clusters of which a subpopulation was completely encapsulated by the extracellular matrix. Since the extracellular matrix is largely responsible for the increased resistance of a biofilm, different and/or more severe (combinations of) disinfection treatments might be needed to eradicate biofilms formed at different environmental conditions. Additionally, repeated exposure of *Salmonella* (biofilm) cells to specific disinfectants can result in increased tolerance for the disinfectant used. Therefore both these aspects can affect persistence of *Salmonella* in food processing sites, and this information should be taken into account during the development of process hygiene and disinfection strategies applied in meat industry.

REFERENCES

Anriany Y, Sahu SN, Wessels KR, McCann LM, Joseph SW. 2006. Alteration of the rugose phenotype in *waaG* and *ddhC* mutants of *Salmonella enterica* serovar Typhimurium DT104 is associated with inverse production of curli and cellulose. Appl Environ Microbiol 72(7):5002-5012.

Berge AC, Wierup M. 2012. Nutritional strategies to combat *Salmonella* in mono-gastric food animal production. Animal 6(4):557-564.

Berger CN, Sodha SV, Shaw RK, Griffin PM, Pink D, Hand P, Frankel G. 2010. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environ Microbiol 12(9):2385-2397.

Bokranz W, Wang X, Tschape H, Römling U. 2005. Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. J Med Microbiol 54(12):1171-1182.

Bore E, Hébraud M, Chafsey I, Chambon C, Skjæret C, Moen B, Møretrø T, Langsrud Ø, Rudi K, Langsrud S. 2007. Adapted tolerance to benzalkonium chloride in *Escherichia coli* K-12 studied by transcriptome and proteome analyses. Microbiol 153(4):935-946.

Borowsky L, Corção G, Cardoso M. 2009. Mannanoligosaccharide agglutination by *Salmlonella enteria* strains isolated from carrier pigs. Braz J Microbiol 40:458-464.

Borucki MK, Peppin JD, White D, Loge F, Call DR. 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl Environ Microbiol 69(12):7336-7342.

Botteldoorn N, Heyndrickx M, Rijpens N, Grijspeerdt K, Herman L. 2003. *Salmonella* on pig carcasses: positive pigs and cross contamination in the slaughterhouse. J Appl Microbiol 95(5):891-903.

Braoudaki M, Hilton AC. 2005. Mechanisms of resistance in *Salmonella enterica* adapted to erythromycin, benzalkonium chloride and triclosan. Int J Antimicrobial Agents 25(1):31-37.

Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F. 2011a. Resistance of bacterial biofilms to disinfectants: a review. Biofouling 27(9):1017-1032.

Bridier A, Dubois-Brissonnet F, Greub G, Thomas V, Briandet R. 2011b. Dynamics of the action of biocides in *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemoth 55(6):2648-2654.

Clayton DJ, Bowen AJ, Hulme SD, Buckley AM, Deacon VL, Thomson NR, Barrow PA, Morgan E, Jones MA, Watson M. 2008**.** Analysis of the role of 13 major fimbrial subunits in colonisation of the chicken intestines by *Salmonella enterica* serovar Enteritidis reveals a role for a novel locus. BMC Microbiol 8(1):228.

Clegg S, Hancox LS, Yeh KS. 1996. *Salmonella typhimurium* fimbrial phase variation and FimA expression. J Bacteriol 178(2):542-545.

Coenye T. 2010. Response of sessile cells to stress: from changes in gene expression to phenotypic adaptation. FEMS Immunol Med Microbiol 59(3):239-252.

Condell O, Sheridan Á, Power KA, Bonilla-Santiago R, Sergeant K, Renaut J, Burgess C, Fanning S, Nally JE. 2012. Comparative proteomic analysis of *Salmonella* tolerance to the biocide active agent triclosan. J Proteom 75(14):4505-4519.

Davison WM, Pitts B, Stewart PS. 2010. Spatial and temporal patterns of biocide action against *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemoth 54(7):2920-2927.

de Busser V, Maes D, Houf K, Dewulf J, Imberechts H, Bertrand S, De Zutter L. 2011. Detection and characterization of *Salmonella* in lairage, on pig carcasses and intestines in five slaughterhouses. International J Food Microbiol 145(1):279-286.

de Rezende CE, Anriany Y, Carr LE, Joseph SW, Weiner RM. 2005. Capsular polysaccharide surrounds smooth and rugose types of *Salmonella enterica* serovar Typhimurium DT104. Appl Environ Microbiol 71(11):7345-7351.

Díez-García M, Capita R, Alonso-Calleja C. 2012. Influence of serotype on the growth kinetics and the ability to form biofilms of *Salmonella* isolates from poultry. Food Microbiol 31(2):173-180.

Dwyer BE, Newton KL, Kisiela D, Sokurenko V, Clegg S. 2011. Single nucleotide polypmorphisms of *fimH* associated with adherence and biofilm formation by serovar of *Salmonella enteriza*. Microbiol 157:3162-3171.

European Centre for Disease and Control (ECDC). 2011. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. EFSA J 9(3):378.

ECDC. 2010. Annual Epidemiological Report on Communicable Diseases in Europe 2010. Epidemiological Report on Communicable Diseases in Europe 2010. Stockholm 2010.

European Food Safety Authority (EFSA). 2008. Report of the task force on zoonoses data collection on the analysis of the baseline survey on the prevalence of *Salmonella* in slaughter pigs, Part A. EFSA J 135:1-111.

EFSA panel on Biological Hazards. 2010. Scientific opinion on a quantitative microbiological risk assessment of *Salmonella* in slaughter and breeder pigs. EFSA J 8(4):80.

Espinal P, Martí S, Vila J. 2012. Effect of biofilm formation on the survival of *Acinetobacter baumannii* on dry surfaces. J Hosp Infect 80(1):56-60.

Freitag CS, Abraham JM, Clements JR, Eisenstein BI. 1985. Genetic analysis of the phase variation control of expression of type 1 fimbriae in *Escherichia coli*. J Bacteriol 162(2):668-675.

Gally DL, Bogan JA, Eisenstein BI, Blomfield IC. 1993. Environmental regulation of the *fim* switch controlling type 1 fimbrial phase variation in *Escherichia coli* K-12: effects of temperature and media. J Bacteriol 175(19):6186-6193.

Gerstel U, Römling U. 2003. The *csgD* promoter, a control unit for biofilm formation in *Salmonella typhimurium*. Res Microbiol 154(10):659-667.

Gibson GR, McCartney AL, Rastall RA. 2005. Prebiotics and resistance to gastrointestinal infections. Brit J Nutr 93 (suppl. 1), 31–34.

Gibson DL, White AP, Snyder SD, Martin S, Heiss C, Azadi P, Surette M, Kay WW. 2006. *Salmonella* produces an O-antigen capsule regulated by AgfD and important for environmental

persistence. J Bacteriol 188(22):7722-7730.

Gradel KO, Randall L, Sayers AR, Davies RH. 2005. Possible associations between *Salmonella* persistence in poultry houses and resistance to commonly used disinfectants and a putative role of mar. Vet Microbiol 107(1–2):127-138.

Gruzdev N, Pinto R, Sela S. 2011. Effect of desiccation on tolerance of *Salmonella enterica* to multiple stresses. Appl Environ Microbiol 77(5):1667-1673.

Guo A, Cao S, Tu L, Chen P, Zhang C, Jia A, Yang W, Liu Z, Chen H, Schifferli DM. 2009. FimH alleles direct preferential binding of *Salmonella* to distinct mammalian cells or to avian cells Vol. Microbiol 155:1623-1633.

Habimana O, Steenkeste K, Fontaine-Aupart MP, Bellon-Fontaine MN, Kulakauskas S, Briandet R. 2011. Diffusion of nanoparticles in biofilms is altered by bacterial cell wall hydrophobicity. Appl Environ Microbiol 77(1):367-368.

Hamilton S, Bongaerts R, Mulholland F, Cochrane B, Porter J, Lucchini S, Lappin-Scott H, Hinton J. 2009. The transcriptional programme of *Salmonella enterica* serovar Typhimurium reveals a key role for tryptophan metabolism in biofilms. BMC Genom 10(1):599.

Hermans K, Nguyen TL, Roberfroid S, Schoofs G, Verhoeven T, De Coster D, Vanderleyden J, De Keersmaecker SC. 2011. Gene expression analysis of monospecies *Salmonella typhimurium* biofilms using differential fluorescence induction. J Microbiol Methods 84(3):467-478.

Hiramatsu R, Matsumoto M, Sakae K, Miyazaki Y. 2005. Ability of Shiga toxin-producing *Escherichia coli* and *Salmonella spp*. to survive in a desiccation model system and in dry foods. Appl Environ Microbiol 71(11):6657-6663.

Hu WS, Chen HW, Zhang RY, Huang CY, Shen CF. 2011. The expression levels of outer membrane proteins STM1530 and OmpD, which are influenced by the CpxAR and BaeSR two-component systems, play important roles in the ceftriaxone resistance of *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemother 55(8):3829-3837.

Iibuchi R, Hara-Kudo Y, Hasegawa A, Kumagai S. 2010. Survival of *Salmonella* on a polypropylene surface under dry conditions in relation to biofilm-formation capability. J Food Protect 73(8):1506-1510.

Ishikawa S, Matsumura Y, Yoshizako F, Tsuchido T. 2002. Characterization of a cationic surfactant-resistant mutant isolated spontaneously from *Escherichia coli*. J Appl Microbiol 92(2):261-268.

Jacela JY, DeRouchey JM, Tokach MD, Goodband RD, Nelssen JL, Renter DG, Dritz SR. 2010. Feed additives for swine: Fact sheets – prebiotics and probiotics, and phytogenics. J Swine Health Prod 18(3):132-136.

Joynson JA, Forbes B, Lambert RJW. 2002. Adaptive resistance to benzalkonium chloride, amikacin and tobramycin: the effect on susceptibility to other antimicrobials. J Appl Microbiol 93(1):96-107.

Karatzas KAG, Randall LP, Webber M, Piddock LJV, Humphrey TJ, Woodward MJ, Coldham NG. 2008. Phenotypic and proteomic characterization of multiply antibiotic-resistant vari-

ants of *Salmonella enterica* serovar Typhimurium selected following exposure to disinfectants. Appl Environ Microbiol 74(5):1508-1516.

Kisiela D, Sapeta A, Kuczkowski M, Stefaniak T, Wieliczko A, Ugorski M. 2005. Characterization of FimH adhesins expressed by *Salmonella enterica* serovar Gallinarum biovars Gallinarum and Pullorum: reconstitution of mannose-binding properties by single amino acid substitution. Infect Immun 73(9):6187-6190.

Kroupitski Y, Pinto R, Brandl MT, Belausov E, Sela S. 2009. Interactions of *Salmonella enterica* with lettuce leaves. J Appl Microbiol 106(6):1876-1885.

Langsrud S, Sundheim G, Holck AL. 2004. Cross-resistance to antibiotics of *Escherichia coli* adapted to benzalkonium chloride or exposed to stress-inducers. J Appl Microbiol 96(1):201-208.

Latasa C, Roux A, Toledo-Arana A, Ghigo J-M, Gamazo C, Penadés JR, Lasa I. 2005. BapA, a large secreted protein required for biofilm formation and host colonization of *Salmonella enterica* serovar Enteritidis. Mol Microbiol 58(5):1322-1339.

Loughlin MF, Jones MV, Lambert PA. 2002. *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. J Antimicrobial Chemoth 49(4):631-639.

Malcova M, Hradecka H, Karpiskova R, Rychlik I. 2008. Biofilm formation in field strains of *Salmonella enterica* serovar Typhimurium: Identification of a new colony morphology type and the role of SGI1 in biofilm formation. Vet Microbiol 129(3-4):360-366.

Mangalappalli-Illathu AK, Vidovic S, Korber DR. 2008. Differential adaptive response and survival of *Salmonella enterica* serovar enteritidis planktonic and biofilm cells exposed to benzalkonium chloride. Antimicrob Agents Chemother 52(10):3669-3680.

Møretrø T, Heir E, Nesse LL, Vestby LK, Langsrud S. 2012. Control of *Salmonella* in food related environments by chemical disinfection. Food Res Int 45(2):532-544.

Rosen DA, Pinkner JS, Walker JN, Elam JS, Jones JM, Hultgren SJ. 2008. Molecular variations in *Klebsiella pneumoniae* and *Escherichia coli* FimH affect function and pathogenesis in the urinary tract. Infec Immun 76(7):3346-3356.

Saini S, Pearl JA, Rao CV. 2009. Role of FimW, FimY, and FimZ in regulating the expression of type 1 fimbriae in *Salmonella enterica* serovar Typhimurium. J Bacteriol 191(9):3003-3010.

Scher K, Römling U, Yaron S. 2005. Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. Appl Environmen Microbiol 71(3):1163-1168.

Solano C, García B, Valle J, Berasain C, Ghigo J-M, Gamazo C, Lasa I. 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. Mol Microbiol 43(3):793-808.

Solomon EB, Niemira BA, Sapers GM, Annous BA. 2005. Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. J Food Protect 68:906-912.

Stepanovi S, Irkovi I, Ranin L, Svabi-Vlahovi M. 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. L Appl Microbiol 38(5):428-432.

Stewart PS. 1998. A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. Biotech and Bioeng 59(3):261-272.

Takenaka S, Trivedi HM, Corbin A, Pitts B, Stewart PS. 2008. Direct visualization of spatial and temporal patterns of antimicrobial action within model oral biofilms. Appl Environ Microbiol 74(6):1869-75.

Tinker JK, Clegg S. 2000. Characterization of FimY as a coactivator of type 1 fimbrial expression in *Salmonella enterica* serovar Typhimurium. Infect Immun 68(6):3305-3313.

van Hoek AHAM, de Jonge R, van Overbeek WM, Bouw E, Pielaat A, Smid JH, Malorny B, Junker E, Löfström C, Pedersen K et al. 2012. A quantitative approach towards a better understanding of the dynamics of *Salmonella spp*. in a pork slaughter-line. Int J Food Microbiol 153(1–2):45-52.

van Houdt R, Michiels CW. 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. J Appl Microbiol 109(4):1117-1131.

Vestby LK, Møretrø T, Langsrud S, Heir E, Nesse LL. 2009a. Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal- and feed factories. BMC Vet Res 5:20.

Vestby LK, Møretrø T, Ballance S, Langsrud S, Nesse LL. 2009b. Survival potential of wild type cellulose deficient *Salmonella* from the feed industry. BMC Vet Res 5:43.

Webber MA, Coldham NG, Woodward MJ, Piddock LJV. 2008. Proteomic analysis of triclosan resistance in *Salmonella enterica* serovar Typhimurium. J Antimicrobial Chemoth 62(1):92-97.

Werner E, Roe F, Bugnicourt A, Franklin MJ, Heydorn A, Molin S, Pitts B, Stewart PS. 2004. Stratified growth in *Pseudomonas aeruginosa* biofilms. Appl Environ Microbiol 70(10):6188-6196.

White AP, Gibson DL, Grassl GA, Kay WW, Finlay BB, Vallance BA, Surette MG. 2008. Aggregation via the Red, Dry, and Rough morphotype iIs not a virulence adaptation in *Salmonella enterica* serovar Typhimurium. Infect Immun 76(3):1048-1058.

Yang Y, Iji PA, Kocher A, Thomson E, Mikkelsen LL, Choct M. 2008. Effects of mannanoligosaccharide in broiler chicken diets on growth performance, energy utilisation, nutrient digestibility and intestinal microflora. Brit Poultry Sci 49(2):186-194

Yeh KS, Hancox LS, Clegg S. 1995. Construction and characterization of a *fimZ* mutant of *Salmonella* typhimurium. J Bacteriol 177(23):6861-6865.

Yue M, Rankin SC, Blanchet RT, Nulton JD, Edwards RA, Schifferli DM. 2012. Diversification of the *Salmonella* fimbriae: a model of macro- and microevolution. PLoS One 7(6):12.

Summary

SUMMARY

Salmonella is one of the most common and widely distributed bacterial pathogens. It is a major public health problem, and millions of salmonellosis cases are reported worldwide every year. In general, *Salmonella* infections in humans are food-borne. Especially food products of animal origin, such as meat and poultry are an important source of salmonellosis. The meat and poultry can get contaminated during the slaughtering process in two ways. One route is directly through the contact with the content of intestines from infected animals and the other one is indireclty through the contact with contaminated food processing equipment. The food processing equipment can be contaminated with biofilms. A biofilm is a population of bacterial cells attached to a surface embedded in an extracellular matrix. If *Salmonella* manage to establish biofilm growth on food processing equipment they can form a persistent source of contamination, since biofilms are hard to eradicate.

The aim of this thesis project is to obtain more insight in the processes involved in *Salmonella* biofilm formation. The effect of substratum, medium composition, temperature and strain origin on biofilm formation was examined for the *Salmonella* serovar Typhimurium. Furthermore the surface behaviour of different *Salmonella* serovars relevant for the pork industry was studied in relation to the survival on dry surfaces and susceptibility to disinfection treatments. In addition it was investigated if repeated exposure of *S.* Typhimurium to benzalkonium chloride (BKC), a disinfectant commonly used in the food industry, results in the selection of resistant variants. Selected BKC resistant variants were phenotypically characterized to identify the mechanisms contribution to the enhanced BKC tolerance. Increasing our knowledge on these different topics may contribute to the development of better strategies for *Salmonella* control in food processing environments.

In chapter 2, biofilm forming capacity of 51 *S.* Typhimurium strains, from medical, retail and industrial settings, was analysed under different environmental conditions. The results indicated that biofilm formation is influenced by strains origin, nutrient availability and temperature. In general, clinical, outbreak-related and retail-product isolates showed dense biofilm formation in nutrient-rich media at ambient and human body temperatures. However, in a nutrient-low medium dense biofilm formation was only observed at ambient temperatures. This was also the only condition at which industrial isolates exhibited dense biofilm formation. In addition, nutrient availability and temperature also influenced the biofilm morphology and the composition of the biofilm extracellular matrix. In a nutrient-rich medium, biofilms were composed of a dense layer of cells with only small amounts of extracellular matrix. However, in a nutrient-low medium at ambient temperatures biofilms were composed of large cell clusters entangled by curli fimbriae imbedded in an extracellular matrix mainly composed of cellulose.

In chapter 3 it was investigated which mechanisms contribute to the formation of dense biofilms in a nutrient-rich medium at 37 °C. Next to curli fimbriae, 10 other putative functional fimbriae gene clusters are present in the genome of *S.* Typhimurium. An expres-

sion analsyes of all these putative functional fimbriae gene clusters revealed that only type 1 fimbriae were significantly higher expressed in strains exhibiting dense biofilm formation at this culture condition. The presence of type 1 fimbriae on the cell surface was confirmed by a hemagglutination assay, transmission and scanning electron microscopy. The latter two techniques revealed that type 1 fimbriae were expressed by a subpopulation of cells only. This was in agreement with zeta potential measurements that indicated two subpopulations in cell cultures of dense biofilm formers, with 20% of cells displaying higher electrophoretic mobility conceivably associated with the presence of negatively charged type 1 fimbriae. The strains that expressed type 1 fimbriae at their cell surface showed next to dense biofilm formation also increased initial attachment compared to the poor biofilm formers. The initial attachment of the dense biofilm formers could be decreased to the level of the poor biofilm formers by addition of mannose to the growth medium. Since type 1 fimbriae are mannose sensitive, these results indicated that type 1 fimbriae contribute to the initial attachment and that this determines the subsequent level of biofilm formation in nutrient-rich medium at 37 °C. The mechanisms involved in attachment and biofilm formation in a nutrient-rich environment at ambient temperatures remain to be elucidated.

Next to *S.* Typhimurium, also *S.* Derby, *S.* Brandenburg and *S.* Infantis are commonly found in meat processing environments. Within these environments, cross-contamination greatly contributes to the contamination of meat products. Therefore, in chapter 4, characteristics important for survival on surfaces in meat processing environments were analysed. This revealed variation in biofilm forming capacity between and within serovars. Furthermore a relation between biofilm forming capacity and survival on dry stainless steel surfaces was found. On such surfaces, biofilms showed greater and longer survival than planktonic cells, and they were less susceptible to peracetic acid disinfection treatments. However, the latter effect was only observed in the presence of organic material, which drastically decreased the activity of peracetic acid. This chapter provided information that suggests that biofilm formation contributes to the survival of *Salmonella* in food processing environments.

An efficient disinfection strategy within the food industry is needed to ensure food safety and prevent the spreading of foodborne pathogens such as *S.* Typhimurium. However, several factors negatively affect the efficiency of disinfection treatments resulting in low level exposure of the bacterial flora facilitating survival of bacterial cells. A disinfectant commonly used within the food industry is benzalkonium chloride (BKC). It has been reported that adaption to BKC during constant exposure results in increased resistance and cross-protection to antibiotics, which might be a risk for public health. However in industrial settings bacterial cells are not constantly, but more likely repeatedly exposed to the disinfectant of use due to the periodic disinfection of the processing environment and equipment. Therefore in chapter 5 the effect of repeated sub-lethal BKC exposure of *S.* Typhimurium cells was analysed. The results showed that one single BKC exposure results in tailing of the inactivation curve indicating variation in BKC susceptibility within the initial population.

Repeated exposure resulted in the enrichment of a more resistant population and variants with increased BKC resistance were isolated. Phenotypical characterization of these resistant variants showed loss of motility, reduced biofilm forming capacity and reduced outer cell membrane permeability pointing to modification of cell surface properties. So, repeated exposure of *S.* Typhimurium to sub-lethal BKC concentrations rapidly selects for resistant variants that might become a part of the house flora within food processing environments.

In conclusion, the results in this thesis show that biofilm forming behaviour is greatly affected by environmental cues and that it may contribute to *Salmonella* survival in food processing environments. In addition, variation was observed in the biofilm forming capacity between strains from different origin and between and within *Salmonella* serovars. These results highlight that development of cleaning and disinfection strategies for eradicating of *Salmonella* cells and/or biofilms that can be present in industrial settings should be validated and verified for relevant conditions and a variety of relevant *Salmonella* serovars. Furthermore, the repeated use of the same disinfectant for a long period of time can result in the selection of variants with increased resistance. This denotes that the disinfection strategy should also be validated and verified on a frequent basis.

Samenvatting

SAMENVATTING

Salmonella is een van de meest voorkomende ziekmakende bacteriën. Het vormt zodoende een groot probleem voor de volksgezondheid en wereldwijd vinden er elk jaar miljoenen gevallen van *Salmonella*-infecties plaats. Een *Salmonella*-infectie wordt meestal veroorzaakt door de consumptie van besmette eieren of vlees. Tijdens de slacht kan het vlees besmet raken door contact met de inhoud van de darmen van bemette dieren, of door contact met besmette slachtapparatuur. Slachtapparatuur kan besmet raken met *Salmonella* doordat cellen zich kunnen hechten aan het oppervlak door de vorming van een bioflm. Een biofilm is een opeenhoping van bacteriecellen op een oppervlak die omgeven is met een door de bacteriën geproduceerde ´lijmlaag´. Deze ´lijmlaag´ is de extracellulaire matrix van de biofilm. Mede door deze matrix zijn biofilms moeilijk te verwijderen met reinigingsmethodes zoals gebruikt binnen een slachterij waardoor ze een constante besmettingsbron kunnen vormen.

Het doel van deze thesis was om meer inzicht te krijgen in de processen die betrokken zijn bij de biofilmvorming van *Salmonella*. Hiervoor werd het effect van verschillende factoren, zoals het oppervlak, het medium, de temperatuur en de afkomst van een *Salmonella* stam, op biofilmvorming geanalyseerd. Deze experimenten werden uitgevoerd met *Salmonella* Typhimurium. Naast *S.* Typhimurium is ook het oppervlaktegedrag van andere serovars die veelvuldig gevonden worden in slachterijen bestudeerd. Verder is er onderzocht of herhaaldelijk gebruik van benzalkoniumchloride (BKC), een disinfectant wat frequent gebruikt wordt in de levensmiddelenindustrie, resulteert in de selectie van resistente-varianten. Meer informatie over bovengenoemde onderwerpen zou bij kunnen dragen aan de ontwikkeling van betere reinigingsmethodes om *Salmonella* te elimineren en te beheersen in de levensmiddelenindustrie.

In hoofdstuk 2 is de biofilmvorming van 51 *S.* Typhimurium stammen met een verschillende afkomst onder verschillende kweekcondities onderzocht. De resultaten toonde aan dat stammen met een klinische of uitbraak-gerelateerde achtergrond of geïsoleerd uit besmette levensmiddelen een biofilm vormden in een nutriëntenrijk medium bij 25 °C en 37 °C. Echter, in een nutriëntenarm medium vond biofilm vorming alleen plaats bij 25 °C. Onder deze kweekconditie vormden ook industriële stammen een biofilm. Verder werd er aangetoond dat de temperatuur en de nutriënten-concentratie effect hadden op de morfologie van de biofilm en de samenstelling van de extracellulaire matrix. In een nutriëntenrijk medium vormde *S.* Typhimurium een biofilm bestaande uit een enkele cellaag met weinig tot geen extracellulaire matrix. Biofilms gevormd in een nutriëntenarm medium daarentegen bestonden uit losliggende clusters van cellen wel compleet omgeven met een extracellulaire matrix. Bij de vorming van deze extracellulaire matrix speelden curli fimbriae en cellulose en belangrijke rol.

Hoofdstuk 3 beschrijft welke factoren/mechanismes betrokken zijn bij de biofilm vorming op een abiotisch oppervlak in een nutriëntenrijk medium bij 37 °C. Naast curli fim-

briae omvat het genoom van *S.* Typhimurium nog 10 andere operons die coderen voor functionele fimbriae. Een analyse van de expressie van al deze fimbriae toonde aan dat alleen type 1 fimbriae hoger tot expressie kwamen in stammen die een dichte biofilm vormden in een nutriëntenrijk medium bij 37 °C. Naast een verhoogde expressie, werden type 1 fimbriae ook aangetoond op het celoppervlak van deze stammen door middel van een hemagglutinatie-analyse,transmissie- en scanning-elektronenmicroscopie. Deze laatste twee technieken brachten ook aan het licht dat type 1 fimbriae enkel op een sub-populatie van de celculturen aanwezig waren. Dit kwam goed overeen met zeta-potentiaal metingen waarbij ook twee populaties met een verschillende elektromobiliteit gevonden werden. 20% van de cellen had een hogere elektromobiliteit wat gerelateerd zou kunnen zijn aan de aanwezigheid van negatief geladen type 1 fimbriae op het celoppervlak. De stammen die type 1 fimbriae tot expressie brachten vormden niet alleen een dichtere biofilm op een abiotisch oppervlak in het nutriëntenrijk medium bij 37 °C, maar vertoonden ook meer initiële celaanhechting. Dit kon gereduceerd worden tot het niveau van de stammen die geen dichte biofilm vormde door de toevoeging van mannose aan het kweekmedium. Aangezien de tip van type 1 fimrbiae bindt aan mannose-residuën tonen deze resultaten aan dat type 1 fimbriae betrokken zijn bij de aanhechting van *S.* Typhimurium cellen aan een abiotisch oppervlak en dat dit de verdere mate van biofilm vorming beïnvloed.

Naast *S.* Typhimurium, worden ook *S.* Derby, *S.* Brandenburg en *S.* Infantis met regelmaat gedetecteerd in varkensslachterijen op zowel carcassen als op de slachtapparatuur. Daarom is in hoofdstuk 4 het oppervlakte gedrag van deze vier serovars bestudeerd. Dit zou meer inzicht kunnen geven in de factoren die een rol spelen bij de overleving van *Salmonella* in een slachterij. De resultaten toonden aan dat de biofilmvormende capaciteit varieert tussen, maar ook binnen *S.* Typhimurium, *S.* Derby, *S.* Brandenburg en *S.* Infantis én dat deze gerelateerd is aan de overleving op een droog roestvast stalen oppervlak. Verder werd er aangetoond dat cellen in een biofilm langer overleven op z`n oppervlak in vergelijking met planktonische cellen en dat ze resistenter zijn tegen een perazijnzuur behandeling als er organisch materiaal aanwezig is. Er kan dus geconcludeerd worden dat biofilmvorming bijdraagt aan de overleving van relevante *Salmonella* serovars in een slachterij.

Om *Salmonella* te elimineren in de levensmiddelenindustrie en de voedselveiligheid te kunnen garanderen is een gedegen reiningsmethode van belang. Echter, de effectiviteit van een reiningsmethode kan door verschillende factoren negatief beïnvloed worden waardoor de bacteriën niet worden blootgesteld aan de benodigde desinfectanten-concentratie. Een blootstelling aan een lagere concentratie kan resulteren in de overleving van bacteriën. Deze 'overlevers' worden hierdoor meerdere malen blootgesteld aan het desingfectant. Hoofdstuk 5 beschrijft het effect van een herhaaldelijke blootstelling aan het desinfectant BKC. Een enkele blootsteling aan een lage BKC -concentratie toonde 'tailing'van de inactivatiecurve aan. Dit betekent dat een enkele blootstelling niet de gehele populatie af doodt. Herhaaldelijke blootstelling van de overlevers in de 'tail' resulteerde in de isolatie van varianten met een verhoogde BKC resistentie. Fenotypische karakterisatie van deze

resistente-varianten liet zien dat ze in het algemeen minder tot geen motiliteit vertoonden, een lagere biofilm vormende capaciteit hadden, en dat het celmembraan minder permeabel was. Dit laatste was echter niet gerelateerd aan een verhoogde expressie en/of activiteit van de effluxpompen in het celmembraan en een verhoogde resistentie tegen antibiotica. Dit suggereert dat het mechanisme verantwoordelijk voor de verhoogde BKC resistentie specifiek was.

Het onderzoek beschreven in dit proefschrift heeft aangetoond dat biofilmvorming van *Salmonella* beïnvloed wordt door omgevingsfactoren zoals de nutriënten-concentratie en temperatuur. Ook is er aangetoond dat er variatie is in de biofilmvormende capaciteit tussen stammen met een verschillende achtergrond en niet alleen tussen, maar ook binnen *Salmonella* serovars. Voor de levensmiddelenindustrie is het ook belangrijk om te weten dat biofilmvorming bijdraagt aan de overleving van *Salmonella* in een productieomgeving. De resultaten geven ook het belang aan van een gedegen reinigingsmethode waarmee de aanwezige bacteriële flora met één blootstelling afgedood wordt, omdat herhaaldelijke blootstelling kan resulteren in de selectie van resistente-varianten. Door regelmatige validatie en verificatie van een reinigingsmethode zou eventuele opgebouwde resistentie tijdig gedetecteerd kunnen worden en de reinigingsmethode hier op aangepast kunnen worden. Dit zou bijdragen aan een betere beheersig van *Salmonella* in de levensmiddelenindustrie.

Acknowledgements

ACKOWLEDGEMENTS/DANKWOORD

Eindelijk is het dan zover, mijn proefschrift is af en het is tijd om misschien wel het meest gelezen hoofdstuk te schrijven; het dankwoord. Een promotie-onderzoek doe je namelijk niet alleen en daarom wil ik de mensen die een bijdrage hebben geleverd, direct of niet direct, graag bedanken.

Als eerste wil ik mijn promotoren Tjakko en Marcel, en mijn co-promoter Roy bedanken dat ze mij de kans hebben gegeven om aan dit promotie-onderzoek te beginnen. Door jullie adviezen en input vanuit verschillende invalshoeken was het een zeer leerzaam traject. Heren bedankt!

Stijn, bedankt voor jouw bijdrage tijdens de eerste 2 jaar van mijn onderzoek. Zonder jouw advies over zowel experimentele zaken op het lab als over het schrijven van wetenschappelijke publicaties had dit proefschrift er anders uitgezien. Next to Stijn, I would also like to thank all the other office and lab mates I had over the years. So Monica, Hasmik, Renata, Masayuki and Sachin thanks for all the discussions and fun time we spent together in lab 422 or office 426. And I hope that singing along with the radio stays a tradition. Also great thanks to the students Caroll and Jo-Ann whom contributed to my PhD project during their internship and master thesis. Thanks for your hard work.

Clint, Sachin (Kadam), Xiao and Lisa, we all started our PhD project in 2008 at the laboratory of food microbiology and furthermore we organized the PhD-trip to Switzerland together. Therefore we experienced the same obstacles, but we were always willing to help each other with practical, theoretical, computer- or side-related issues. I am very grateful for this and I enjoyed the many talks and jokes we made together.

Furthermore I would like to thank all the other colleagues for the great time I had at the laboratory of food microbiology. I hope you can keep the nice work atmosphere in the new building as well.

Voor enkele proeven moest ik uitwijken naar ander afdelingen. Daarom wil ik ook de medewerkers van de biomedical engineering afdeling van de Universiteit van Groningen bedanken. In het bijzonder dank aan Bastiaan voor zijn hulp en adviezen betreffende de zeta potentiaal metingen.

Tijdens mijn promotie heb ik ook veel steun gehad van mensen buiten het werk. Om te beginnen kon ik in Wageningen de nodige afleiding krijgen en promotieperikelen uiten bij Linda. We kennen elkaar van de HLO, en geheel toevallig gingen we beide studeren in Wage-

ningen en begonnen we ook nog eens op dezelfde dag aan onze promotie. Hierdoor konden we elkaar vaak helpen met praktische tips. Zeker aan het einde heb je me veel geholpen met de laatste details van mijn proefschrift. Hiervoor veel dank en ik vind het daarom ook leuk dat jij een van mijn paranimfen bent.

Ook grote dank aan Kim en Bien. Dames ik kon altijd op jullie rekenen voor wat dan ook. Zelfs voor bezoekjes aan het Biotechnion laat in de avond als ik weer eens wat op kweek moest zetten. Jullie zorgde voor de nodige ontspanning naast mijn werk. Kim en Martin ik heb genoten van de lange avonden die we samen op het mooiste balkon in Wageningen hebben door gebracht met een smeulende BBQ. Ik hoop dat er nog vele mogen volgen. Kim ik vind het fantastisch dat ook jij een van mijn paranimfen bent.

Verder wil ik alle gildezusters en -broeders van ´t BSG bedanken voor de gezellige avondjes en goede feestjes die we samen beleefd hebben. In het bijzonder superklusser Peer en zijn Christine, Dirk, Ilse, Lieke en (constant) Stijn. Ook wil ik Marit en Co bedanken voor de vele kopjes koffie die altijd voor me klaar stonden op de Markt.

Maar ook buiten Wageningen zijn er mensen die ik hier graag wil bedanken. In het Roosendaalse waren er mensen die altijd voor mij klaar stonden. Om te beginnen Anton en Talita, en Robin. Bedankt dat ik altijd bij jullie langs mag komen.

Cindy en Sonja, jullie zijn altijd in voor een gezellig uitje of een avondje lekker bijkletsen. Dit bracht de soms nodige afleiding, hiervoor bedankt. Sonja ik ben blij dat je nu wat dichterbij woont zodat ik wat vaker langs kan komen en Cindy ik hoop dat we nog vaak als super-kook-duo aan de slag gaan.

En natuurlijk DE meiden, alias de kanjers uit Tullepetaonsestad; As, Es, Juud, Mies en Veer. Onze vriendschap gaat al terug naar het Gertrudis College. Wat hebben we daar samen een leuke tijd gehad! En elke keer als we elkaar zien is het net of de tijd heeft stil gestaan en is het ouderwets gezellig. Ik kijk er elke keer naar uit. Ik heb in het bijzonder genoten van elke carnaval (met name vrijdagavond) en natuurlijk het samen onveilig maken van Lowlands. Dames bedankt voor jullie luisterend oor en steun de afgelopen jaren. En Mies en Es, zonder jullie hulp had de kaft er niet zo mooi uitgezien!

Ook wil ik de familie Roks, Castelijn en van Stiphout bedanken. In het bijzonder dank aan mijn schoonzusjes Judith en Linda, en mijn schoonouders Leon en Antonet. Ook bedankt voor het warme welkom in Helmond, al moet ik er nog een beetje wennen.

Natuurlijk wil ik ook ons ma en onze Jan bedanken. Helaas waren de laatste jaren niet de makkelijkste, maar opgeven dat doen we niet en daar ben ik heel trots op. Ik wil jullie bedanken voor het vertrouwen dat jullie in mij hebben en de interesse en motiverende woorden ondanks dat jullie niet altijd helemaal begrepen waar ik nu mee bezig was. Ik mag heel blij zijn met zo een moeder en broer.

Lieve Ruud, ik wil je bedanken voor het aanhoren van de frustraties de afgelopen jaren die komen kijken bij een promotie-onderzoek. Vaak wist je me weer snel te motiveren om door te zetten. Bedankt voor je geduld, hulp en liefde. Jij bent mijn maatje.

En pa, helaas heb je alleen de eerste 2 maanden van mijn promotie mee mogen maken, maar ik hoop dat je af en toe op wat voor manier dan ook over mijn schouder hebt meegekeken.

Greetje

Personalia

Curriculum vitae

List of publications

Education certificate

LIST OF PUBLICATION

Castelijn GAA, van der Veen S, Zwietering MH, Moezelaar R, Abee T. 2012. Diversity in biofilm formation and production of curli fimbriae and cellulose of *Salmonella* Typhimurium strains of different origin in high and low nutrient medium. Biofouling 28(1):51-63.

Castelijn GAA, Parabirsing J, Zwietering MH, Moezelaar R, Abee T. 2013. Surface behaviour of *S.* Typhimurium, *S.* Derby, *S.* Brandenburg and *S.*Infantis. Vet Microbiol 161:305-314.

Castelijn GAA, van der Veen S, Krom BP, Zwietering MH, Moezelaar R, Abee T. 2013. Type 1 fimbriae are involved in the attachement of *Salmonella* Typhimurium to abiotic surfaces and biofilm formation. Submitted for publication.

Castelijn GAA, van der Veen S, Zwietering MH, Moezelaar R, Abee T. 2013. Isolation and phenotypic characterization of resistant variants from *Salmonella* Typhimurium cell cultures treated with benzalkonium chloride. Submitted for publication.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Courses
Systems biology course: Statistics of omics data analysis, VLAG (2008)
Training period at the University of Groningen at the BME department (2008)
Genetics and physiology of food-associated micro-organisms, VLAG (2010)

Meetings
ASM 3rd Conference on *Salmonella* (+poster), Aix-en-Provence, France (2009)
Safepork Conference (+poster), Maastricht, The Netherlands (2011)
KNVM & NVvM The scientific spring meeting (+oral presentation), Papendal, The Netherlands (2011)
KNVM & NVvM The scientific spring meeting, Papendal, The Netherlands (2012)

General courses

VLAG PhD week (2008)
Working with Endnote X2, Wageningen University (2009)
NWO Talent Day, Utrecht, The Netherlands (2010)
Course Supervision and Organisation of MSc thesis work, Wageningen University (2010)
Effective behaviour in your professional surroundings, Wageningen Graduate Schools (2011)
Writing for academic publication, Linda McPhee Consulting (2011)
Career perspectives, Wageningen Graduate Schools (2012)

Other activities

Preparation PhD research proposal (2008)
Participation seminars Laboratory of Food Microbiology (2008-2012)
Participation TIFN C-1056: Spores and Biofilms project meetings (2008-2012)
FHM PhD trip to Canada (2008)
Organisation of the FHM PhD trip to Switzerland (2010-2011)
FHM PhD trip to Switzerland (2011)

CURRICULUM VITAE

Greetje Castelijn was born on February 14, 1984 in Roosendaal, The Netherlands. She received her ´HAVO-diploma´ with the specialization in nature and health at the Gertrudis College in Roosendaal in 2001. From 2001 until 2005, she studied biology and medical laboratory science at the Avans Hogeschool in Etten-leur. As part of her bachelor study an internship was carried out at the Pulmonology department of the University of Maastricht and a research project was carried out at Unilever R&D in Vlaardingen. After her graduation in 2005 she started her master study in molecular life sciences at the Wageningen University, specializing in biomedical research. As part of her master study she contributed to two research projects. The first project was conducted at the Wageningen University at the cell biology and immunology department, and the second one was conducted at Rijksinstituut voor volksgezondheid en milieu (National institute of public health and environment) at the laboratory for infectious diseases and prenatal screening. She graduated in 2005, whereafter she started her PhD project at the Wageningen University at the laboratory of food microbiology. Her PhD project was entitled ´*Salmonella* biofilms´. The results of that project are described in this thesis. From December 2012, Greetje is employed as head of the microbiology laboratory at the quality control department of Bavaria at Lieshout, The Netherlands.

The study presented in this thesis was performed within the framework of TI Food&Nutrition.

Financial support from Wageningen Univeristy and TI Food&Nutrition for printing this thesis is gratefully acknowledged.

Cover design by Esther van Eekelen and Michelle van Vliet.

Printed by van Stiphout Grafische Communicatie.