SYMPOSIUM: BIOFILMS: DEVELOPMENT AND CONTROL

Structure and Functional Characteristics of Bacterial Biofilms in Fluid Processing Operations

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ABSTRACT

Bacterial biofilms create a number of serious problems for industrial fluid processing operations. Mechanical blockages, impedance of heat transfer processes, and biodeterioration of the components of metallic and polymeric systems result in billions of dollars in losses each year. Product spoilage and possible risks to public health are also consequences of biofilm-mediated contamination. Fundamentally, these biofouling activities can be described in terms of the physicochemical properties that are associated with bacterial metabolism and biofilm development. Treatment of biofouling is also complicated by the unique structural attributes of biofilms: extracellular polymeric substances create diffusional barriers to antimicrobial agents, protecting labile cellular targets from both oxidizing and nonoxidizing compounds. The mechanisms associated with the initial events of bacterial adhesion to engineered surfaces and subsequent fouling of biofilm formation are poorly understood. However, studies of bacterial biofilm architecture have been greatly facilitated by the application of confocal laser microscopy, scanning or transmission electron microscopy, and Fourier transform infrared spectroscopy. This paper reviews the genesis of biofilm formation and describes the influence of structure on biofouling activities in industrial fluid handling systems.
(**Key words**: adhesion, microbial contamination, biofouling)

Abbreviation key: **EPS** = extracellular polymeric substances.

INTRODUCTION

To a great extent, the science of public health has evolved from efforts to control various milkborne pathogens. Pasteurization and other procedures for disinfection of finished products have been very effective in controlling a broad spectrum of bacterial and rickettsial pathogens in milk and milk products. Despite the generally high quality of milk products in North America, recent reports show that bacterial contamination continues to present a significant threat to product quality and systems operations. Several factors have accounted for the current heightened concern over food product safety, including the recent and highly publicized contamination of hamburger meats in the Pacific Northwest by *Escherichia coli*, emergence (or reemergence) of *Listeria* spp. in milk and soft cheese processing operations (10, 16, 22), and bacterial outbreaks in raw milk supplies (31).

Although some of these outbreaks can be attributed to poor quality assurance and sanitization procedures, other contamination problems occur despite the application of normal preventive maintenance and treatment regimens. An important reservoir of microbial contamination that has received relatively little attention is the microbial biofilm. In dairy processing operations (29), as well as in numerous other industrial systems (26), most bacteria are associated with surfaces. In addition to creating problems associated with public health and product spoilage, biofilms are responsible for mechanical blockages and the impedance of heat transfer processes. During plant operations, microbial biofilms are often difficult to detect and treat. By virtue of a number of unique survival strategies, bacteria and other organisms within biofilms are able to resist disinfectants and biocides, which are otherwise effective against their free-floating counterparts (7). This apparent resistance has been implicated in the survival of *Listeria* spp. in dairy product processing operations (18, 19).

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The development of bacterial biofilms is a major cause of process fluid contamination leading to product deterioration. The inherent resistance of bacteria in biofilms leads to cycles of regrowth following system disinfection procedures. A recent review of biofilms in the dairy processing industry has been prepared by Flint et al. (11). Those scientists describe problems that are unique to pasteurization processes, in particular, *Streptococcus thermophilus* survival in biofilms on plate heat exchangers.

BACTERIAL BIOFILM DEVELOPMENT

In natural aquatic systems, the majority of bacteria are attached to surfaces. Indeed, surface area is a major limiting factor for microbial growth in nearly every freshwater and marine environment (28). The ratio of planktonic (free-floating) bacteria to biofilm bacteria is a function of several interrelated factors, including surface energetics (34), materials of construction (19, 35), topography, hydraulic factors, and biofilm chemistry (8).

Bacterial attachment and the formation of a biofilm appear to take place in a three-stage process. During the first stage, surfaces are rapidly coated with an organic conditioning film. This film might consist in blood of proteinaceous compounds such as albumin (33), in freshwater environments of humic substances (20), and in dairy operations of proteinaceous components of milk and milk products. This first stage occurs within the first 5 to 10 s after an otherwise clean surface is placed into a fluid environment. During the second stage of adhesion, single bacterial cells are transported to surfaces, and reversible bonds are formed between the cell wall and the substratum. Bacterial extracellular polymeric substances (**EPS**) appear to mediate the attachment of primary colonizers to organic conditioning films that are associated with animate and inanimate substrata (21).

The mature, third-stage biofilm consists of the organic conditioning film, a succession of colonizing bacterial consortia with their associated EPS and various detrital particles and ionic species. It is this structure that gives rise to the planktonic bacteria and their byproducts (e.g., endotoxins).

The question of whether differing substratum surface properties are communicated to the initial or succeeding organisms through the conditioning film is of great interest. Some workers (33) have suggested that substratum properties can be transferred by an adsorbed protein film to adhering eucaryotic or prokaryotic cells. This supposition is based on their finding that the amount and surface structure of albumin adsorbed onto inanimate surfaces was a function of substratum wettability (surface free energy). Conversely, Flint et al. (11) found that washed cells of *S. thermophilus* and *Bacillus cereus* attach to clean stainless steel surfaces within 60 s in the apparent absence of a conditioning film. Gasket materials, including Buna-n and Teflon, have been found to accrete significant bacterial biofilms in a milk processing operation (2). Similar biofilms were found on surfaces exposed to both raw and pasteurized milk. Despite the recognition of the importance of conditioning films as precursors to biological fouling activities, treatments have not been developed for their control or modification.

ADAPTIVE ADVANTAGES: LIFE IN A BIOFILM

Several adaptive advantages have been ascribed to life within a biofilm. In 1943, Zobell (39) proposed that solid surfaces act not only to concentrate nutrients by adsorption but also to retard the diffusion of exoenzymes away from the cell, thus promoting the uptake of substrates that must be hydrolyzed extracellularly. Attachment appears to be an important adaptive mechanism in what Morita (27) has termed the starvation-survival mechanism of bacteria in extreme environments. A decrease in the concentrations of bulk phase carbon sources promotes the attachment of marine and freshwater bacteria (20). Bacteria in milk transmission (29) and industrial purified water systems (24) also show a preference for surfaces.

Biofilm organisms are afforded a measure of protection from the antagonistic agents that are present in bulk-phase environments. Protection from lytic bacteria such as *Bdellovibrio* spp. (36), the toxic effects of heavy metals (23), and bactericidal agents (8, 15, 29) are important advantages afforded to microorganisms within biofilms. This protective feature is a significant factor in many disease processes and biological fouling activities in industrial systems. Bacteria that are associated with biofilms are more resistant to antibiotic treatments that would otherwise prove effective against free-living populations (15). In vitro studies (12) demonstrated differential resistance of *Listeria monocytogenes* biofilms to a combination treatment of sodium hypochlorite and heat. Mean reduction values in viable counts following treatment were about 100 times lower for biofilm than for planktonic cells. Although the mechanisms of this apparent antimicrobial resistance are poorly understood, EPS appear to have an important role.

Resistance is likely a function of several interrelated factors, including diffusion barriers, differential metabolic activity, and cell-wall ultrastructure.

DETECTION

To a great extent, the availability of effective detection techniques has limited the progress in understanding and resolving the problems related to biofilms. For 100 yr, microbiologists, for the most part, have virtually ignored the relationship of surface-associated bacteria and other microorganisms to the overall population. Although planktonic samples can be obtained relatively easily from a water system or milk distribution line spigot, samples from pipeline and storage tank surfaces are more difficult to sample reproducibly. The acquisition of representative samples from surfaces in distribution and storage systems is particularly important for evaluations of disinfectant and biocide efficacy.

The Robbins Device (Tyler Instruments, Calgary, AB, Canada) consists of a series of sample coupons that are flush-mounted in a rectangular flow channel. This biofilm sampler was designed to be side-streamed to an existing distribution system to enable process control testing (9). Biofilms may be quantitatively removed from the Robbins Device by a combination of scraping and sonication and then enumerated. Laminar flow adhesion cells for sampling bacterial biofilms have been described (25). Reproducible colonization of aerobic and anaerobic bacterial isolates has been obtained with these devices, which also provide for real-time image analysis of colonizing organisms.

Geesey and White (13) and Pedersen (30) have reviewed techniques for the sampling and isolation of bacteria associated with various surfaces. Fourier-transform infrared spectroscopy in the attenuated reflectance mode has been applied to the real-time analysis of developing biofilms. In addition to changes in biomass, metabolic status can be monitored (e.g., the production of poly-β-hydroxyalkanoates). The application of quartz crystal microbalance gravimetric measurements to on-line monitoring of biomass has been described (13). This technique enables real-time analysis of cell numbers with a detection limit in the range of 10^5 cells·cm^{-2}. Measurements of open circuit potential detected the onset of biofilm formation on 316 stainless steel surfaces (25).

Wong and Cerf (38) have reviewed monitoring techniques that are specific for biofilms in dairy operations. Some of these techniques, including ATP-based bioluminescence, are subject to interferences from nonmicrobial biomass. There is a clear need for on-line tools to monitor bacterial biofilm development and cleaning efficacy in food processing industries.

TREATMENT OPTIONS

For the most part, the efficacy of disinfectant and biocide treatment has been evaluated on the basis of a bottle test. In this assay, bacteria are grown in laboratory culture (very often through multiple passages) and then challenged with an antimicrobial solution. System treatment concentrations, contact times, and environmental conditions (e.g., temperature) are based upon these types of laboratory studies. A number of workers (17, 29) have shown that the success of an antimicrobial agent is dependent upon its ability to inactivate and remove biofilm organisms. Indeed, low concentrations of sodium hypochlorite in the range of 0.5 to 5 ppm are only inhibitory to biofilms associated with stainless steel surfaces; concentrations exceeding 50 ppm were required for inactivation under process control conditions (5). Particular attention should be paid to gasket surfaces that contact the product because biofilm bacteria can remain viable on those areas despite otherwise effective clean-in-place treatments (2).

As was mentioned previously, EPS appear to afford bacteria protection from antagonistic agents. For example, endemic strains of *Staphylococcus aureus* that were isolated from poultry equipment were eight times as resistant to chlorine as were *S. aureus* strains that were isolated from normal skin (4). The major phenotypic difference between these strains was the extensive EPS associated with the poultry equipment isolates and their ability to form macro clumps. Others (1) have shown that *Pseudomonas aeruginosa* survived within biofilms associated with polyvinyl chloride piping after 7 d of exposure to iodophors and phenolic antimicrobial solutions. These researchers (1) suggested that these organisms survived within EPS masses on the interior walls of the piping. The structure of EPS acts as a diffusional barrier to antimicrobial penetration. Suci et al. (32) found that the in vitro penetration rate of ciprofloxacin, an antibiotic, was significantly impeded by *P. aeruginosa* biofilms. The structure and composition of EPS apparently influence both diffusional resistance and oxidizing chemical demand.

Sodium hypochlorite concentrations of 100 mg·L^{-1} heated to 65°C for 5 min or to 72°C for 1 min were required to inactivate *L. monocytogenes* biofilms as-

sociated with stainless steel surfaces (18). Treatment with 100 mg·L^{-1} of sodium hypochlorite for 30 s, followed by heat treatment at 65°C for 30 s, was not effective. With chlorine compounds, pH is an important treatment variable. The pk_a of hypochlorous acid, which has far stronger bactericidal activity than the hypochlorite ion, is approximately 7.5. Therefore, treatment pH should be maintained in the acidic range to provide maximal efficacy. Characklis (6) suggests that chlorination programs might be improved by increasing chlorine concentration at the water-biofilm interface, increasing fluid shear stress at the water-biofilm interface, and controlling pH. High pH favors the hypochlorite ion promotion of detachment of mature biofilms, and low pH enhances hypochlorous acid disinfection of thin films. Other biocides used for biofilm control in the dairy industry include iodine, ozone, and chloramines. The isothiazolone microbicide, 2-methyl-5-chloro-2-methyl isothiazolone, has been employed at 10 mg·L^{-1} for the successful control of *L. monocytogenes* that is associated with conveyer systems in a dairy processing and packaging operation. As with some oxidizing biocides applied for suboptimal contact times (29), biofilm-mediated resistance to quaternary ammonium and iodophor compounds has been reported (37).

Ozone has shown promise for disinfecting surfaces that come in contact with milk. Ozone is prepared onsite by passing dry oxygen or air through high voltage corona arc discharge electrodes. Like chlorine, ozone is a powerful oxidizing agent; bacterial membrane lipids, carbohydrates, and proteins are oxidized, resulting in cell death. Greene et al. (14) compared ozonation with chlorination in disinfecting stainless steel plates colonized with *P. aeruginosa* or *Alcaligenes faecalis*. They found that the killing efficiencies of these two oxidizing agents were comparable.

A relatively new disinfection technology that might have applications for the dairy and food products industries has been described. The technology involves passage of low level electrical fields (5 V cm^{-1}; 15 μA·cm^{-2}) through biofilms in the presence of antimicrobials (3). Enhancement of biofilm bacterial inactivation occurred with both antibiotics and industrial biocides. Although viable numbers of bacteria did not decrease as a function of current application alone, killing activities of the biocides were enhanced by several orders of magnitude in the presence of these low level electric fields. Although mechanisms for this effect have not yet been established, they may involve alterations in EPS charge or cell membrane transport processes that facilitate transport of the antimicrobials to labile cellular components.

CONCLUSIONS

Microbial biofilms are a major source of most of the pathogenic and spoilage bacteria in the dairy processing industry. The physiology of attached bacteria differs from that of planktonic bacteria, which might be an important factor in their apparent differential antimicrobial resistance. Biofilm formation can be described as a three-step process: development of a conditioning film, primary colonization, and mature biofilm development with associated EPS and detrital materials. A number of novel techniques have recently been employed for the detection of bacteria associated with surfaces, including Fourier-transform infrared spectroscopy and quartz crystal balance microgravimetry. The structure of EPS provides bacteria within biofilms with protection from a broad range of antimicrobial agents. The combination of diffusional barriers, nonspecific binding to charged moieties associated with EPS, and an intrinsic oxidizing biocide demand reduces antimicrobial efficacy dramatically. Treatment efficacy should be evaluated based upon biofilm challenges, which present a more conservative test. Treatment agents that are active against planktonic organisms might have little or no activity against those organisms present within biofilms. Prevention of biofilm development is the key to control: frequent system cleaning, application of combination treatments (oxidizing agents and surface-active compounds), and frequent surface monitoring are important for an effective preventive maintenance program.

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