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REVIEW ARTICLE

Biofilm formation and the food industry, a focus on the bacterial outer surface

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Summary

The ability of many bacteria to adhere to surfaces and to form biofilms has major implications in a variety of industries including the food industry, where biofilms create a persistent source of contamination. The formation of a biofilm is determined not only by the nature of the attachment surface, but also by the characteristics of the bacterial cell and by environmental factors. This review focuses on the features of the bacterial cell surface such as flagella, surface appendages and polysaccharides that play a role in this process, in particular for bacteria linked to food-processing environments. In addition, some aspects of the attachment surface, biofilm control and eradication will be highlighted.

Introduction

The ability to stick to surfaces and to engage in a multistep process leading to the formation of a biofilm is almost ubiquitous among bacteria. Therefore, biofilm formation has substantial implications in fields ranging from industrial processes like oil drilling, paper production and food processing, to health-related fields like medicine and dentistry. The cellular mechanisms underlying microbial biofilm formation and behaviour are beginning to be understood and are targets for novel specific intervention strategies to control problems caused by biofilm formation in these different fields and in particular for the food-processing environments. Food spoilage and deterioration not only results in huge economic losses, food safety is a major priority in today's globalizing market with worldwide transportation and consumption of raw, fresh and minimally processed foods.

Biofilm formation depends on an interaction between three main components: the bacterial cells, the attachment surface and the surrounding medium (Davey and O'Toole 2000; Donlan 2002; Dunne 2002; Stoodley *et al.* 2002). This review will focus on the bacterial surface, which is the interface of the bacterium with its surroundings, and on the properties of the attachment surface influencing biofilm formation. Both are discussed in a context of food-processing environments; therefore, aspects dealing with biofilm prevention, control and eradication are also highlighted.

Properties of the bacterial and the abiotic surface affecting biofilm formation

The bacterial cell surface

Bacterial attachment to surfaces or other cells can be seen as a physicochemical process determined by Van der Waals, electrostatic and steric forces acting between the cells and the attachment surface. A theory to quantitatively describe this interaction of charged surfaces through

a liquid medium, designated the Derjaguin, Verwey, Landau and Overbeek (DLVO) theory, has been developed in the 1940s. Later, an extended DLVO theory was developed, which incorporated besides these long-range forces also hydrophilic/hydrophobic and osmotic interactions, resulting in more accurate predictions of bacterial adhesion [reviewed by Strevett and Chen (2003)]. These theories are not reviewed here, instead the wide variety of individual outer cell surface structures and molecules that are exposed on, or protrude from, the cell surface are described in detail. These structures shape the physicochemical surface properties of bacterial cells, and hence determine attachment and biofilm formation properties. However, the presence or absence of a certain structure on initial attachment or biofilm formation should be evaluated with care because multiple structures can be present, each with their own specific effects, and different structures could have diverse roles depending on the bacterium and the attachment surface.

Flagella

Many bacteria are motile by virtue of peritrichous or polar flagella, and motility is generally regarded as a virulence factor facilitating the colonization of host organisms or target organs by pathogenic bacteria. Flagellar motility is critical for initial cell-to-surface contact and normal biofilm formation under stagnant culture conditions for *Escherichia coli* (Pratt and Kolter 1998), *Listeria monocytogenes* (Vatanyoopaisarn *et al.* 2000; Lemon *et al.* 2007; Todhanakasem and Young 2008) and *Yersinia enterocolitica* (Kim *et al.* 2008). Although lack of flagella also affected initial attachment under flow conditions for *Y. enterocolitica* and *L. monocytogenes*, further maturation was unaffected for *Y. enterocolitica* (Kim *et al.* 2008), and the formation of high density biofilms was not suppressed for *L. monocytogenes* (Todhanakasem and Young 2008). For *Pseudomonas fluorescens*, mutants lacking flagella showed a decreased attachment to a variety of plant seeds and inert surfaces such as sand (Deflaun *et al.* 1990, 1994) and a decreased colonization of potato roots (De Weger *et al.* 1987). Finally, initial attachment of *L. monocytogenes* to stainless steel can also be affected by flagella *per se* (Vatanyoopaisarn *et al.* 2000). These observations indicate that flagella can affect adherence and biofilm formation via different mechanisms depending on the type of bacterium. First, motility can be necessary to reach the surface by allowing the cell to overcome the repulsive forces between the cell and the surface. This mechanism is possibly more important under stagnant than under flow conditions. In addition, motility can be required to move along the surface, thereby, facilitating growth and spread of a developing biofilm. Finally, flagella themselves (as surface appendages) can directly mediate attachment to surfaces.

Surface appendages

Fimbriae, thread-like structures that protrude from the cell surface, are classified on the basis of their adhesive, antigenic or physical properties, or on the basis of similarities in the primary amino acid sequence of their major protein subunits (Low *et al.* 1996). Type 1 fimbriae, which are rod-shaped and approximately 7-nm wide and 1-μm long, are the most common adhesins found in both commensal and pathogenic *E. coli* as well as in other Enterobacteriaceae (Klemm and Krogfelt 1994). Their role in biofilm formation has been studied exhaustively, demonstrating a critical role in initial stable cell-to-surface attachment for numerous *E. coli* strains (Pratt and Kolter 1998; Beloin *et al.* 2004; Ren *et al.* 2004) including Shiga toxin-producing strains (Cookson *et al.* 2002), in adherence to Teflon and stainless steel for *Salmonella enterica* serovar Enteritidis (Austin *et al.* 1998), and in promoting biofilm formation on abiotic surfaces (polystyrene) for *Klebsiella pneumoniae* (Schembri *et al.* 2005).

Besides Type 1 fimbriae, other types of fimbriae have been shown to affect biofilm formation. For example, Di Martino *et al.* (2003) showed that for a *Kl. pneumoniae* strain, which produced both Type 1 and Type 3 fimbriae, the latter constituted the main factor facilitating adherence to both glass and polypropylene, and the formation of a full-grown biofilm on polystyrene. Type 4 fimbriae promoted the rapid formation of strongly adherent biofilms for the opportunistic pathogen *Aeromonas caviae* (Bechet and Blondeau 2003), commonly found in water and foods (Neyts *et al.* 2000), and affected the binding of *Pseudomonas aeruginosa* to stainless steel, polystyrene and polyvinylchloride (Giltner *et al.* 2006). Genes involved in the biogenesis, regulation and secretion of Type 4 fimbriae were found to be up-regulated within 6 h of attachment to silicone tubing for *Pseudomonas putida* (Sauer and Camper 2001), often associated with spoilage of fresh milk and vegetables (Ternstrom *et al.* 1993; Garcia-Gimeno and Zurera-Cosano 1997). Type 4 fimbriae also played a role in the colonization and persistence of *Vibrio vulnificus* in oysters (Paranjpye *et al.* 2007). *Vibrio vulnificus* is a pathogen associated with human infections caused by raw oyster consumption (Blake *et al.* 1979) and an important cause of reported deaths from food-borne illness in Florida (Hlady *et al.* 1993). Furthermore, for enterohemorrhagic *E. coli* O157:H7, these structures not only affected attachment and biofilm formation but have also been implicated in virulence and transmission (Xicohtencatl-Cortes *et al.* 2009).

Curli fimbriae (called thin aggregative fimbriae in *Salmonella*) are proteinaceous, coiled filamentous surface

structures, which are assembled by an extracellular nucleation/precipitation pathway (Olsen *et al.* 1989). The effect of curli on attachment and biofilm formation of *E. coli* O157:H7 appears to be variable. In one study, curli production enhanced the biofilm-forming capacity of a particular strain to stainless steel (Ryu *et al.* 2004b), although initial attachment was unaffected (Ryu and Beuchat 2005). In another study, different Shiga toxin-producing and enterohaemorrhagic *E. coli* strains showed an enhanced attachment to abiotic surfaces such as polystyrene and stainless steel when curli were produced (Cookson *et al.* 2002; Pawar *et al.* 2005). Probably, this increased attachment is strain dependent as shown in a study comparing the attachment of curli-producing and noncurli-producing *E. coli* O157:H7 strains to lettuce (Boyer *et al.* 2007). Interestingly, it cannot be excluded that the observed differences are not only strain dependent, but are also induced by other (nonevaluated) mechanisms or by the occurrence of dissimilar environmental triggers in the experiments.

In addition to curli, cellulose is also usually associated with biofilms of various salmonellae, including strains of the serovar Typhimurium (Solano *et al.* 2002; Jain and Chen 2007). The simultaneous production of cellulose and curli leads to the formation of a highly inert, hydrophobic extracellular matrix in which the cells are embedded (Zogaj *et al.* 2001). However, other capsular polysaccharides can be present in the extracellular biofilm matrix of *Salmonella* strains (de Rezende *et al.* 2005), and the exact composition depends upon the environmental conditions in which the biofilms are formed (Prouty and Gunn 2003). A variety of environmental cues such as nutrients, oxygen tension, temperature, pH, ethanol and osmolarity can influence the expression of the transcriptional regulator CsgD, which regulates the production of both cellulose and curli (Gerstel and Romling 2003). In addition, a study of 122 *Salmonella* strains indicated that all had the ability to adhere to plastic microwell plates and that, generally, more biofilm was produced in low nutrient conditions, as can be found in specific food-processing environments, compared to high nutrient conditions (Stepanovic *et al.* 2004).

Pili are structurally similar to fimbriae and are involved in a process of horizontal gene transfer called conjugation. Mostly, the transferred DNA is a conjugative plasmid encoding the formation of the conjugative pilus itself, and thereby mediates an intimate cell-to-cell contact. This conjugation process can stimulate biofilm development, because the conjugative pilus can act as an adhesion factor allowing nonspecific cell-solid surface or cell–cell contacts (Ghigo 2001; Reisner *et al.* 2003). *Vice versa*, the high density of bacterial populations in biofilms can stimulate conjugation and plasmid dispersal (Hausner and Wuertz 1999; Molin and Tolker-Nielsen 2003) and can therefore contribute to the spread of resistance genes, which are often also carried on the plasmid (Bower and Daeschel 1999). Luo *et al.* (2005) have demonstrated that conjugation enhanced the expression of CluA, a surface-bound clumping protein encoded by the chromosomally embedded sex factor, and subsequently facilitated biofilm formation in *Lactococcus lactis*. Furthermore, this enhanced biofilm-forming trait is transmissible by conjugation.

In addition to proteinaceous organelle-type surface appendages, some Gram-negative bacteria can produce autotransporter proteins. These are secretory proteins that contain in their primary structure all the information necessary to direct their own secretion across the cytoplasmic and outer membrane to the bacterial cell surface. Adhesive phenotypes such as aggregation and biofilm formation have been attributed to a subfamily of *E. coli* autotransporters, including antigen 43 (Ag43) (Danese *et al.* 2000a; Kjaergaard *et al.* 2000), the AIDA adhesin associated with some diarrheagenic *E. coli* (Sherlock *et al.* 2004), and the TibA adhesin/invasin from enterotoxigenic *E. coli* (Sherlock *et al.* 2005).

Surface polysaccharides

The lipopolysaccharide (LPS) outer layer of Gram-negative bacteria typically consists of a surface exposed O-antigen, a core structure and a lipid A moiety that is embedded in the outer membrane lipid bilayer. The LPS layer not only affects the bacterium's susceptibility to disinfectants, antibiotics and other toxic molecules (Russell and Furr 1986), it also plays a role in biofilm formation. For example, O-antigen mutants of *Salmonella enterica* serovar Typhimurium showed reduced capacities to attach and colonize alfalfa sprouts (Barak *et al.* 2007). Alterations in the LPS of *Salm.* Typhimurium had also osmolyte-dependent effects on biofilm formation (Anriany *et al.* 2006). For *E. coli*, truncation of LPS (deep-rough phenotype) did not affect adhesion *per se*, but had a pleiotropic effect on the biosynthesis of Type 1 fimbriae and flagella, resulting in a reduced adherence (Genevaux *et al.* 1999). Alterations in the peptidoglycan structure exposed at the surface of Gram-positive bacteria can also have an effect on attachment, as shown by analysis of *L. monocytogenes* rough colony variants. The latter, characterized by an impaired cellular localization of several peptidoglycan-degrading enzymes such as the cell wall hydrolase A (CwhA), showed enhanced attachment to stainless steel (Monk *et al.* 2004).

Many bacteria produce and secrete extracellular polysaccharides (EPS). The polysaccharide-containing layers outside the cell are collectively defined as glycocalyx, but when the layers are rigid and organized in a tight matrix

that excludes particles, the term capsule is used. If the layers do not exclude particles and are more easily deformed and detached, the term slime is used. These EPS are an important constituent of the extracellular matrix characteristically produced by many biofilms. The matrix often contains additional constituents, such as nucleic acids, proteins, glycoproteins and lipoproteins.

For *Kl. pneumoniae*, the capsule is considered to be a dominant virulence factor, and its synthesis blocked Type 1 fimbriae-promoted biofilm formation on abiotic surfaces (see above), thereby, actually reducing the bacterial adhesion to such surfaces (Schembri *et al.* 2005). For *V. vulnificus*, expression of capsular polysaccharides also inhibited attachment and biofilm formation on abiotic surfaces (plastic) (Joseph and Wright 2004). The EPS colanic acid (or M antigen) produced by most *E. coli* strains as well as by other species of the Enterobacteriaceae appears to be important for establishing the complex structure and depth of *E. coli* biofilms, but not for initial attachment to abiotic surfaces (Danese *et al.* 2000b; Prigent-Combaret *et al.* 2000). Overproduction of EPS can even inhibit initial attachment of *E. coli* O157:H7 to stainless steel (Ryu *et al.* 2004a). The unbranched polysaccharide, β-1,6-poly-*N*-acetyl-D-glucosamine (PGA), is involved not only in adhesion by staphylococci, but also in attachment to abiotic surfaces, intercellular adhesion and biofilm formation of *E. coli* (Wang *et al.* 2004). Furthermore, depolymerization of PGA led to dispersal of biofilms (Itoh *et al.* 2005). Colanic acid, PGA and cellulose production, but not LPS production, affected binding of *E. coli* O157:H7 to alfalfa sprouts as shown by mutational analysis (Matthysse *et al.* 2008).

These observations indicate contrasting roles for EPS (and LPS) in biofilm formation of different bacteria. The particular function of EPS in biofilm formation may depend on its structure, relative quantity and charge and on the properties of the abiotic surface and surrounding environment. Furthermore, EPS play a role not only in biofilm formation but also in the increased resistance of biofilm bacteria to biocides as described in section Implications of biofilm formation.

Factors affecting the bacterial cell surface

The attachment and biofilm-forming capabilities of bacteria depend on multiple factors including the attachment surface (see below), the presence of other bacteria, the temperature, the availability of nutrients and pH. Although the mechanisms underlying these effects are not always explained, biofilm formation can in some cases be influenced through alterations of the bacterial cell surface. For instance, curli expression and attachment to plastic surfaces by enterotoxin-producing *E. coli* strains was found to be higher at 30°C than at 37°C (Szabo *et al.* 2005). Similarly, expression of thin aggregative fimbriae in *Salm.* Typhimurium and of fimbriae in *Aeromonas veronii* strains isolated from food was affected by temperature, with a lower temperature (28 and 20°C, respectively) favouring expression (Kirov *et al.* 1995; Romling *et al.* 1998). Production of these outer surface structures at low(er) temperatures could enhance the attachment to surfaces and hence facilitate persistence and survival in food-processing environments. The adhesion of *L. monocytogenes* to polystyrene after growth at pH 5 was lower than after growth at pH 7, and this could be attributed to the down-regulation of flagellin synthesis (Tresse *et al.* 2006).

The large cell densities existing in biofilms create a local environment suitable for cell density-dependent bacterial communication. Bacteria throughout the bacterial kingdom have evolved the ability to steer the behaviour of individual cells, populations or communities by using various modes of communication. One of the best studied communication mechanisms in bacteria is quorum sensing, which is based on the production of low-molecular-mass signalling molecules. When the bacterial cell density is low, the extracellular concentration of the signals will also be low and remain undetected. However, as the cell density increases in a growing (biofilm) population, a critical signal concentration will be reached, allowing the signalling molecule to be sensed and enabling the bacteria to respond. The nature of the signalling molecules is diverse. While most Gram-negative bacteria use *N*-acyl-homoserine lactones (AHL) as signalling molecules (Lazdunski *et al.* 2004), Gram-positive bacteria commonly use amino acids and short post-translationally processed peptides (Sturme *et al.* 2002). Additional families of bacterial signalling molecules have been identified such as Autoinducer-2 (AI-2) for both Gram-negative and Gram-positive bacteria (Schauder and Bassler 2001; Xavier and Bassler 2003).

These communication mechanisms control various functions such as virulence, biofilm development and the production of antimicrobial compounds and several other secondary metabolites. As such, quorum sensing can affect the establishment of bacteria in a mixed biofilm community (Moons *et al.* 2006), their food spoilage potential (Ammor *et al.* 2008; Wevers *et al.* 2009), or their survival in particular (food-processing related) stressful environments (Van Houdt *et al.* 2006, 2007a). Also, the production of surface appendages and motility, putatively affecting biofilm formation, can be regulated by quorum sensing (Daniels *et al.* 2004; Van Houdt *et al.* 2007b).

Although quorum sensing has been shown to play a role in biofilm formation for several bacteria, this is not always the case, and no consistent correlation was found

between AHL or AI-2 production and biofilm-forming capacity of 68 Gram-negative strains isolated from an industrial kitchen (Van Houdt *et al.* 2004).

The attachment surface and environmental parameters

The properties of the attachment surface are important factors that affect and determine the biofilm formation potential together with the bacterial cells. The choice of material is therefore of great importance in designing food contact and processing surfaces. Properties such as surface roughness, cleanability, disinfectability, wettability (determined by hydrophobicity) and vulnerability to wear influence the ability of cells to adhere to a particular surface and thus determine the hygienic status of the material. In addition, materials in direct contact with foods have to meet certain specifications and are subject to official approval procedures before they can be used. Materials often used in the food industry include plastics, rubber, glass, cement and stainless steel. The degree of biofilm formation on different materials for *Legionella pneumophila* has been ranked by Rogers *et al.* (1994) and by Meyer (2001) with the capacity to support biofilm growth increasing from glass, stainless steel, polypropylene, chlorinated PVC, unplasticized PVC, mild steel, polyethylene, ethylene-propylene to latex.

However, general predictions for the degree of biofilm formation on a particular material cannot be made because the biofilm-supporting capacity of any material also depends on bacteria and on environmental factors. For instance, temperature and nutrient availability can influence the ability of *L. monocytogenes* to adhere to polyvinyl chloride, buna-n rubber and stainless steel, because of altered bacterial surface physicochemical properties like hydrophobicity/hydrophilicity and surface charge (Briandet *et al.* 1999; Norwood and Gilmour 1999; Moltz and Martin 2005).

In food-processing environments, bacterial attachment is additionally affected by food matrix constituents. Residues from ready-to-eat meat products such as small amounts of meat extract, frankfurters or pork fat, initially reduced biofilm formation of *L. monocytogenes*, but with time supported increased biofilm cell numbers and prolonged survival on a variety of materials including stainless steel, conveyor belt rubber, and wall and floor materials (Somers and Wong 2004). Skim milk and milk proteins such as casein and lactalbumin were found to significantly reduce the attachment of *Staphylococcus aureus*, *Serratia marcescens*, *Pseudomonas fragi*, *Salm.* Typhimurium, spores and vegetative cells of thermophilic bacilli, and *L. monocytogenes* to stainless steel (Helke *et al.* 1993; Wong 1998; Barnes *et al.* 1999; Parkar *et al.* 2001) and buna-n rubber gaskets (Helke *et al.* 1993; Wong 1998). Not only physicochemical, but also nutritional properties of the food matrix affect attachment and persistence. For instance, Allan *et al.* (2004a,b) showed that survival rates of *L. monocytogenes* on several surfaces, including stainless steel, acetal resin, mortar and fibreglass reinforced plastic, were higher in the presence of biological soil (porcine serum). Finally, the presence of a mixed microbial community adds additional complexity to attachment and biofilm formation under certain conditions. The presence of *Staphylococcus xylosus* and *Ps. fragi* affected the numbers of *L. monocytogenes* found in biofilms on stainless steel (Norwood and Gilmour 2001). Similarly, bacteriocin-producing *L. lactis* as well as several endogenous bacterial strains isolated from food-processing plants influenced the establishment of *L. monocytogenes* on stainless steel, suggesting that the 'house microflora' can have a strong suppressing effect on *L. monocytogenes* establishment in biofilms in a food-processing environment (Leriche *et al.* 1999; Carpentier and Chassaing 2004).

Stainless steels, in particular austenitic grades 304 and 316, are probably the most commonly used food contact surfaces because of their chemical and mechanical/physical stability at various food-processing temperatures, cleanability and high resistance to corrosion (Zottola and Sasahara 1994). The grade, which reflects the composition and to a lesser extent the finish (pickling, bright annealed), significantly affected the hygienic status of stainless steel as measured by the number of residual adhering *Bacillus cereus* spores after a complete run of soiling followed by a cleaning-in-place procedure (Jullien *et al.* 2003). Grade 316 has nearly the same mechanical and physical characteristics as 304 but has a higher resistance to corrosion by foods, detergents and disinfectants, because of the anticorrosive properties of the added molybdenum. Food contact surfaces are commonly treated with disinfectants and cleaning agents that contain peroxides, chloramines or hypochlorites. In particular, the latter can be very aggressive to stainless steels depending on the prevailing pH. The liberation of free chlorine can cause pitting, characterized by local breakdown of the protective 'passive' oxide surface layer and formation of local deep pits on these free surfaces, thereby facilitating bacterial adhesion and biofilm formation. Therefore, the duration and operating temperature of cleaning and disinfection treatments should be carefully controlled, and thorough rinsing with water should always be performed as a last step (BSSA 2001).

Implications of biofilm formation

Biofilms formed in food-processing environments are of special importance as they have the potential to act as a persistent source of microbial contamination that may lead to food spoilage or transmission of diseases. It is

generally accepted and well documented that cells within a biofilm are more resistant to biocides than their planktonic counterparts. Numerous reports indicate that the antimicrobial efficacy of various aqueous sanitizers is lower for biofilm-associated than for planktonic *Salmonella* spp. Nine disinfectants commonly used in the feed industry and efficient against planktonic *Salmonella* cells showed a bactericidal effect that varied considerably for biofilm-grown cells with products containing 70% ethanol being most effective (Moretro *et al.* 2009). Other studies similarly indicated that compared to planktonic cells, biofilm *Salmonella* were more resistant to trisodium phosphate (Scher *et al.* 2005) and to chlorine and iodine (Joseph *et al.* 2001). *Listeria monocytogenes* biofilms were more resistant to cleaning agents and disinfectants including trisodium phosphate, chlorine, ozone, hydrogen peroxide, peracetic acid (PAA) and quaternary ammonium compounds (Frank and Koffi 1990; Lee and Frank 1991; Somers *et al.* 1994; Sinde and Carballo 2000; Stopforth *et al.* 2002; Somers and Wong 2004; Robbins *et al.* 2005). *Lactobacillus plantarum* ssp. *plantarum* biofilms showed increased resistance towards various organic acids, ethanol and sodium hypochlorite (Kubota *et al.* 2009).

Which disinfectant is the most effective in a particular situation depends on numerous factors including the nature of the attachment surface, temperature, exposure time, concentration, pH and bacterial resistance (Mafu *et al.* 1990; Bremer *et al.* 2002). Resistance is attributed to different mechanisms: a slow or incomplete penetration of the biocide into the biofilm, an altered physiology of the biofilm cells, expression of an adaptive stress response by some cells, or differentiation of a small subpopulation of cells into persister cells.

Biofilm resistance to chlorine is still incompletely understood, but is at least partly because of hindered penetration of the biocide into the biofilm (De Beer *et al.* 1994; Chen and Stewart 1996; Xu *et al.* 1996). Active chlorine concentrations as high as 1000 ppm are necessary for a substantial reduction in bacterial numbers in multispecies biofilms (formed by *L. monocytogenes*, *Ps. fragi* and *Staph. xylosus*) compared to 10 ppm for planktonic cells (Norwood and Gilmour 2000). Chlorine concentrations measured in biofilms of *Kl. pneumoniae* and *Ps. aeruginosa* were typically only 20% or less of the concentration in the bulk liquid (De Beer *et al.* 1994). The slow or incomplete penetration of the biocide into the biofilm is partly because of diffusion limitation in the exopolymeric matrix, but primarily because of neutralization of the active compound in the outermost regions of the matrix. The active chlorine species react with organic matter in the surface layers of the biofilm faster than they can diffuse into the biofilm interior (Chen and Stewart 1996; Xu *et al.* 1996). This explains that an exopolysaccharide-overproducing curli-producing *E. coli* O157:H7 strain showed an increased resistance to chlorine (Ryu and Beuchat 2005). Solano *et al.* (2002) demonstrated that the biofilm matrix protected *Salm.* Enteritidis cells to chlorine as cellulose-deficient mutants were more sensitive to chlorine treatments.

Biofilm cells, especially those buried deep in the biofilm, exhibit decreased growth rates because of oxygen and nutrient gradients (Brown *et al.* 1988). This results in a quasi-dormant state that in turn causes an increased resistance to biocides (Gilbert *et al.* 1990; Evans *et al.* 1991). Concordant with these observations, older biofilms appear to be more resistant against various disinfectants than younger biofilms (Frank and Koffi 1990; Lee and Frank 1991). The observed differences between planktonic and biofilm bacteria reflect important physiological alterations taking place subsequent to attachment. There is increasing evidence that these alterations are caused by unique gene expression patterns in biofilm bacteria, which are not observed in planktonic cells (Prigent-Combaret *et al.* 1999; Stoodley *et al.* 2002; Beloin *et al.* 2004; Ren *et al.* 2004), and which are at the basis of the biofilm-specific adaptive response. For instance, higher numbers of *Salm.* Enteritidis biofilm cells survived a lethal benzalkonium chloride treatment compared to planktonic cells when cells were previously exposed to sublethal concentrations of the agent (Mangalappalli-Illathu *et al.* 2008). *Salm.* Enteritidis isolates that survived better on surfaces also survived better in acidic conditions and in the presence of hydrogen peroxide and showed enhanced tolerance towards heat (Humphrey *et al.* 1995; Mangalappalli-Illathu *et al.* 2008).

Another possible mechanism of biocide resistance is based on the observation that some of the biofilm cells are able to sense the biocide challenge and actively respond to it by deploying protective stress responses more effectively than planktonic cells (Szomolay *et al.* 2005). Sanderson and Stewart (1997) reported that when *Ps. aeruginosa* biofilms were repeatedly exposed to monochloramine, the second dose was less effective than the first. *Pseudomonas aeruginosa* biofilms also showed increased catalase (*katB*) expression during treatment with hydrogen peroxide at a concentration sublethal for biofilm cells but lethal for planktonic cells (Elkins *et al.* 1999). Other studies reported that exposure of biofilm cells to antibiotics elicited a response resulting in increased synthesis of EPS, resulting in a more proliferous biofilm matrix (Sailer *et al.* 2003; Bagge *et al.* 2004).

Persisters, a small fraction of essentially invulnerable cells, are phenotypically variant cells that neither grow nor die in the presence of bactericidal agents, but that are largely responsible for the recalcitrance of infections caused by bacterial biofilms [for review see Lewis (2001, 2005,

2007)]. Persister formation has been attributed to specific cellular toxins, proteins that block cellular processes like translation, thus rendering the cell resistant against biocides that act only against active cells (Lewis 2001, 2005, 2007).

Prevention, control, removal and eradication of biofilms in the food industry

Prevention and control

Microbial attachment to (food-processing) surfaces is a rather fast process, and therefore, it is for most applications not possible to clean and disinfect frequently enough to avoid attachment. Nevertheless, an adequate frequency of disinfection should be carefully determined to avoid biofilm maturation and build-up of absorbed organic material (product residues), which can influence the hygienic status of the material and the availability of nutrients. Sharma *et al.* (2003) recommended to control the operating time between cleaning and sanitation to prevent mixed species biofilm formation in pasteurization lines of commercial and experimental dairy plants. Cleaning and sanitation of food-processing surfaces with short intervals was proposed as an effective approach to prevent or limit sporulation in biofilms formed by vegetative *Bacillus subtilis* cells (Lindsay *et al.* 2005).

Rational equipment design that minimizes laminar product flow, reduces static product and facilitates cleaning and cleaning-in-place processes can result in a reduced bacterial attachment to the processing equipment. As described in Introduction, the choice of material herein is crucial in terms of biofilm formation. The hygienic properties of the material can be altered by specific modifications to render it intrinsically antibacterial and/or less susceptible to attachment. For example, the deposition of antifouling layers on stainless steel can influence their hygienic status, as demonstrated by the 81–96% decrease in *L. monocytogenes* attachment and biofilm formation on polyethylene glycol-modified stainless steel. The modified surface properties were obtained by plasma-enhanced cross-linking of polyethylene glycol on stainless steel. This promising technique reduced bacterial deposition in food-processing environments (Dong *et al.* 2005), with PEG-deposition stable to cleaning and storage for up to 2 months (Wang *et al.* 2006). Guerra *et al.* (2005) showed that nisin, an antimicrobial peptide also used as food preservative, adsorbed to stainless steel, rubber and polyethyleneterephthalate (PET) surfaces, and upon doing so retained its antibacterial activity and inhibited the growth of *Enterococcus hirae*. Moreover, nisin-coated PET bottles significantly reduced the total aerobic plate counts in skim milk compared to uncoated bottles, although it was not clear whether the effect was because of adsorbed nisin or nisin released in the bulk. Nevertheless, this PET-based bioactive packaging extended the shelf-life and consequently could be a promising technique for extending the shelf-life of various packaged foods (Guerra *et al.* 2005). The incorporation of transition metal catalysts into polymer surfaces promotes the formation of active oxygen species from peroxides and persulfates, thereby targeting particularly the cells nearest to the surface. This localized antibacterial action at the surface is believed to also affect the adhesion properties of the biofilm cells (Wood *et al.* 1996, 1998). The application of such surface-active systems is restricted to some specific food contact materials, and their durability and application costs need to be carefully considered.

An efficient control programme evidently relies on adequate detection systems for biofilms. Several methods are commonly used like conventional total viable count, different microscopy and spectroscopy techniques, impedance measurements and ATP determination [reviewed by Wirtanen *et al.* (2000); Verran *et al.* (2002); Janknecht and Melo (2003)]. Each technique has its advantages and constraints, and a well-chosen combination of detection methods guarantees the most efficient detection.

Removal and eradication

Cleaning processes

The primary objective of a cleaning process is the removal of product residues. Indirectly, removal of these residues is also a first critical point in the removal, killing and control of biofilms. Adequate methods that break up and remove the product deposited on the contact surface as well as existing biofilm matrix are important for the food-processing industry (Zottola and Sasahara 1994), because incomplete removal facilitates the reattachment of bacteria to the surface and formation of a novel biofilm even if the bacteria from the previous biofilm were killed (Gibson *et al.* 1999). Moreover, disinfectants are less effective when food particles or dirt is present on the surfaces (Holah and Thorpe 1990; Sinde and Carballo 2000). The standard methods used in many food-processing industries, such as alkali-based and acid-based cleaning, are only adequate in removing the extracellular polymeric biofilm matrix when the correct process parameters, i.e. appropriate formulations, concentrations, time, temperature and kinetic energy (flow) are applied, and suboptimal process parameters will drastically affect the overall outcome (Parkar *et al.* 2004; Antoniou and Frank 2005). The removal of biofilms is also significantly facilitated by the application of mechanical force (like brushing and scrubbing) to the surface during cleaning (Wirtanen *et al.* 1996). Sadoudi *et al.* (1997) demonstrated that pulsed laser beams could be used as an alternative cleaning method for reduction of

the microbial load on surfaces. However, although efficient, the removal resulted in the transfer of bacteria to the air in the form of an aerosol, and additional measures will therefore be necessary to prevent the spread of surviving bacteria. This is one of the reasons why the use of high pressure sprays has been replaced by foam or gel cleaning.

Chemical disinfectants

A wide range of chemical disinfectants is used in the food industry, which can be divided into different groups according to their mode of action: (i) oxidising agents including chlorine-based compounds, hydrogen peroxide, ozone and PAA, (ii) surface-active compounds including quaternary ammonium compounds and acid anionic compounds, and (iii) iodophores. The efficiency of disinfection is influenced by pH, temperature, concentration, contact time and interfering organic substances like food particles and dirt (Holah 1992; Mosteller and Bishop 1993). Therefore, cleaning agents like detergents and enzymes are frequently combined with disinfectants to synergistically enhance disinfection efficiency (Jacquelin *et al.* 1994; Johansen *et al.* 1997). The increased resistance of biofilm cells to biocides, which is at least partially because of interference of the exopolymeric matrix (described in section Properties of the bacterial and the abiotic surface affecting biofilm formation), explains why the disinfectant most effective to planktonic cells is not necessarily the most active against biofilm cells. Holah *et al.* (1990) and Meyer (2001) ranked the efficiency of disinfectants to kill biofilm cells and concluded that the effectiveness increased from quaternary ammonium compounds over amphoterics, chlorine, biguanides to peroxy acids. Fatemi and Frank (1999) reported similarly that peroxy acid disinfectants were more effective than chlorine for inactivating multispecies biofilms of *Pseudomonas* sp. and *L. monocytogenes* on stainless steel. This difference in effectiveness was even more pronounced in the presence of an organic challenge. However, Mosteller and Bishop (1993) reported no superior efficiency of PAA on *Ps. fluorescens*, *L. monocytogenes* and *Y. enterocolitica* biofilms on both rubber and Teflon(R) surfaces; and in a comparative study, Rossoni and Gaylarde (2000) found sodium hypochlorite to be more effective than PAA in killing or removing *E. coli*, *Ps. fluorescens* and *Staph. aureus* adhering to stainless steel. Trachoo and Frank (2002) demonstrated that chlorine was more effective than PAA and than a PAA/peroctanoic acid mixture against *Campylobacter jejuni* in multispecies biofilms. Moreover, the presence of the biofilm enhanced attachment of *Camp. jejuni* and decreased disinfectant effectiveness. Similarly, *Listeria innocua* cells were much more resistant to sodium hypochlorite and PAA in a multispecies biofilm with *Ps. aeruginosa* than in a pure-culture biofilm on stainless steel, Teflon(R) and rubber (Bourion and Cerf 1996).

The application of ozone as an alternative for sanitation has gained interest in the food industry. This trioxygen molecule with strong oxidizing properties (52% stronger than chlorine) has been shown to be effective over a much wider spectrum of micro-organisms than chlorine and other disinfectants and could be used as a disinfectant for both planktonic and biofilm bacteria. However, more information needs to be collected regarding the efficacy of ozone on food pathogens adherent to different material surfaces and concerning the effects of process parameters, e.g. temperature, pH, contact time, to further substantiate that ozone is an efficient disinfectant [reviewed by Guzel-Seydim *et al.* (2004)].

Finally, it deserves mention that much research and many new developments are currently ongoing in the field of biofilm disinfection, including the development of molecules that interfere with quorom sensing (Girennavar *et al.* 2008; Steenackers *et al.* 2008; Pan and Ren 2009), and naturally occurring biocides with either a wide action spectrum (Lebert *et al.* 2007; Chorianopoulos *et al.* 2008) or a more specific action against particular pathogenic and spoilage bacteria (Ammor *et al.* 2004; Lebert *et al.* 2007). It can be anticipated that a case-by-case evaluation of these novel approaches will be necessary because their efficacy, similar to that of established methods, will be affected by process parameters and the prevailing microbial population to be eradicated.

All these studies indicate that the statement: 'the disinfectant most effective to planktonic cells is not necessarily the most active against the biofilm cells' illustrated above, needs to be extended to 'furthermore the most active disinfectant against pure culture biofilm is not necessarily the most active against multispecies biofilms in challenging (food-processing) environments'. Nevertheless, active chlorine is probably the most widely used compound because chlorine-based compounds are easy to prepare and apply, and are generally the most cost-efficient.

Physical methods

Physical treatments have been studied as alternatives for the use of chemical disinfectants in the food industry in particular for the sanitation of surfaces. Niemira and Solomon (2005) showed that ionizing radiation was equally or more effective against *Salmonella* spp. biofilm cells than against planktonic cells and could therefore be a useful sanitization treatment on a variety of foods and contact surfaces. A relatively recent technique called atmospheric plasma inactivation makes use of reactive oxygen species and radicals generated by high voltage atmospheric pressure glow discharges to inactivate microorganisms. The technique appears to be effective against

both biofilm and planktonic micro-organisms (Vleugels *et al.* 2004). Oulahal-Lagsir *et al.* (2003) used a combined treatment of ultrasound and enzyme preparations for effectively removing *E. coli* biofilms on stainless steel sheets in milk. Ultrasound can also be used to increase the efficacy of biocides such as ozone (Bott and Tianqing 2004; Baumann *et al.* 2009). Another technique for enhancing the efficiency of biocides and antibiotics is the use of electric fields. This so-called bioelectric effect is based on an improved penetration of the active compound through the biofilm, thereby reducing the concentrations needed to eradicate biofilm bacteria to levels very close to those effective against planktonic bacteria (Costerton *et al.* 1994). The applicability of these combined disinfection systems should be comprehensively and systematically examined, considering also their economic costs and regulatory aspects.

Concluding remarks

Bacterial biofilms are ubiquitous in nature, and the food industry does not escape from the problems they can cause. In particular, biofilms formed on food-processing equipment and other food contact surfaces act as a persistent source of contamination threatening the microbiological quality and safety of food products, and resulting in food-borne disease and economic losses. Biofilm prevention and control is therefore a priority in the food industry, and this industry should be stimulated to:

- Develop and plan cleaning and disinfection programmes, which can prevent and/or eradicate biofilms and monitor their efficacy.
- Include the biofilm-supporting properties of food contact materials, in addition to their thermal, mechanical and chemical resistance, as an element of the hygienic design of equipment and utensils.
- Identify biofilm-prone areas in existing process lines and systematically monitor organic and microbial load in these areas.
- Invest in research on the efficacy of cleaning agents and disinfectants, the factors involved in attachment and biofilm formation, the decreased sensitivity of biofilm bacteria to disinfectants, and on developing novel biofilm prevention or control strategies.

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